*Molecular and Cellular Biochemistry* **265:** 123–132, 2004.


# Diethyldithiocarbamate can induce two different type of death: Apoptosis and necrosis mediating the differential MAP kinase activation and redox regulation in HL60 cells

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Received 26 December 2003; accepted 15 March 2004

## Abstract

Although apoptosis and necrosis have been considered different pathways to cell death, only one compound induces both types of cell death. Diethyldithiocarbamate (DDC) has been shown to have antioxidant or prooxidant effects in several different systems. We observed in our present study that DDC induced not only apoptosis but also necrosis depending on its dosage in HL60 premyelocytic leukemia cells. Moreover, in hypoxia cell culture conditions, DDC-induced necrotic cells decreased but DDC-induced apoptosis continued. We investigated the DDC-induced different cell death mechanisms as they are correlated with reactive oxygen species (ROS). High-dose DDC-induced necrotic cell death is thought to depend on the increase of intracellular ROS, while low-dose DDC-induced apoptosis is thought to depend on changes of the intracellular redox state by the transporting of external metal ions. There was no sequential or quantitative change of Bcl-2 family proteins in DDC-induced apoptotic or necrotic pathways. However, the mitochondrial transmembrane potential was remarkably decreased in the DDC-induced necrosis. Finally, duration of c-Jun N-terminal kinase (JNK) activation resulted in different types of cell death. (Mol Cell Biochem **265:** 123–132, 2004)

*Key words*: diethyldithiocarbamate, apoptosis, necrosis, ROS, JNK

## Introduction

Cell death is generally classified into two large categories: apoptosis and necrosis. Based on morphological changes, apoptosis is characterized by plasma membrane blebbing, condensation, and fragmentation of cells and nuclei, as well as extensive degradation of chromosomal DNA into nucleosomal units, while necrosis is accompanied by the swelling of cells and organellas, in addition to the ultimate disintegration of the plasma membrane [1]. It is widely believed that apoptosis represents active, programmed cell death, while necrosis represents passive cell death without underlying regulatory mechanisms. However, recent studies have shown that necrotic cell death may also be regulated. For example, inhibiting caspases, which belong to the family of cysteine protease and which play a central role in the regulation of apoptosis, resulted in necrotic-like morphology [2]. Moreover, in some cell lines tumor necrosis factor (TNF) can

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activate the necrotic death program, although it induces typical apoptotic cell death in many other cell types [3].

There are various signal transduction pathways in the cells to transmit appropriately in response to extracellular signals. The mitogen-activated protein (MAP) kinase signaling pathway mediates a variety of biological events, including mitogenesis, differentiation, and cell death [4]. Bcl-2 family proteins are arbiters of pro- or anti-apoptotic cell death via mitochondria [5]. Caspases, as mentioned above, act as initiators or effectors of apoptotic signals [6]. Moreover, there is much evidence that reactive oxygen species (ROS) are the key mediators of not only apoptotic but also necrotic cell death [3].

Diethyldithiocarbamate (DDC), a potent copper chelating agent, is widely used as a therapeutic agent for treating alcoholism [7], metal poisoning [8], AIDS [9], and cancer [10]. Many studies have been reported that DDC prevents apoptosis because of its antioxidant property [11–13]. As a result, there are many experimental treatment trials to test the efficacy of DDC against oxidative stress-related diseases [14–16]. However, DDC also has a pro-oxidant effect on cell damage [17]. In our present study, DDC induced not only apoptosis but also necrosis in human promyelocytic leukemia (HL60) cells depending on its concentration. Similarly, it is known that anticancer drugs and also irradiation induce apoptosis and also necrosis depending on the dosages [18, 19]. However, it has not been clear why a single substance can induce two types of cell death or what the difference is between the apoptotic and necrotic cell death mechanisms. Since DDC has both anti- and pro-oxidant effects and can induce different types of cell death, it is very difficult to know whether the clinical use of this drug will be beneficial or toxic. Therefore, it is very important to reveal how DDC induces different types of cell death. We now report that for HL60 cells, differential DDC effects on cell death are mediated by the differential MAP kinase activation and intracellular redox regulation.

## Materials and methods

*Materials*

Diethyldithiocarbamate (DDC), N-acetylcystein (NAC), and bathocuproine dislfonic acid (BCPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Benzyloxycarbonyl-2,6-dichlorobenzene (z-Asp-$CH_3$-DHB) was from the Peptide Institute, Inc, Osaka, Japan.

The primary antibodies used for Western blot analysis were polyclonal anti-Bcl-2, anti-Bax (DakoCytomation A/S, Denmark), anti-Bcl-xL (Sigma-Aldrich), anti-phospho-JNK, and anti-phospho-ERK (Promega, Madison, WI, USA).

*Cell culture conditions and cellular treatment*

The human promyelocytic leukemia (HL60) cell line was obtained from the Riken Cell Bank (Ibaraki, Japan) and was maintained in RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. DDC was dissolved in phosphate-buffered saline without $Ca^{2+}$, $Mg^{2+}$ (PBS(-), pH 7.4) and was added to the cell culture medium at the indicated concentrations. In all the experiments performed, only freshly prepared DDC solutions were used. To examine the effect of various substances on the DDC-induced cell death, cells were preincubated for 24 h with NAC (5 mM) and for 2 h with BCPS (50 $\mu$M) before treatment with DDC. z-Asp-$CH_3$-DHB (50 $\mu$M) was coadministrated with DDC.

*Hypoxia cell culture conditions*

Before treatment with DDC, hypoxia culture conditions were prepared by replacement of air with nitrogen gas in the cell culture flask. After treatment with or without DDC, nitrogen gas was again pumped into the culture flask, and then flasks were incubated with a disposable $O_2$-absorbing and $CO_2$-generating agent, AnaeroPack (Mitsubishi Gas Chemical Company, Inc, Tokyo, Japan), in a specially designed box at 37 °C for 24 h.

*Cell viability*

After treating the HL60 cells with 1 mM or 1 $\mu$M DDC at 37 °C for 24 h, we photographed the HL60 cell morphology in four visual fields at 100× magnification (over 100 cells/field) using a phase contrast microscope (Nikon) and counted the typical apoptotic and necrotic morphological cells. Treated cells were stained at 37 °C for 10 min with 200 $\mu$M Hoechst 33258 (Wako, Tokyo, Japan), and fragmentation of the nuclei was observed using a fluorescence microscope.

*Superoxide dismutase (SOD) activity*

HL60 cells were treated with or without DDC sequentially. Then, cells were collected, washed in PBS(-), and lysed by sonication. Solutions of cell lysates were centrifuged at 500*g* for 10 min, and the supernatants were used for assay. SOD activity was assayed as described previously [20]. Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL, USA).

*Flow cytometry*

*Determination of intracellular ROS*

Cells ($5 \times 10^4$/ml) treated with or without DDC were incubated with 2′,7′-dichlorofluorescein diacetate (5 $\mu$M DCFH-DA in DMSO, Molecular Probes, Eugene, OR, USA) for 15 min at 37 °C followed by analysis on a flow cytometer (FACScan, Becton Dickinson). We recorded 2′,7′-dichlorofluorescein fluorescence, which is oxidized DCFH, in FL1.

*Determination of mitochondrial transmembrane potential $\Delta\psi m$*

To evaluate mitochondrial transmembrane potential $\Delta\psi m$, we incubated cells ($5 \times 10^4$/ml) treated with or without DDC with 3,3-dihexyloxacarbocyanine iodide (40 nM DiOC6 in DMSO, Molecular Probes, Eugene, OR, USA) for 5 min at 37 °C followed by analysis on a flow cytometer (FACScan, Becton Dickinson). DiOC6 fluorescence was recorded in FL1.

*Western blotting*

HL60 cells treated with or without DDC sequentially were washed in PBS(-), suspended in 50 mM Tris-HCl (pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu$M pepstatin A, and 2 $\mu$M leupeptin, and lysed by sonication. A solution of cell lysates was centrifuged at 500$g$ for 10 min, and the supernatants were used for assay. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% gel on which 10 $\mu$g of total protein/well was loaded. After SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 0.5% Tween 20-TBS (0.01 mM Tris-HCl, pH 7.4, with 0.15 M NaCl) containing 3% nonfat dry milk, followed by successive incubations with primary and secondary antibodies. The bound antibody was detected using the ECL kit (Amersham Pharmacia Biotech, UK).

*Statistical analysis*

Statistical analysis was performed with the Mann-Whitney U-test and a value of $p < 0.01$ was considered statistically significant.

# Results

*Treatment with DDC-induced apoptotic and necrotic cell death in HL60 cells*

When 1 mM DDC was added to HL60 cells for 24 h, nearly 100% of the cells showed typically necrotic morphology, that is, the swelling of cell membrane and condensation of the nuclei. And, all these cells show cell membrane destruction and pyknosis at and after 24 h. It was rare to see apoptotic cells. In contrast, treatment of the cells with 1 $\mu$M DDC for 24 h induced apoptosis, characterized by plasma membrane blebbing and fragmentation of cells and nuclei in about 80% of the cells. Both necrotic and apoptotic morphological changes were observed after treatment with DDC for 6 h (Fig. 1).

*Treatment with DDC in hypoxia conditions*

In the hypoxia cell culture conditions, necrotic cells treated with 1 mM DDC decreased by half in the normal conditions, with the rest of the cells showing normal morphology. However, apoptotic cells treated with 1 $\mu$M DDC did not decrease, even in the hypoxia condition (Fig. 2).

*Effect of DDC on ROS production*

To examine whether DDC-induced necrotic and apoptotic cell death was concerned with ROS production, we first determined Cu/Zn-SOD activity. SOD is the enzyme catalyzed from oxygen radicals to hydrogen peroxide. DDC inhibits activity of Cu/Zn-SOD [21], which is the isoform of SOD, by withdrawal of essential metal from the enzyme. When 1 mM DDC was added, Cu/Zn-SOD activity was remarkably decreased after treatment for 1 h. There was minor activity after treatment for 18 h. In the case of treatment with 1 $\mu$M DDC, Cu/Zn-SOD activity stayed at a 60% level (Fig. 3a).

We next examined the production of intracellular ROS by HL60 cells treated with DDC using flow cytometry. Cells treated with 1 mM DDC for 30 min and 1 h showed decreased ROS contents. On the other hand, cells treated with 1 $\mu$M DDC showed slightly increased ROS contents (Fig. 3b).

*Blocking of DDC-induced apoptotic cell death but not DDC-induced necrosis by NAC and BCPS*

NAC is a glutathione precursor and reducing agent. If DDC acts as pro-oxidant property to the cells, NAC could inhibit the effect of DDC as an antioxidant. Therefore, we examined whether NAC could affect DDC-induced cell death. NAC completely blocked apoptosis treated with 1 $\mu$M DDC, but it did not block necrosis treated with 1 mM DDC, while the morphology of the cells changed from necrotic to apoptotic (Fig. 4a).

Since DDC is a membrane-permeable copper-specific chelator, we considered that the change of the intracellular redox state by metal ions was probably involved in the DDC-induced cell death. BCPS is a non-permeable

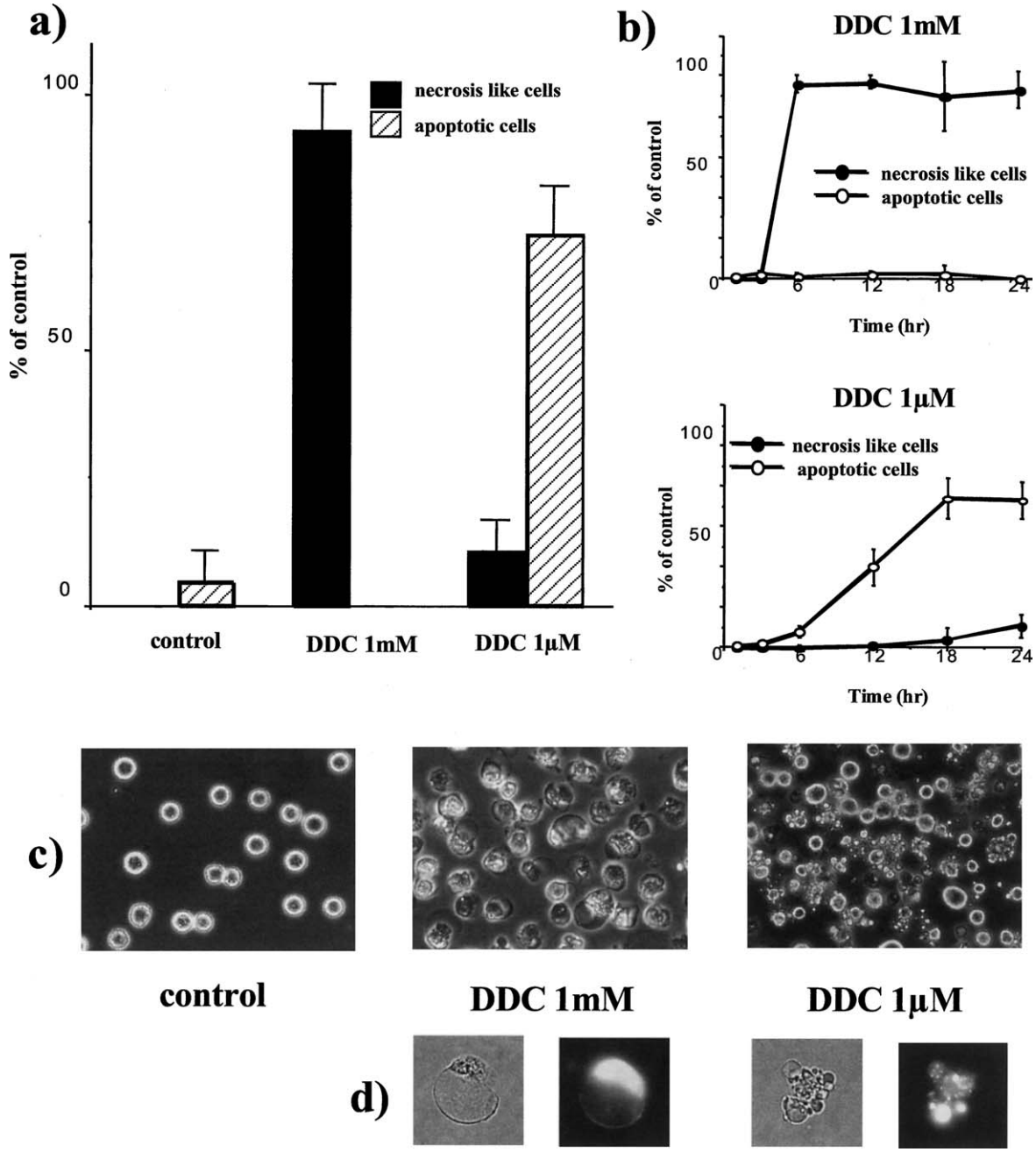


*Fig. 1.* DDC-induced necrotic and apoptotic cell death. (a) HL60 cells were incubated with 1 mM and 1 $\mu$M DDC for 24 h at 37 °C, and the typical necrotic and apoptotic morphological cells were counted. Values are means ± S.D. ($n = 4$). (b) Time-dependent change of the DDC-induced necrotic and apoptotic cells. Values are means ± S.D. ($n = 4$). (c) HL60 cells were incubated with 1 mM and 1 $\mu$M DDC for 24 h at 37 °C, and the cells are shown by phase contrast microscopy (×100). (d) The cells stained with Hoechst 33258 are shown by fluorescence microscopy (×200).

monovalent copper-specific chelator. When cells were treated with BCPS before being treated with 1 $\mu$M DDC, apoptotic cell death was completely blocked. However, BCPS did not block necrosis treated with 1 mM DDC, and the morphology of the cells changed from necrotic to apoptotic (Fig. 4b).

*Effect of a broad-spectrum caspase inhibitor (z-Asp-$CH_3$-DAB) on DDC-induced necrotic and apoptotic cell death*

To clarify the downstream DDC-induced necrotic and apoptotic cell death signaling pathway, we examined the

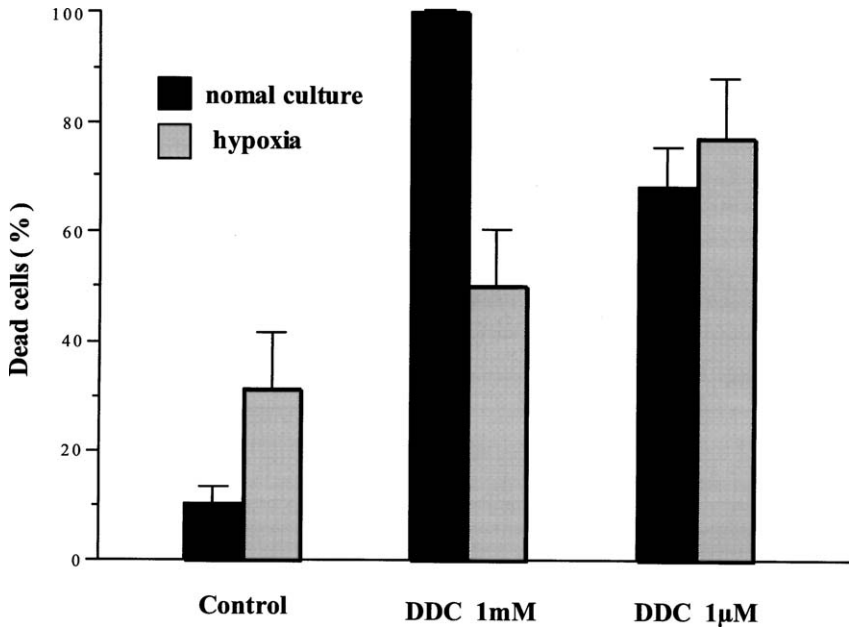


*Fig. 2.* HL60 cells were incubated with 1 mM and 1 $\mu$M DDC in normal culture conditions and in hypoxia for 24 h at 37 °C. Dead cells were counted. Values are means ± S.D. ($n = 7$).

involvement of caspases. A broad-spectrum caspase inhibitor, z-Asp-$CH_3$-DAB, completely blocked apoptotic cell death treated with 1 $\mu$M DDC; however, it had no effect on necrotic cell death treated with 1 mM DDC (Fig. 4c).

*Change of mitochondrial transmembrane potential ($\Delta\psi m$)*

The loss of mitochondrial transmembrane potential ($\Delta\psi m$) has been observed in the apoptosis induced by various stimuli [22]. In our study, when cells were treated with 1 mM DDC for 6 h, $\Delta\psi m$ was remarkably decreased. However, when cells were treated with 1 $\mu$M DDC, there was no alteration of $\Delta\psi m$ (Fig. 5).

*No correlation of Bcl-2 family proteins in the DDC-induced cell death*

Bcl-2 family proteins are key regulators of mitochondrial-dependent apoptosis [5]. Bcl-2 and Bcl-xL are anti-apoptotic, and Bax is pro-apoptotic. We examined whether DDC-induced cell death was regulated by the amount of several Bcl-2 subfamily proteins. There was no sequential change of any proteins at 6, 12, 18, or 24 h after treatment with 1 mM DDC or with 1 $\mu$M DDC (Fig. 6a).

*Differential effect on the activation of MAP kinase between DDC-induced necrosis and apoptosis*

C-Jun N-terminal kinase (JNK), a member of the MAP kinase family, is activated in response to a variety of cellular stresses and has been reported to be involved in apoptosis [4]. We measured the activation of JNK by detecting its phosphorylation. There was no activated JNK in the control cells or in treated cells 5 min–1 h after treatment with DDC. When cells were treated with 1 mM DDC for 3 h, we could observe the activation of JNK. At 6 h after treatment with 1 mM DDC, we observed down-regulation of phosphorylated JNK. On the other hand, when cells were treated with 1 $\mu$M DDC for 3 h, we could also see the activation of JNK. Moreover, the phosphorylation of JNK continued at 6 h after the treatment with 1 $\mu$M DDC (Fig. 6b).

## Discussion

Apoptosis and necrosis have long been considered to be two different cell death processes [1, 3]. In our present study, we observed that DDC could induce both types of cell death in HL60 cells, depending on the dosage. We examined the difference between DDC-induced necrotic and apoptotic signal transduction pathways. JNK was activated in the apoptosis as well as in the necrosis, but a different type of activation was observed. The phosphorylation was transient in the necrosis but sustained in the apoptosis, which indicated the possibility that DDC-induced cell death was regulated by only one type of JNK signal pathway and that cells make decisions regarding the type of cell death based on differences in the duration of JNK activation. There is precedence for this type of behavior. In PC12 cells, for example, it has been reported that the decision about proliferation or differentiation was regulated

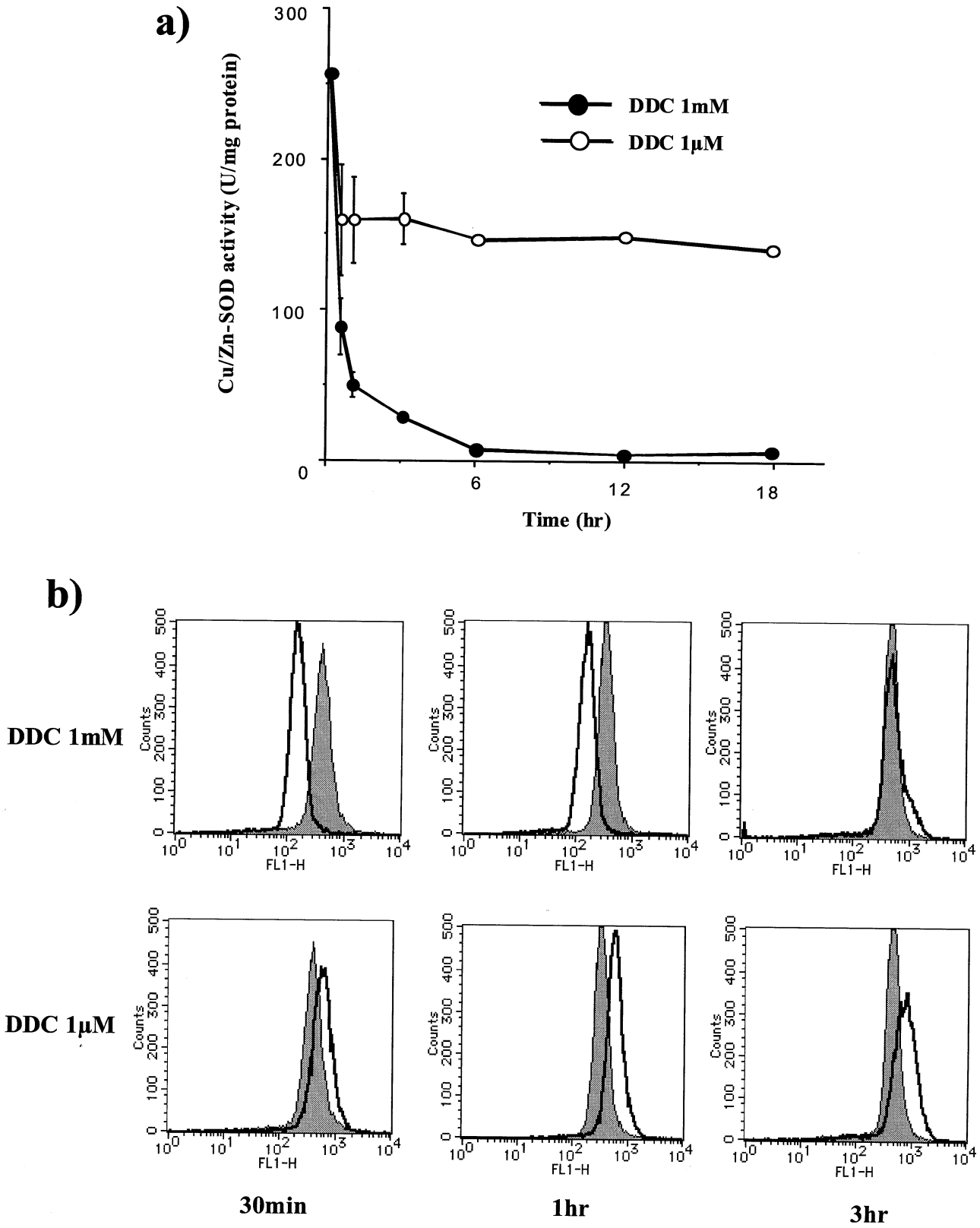


*Fig. 3.* (a) Time-dependent change of Cu/Zn-SOD activity on HL60 cells treated by 1 mM and 1 $\mu$M DDC. Values are means $\pm$ S.D. ($n = 4$). (b) Intracellular reactive oxygen species determination by flow cytometry. Gray areas indicate control cells; open areas with black lines indicate DDC-treated cells.

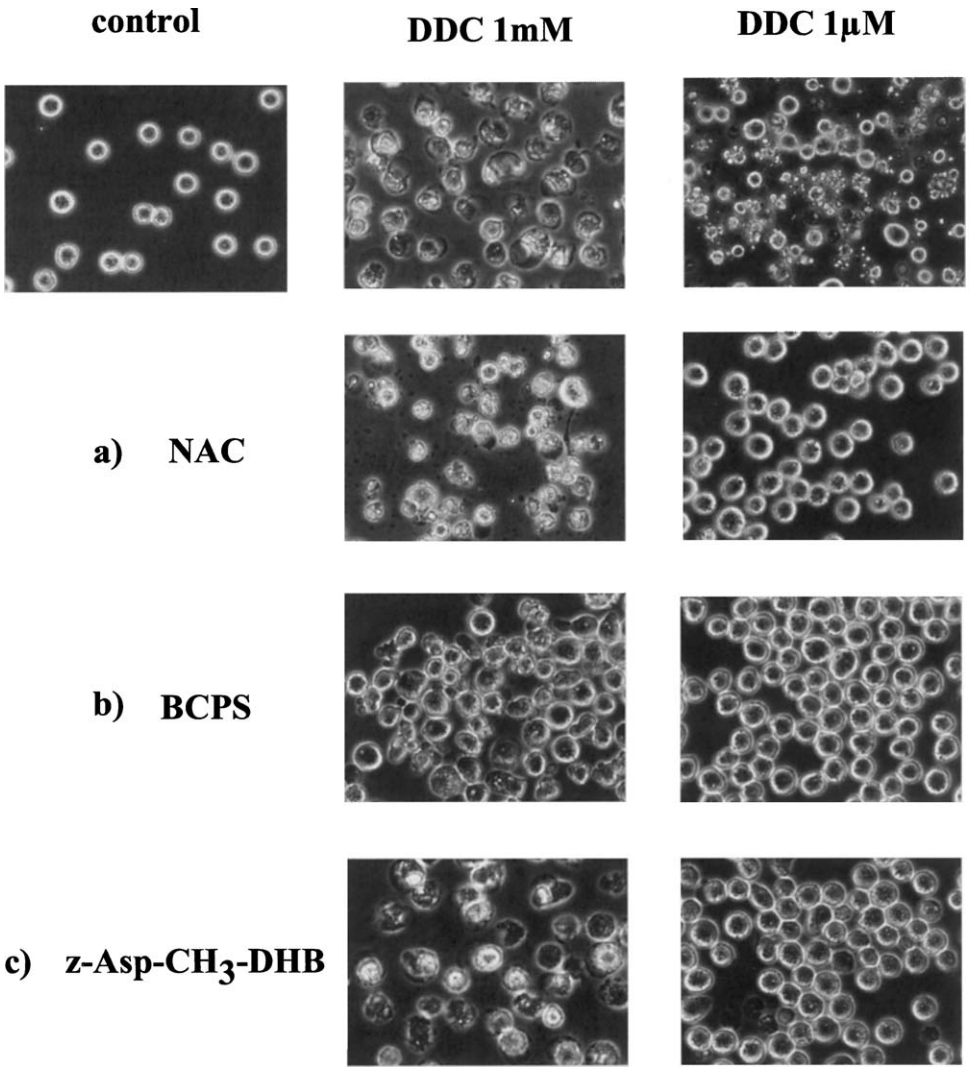


*Fig. 4.* Effect of NAC (5 mM), BCPS (50 $\mu$M), and a broad-spectrum caspase inhibitor (z-Asp-$CH_3$-DAB(50 $\mu$M)) on DDC-induced necrotic and apoptotic cell death. HL60 cells were incubated with 1 mM and 1 $\mu$M DDC for 24 h at 37 °C. Before treatment with DDC, HL60 cells were preincubated for 24 h with NAC and for 2 h with BCPS. Z-Asp-$CH_3$-DHB was coadministrated with DDC. The cells are shown by phase contrast microscopy ($\times$100).

by the duration of ERK, also a member of the MAP kinase family, activation [23].

Many reports have suggested that apoptosis is regulated by Bcl-2 family proteins through mitochondrial signaling [5, 22]. However, in the DDC-induced apoptotic as well as necrotic pathway, there was no sequential or quantitative change of either anti-apoptotic Bcl-2 and Bcl-xL proteins or pro-apoptotic Bax protein. This result indicated that DDC-induced cell death in HL60 cells was not regulated by Bcl-2 family proteins. Mitochondrial transmembrane potential ($\Delta\psi m$) was remarkably decreased in the DDC-induced necrosis, which indicated the possibility that the DDC signaling pathway to necrosis works directly on the mitochondria rather than via a change of Bcl-2 family protein contents. On the other hand, there is no alteration of $\Delta\psi m$ in DDC-induced apoptosis. Ragione et al. [24] observed cytochrome *c* release from mitochondria in pyrrolidinedithiocarbamate (PDTC)-induced apoptosis. There have also been reports that cytochrome *c* release does not depend on the decrease of $\Delta\psi m$ [25, 26]. Therefore, we cannot assert in our present study whether mitochondria is the target organ of DDC-induced apoptosis.

Our present study indicated that DDC-induced apoptosis was a caspase-dependent cell death, because it was completely blocked by a broad-spectrum caspase inhibitor (z-Asp-$CH_3$-DAB). However, DDC-induced necrosis was not independent of caspase that is inhibited by z-Asp-$CH_3$-DAB. A recent study has noted that Fas-mediated apoptotic cell death was blocked by a broad caspase inhibitor (z-VAD-fmk); however, the cells still died as a result of necrotic

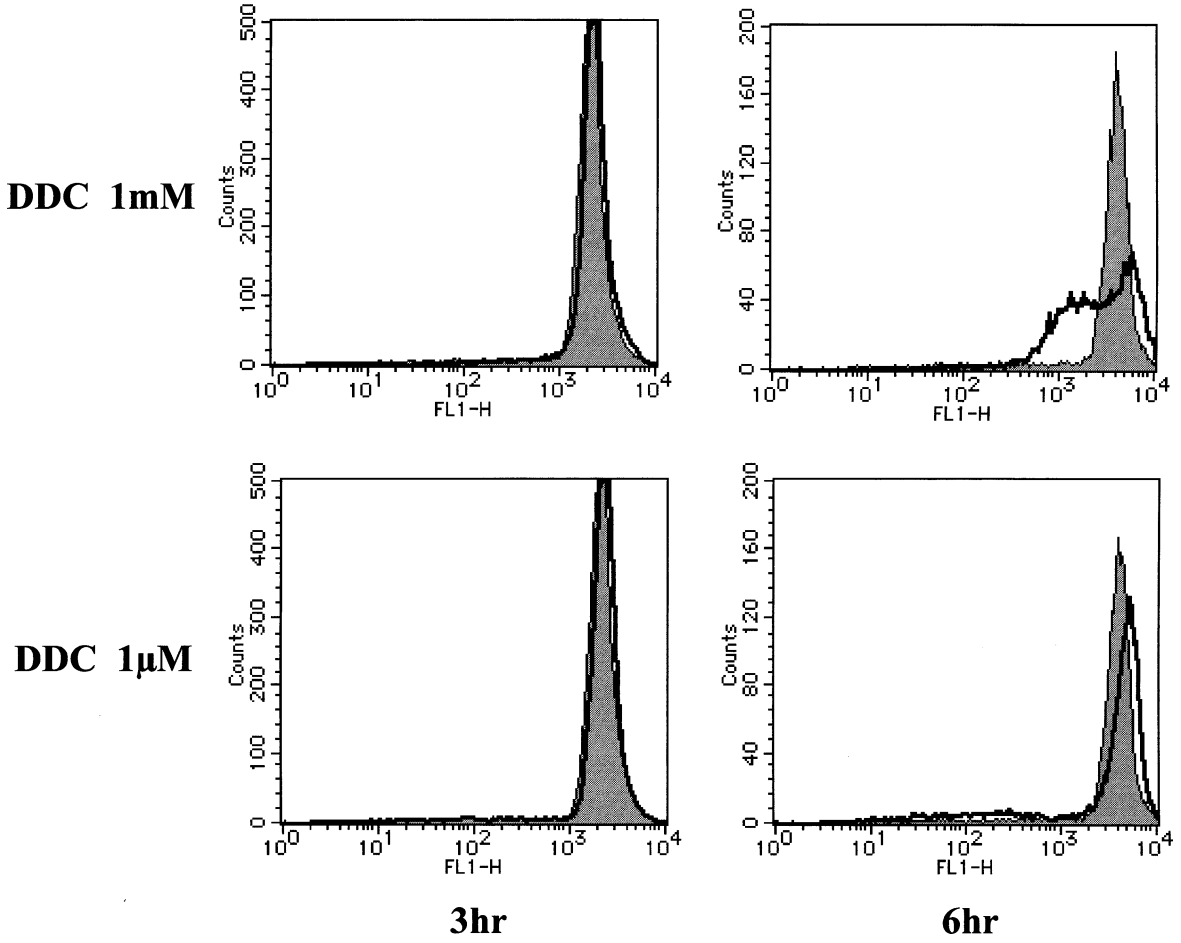


*Fig. 5.* Effect of 1 mM and 1 $\mu$M DDC on the mitochondrial transmembrane potential. Gray areas indicate control cells; open areas with black lines indicate DDC-treated cells.

morphological change [27]. We believe that DDC-induced necrotic cell death is also caspase-independent, but necrosis does not occur because of the inhibition of apoptosis, as mentioned above.

DDC is a metal chelating agent with thiol groups. DDC is known to exert not only antioxidant but also pro-oxidant effects on cells. Therefore, we hypothesized that DDC-induced cell death was involved with ROS. Antioxidant NAC completely blocked DDC-induced apoptotic cell death, which supported our hypothesis. We next examined SOD activity. SOD regulates the amount of superoxide radicals ($O_2^-$), which are the source of various ROS and are located the upstream of ROS formation cascade. DDC is known to inhibit Cu/Zn-SOD activity [21]. Cu/Zn-SOD activity was remarkably decreased at the dose of DDC-induced necrosis, but at the dose of DDC-induced apoptosis Cu/Zn-SOD activity continues at a 60% level. We also determined intracellular ROS by flow cytometry. A decrease of ROS was observed in the case of necrosis, while only a slight increase of ROS was observed in the case of apoptosis. Since ROS was detected as mainly hydrogen peroxide ($H_2O_2$) in our present study, this result indicated the possibility that in DDC-induced necrosis the conversion from $O_2^-$ to $H_2O_2$ was inhibited because of low Cu/Zn-SOD activity and thereby $O_2^-$ accumulated excessively in the cells.

BCPS could completely block DDC-induced apoptosis. We thought this was because BCPS is a non-permeable monovalent copper-specific chelator, and when cells were treated with BCPS before being treated with DDC, BCPS chelated extracellular copper ions and so DDC could not carry copper ions into the cells. In order for this hypothesis to be supported, detailed experiments that include the measurement of copper in the cells are required. And, This result indicated, as Novel et al. [28] suggested, that DDC-induced apoptosis was dependent on a change of the intracellular redox state by transporting external copper ions into the cells, which finally led to activation of the JNK pathway. We consider the possibility that DDC-induced apoptosis involves apoptosis signal-regulating kinase 1 (ASK1) on the way to the downstream signal transduction pathway. ASK1 is present as an

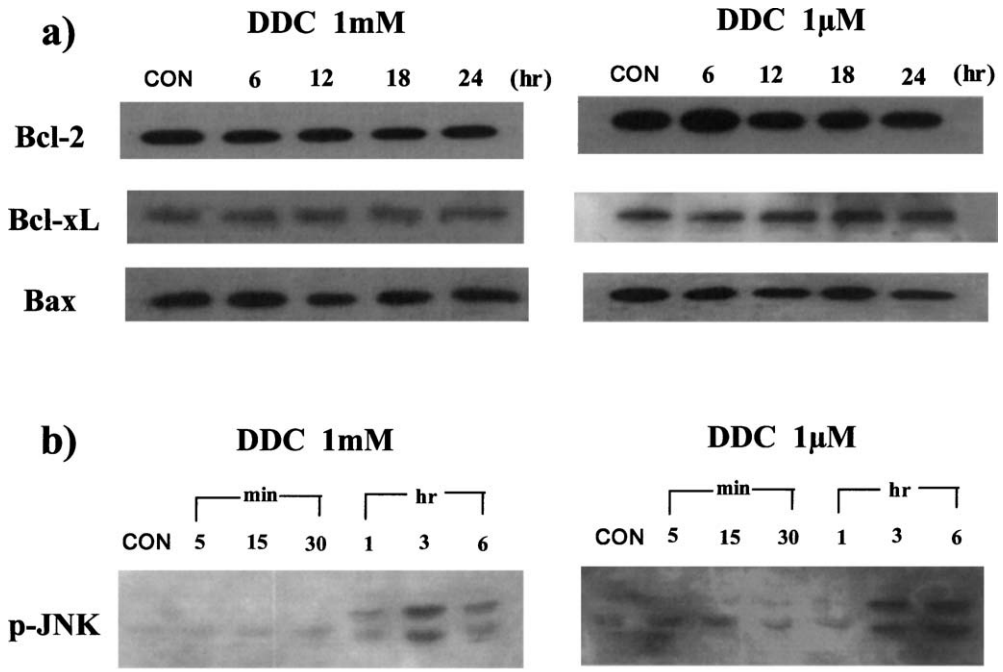


*Fig. 6.* (a) Western blot analysis of protein for Bcl-2 family and (b) phospho-JNK on HL60 cells treated with 1 mM and 1 $\mu$M DDC.

inactive complex with reduced-form thioredoxin. When the cells are exposed to oxidative stress, thioredoxin change to oxidized-form and unbound from ASK1, enabling activation of ASK1. ASK1 has been identified as an upstream activator of the JNK pathway [29–31]. Further study is necessary to investigate this hypothesis.

NAC and BCPS did not block DDC-induced necrosis, while the morphology of the cells changed from necrotic to apoptotic. We believe this is because the effect of high-dose DDC was attenuated by each compound, sequentially, the same pathway as the low-dose DDC would proceed.

Given the results of our study, we believe that high-dose DDC-induced necrotic cell death can be attributed to the increase of intracellular ROS, especially $O_2^-$. We believe that this $O_2^-$ was not directly cytotoxic but rather was involved as a second messenger to downstream signals. On the other hand, it appears that low-dose DDC-induced apoptosis was not dependent on an increase of intracellular ROS and could be attributed to the change of the intracellular redox state by transporting external copper ions into the cells. We also conclude that the duration of JNK activation may cause the different types of cell death.

It was interesting to note that DDC-induced cell death was affected by whether the cell culture condition was hypoxic. The number of DDC-induced necrotic cell deaths decreased in hypoxia because the triggering of necrosis might be dependent on the increase of intracellular ROS. On the other hand, DDC-induced apoptosis occurred even in hypoxia because the trigger might be dependent on the change of the intracellular redox state rather than on the ROS contents. For example, we believe that the regulation of thioredoxin, which oxidized the two active site cysteine residues to disulfide bond formation, enabling activation of ASK1 as described above, could occur if electrons are delivered in the cells, even if there is no increase of ROS.

Fiers et al. [3] reported that several pathways of cell death can be roughly distinguished. It is possible that more than one pathway can be latently present in the same cell and be switched on specifically, depending on the stimulus, and that even if the primary signals are different, the signals can directly or indirectly connect to the same pathway. Clarifying DDC-induced necrotic and apoptotic pathways in detail would help to elucidate the mechanism of anticancer drugs and irradiation-induced necrosis and apoptosis. Moreover, primary signals of these drugs may be similar to the signals of DDC, as one of their functions is ROS production in the cells.

Recently, DDC has been used to inhibit apoptosis, and there are many experimental treatment trials in which DDC is being used against oxidative stress-related diseases such as hypertension [14], cardiac infarction [15], and lung reperfusion injury [16]. However, since we observed in the present study that DDC itself has a cytotoxic effect and that it can have both necrotic and apoptotic actions, we urge caution in its usage. Kanno et al. [32] have reported that DDC induced different types of cytotoxicity in various murine and human

leukemia cell lines. Therefore, the DDC dosage, kinds of cells, and cell culture conditions must be chosen very carefully.

There have been many reports that DDC prevents cell death. Moreover, interestingly, when the HL60 cells were treated with an intermediate dose of DDC between DDC-induced necrosis and apoptosis, the cell morphology appeared normal and the cells remained alive in our present study (data not shown). We believe that further studies are necessary to reveal where the divergence point is between DDC-induced cell death and cell death prevented by DDC and to determine what the key molecule is that makes possible the opposite survival mechanisms of DDC.

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