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PYRROLIDINE DITHIOCARBAMATE, A POTENT INHIBITOR OF NUCLEAR FACTOR κB (NF-κB) ACTIVATION, PREVENTS APOPTOSIS IN HUMAN PROMYELOCYTIC LEUKEMIA HL-60 CELLS AND THYMOCYTES

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Abstract—We examined the effect of pyrrolidine dithiocarbamate (PDTC), which potently blocks the activation of nuclear factor κB (NF-κB), on the induction of apoptosis by a variety of agents. Treatment of a human promyelocytic leukemia cell line, HL-60, with 10 μg/mL etoposide or 2 μM 1-β-D-arabinofuranosylcytosine induced NF-κB activation within 1 hr and subsequently caused apoptosis within 3–4 hr. The simultaneous addition of 50–500 μM PDTC with these agents blocked NF-κB activation and completely abrogated both morphologically apoptotic changes and internucleosomal DNA fragmentation for up to 6 hr. However, PDTC failed to inhibit the endonuclease activity contained in the whole cell lysates. The inhibitory effect of PDTC was also observed in etoposide- and dexamethasone-induced apoptosis in human thymocytes at a concentration of 1–10 μM. Since PDTC has both antioxidant and metal-ion chelating activities, we tested the effects of *N*-acetyl-L-cysteine (NAC) (antioxidant) or *o*-phenanthroline (OP) (metal-ion chelator) on the induction of apoptosis. Pretreatment of HL-60 cells or thymocytes with 100–500 μM OP for 2 hr, but not 10–60 mM NAC, suppressed subsequent occurrence of apoptosis induced by etoposide. These results suggest that the activation of NF-κB plays an important role in the apoptotic process of human hematopoietic cells.

Key words: apoptosis; pyrrolidine dithiocarbamate; NF-κB; transcriptional factor; DNA fragmentation

Apoptosis has been widely documented to occur in a number of cell types in response to various physiological and pathological stimuli [1, 2]. One of the biochemical hallmarks is internucleosomal DNA fragmentation, which is considered to be caused by the activation of endogenous endonuclease [3]. In cells undergoing apoptosis, the activation of numerous genes including c-*jun*, c-*fos*, TRPM-2, transglutaminase, and sulfated glycoprotein has been described [4]. The first two genes code for proteins that form complexes with properties of transcriptional factors [5, 6]. Despite the identification of genes necessary for cell death and their possible ability to regulate apoptosis, the essential biochemical events in apoptotic cell death remain largely unknown.

NF-κB† was originally discovered as a B cell specific transcription factor which bound to an enhancer sequence located in the J–C intron of the κ light chain [7]. NF-κB activation is induced by a wide variety of stimuli in different cell types [8]. Recently investigators have shown that NF-κB activation can be achieved by ara-C in KG-1 cells [9] or by tumor necrosis factor α in HL-60 cells [10], and by glucocorticoids in rat thymocytes [11]. It is noteworthy that these agents were previously reported to cause apoptosis in respective cells [12–14]. However, the exact role of NF-κB activation in the apoptotic process remains unclear. In the present study, we aimed to determine the role of NF-κB activation in apoptosis by using a novel, potent inhibitor of NF-κB, PDTC [15]. We found that the simultaneous addition of micromolar PDTC completely abrogated both morphologically apoptotic changes and DNA fragmentation induced by different classes of apoptosis-inducers in a human promyelocytic leukemia cell line and human thymocytes.

MATERIALS AND METHODS

Chemicals. We purchased RNase, A23187, camptothecin, and ATA from the Sigma Chemical Co. (St Louis, MO, U.S.A.), and PDTC, NAC and OP from Nacalai (Kyoto, Japan). PDTC and OP were dissolved in PBS and 30% ethanol, respectively, and NAC was dissolved in water and adjusted to pH 7.4 by the addition of 1 N NaOH. Etoposide, ara-C, and dexamethasone were kindly donated by

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† Abbreviations: NF-κB, nuclear factor κB; ara-C, 1-β-D-arabinofuranosylcytosine; PDTC, pyrrolidine dithiocarbamate; ATA, aurintricarboxylic acid; NAC, *N*-acetyl-L-cysteine; OP, *o*-phenanthroline.

Nippon Kasei Co. (Tokyo, Japan), Nippon Shinyaku Co. (Kyoto, Japan), and Banyu Pharmaceutical Co. (Tokyo, Japan), respectively.

Cell culture and thymocyte preparation. A human promyelocytic leukemia cell line, HL-60, was obtained from the Japanese Cell Research Bank (Tokyo, Japan). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (regular medium) in a humidified atmosphere in 5% CO_2/95% air at 37°. Thymus glands were obtained from patients of less than 5 years of age undergoing corrective cardiac surgery. Single cell suspensions were prepared by mincing thymus through a stainless steel mesh and washing twice by PBS. The thymocytes were resuspended in regular medium at a density of 5×10^6 cells/mL.

Assessment of apoptotic cells. The logarithmically growing HL-60 cells or freshly prepared thymocytes were incubated with various combinations of drugs described below for the indicated times. The apoptotic cells were defined as those with condensed and fragmented nuclei in May–Giemsa-stained preparations. More than 500 cells were counted.

DNA extraction and electrophoretic analysis. DNA was prepared and analysed by gel electrophoresis with a slight modification of established methods [16]. Briefly, 3×10^6 cells were lysed by incubation at 60° overnight in digestion buffer containing 150 mM NaCl, 25 mM EDTA, 100 μg/mL proteinase K, and 0.2% SDS. The DNA was extracted twice with phenol/chloroform and once with chloroform and precipitated in ethanol with 0.1 M CH_3COONa. The DNA was then dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) and treated with RNase. The procedures for DNA extraction and precipitation were repeated. Approximately 10 μg DNA were electrophoresed on 1.8% agarose gel containing 0.5 μg/mL ethidium bromide at 1 V/cm for 10 hr. The DNA was visualized under UV light and photographed.

Preparation of nuclear extracts. Nuclear extracts were prepared according to the methods described by Li *et al.* [17] with slight modifications. Briefly, 5×10^7 cells were harvested and washed once by PBS and once with 200 μL of ice-cold buffer A (10 mM Hepes–NaOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, pH 7.9). The cells were lysed in 200 μL of buffer A by gently passing the cell suspension through a 27-gauge needle. The nuclei were collected by centrifuging for 8 sec. in an Eppendorf microcentrifuge at 12,000 r.p.m. and washed twice by buffer A. Crude nuclei were extracted with ice-cold buffer B (20 mM HEPES–NaOH, 25% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9) for 30 min on ice. Buffer C (100 μL) (20 mM Hepes–NaOH, 20% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9) was added, and the mixture was centrifuged for 15 min. Supernatants were collected and the protein concentration was determined by using Bio-Rad protein assay kit (Bio-Rad Laboratories).

Electrophoretic mobility shift assay. A 26-bp synthetic oligonucleotide (5′-GATCCAGAGGGG-ACTTTCCGAGAGGC-3′) containing NF-κB consensus sequence (GGGGACTTTCC) [18] was end-labeled with [γ-^{32}P]dGTP and Klenow enzyme. The labeled DNA was purified through a NICK Column (Pharmacia). Binding reactions with equal amounts of nuclear extracts (4 μg/reaction) were performed in 10 μL final volume of 12% glycerol, 12 mM HEPES–NaOH, 4 mM Tris–HCl, 60 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, pH 7.9, containing 10,000 cpm probe, 2 μg poly dIdC, and 3 μg bovine serum albumin. After incubation for 30 min at room temperature, the mixtures were loaded on a 4% polyacrylamide gel in TAE buffer (1 × TAE: 6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.9). After electrophoresis, dried gel was autoradiographed. For competition assay, unlabeled NF-κB oligonucleotide was added at 100-fold excess compared with the end labeled fragment.

Preparation of whole cell lysates. For the preparation of whole cell lysates from HL-60 cells, 10^7 cells were lysed in ice-cold STM buffer (250 mM sucrose, 10 mM Tris–HCl, 5 mM $MgSO_4$, pH 7.5) containing 1% Nonidet P-40 for 10 min [12]. Then the lysates were incubated with or without various concentrations of PDTC, 1 mM $ZnSO_4$, or 300 μM ATA for 3 hr. The DNA was then extracted and analysed as described above.

RESULTS

Inhibition of apoptosis induced by different stimuli in HL-60 cells by PDTC

We first examined the morphology of HL-60 cells treated with etoposide in the presence or absence of PDTC. As shown in Fig. 1A, cells showing apoptotic features became evident after 3 hr of incubation with 10 μg/mL etoposide. However, the addition of 100 μM PDTC from the start of the culture completely abrogated apoptotic changes for up to 6 hr. The effect of PDTC was dose dependent, demonstrating a complete rescue from apoptosis at 50 μM (Fig. 1B). A kinetic study for the time of PDTC addition revealed that this agent was fully effective in preventing apoptosis, even if added 30 min after the start of incubation with 10 μg/mL etoposide (Fig. 1C). These morphological changes were in accordance with the appearance of DNA ladder patterns on electrophoretic analysis (Fig. 2). Etoposide on its own could induce the visible DNA ladder pattern after 3 hr of culture. Coincubation with 50 μM or 100 μM PDTC, but not 10 μM PDTC, completely blocked DNA fragmentation. Similar results were obtained when HL-60 cells were incubated with 2 μM ara-C for 4 hr with or without various concentrations of PDTC (Fig. 3A and B). PDTC also protected these cells from apoptosis induced by camptothecin (1 μM), UV irradiation (20 J/m^2), or A23187 (1 μM) (data not shown).

NF-κB activation by etoposide or ara-C, and its inhibition by simultaneous treatment with PDTC in HL-60 cells

To examine the effect of PDTC on NF-κB activation by etoposide or ara-C in HL-60 cells, we employed the electrophoretic mobility shift assay.

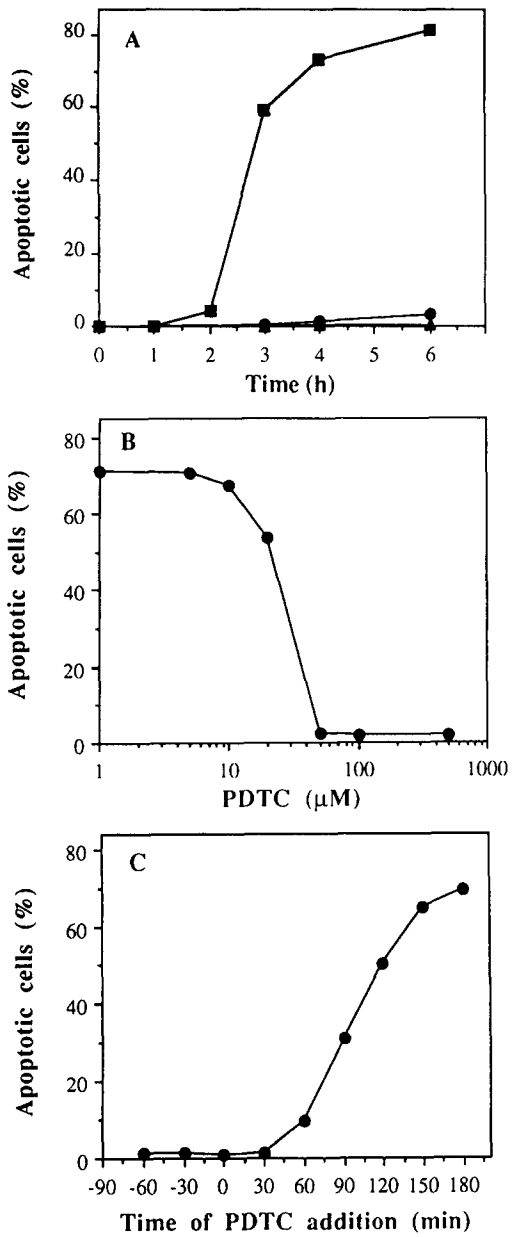

Fig. 1. Effects of PDTC on the induction of apoptosis treated with 10 μg/mL etoposide in HL-60 cells. Data represent the means of three experiments. (A) Time course of the appearance of morphologically apoptotic cells after treatment with etoposide (■), 100 μM PDTC (▲), or both (●). (B) Dose-dependent inhibition of PDTC evaluated after 4 hr of treatment. (C) Kinetics of the time of addition of 100 μM PDTC during 4 hr of incubation with etoposide. Etoposide was added to the culture at time 0.

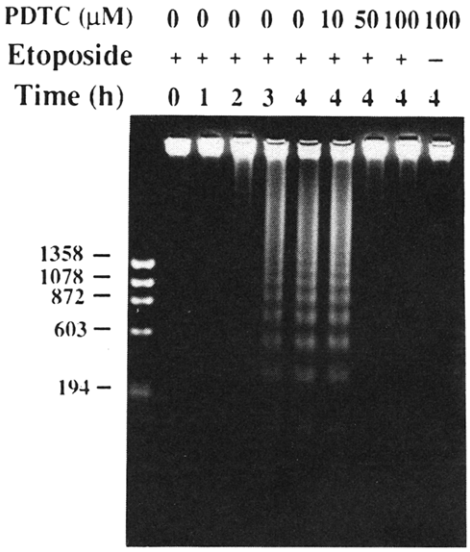

Fig. 2. Electrophoretic DNA analysis. HL-60 cells were treated with 10 μg/mL etoposide in the presence or absence of various concentrations of PDTC for the times indicated. The ordinate shows the sizes of φX174 *Hae* III-digested fragments in base pairs.

Figure 4 demonstrated that treatment with 10 μg/mL etoposide or 2 μM ara-C stimulated NF-κB mobilization within 1 hr, which was completely blocked by the addition of cold competitors. Simultaneous treatment of these cells with 100–300 μM PDTC clearly blocked NF-κB activation (Fig. 4).

Inhibition of dexamethasone- and etoposide-induced apoptosis by PDTC in human thymocytes

Next we examined whether PDTC can also protect apoptosis in human thymocytes. We used unfractionated thymocytes since glucocorticoids caused apoptotic cell death in both human cortical and medullary thymocytes with similar kinetics [19]. The thymocytes treated with the synthetic glucocorticoid, dexamethasone (1 μM) or etoposide (40 μg/mL) underwent apoptosis after 4 hr of incubation. The simultaneous addition of 1–10 μM PDTC substantially inhibited both morphological changes and DNA fragmentation induced by each treatment for up to 8 hr of culture (Fig. 5).

Inhibition of apoptosis in HL-60 cells and thymocytes by OP, but not NAC

PDTC has at least two chemical properties: one is antioxidant activity and the other is metal-ion chelating activity [15, 20]. Thus we tested the effects of an antioxidant, NAC, and a metal-ion chelator, OP, on the induction of apoptosis in HL-60 cells. As shown in Table 1, pretreatment with 100–500 μM OP for 2 hr before etoposide addition significantly inhibited the subsequent appearance of etoposide-induced apoptotic cells. On the other hand, 10–60 mM NAC treatment demonstrated no inhibitory effect on the induction of apoptosis. A similar inhibitory effect by OP (but not NAC) was observed in etoposide- or dexamethasone-induced apoptosis in human thymocytes (Table 2).

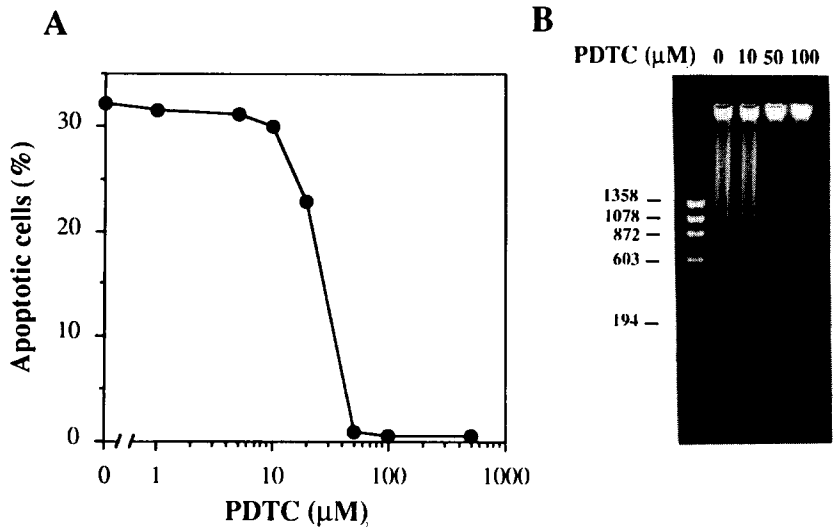

Fig. 3. Effects of PDTC on ara-C-induced apoptosis. HL-60 cells were treated with 2 μM ara-C for 4 hr in the presence or absence of various concentrations of PDTC. (A) Morphologically apoptotic cells. (B) DNA fragmentation. Data in panel A represent the means of three experiments.

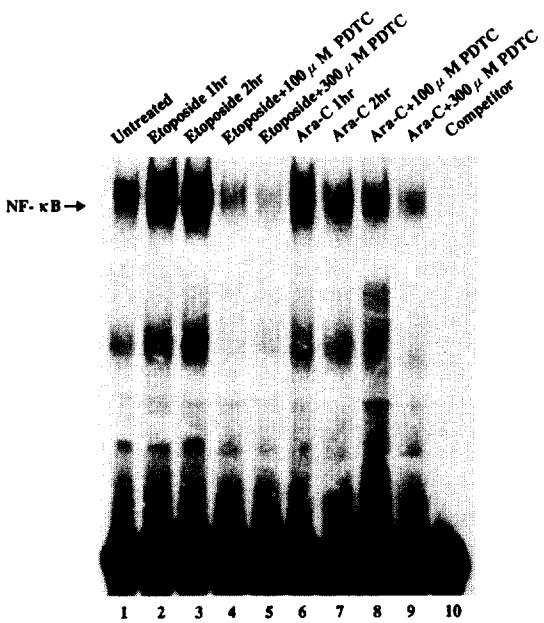

Fig. 4. Induction and specificity of NF-κB binding by etoposide or ara-C, and its blocking by PDTC in HL-60 cells. Lane 1: untreatment; lanes 2 and 3: treatment with 10 μg/mL etoposide for 1 and 2 hr, respectively; lanes 4 and 5: co-treatment with etoposide and 100 or 300 μM PDTC, respectively, for 1 hr; lanes 6 and 7: treatment with 2 μM ara-C for 1 and 2 hr, respectively; lanes 8 and 9: co-treatment with ara-C and 100 or 300 μM PDTC, respectively, for 1 hr; lane 10: treatment with 10 μg/mL etoposide for 2 hr, followed by addition of cold competitor to the nuclear extract.

Evaluation of PDTC action as an endonuclease inhibitor

We recently demonstrated that endonuclease activity was found in whole cell lysates of various myelogenous leukemia cell lines, and that it was inhibited by the addition of putative endonuclease inhibitors, such as 1 mM $ZnSO_4$ or 300 μM ATA [21]. These observations led us to examine the possibility that PDTC works as an inhibitor of endonuclease in crude cell lysates from HL-60 cells. However, the addition of 10 μM-1 mM PDTC to whole cell lysates did not prevent the appearance of DNA ladder patterns (Fig. 6).

DISCUSSION

NF-κB, an inducible transcription factor, plays a pivotal role in the nuclear/cytoplasmic signalling pathway [8]. Recent studies have shown that *cis*-acting elements binding NF-κB are functional in many inducible genes encoding cytokines, cell surface receptors, acute phase response proteins and viruses [8, 15]. Rapid induction of NF-κB nuclear activity is regulated at a post-transcriptional level, involving the release of an inhibitory factor, IκB, from a preformed cytoplasmic protein complex [8]. NF-κB activation has also been observed in KG-1 cells within 30 min when treated with 0.1 μM to 1 mM ara-C [9], in HL-60 cells treated with 5 pM tumor necrosis factor α [10]. These agents are capable of causing apoptosis in respective cells [12, 13]. Furthermore, Sikora *et al.* [11] demonstrated that NF-κB was activated in rat thymocytes undergoing apoptosis after dexamethasone or heat treatment. Oxidative stress is also proposed as a mediator of apoptosis probably through the activation of NF-κB [22]. Most recently, high levels of c-*rel*

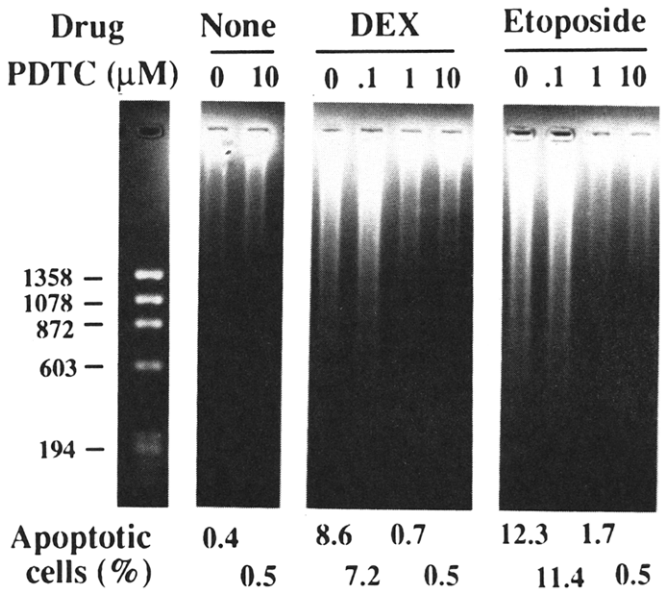

Fig. 5. Effects of PDTC on apoptosis in human thymocytes. Thymocytes were treated with or without 1 μM dexamethasone (DEX) or 40 μg/mL etoposide for 8 hr in the presence or absence of various concentrations of PDTC. Data shown are one of three experiments with identical results.

Table 1. Effects of OP and NAC on the etoposide (Etp)-induced apoptosis in HL-60 cells

	NAC (mM)	0	10	30	60
Apoptotic cells (%)	NAC + Etp	61.9 ± 5.5	59.5 ± 4.5	56.9 ± 5.9	59.3 ± 6.1
	NAC alone	0.1 ± 0.1	0.2 ± 0.1	8.2 ± 2.2	21.2 ± 4.9
	OP (μM)	0	100	250	500
Apoptotic cells (%)	OP + Etp	61.9 ± 5.5*†	41.1 ± 2.0*	33.0 ± 3.5*	4.8 ± 1.2†
	OP alone	0.1 ± 0.1	0.9 ± 0.1	1.8 ± 0.8	2.5 ± 0.9

Data represent means ± SD of three experiments. NAC or OP was added at −2 hr relative to 10 μg/mL etoposide addition (time 0), and the apoptotic cells were enumerated after 4 hr of incubation. The differences in apoptotic counts between the OP plus etoposide co-treated cells and the etoposide treated cells were significant; *$P < 0.05$, †$P < 0.01$ (Student's *t*-test).

Table 2. Effects of OP and NAC on the etoposide-(Etp) or dexamethasone-(DEX) induced apoptosis in human thymocytes

	NAC (mM)	0	10	30	60
Apoptotic cells (%)	NAC + Etp	20.6 ± 2.6	20.1 ± 1.7	20.3 ± 2.5	19.3 ± 2.9
	NAC + DEX	22.5 ± 3.4	19.8 ± 2.2	21.3 ± 2.7	24.8 ± 4.2
	NAC alone	0.8 ± 0.4	1.3 ± 0.9	3.0 ± 1.4	2.8 ± 1.2
	OP (μM)	0	10	100	500
Apoptotic cells (%)	OP + Etp	20.6 ± 2.6*†	10.3 ± 3.9*	5.4 ± 1.0†	3.9 ± 1.1†
	OP + DEX	22.5 ± 3.4†	4.6 ± 1.4†	3.7 ± 1.8†	3.3 ± 1.2†
	OP alone	0.8 ± 0.4	1.3 ± 0.2	2.5 ± 0.9	1.3 ± 0.9

Data represent means ± SD of three experiments. NAC or OP was added at −2 hr relative to 40 μg/mL etoposide or 1 μM dexamethasone addition (time 0), and the apoptotic cells were enumerated after 8 hr of incubation. The differences in apoptotic counts between the OP plus etoposide or dexamethasone co-treated cells and the etoposide or dexamethasone treated cells were significant; *$P < 0.05$, †$P < 0.01$ (Student's *t*-test).

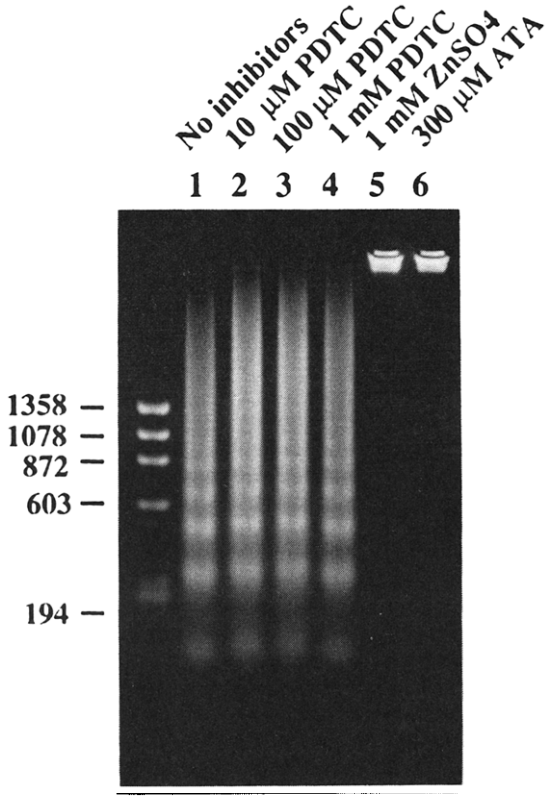

Fig. 6. DNA gel analysis following incubation of whole cell lysates from HL-60 cells for 3 hr without inhibitors (lane 1), or in the presence of 10 μM PDTC (lane 2), 100 μM PDTC (lane 3), 1 mM PDTC (lane 4), 1 mM $ZnSO_4$ (lane 5), and 300 μM ATA (lane 6).

expression were reported to be associated with apoptosis in the developing avian embryo and in bone marrow cells *in vitro* [23]. Like NF-κB, c-Rel binds consensus κB oligonucleotides and has been postulated to regulate transcription of a number of different genes [8]. Although these reports suggest a functional link between apoptosis and NF-κB, the precise mechanism remains to be clarified, especially in human cells. In the present study, we used PDTC, a known potent inhibitor of NF-κB activation, in micromolar concentrations [15] to test whether NF-κB activation participates in apoptosis in human hematopoietic cells. We clearly demonstrated that PDTC inhibits apoptosis in HL-60 cells induced by different stimuli, including etoposide [24], ara-C [25], camptothecin [12], UV irradiation [26], and calcium ionophore [21], and apoptosis in thymocytes triggered by etoposide [27] and dexamethasone [14, 19]. Concomitantly, PDTC blocked NF-κB mobilization to nucleus induced by etoposide or ara-C. This may suggest the association of NF-κB activation with induction of apoptosis in human hematopoietic cells. We observed that the inhibition of apoptosis required considerably lower PDTC concentrations in thymocytes than in HL-60 cells (1 μM versus 100 μM). This disparity is probably due to the difference in the requirement to inhibit NF-κB activation as seen between primary T cells (1 μM) and tumoral cell line, Jurkatt cells (100 μM) [15, 28].

The molecular basis for the action of PDTC may involve interference with reactive oxygen metabolism, chelation of divalent heavy metal ions, or both of these mechanisms [15, 20]. We also tested the effect of NAC, since this compound has antioxidant activity comparable to that of PDTC [15, 29]. No inhibitory effects of 10–60 mM NAC on the induction of apoptosis of HL-60 cells were observed. However, NAC can prevent apoptosis in HIV-chronically infected U937 cells [30] and apoptosis induced by growth factor deprivation in an IL-3-dependent cell line at these concentrations [31]. As to chelation of metal ions, we investigated the effect of OP, another metal ion chelator, on the induction of apoptosis. Treatment of HL-60 cells with 500 μM OP at −2 hr relative to 10 μg/mL etoposide addition almost completely suppressed apoptosis after 4 hr of co-treatment. Essentially identical results were obtained in the study using human thymocytes. On the other hand, pretreatment of these cells for 2 hr with 50–500 μM desferal, a strong chelator of Fe ion, failed to suppress apoptosis induced by etoposide (data not shown). Therefore, it may be speculated that PDTC inhibits apoptosis by inhibiting NF-κB activation through metal ion, probably other than Fe ion, mediated mechanisms.

Recently, the association of another inducible transcription factor, c-*jun* and c-*fos*, with the apoptotic process was identified with the use of antisense oligonucleotides [32]. Furthermore, Abbadie *et al.* [23] showed that c-*rel* levels were overexpressed in the avian cells undergoing apoptosis using monoclonal antibodies and transfected cells. Thus, for further elucidation of the role of NF-κB activation in apoptosis in human cells, the more precise molecular approach is necessary. At any rate, our findings that PDTC inhibits apoptosis in HL-60 cells and thymocytes induced by a wide variety of agents suggest that NF-κB-dependent regulation plays an important role in a common pathway of the apoptotic process.

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