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Effect of diethyldithiocarbamate on proliferation, redifferentiation, and apoptosis of human hepatoma cells[1]

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KEY WORDS diethyldithiocarbamate; hepatocellular carcinoma; cell division; cell differentiation; apoptosis; agar gel electrophoresis; electron microscopy; flow cytometry

ABSTRACT

AIM: To examine the effects of diethyldithiocarbamate (DDC) on the proliferation, redifferentiation, and apoptosis in human hepatoma cells. **METHODS**: Cell surface charge, biochemical changes, cell growth in soft agar, single cell electrophoresis, electron microscopy examination, and flow cytometry analysis were measured. **RESULTS**: After being treated with DDC 3 mmol/L the growth curve and mitotic index of human hepatoma cells decreased remarkably, and the cellular growth inhibitory rate amounted to 52.4 %. The indices related with cell malignancy were alleviated significantly, such as the cell surface charge decreased significantly, the electrophoresis rate dropped from 1.6 to 0.8 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$, the average value of α-fetoprotein (α-FP) content decreased from 314 to 95 μg/g (protein), and γ-glutamyl-transpeptidase (γ-GT) activity from 0.9 to 0.14 U/g (protein). The cell differentiation index increased significantly, such as the average levels of tyrosine-α-ketoglutarate transaminase (TAT) activity increased from 11.6 to 36 μmol/g (protein), and the colonogenic potential decreased by 95.6 %. The apoptotic bodies, detached cells, and apoptotic morphological features appeared, and the treated cells' DNA was fragmented as observed by the comet assay. The flow cytometric results showed that a 42.9 % fractional DNA content existed in the treated cells. **CONCLUSION**: DDC can inhibit human hepatoma cells proliferation, and can induce redifferentiation as well as apoptosis.

INTRODUCTION

Cancer involves deregulated cell proliferation, abnormal differentiation, and abnormal apoptosis. Reactive oxygen species (ROS) may play a crucial role in the mechanisms underlying the regulation of cell proliferation, differentiation, and apoptosis. Most tumor cells appear with relatively incomplete differentiation. Experimental and epidemiological evidences implicate that lower differentiated tumor cells exhibited lower levels of antioxidative activity and were more sensitive to ROS than their normal, fully differentiated counterparts. ROS are postulated to be the causal factors and may be a general rather than an isolated phenomenon during cellular differentiation and apoptosis[1]. A number of studies showed that malignant tumor cells could be induced to redifferentiation and apoptosis by the addition of ROS or depletion of cellular antioxidants[2-4]. We have reported that oxidative stress generated from Fe^{2+}/Vit C system can inhibit the proliferation, induce redifferentiation and apoptosis of human hepatoma cell line SMMC-7721[5]. Therefore, the idea of a pro-oxidant therapy is clearly an attractive possibility for treating malignancy.

Cleveland *et al*[6] recently reported that a compound killed leukemia cells by inhibiting an antioxidative enzyme that kept down the level of ROS. DDC is a strong prooxidant which can promote significant generation of O_2^- *in vivo* by inhibiting superoxide dismutase (SOD)[7]. It has been used in humans for treating nickel poisoning or as an immune modulator, and its toxicity is low[8]. To investigate whether DDC can induce differentiation and apoptosis of tumor cells may offer a general clinical agent for combating cancer by normalizing tumor cells or inducing apoptosis in them.

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MATERIALS AND METHODS

Reagents Culture media was purchased from Gibco Laboratories (Santa Clara, CA). Bovine serum was obtained from Sijiqing Biotechnology Co (Hangzhou, China). Diethyldithiocarbamate (DDC), trypsin, RNase A, propidium iodide (PI), proteinase K, nonidet NP-40, dimethyl sulfoxide were obtained from Sigma (St Louis, MO). Acridine orange (AO), mercaptoethanol, and ethidium bromide (EB) were purchased from Fluka (Buchs, Switzerland). All other reagents were of analytical reagent quality.

Cell culture Human hepatoma cells SMMC-7721 set up by the Second Military Medical University, Shanghai, China, were cultured in RPMI-1640 medium supplemented with 10 % inactivated bovine serum, benzylpenicillin 100 mU/L, streptomycin 100 ng/L, and $NaHCO_3$ 2.0 g/L at 37 ℃. Cells 8×10^7 L^{-1} were cultured for 24 h, and the culture medium was aspirated and replaced with the culture medium containing DDC 1 or 3 mmol/L. The culture medium was replaced with a fresh medium as the control against DDC treated groups.

Determination of cell growth curve and mitotic index Cells 8×10^7 L^{-1} were cultured in 15 mL culture flasks and small bottles with a cover slide. The culture flask contained a cellular suspension 2 mL and the small bottles 1 mL. DDC 1 or 3 mmol/L were added after subculturing for 24 h. Three flasks from treatment and control groups were collected every day in the first 6 d, and the viable cells were counted using trypan blue dye exclusion method. Three small bottles for each group were taken at the same time. Cells were fixed in Bouin-Hollonde solution and stained with hematoxylin-eosin. The mitotic cells were counted from 1000 cells every day.

Cell electrophoresis The cells treated with DDC 1 or 3 mmol/L for 72 h and the control cells were collected and washed with *D*-Hanks' solution twice, then resuspended at a density of 1×10^9 L^{-1}. The cell electrophoresis determination was performed on a round plastic tube with electric-bridge filled with NaCl 10 %-agar 1 % and Ag-AgCl electrodes at a direct current voltage of 40 V at 24 ℃, taking sucrose 9 % as the electrophoretic medium and using a microcapillary electrophoresis apparatus. The results are expressed as the average time (s) taken by a cell to move over a distance of 120 μm and 40 cells in each group were thus determined. The experiments were repeated thrice with similar results, and the result of one experiment chosen as the prototype.

Assays for α-fetoprotein (α-FP) and α-glutamyltranspeptidase (γ-GT) Two assays were performed at the end of the 72-h treatment. Cell suspension (5×10^8 cells) 0.2 mL was transferred into 0.3 mL of ice-cold lysis buffer containing Tris 5 mmol/L, edetic acid 20 mmol/L, and Triton X-100 0.5 % (v/v), pH 8.0, and was left to lyse for 30 min on ice before centrifugation for 10 min at $3000 \times g$. The α-FP in the supernatant was determined by an α-FP reagent kit (Biological Reagent Research Institute, Lanzhou, China) using the ELISA bi-antibody with α-FP. γ-GT was determined by the γ-GT reagent kit (Chemical Reagent Research Institute, Shanghai, China) using the azo-coupling reaction. All protein content was measured with the Folin phenol reagent using Lowry's method.

Assay for tyrosine-α-ketoglutarate transaminase (TAT) The TAT activity was detected in the whole cells by the method of Diamondstone[9]. Cell suspension 0.2 mL (in KCl 0.14 mmol/L, 5×10^7 cells) was transferred into 0.3 mL of ice-cold lysis buffer described above, and samples were allowed to lyse for 30 min at 0 ℃, $31\,000 \times g$. *p*-Hydroxybenzaldehyde 1 μmol produced in the reaction system at 37 ℃ for 30 min was defined as 1 unit of TAT.

Colonogenic assay After 48-h treatment, the treated and untreated cells were washed with RPMI-1640 medium containing 10 % heat-inactivated bovine serum. Cell counts were performed by a hemocytometer, and viable cells were assayed by trypan blue exclusion method. An underlayer of 0.5 % agar in RPMI-1640 medium containing 10 % bovine serum was prepared (0.5 mL for each well in a 24-well culture plate) and cells were suspended in the plating layer. Cells were routinely plated at a concentration of 6×10^6 L^{-1} on the plating layer 0.5 mL. Cultures were incubated at 37 ℃ in a 5 % CO_2 humidified atmosphere. Cultures were scored 21 d after plating. A colony was defined to be an aggregate of >50 cells[10].

Morphology of apoptosis The acridine orange-stained cells were examined using the fluorescent microscope. After fixed with acetic acid-methanol (1∶3) for 1 h, the cells were stained with acridine orange solution (10 mg/L in PBS, pH 4.2) for 15 min, then decolorized with distilled water. Green fluorescence was detected between 500 nm and 525 nm using an Olympus BH-2 microscope with a fluorescence attachment. The percentage of apoptotic cells was assessed by acridine

orange staining using the morphological criteria of apoptosis. Apoptotic cells were observed to be small and contained fragmented nuclei and condensed chromatin, compared with intact cells with large and well-defined nuclei. Counts were made on a minimum of 200 cells per culture[11].

Electron microscopy Control and treated cells (1×10^7) were pelleted by centrifugation and washed twice with PBS, fixed with 2.5 % glutaraldehyde in cacodylate buffer 0.1 mol/L (pH 7.4), postfixed with 1 % osmium tetroxide for 24 h, embedded with 1 % agar, and dehydrated through graded ethanol. The cells were embedded in an Epon/Araldite mixture. Semithin sections were cut with glass knives, mounted on glass slides, stained with toluidine blue, and viewed under the light microscope for orientation. Ultra-thin sections were cut with diamond knives, floated onto a 200 mesh copper grid, stained with uranyl acetate and lead citrate, and examined under Hitachi H-600 transmission electron microscope[12].

Single cell electrophoresis To detect DNA fragmentation and morphological changes of nuclei in SMMC-7721 cells, a modified method of single-cell gel electrophoresis[13] was used. An aliquot 100 μL of the cell suspension was suspended in 1 mL melted 0.75 % low gelling temperature agarose in PBS at 33 ℃ giving a final cell density of about $1 \times 10^7\ L^{-1}$. The mixture was dispersed on a microscope slide pretreated with agarose of low concentration (0.1 %) in order to improve the adhesion of the 0.75 % agarose onto the slide. After gel formation at 0 ℃, the slides were treated for 15 ~ 60 min in a lysing solution containing 2.5 % SDS, edetic acid 0.025 mmol/L, pH 9.5. An electric field of about 5 V/cm was applied for 5 min and the slides were then washed in distilled water for 5 min, and followed by 1 h drying. The agarose layer was now very thin. The slides were stored at − 18 ℃ for further processing. After a further short wash in distilled water, the DNA was stained by immersing the slide in a solution of acridine orange (AO) 2 mg/L and cells were observed through an Olympus fluorescent microscope.

Detection of apoptosis by flow cytometry analysis Apoptosis was detected by analyzing the reduced fluorescence of the DNA binding dye propidium iodide (PI) in the apoptotic nuclei[14]. In brief, cultured cells (about 1×10^7) (detached and attached) were washed three times with PBS containing edetic acid 5 mmol/L. The cells were fixed in 70 % ethanol at − 20 ℃ overnight. Following fixation, the cells were centrifuged and resuspended in 500 μL PBS containing 0.1 % Triton X-100, edetic acid 0.1 mmol/L, and RNase 100 mg/L, and stored in the dark at 4 ℃ for 3 − 4 h. Then, the cells were washed once with PBS, resuspended in 500 μL of PBS containing PI 50 mg/L and incubated at 4 ℃ for 30 min. Total DNA content per cell was analyzed and stored with Becton Dickinson Facscalibure. A minimum of 1×10^4 cells per sample was analyzed. Data were acquired in list mode and analyzed with Cellquest software (Becton Dickinson).

Statistics Data were expressed as $\bar{x} \pm s$, and compared with t test.

RESULTS

The cell growth rate was slowed down and the doubling time was delayed by DDC at 3 mmol/L after subculturing for 48 h. And the survival rate of cells showed no obvious difference between the DDC 1 and 3 mmol/L treated groups and the corresponding control (Tab 1). The mitotic index showed that cells exhibited vigorous proliferating capability with a division peak on the fifth day after subculturing, and the mitotic index was 5.53 %. After treatment with DDC 3 mmol/L, the mitotic index declined to 2.66 %, and the division peak shifted to the fourth day after subculturing (Fig 1). The results indicated that DDC 3 mmol/L inhibited hepatoma

Tab 1. Effects of DDC on the proliferation and the survival rate of hepatomacytes observed for 48 h. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control group. In the survival rate test, total of 1000 cells were counted in each group.

DDC / mmol·L^{-1}	Cell doubling time/h	10^{-5} × Number of cells Original	Final	Inhibition/%	Survival rate/%
0	23.6 ± 1.4	2.0	132 ± 12		98.4
1	24.8 ± 1.5^a	2.0	116 ± 10^a	13.8^a	98.2^a
3	32.1 ± 1.5^c	2.0	66 ± 8^c	52.4^c	96.5^a

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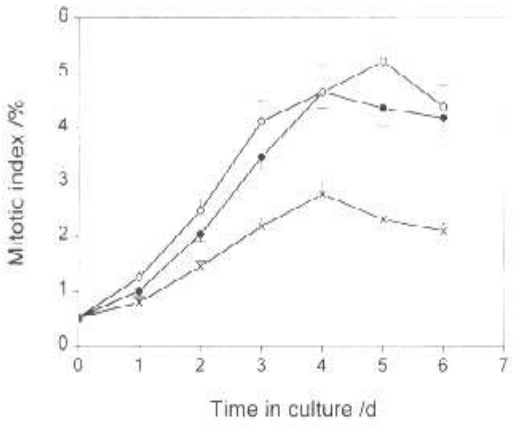

Fig 1. Effect of DDC on the mitotic index of hepatomacytes. Cells were inoculated at a density 1×10^8 L^{-1}. The mitotic rates were counted every day. The cells were cultured in the medium with none (○), or DDC 1 (●) and 3 mmol/L (×) respectively. Means of three parallel experiments were plotted. $n = 3 \times 3$ cultures per condition. $\bar{x} \pm s$.

cells proliferation, but according to survival rate, no cytotoxicity was observed.

After treatment with DDC 3 mmol/L for 6 d, the electrophoresis rate apparently slowed down. The percentage of retardation reached as high as 43.7 % (Tab 2). Both the α-FP content and the γ-GT activity decreased markedly, the average value of TAT activity increased, and the colonogenic potential (CP) of treated cells in soft agar decreased greatly (Tab 3).

Tab 2. Effect of DDC on the cell surface charge of hepatoma cells. $n = 3$ experiments. $\bar{x} \pm s$. $^{\#}P < 0.01$ *vs* control group.

DDC /mmol·L^{-1}	Electrophoresis time/s	Electrophoresis /μm^{-1}·s^{-1}·V^{-1}·cm^{-1}	Retardation /%
0	12.3 ± 0.3	1.6	
3	22.8 ± 0.5	0.8	43.7$^{\#}$

A substantial increase in the number of condensed apoptotic nuclei was observed after exposure to DDC 3 mmol/L for 48 h (Fig 2). The nuclei of these cells were smaller and their chromatin condensed around the periphery of the nucleus. The apoptotic bodies, one of the main morphological characteristics, appeared. The percentage of apoptotic cells in the control group was 1.4 % ± 0.4 %, while it was 22.1 % ± 1.5 % in the DDC 3 mmol/L treated group, 16-fold higher than that of control.

Further characterization of the morphological changes induced by DDC 3 mmol/L at the ultrastructural level were observed, cell surface protrusion (SP) became evident, and some of the cells appeared to be in the process of being pinched off to form apoptotic bodies.

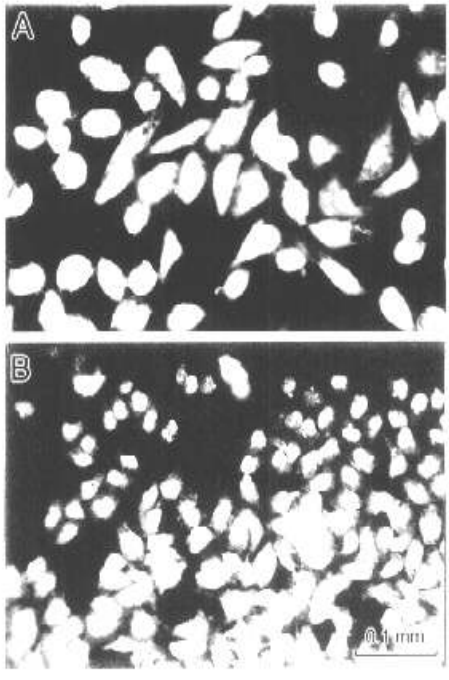

Fig 2. The chromatin conformation in monolayer cells stained with acridine orange. A: Control cells. B: Cells treated with DDC 3 mmol/L for 48 h. Magnification × 132. Arrowhead indicates the apoptotic body.

Tab 3. Effect of DDC on α-FP content, γ-GT, and TAT activities, and clonogenic potential (cp) of hepatoma cells. $n = 3$ experiments. $\bar{x} \pm s$. $^{\#}P < 0.01$ *vs* control group.

DDC/mmol·L^{-1}	α-FP/μg·g^{-1}	γ-GT/IU·g^{-1}	TAT/μmol·g^{-1}	Number of clonies obtained	CP/%
0	311 ± 24	0.9 ± 0.7	11.6 ± 0.8	325 ± 26	100
3	95 ± 8$^{\#}$	0.14 ± 0.10$^{\#}$	36 ± 3$^{\#}$	16 ± 4	4.4$^{\#}$

The chromatin (Ch) of apoptotic cells condensed in large masses in the nucleus. The internal membranes showed dilations sometimes giving the appearance of cytoplasmic "boiling", and as well as cytoplasmic blebbing (CB). The dilation of nuclear membrane (NM) appeared to be the common characteristics of apoptotic cells (Fig 3).

DNA fragmentation, the most characteristic bio chemical feature of apoptosis, was evaluated by single-cell electrophoresis and flow cytometric analysis respectively. The results of single cell gel electrophoresis indicated that treated cells exhibited DNA fragmentation (comet like pattern on single-cell gel electrophoresis) (Fig 4). A subdiploid peak of DNA can be observed in apoptotic cells by flow cytometry after staining with propidium iodide[11]. In order to quantify the extent of the apoptotic process, the DNA content analysis of treated and control cells was carried out by flow cytometry. The analysis of the data (Fig 5) confirmed that the decrease in the rate of cell proliferation associated with the increasing size of the sub-G_1 peak, the index of DNA degradation of apoptosis cells, in the DNA histograms. The percentage of fluorescence intensity detected in the sub-G_1 region was obviously increased in DDC 3 mmol/L treated group (28.3 % ± 2.1 %), as compared with the control (2.10 % ± 0.10 %).

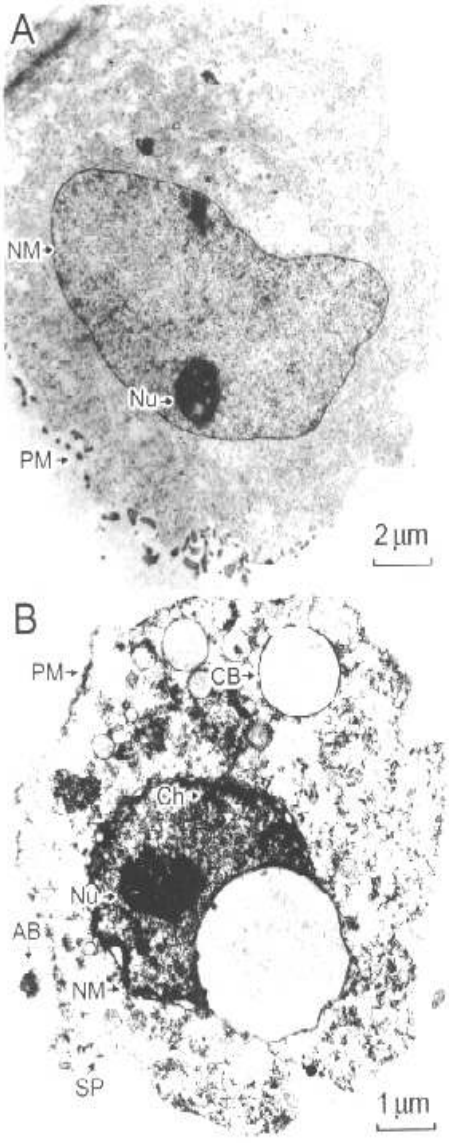

Fig 3. The effect of DDC on the ultrastructure of hepatoma cells. a: Control cells. b: Cells treated with DDC 3 mmol/L for 48 h. Magnification (a: × 4000, b: × 8000). Arrows indicate nucleolus (Nu), nuclear membrane (NM), chromatin (Ch), plasma membrane (PM), apoptotic body (AB), surface protrusions (SP), cytoplasmic blebbing (CB).

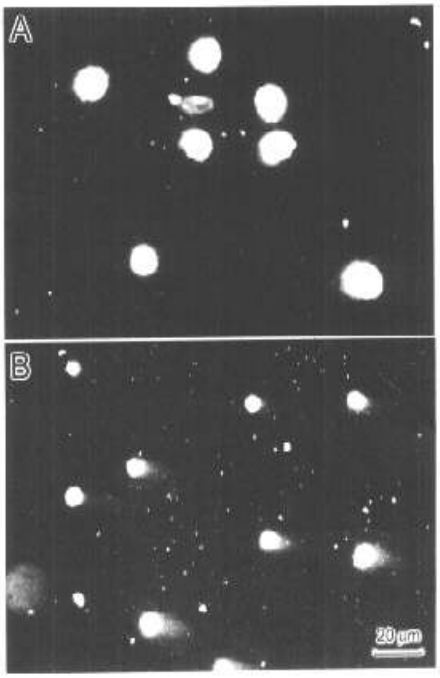

Fig 4. DNA fragmentation in hepatoma cells induced by DDC 3 mmol/L for 48 h detected by single cell gel electrophoresis. a: Intact cells. b: Treated cells. Magnification × 400.

DISCUSSION

Important characteristics of the transforming cells are their continuous division and multiplication. Therefore, the inhibitory effect on the multiplication of tumor cells is

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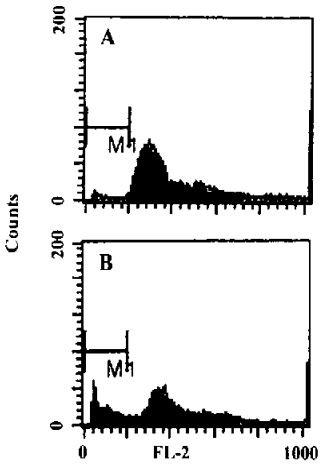

Fig 5. Flow cytometric analysis of hepatoma cells. Cells were fixed with 70 % ethanol and stained with propidium iodide in the presence of RNase A. Cells (1×10^4) were analysed after excitation at 488 nm by argon laser on a Becton-Dickinson Facscalibure, and histograms of DNA content versus cell number were generated using Cellquest software. A: Intact exponentially growing cells. B: Cells treated with DDC 3 mmol/L.

a significant appraisal of redifferentiation. The results of the proliferation of cells and mitotic index confirm that DDC can inhibit the multiplication of hepatoma cells.

The net charges at the tumor cell surface are generally more than those in the corresponding normal cells, thus, the electrophoresis rate of tumor cells should be higher than that of normal cells, and the change in the cell electrophoresis rate be taken as an appraisal of tumor cell differentiation[15,16]. Our results showed that DDC could decrease the amount of hepatoma cell surface negative charge greatly. The increase in α-FP content and γ-GT activity are related with hepatocytes malignancy, while the increase in TAT activity and the decrease in clonogenic potential are the indices of hepatoma redifferentiation. Inspecting the clonogenic potential of cultured cells in soft agar is a very important marker for distinguishing between malignant, benign, or normal cells[5,10,15]. DDC is able to increase the TAT activity, to decrease the content of α-FP, γ-GT activity, and clonogenic potential.

All these changes indicated that the hepatomacytes were inclined towards normalization, and confirmed that DDC did induce the redifferentiation of hepatoma cells and impelled the tumor cells reversion against the malignant phenotype.

The morphological and biochemical data showed that the occurrence of an apoptotic process was simultaneously accompanied with a redifferentiation process in the hepatoma cells. Apoptotic cells rounded up and fragmented into multiple residues. In the nucleus, the chromosome showed condensation and aggregation at the periphery of the nucleus. This was followed by nuclear fragmentation, cell surface protrusion, the internal membranes dilation, cytoplasmic blebbing, and the preservation of cellular membrane and organelles. All the above mentioned morphological changes are the characteristics of apoptosis[11,12].

DNA fragments have been described as a biochemical hallmark of apoptosis. In our study, we have employed the comet assay, which is a sensitive and rapid technique for DNA strand break detection in individual cells to study the DNA fragments. With the apoptotic DNA fragmentation characterized by generation of double strand breaks rather than the random breaks often seen in necrosis, the comet assay can distinguish apoptosis from necrosis and has been used to assess apoptosis[13]. In our experiment, the extent of DNA damage in apoptotic cells was so apparent that they were easily distinguishable visually from the untreated cells. The most obvious aspect of apoptotic comets is the movement of most of the DNA from the head into the tail of the comet.

The extent of the apoptotic process can be demonstrated in DNA histograms by the sub-G_1 peak, which is an established indicator of apoptosis[14]. In the present study, in treated cells, there was a sub-G_1 peak, which compromised of about 28.3 % of the population compared with 2.10 % in the control.

Overall experimental results described above unequivocally demonstrated that hepatoma cells incubated with DDC underwent apoptosis.

A number of studies showed that malignant tumor cells could be induced to redifferentiation and apoptosis by ROS[2-4,15]. We have observed that oxidative stress generated from Fe^{2+}/Vit C system can inhibit the proliferation, induce the redifferentiation and apoptosis of human hepatoma cell line SMMC-7721[5]. DDC is a strong pro-oxidant which can promote significant generation of O_2^- *in vivo* by inhibiting the activity of superoxide dismutase (SOD)[7]. Superoxide in the cell medium may further generate H_2O_2 and ·OH. So its induction of human hepatoma cells' differentiation and apoptosis may be related to oxidative stress. Cleveland *et al*[6] have also reported that leukemia cells can be

killed by inhibiting an antioxidative enzyme that keeps down the level of ROS.

Experimental and epidemiological evidences implicate that redifferentiated tumor cells exhibit lower levels of antioxidant activity and more sensitivity to ROS than their normal, fully differentiated counterparts. ROS may be the causal factors and may be a general rather than an isolated phenomenon during cellular differentiation and apoptosis[1]. This may lead to a new prediction: the tumor cell redifferentiation or apoptosis may be induced by promoting the generation of ROS in tumor cells. Our study offers some evidence to support the above new prediction.

Inducing tumor cells differentiation or apoptosis, rather than killing tumor cells with high cytotoxicity or other side-effects is considered to be a new strategy for combating cancer. Differentiation and apoptosis inducers are expected to be such a type of antitumor agents. DDC has been used in humans and its toxicity is low[8]. We have reported for the first time that DDC could induce the differentiation and apoptosis in tumor cells. These results prove that DDC can be probably used as an useful general clinical apoptosis inducer against cancer, or at least, against some types of cancer. The mechanism of DDC for induction of redifferentiation and apoptosis in hepatoma cells is still not fully clear and needs to be studied further.

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二乙基二硫代氨基甲酸钠对人肝癌细胞增殖、再分化与凋亡的作用[1]

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关键词 二乙基二硫代氨基甲酸钠；肝细胞癌；细胞分裂；细胞分化；细胞凋亡；琼脂凝胶电泳；电子显微镜检查；流式细胞术

目的：测定二乙基二硫代氨基甲酸钠(DDC)对人肝癌细胞增殖、再分化与凋亡的作用. **方法**：采用细胞表面电荷、生化变化、软琼脂细胞生长、单细胞电泳、电子超微结构和流式细胞 DNA 片断分析等

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指标测定细胞分化和凋亡. **结果**: 用 DDC 3 mmol/L 处理后, 肝癌细胞的生长和分裂指数显著下降, 增殖抑制率达 52.4 %. 与恶化有关的指标显著减轻, 如细胞表面电荷明显降低, 电泳率从 1.6 降低到 0.8 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$, 甲胎蛋白由 314 降为 95 μg/g (protein), γ-谷氨酰转氨酶活性由 0.9 降到 0.14 U/g (protein). 与分化相关的酪氨酸-α-酮戊二酸转氨酶活性显著上升, 由 11.6 升高到 36 μmol/g (protein), 细胞克隆形成力降低 95.6 %. 同时, 处理细胞出现了凋亡小体、不贴壁细胞以及其它凋亡特征, 且处理细胞 DNA 发生片断化, 流式细胞分析显示处理组 42.9 % 的 DNA 发生断裂. **结论**: DDC 能够抑制人肝癌细胞增殖, 并诱导细胞分化和凋亡.

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