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INHIBITION OF APOPTOSIS BY ANTIOXIDANTS IN THE HUMAN HL-60 LEUKEMIA CELL LINE

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Abstract—Cell death via apoptosis is an important event involved in a number of immunological processes. Recently, apoptosis has been associated with oxidative stress in a number of cell systems. Here we assessed the inhibitory capacity of different antioxidants on UV- and drug-induced apoptosis in the human leukemic cell line, HL-60. We found that the oxygen radical scavenger, BHA, the radioprotector cysteamine and the metal chelators, pyrrolidinedithiocarbamate (PDTC), diethyldithiocarbamate (DEDTC), and dimethyldithiocarbamate (DMDTC), were able to significantly inhibit nuclear fragmentation and reduce the formation of apoptotic bodies in UV-irradiated human leukemic cells. Both BHA and PDTC were found to reduce DNA fragmentation as assessed by *in situ* DNA nick-end labelling and quantification thereof using fluorescence flow cytometry. In addition to inhibiting UV-induced apoptosis, PDTC was also capable of reducing the amount of apoptosis induced by a range of cytotoxic drugs, such as actinomycin-D, camptothecin, etoposide, and melphalan, whereas BHA and cysteamine were not as effective in these cases after more than four hours in culture when compared to PDTC. To further elucidate the working mechanism of PDTC, we have looked at the effect of PDTC on DNA fragmentation in isolated nuclei, under conditions that promote activation of endogenous endonuclease involved in apoptosis. In contrast to $ZnCl_2$, a potent inhibitor of endonuclease activity, PDTC was unable to inhibit DNA-ladder formation in this assay. Taken together, these results indicate that oxygen radicals may have a central role to play in the induction of apoptosis and that dithiocarbamates can serve as potent inhibitors of apoptosis induced by a wide variety of stimuli.

Key words: apoptosis; antioxidants; dithiocarbamate

Apoptosis is a form of cell death normally activated under physiological conditions [1], such as involution in tissue remodelling during morphogenesis [2], and a number of immunological processes. The latter include the deletion of autoreactive cells in thymic development [3, 4], T-cell mediated cytotoxicity [5], and activation-induced cell death in T-cells [6]. Apart from its role in normal cell regulation, apoptosis can be seen under a wide variety of pathological conditions, and is thought to be involved in a number of auto-immune diseases such as systemic Lupus Erythematosus [7]. Also, recent observations indicate that HIV-infected T-cells die by apoptosis [8–10].

The apoptotic process is characterized by morphological and biochemical hallmarks, including cell shrinkage, chromatin condensation, and internucleosomal degradation of the cell's DNA [11]. A number of oncogenes have been shown to be involved in the regulation of apoptosis: c-myc was shown to enhance activation-induced cell death in T-cell hybridomas [6], and bcr-abl suppresses spontaneous and drug-induced apoptosis in the chronic myelogenous cell line K562 [12]. Moreover, it has been proposed that the bcl-2 protooncogene, which inhibits most types of apoptotic cell death, regulates an antioxidant pathway [13] and, indeed, recent evidence suggests that oxidative stress may play a central role in the regulation of apoptosis [14–16].

The human promyelocytic leukemia line, HL-60, has been widely used as a model to study apoptosis-related aspects in the myelomonocytic lineage, and inducers of apoptosis include differentiation-inducing agents [17], RNA-synthesis inhibitors [17], and topoisomerase inhibitors [18]. Furthermore, the cell line proved very sensitive to chemical and physical treatments known to induce oxidative stress. Thus, UV irradiation [19] and exposure to H_2O_2 [20] were found to be potent inducers of apoptosis in HL-60 cells.

It is evident that inhibitors of apoptosis would have widespread clinical implications. So far, it has proven difficult to find putative inhibitors of apoptosis effective on a wide range of apoptosis-induced agents, and in this respect attention has focused almost entirely on zinc ions. Indeed, it has been shown that addition of zinc ions can inhibit cells from undergoing apoptosis induced by diverse agents such as UV [21], or topoisomerase inhibitors such as camptothecin and etoposide [22]. Furthermore, zinc ions have also been shown to have radioprotection capacity [23].

One prime obstacle in the search for a "wide-spectrum" inhibitor of apoptosis is our poor understanding of the apoptosis-induction pathway, especially of elements common for the various inducing agents. The finding that oxidative stress could be a common step in the apoptotic pathway [16], together with the fact that bcl-2 might act as a natural anti-oxidant, thus preventing apoptosis [13], is of particular interest, as it implies ex-

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Abbreviations: BHA, butylated hydroxyanisole; DTCs, dithiocarbamates; DEDTC, diethyldithiocarbamate; DMDTC, dimethyldithiocarbamate; FSC, forward scatter; GSH-PX, glutathione-peroxidase; PDTC, pyrrolidinedithiocarbamate; SOD, superoxide anion dismutase; SSC, side scatter; TdT, terminal deoxynucleotidyl transferase; TNF, tumor necrosis factor; TUNEL, terminal transferase-mediated biotinylated-UTP nick end labelling.

ogenous anti-oxidants might be putative inhibitors of apoptosis induced by a wide variety of agents. Hence, in this article we have investigated the possible effects of different types of anti-oxidants on apoptosis induced by a range of agents in human myelomonocytic leukemia cell lines.

The anti-oxidants used included the oxygen radical scavenger, butylated hydroxyanisole (BHA); the radioprotector, cysteamine; and the metal chelator, pyrrolidinedithiocarbamate (PDTC). Cysteamine has been shown to protect cells against radiation-induced cell death [23], and is a potent radical scavenger. BHA was previously reported to inhibit the cytotoxic activity of Tumour Necrosis Factor (TNF), supposed to be mediated by mitochondrial radical generation [24]. PDTC is of importance, as it is one of several dithiocarbamates (DTCs) [25] found to have biochemical effects both *in vitro* and *in vivo*. Recently, PDTC has been found to inhibit NF-κB-related gene-expression [26, 27]. Furthermore, DTCs have been reported to inhibit progression of AIDS [28, 29], and to have therapeutic properties in Wilson's disease, systemic lupus erythematosus, and some T-cell deficiencies. Interestingly, DTCs have been shown to protect against radiation damage to bone marrow [30] and to prevent necrosis of tubular epithelial cells in mice treated with cisplatin [31]. The effects of antioxidants on cell morphology and DNA fragmentation in apoptotic cells was monitored both microscopically and by flow cytometry analysis. We also looked at the possible inhibition by PDTC of the endonuclease(s) involved in apoptosis.

MATERIALS AND METHODS

Cell culture conditions

The human promyelocytic HL-60 [32] leukemic cell line was used in this study. These cell lines undergo apoptosis following exposure to a variety of agents including UV irradiation [19]. Cells were cultured in RPMI 1640 medium (Gibco, U.K.), supplemented with 10% FCS (Biochrom KG, Germany) and 1% penicillin/streptomycin (Gibco, U.K.). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cell viability and morphology

Cell number was determined using a Neubauer haemocytometer, and viability was assessed by their ability to exclude trypan blue. Cell morphology was assessed by staining cytocentrifuge preparation with Rapi-Diff II (Paramount reagents Ltd., U.K.). Apoptotic cells were identified as previously described [20]. Forward (FSC) and side-scatter (SSC) properties were assessed on cells fixed for 30 min in ice-cold ethanol using a Facscan flow cytometer (Becton-Dickinson, U.S.A.) equipped with Lysis II software as previously described [33].

UV-induced apoptosis

Apoptosis was induced in HL-60 cells by exposure to UV irradiation as previously described [19]. Briefly, cells (10^6/ml) were seeded in polystyrene 24-well plates (Nunc, Roskilde Denmark), and exposed from below to a 302-nm UV transilluminator source at a distance of 2.5 cm for 15 min at room temperature. Cells were then returned to 37°C and assessed for apoptosis at appropriate time periods.

Drug-induced apoptosis

Cells were pelleted at 200 g and re-suspended at 5×10^5 cells/mL in culture medium. Actinomycin-D (10 μg/ml), camptothecin (10 μg/ml), etoposide (50 μg/mL), and melphalan (50 μg/mL) were added. These agents were supplied by Sigma Chemical Co. (St. Louis), and stock solutions made in dimethylsulfoxide. Stock solutions were diluted by at least 1/1000 to ensure solvent levels of less than 0.1%.

Anti-oxidants and inhibition of apoptosis

Butylated hydroxyanisole (BHA) (Sigma) was dissolved in ethanol before use. BHA-treated cells were pretreated for 2 hours with the antioxidant prior to irradiation. Cysteamine and the dithiocarbamate stocks were prepared in distilled H_2O: pyrrolidinedithiocarbamate (Sigma) (1 M), diethyldithiocarbamate (Aldrich) (1 M), and dimethyldithiocarbamate (Aldrich) (0.1 M). Stocks were prepared immediately before use. DTCs were added immediately after irradiation, and cysteamine immediately before irradiation. In some cases $ZnCl_2$ was used as an apoptosis inhibitor. $ZnCl_2$ (Sigma) was prepared as a 10 mM stock solution in 0.15 mM saline, and sterile filtered through a 0.2 μm acrodisc filter before use. $ZnCl_2$ was added to the culture media at 1 mM prior to irradiation.

Isolation of nuclei

Nuclei were isolated as described previously [34]. Briefly, cells (2×10^6/ml) were pelleted (200 g for 5 min), and nuclei prepared by re-suspension in 1.5 mM $MgCl_2$; nuclei were re-suspended in nuclei incubation buffer (10 mM Tris pH 7.2, 200 mM sucrose). Nuclei were also incubated in the presence of 1 mM $ZnCl_2$ or 40 μM PDTC.

DNA isolation and electrophoresis

DNA was isolated by centrifuging cells at 200 g for 5 min at room temperature. Cell pellets were re-suspended at 2×10^6/ml in lysis buffer (20 μl) containing 20 mM EDTA, 100 mM Tris pH 8.0, 0.8% (w/v) sodium lauryl sarcosinate, and 10 μl of 1 mg/ml RNase A (prepared in 0.1 M Sodium acetate, 0.3 mM EDTA pH 4.8), and these incubated at 37°C for 18 hr. A 10-μl aliquot of 20 mg/ml proteinase K was then added. Extracted DNA was then incubated for a further 1.5–2 hr at 50°C.

DNA electrophoresis was carried out in 1.5% agarose gels. Before gel casting, 3 μl of a 10 mg/ml ethidium bromide solution was added to 100 ml of the 1.5% agarose solution. Prior to electrophoresis, loading buffer (10 mM EDTA, 0.25% (w/v) bromophenol blue and 50% (w/v) Glycerol) was added to each sample. Electrophoresis was carried out for 4 hr at 55 V in (2 mM EDTA pH 8.0, 89 mM Tris, 89 mM boric acid) TBE buffer. Gels were cast in apparatus supplied by CBS Scientific Co. California, U.S.A.

DNA nick-end labelling (TUNEL)

For *in situ* terminal deoxynucleotidyl transferase-mediated labelling of DNA nick-ends, we used an improved version of the assay previously described by Gorczyca *et al.* [35]. Cells (5×10^5/ml) were centrifuged (200 g, 5 min) and re-suspended in 1% paraformaldehyde in PBS (pH 7.4) and left for 15 min on ice. Cells were then washed with PBS before re-suspension in ice cold 70%

ethanol. Fixed cells were stored overnight at –20°C. After rehydration in PBS, cells were re-suspended in 50 µl reaction mixture containing 0.1 mM dithiothreitol, 0.05 mg/ml BSA, 2.5 mM $CoCl_2$, 0.4 mM Bio-16-dUTP, and 0.1 U/µl TdT-enzyme in 0.1 M Na cacodylate (pH 7.0) buffer. This mixture was incubated at 37°C for 30 min. Cells were then washed in PBS and re-suspended in 100 µl staining buffer containing 2.5 µg/ml fluorescinated avidin, 4X concentrated saline-sodium citrate buffer, 0.1% Triton X-100, and 5% (w/v) nonfat dry milk. Cells were incubated for 30 min at room temperature in the dark. Stained cells were rinsed in PBS before analysis for fluorescence using a Becton Dickinson FACScan. Bio-16-dUTP and the TdT-enzyme were obtained from Boehringer Mannheim. All other chemicals were obtained from Sigma.

Measurement of intracellular peroxides

Peroxide levels were assessed using the method of Hockenberry *et al.* [13]; briefly, cells (5×10^5/ml) were loaded with 5 µM DCFH/DA (molecular probes) (dissolved at 2000X in DMSO) for 1 hr at 37°C prior to cytotoxic insult. The apoptosis-inducing agents used were UV irradiation, H_2O_2, actinomycin-D, camptothecin, etoposide, and melphalan. Peroxide levels were measured using a Becton Dickenson FACScan flow cytometer.

RESULTS

BHA and cysteamine prevent apoptosis in UV-irradiated or H_2O_2-treated HL-60 cells, but fail to prevent drug-induced apoptosis

Previous reports have shown that an antioxidant pathway might play a central role in the protection against apoptosis [13, 16]. To further investigate the possible inhibitory role of antioxidants in apoptosis, we looked at the effect of the radical scavenger BHA and the radioprotector cysteamine on apoptosis in HL-60 cells induced by different stimuli. BHA has previously been shown to be a potent inhibitor of TNF-induced cytotoxicity [24]. UV irradiation is a strong inducer of apoptosis in normal, untreated HL-60 cells. Nuclear fragmentation appears after 2 hr, and complete cellular break-up into apoptotic bodies occurs within a 4-hr time span for the majority of the population. However, when HL-60 cells were treated with BHA 2 hr prior to UV irradiation, cells were found to be protected from nuclear and cellular fragmentation as assessed morphologically. Indeed, in the presence of BHA, UV-induced nuclear fragmentation only occurred in about 10% of the population, as compared to 60% for UV-irradiated HL-60 cells in the absence of BHA. This effect was visible for up to 6 hr after irradiation. A similar reduction of apoptosis by BHA was seen in cells subjected to 200 µM H_2O_2. In a similar fashion, both cysteamine and PDTC protected against UV- and H_2O_2-induced cell death, and again protection lasted 6–8 hr (Fig. 1a). However, BHA and cysteamine had only a marginal effect on drug-induced apoptosis (after >4 hr in culture), whereas PDTC significantly inhibited the process (Fig. 1b).

Dose response inhibition of apoptosis by PDTC

It is clear from the dose-response curve that concentrations as low as 10 µM PDTC were able to protect UV-irradiated HL-60 cells from apoptosis (Fig. 2a). In-

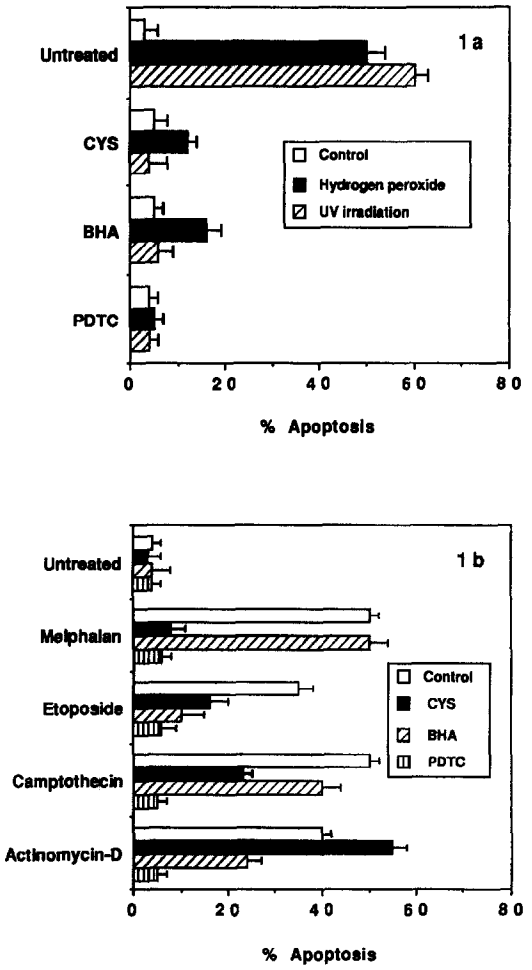

Fig. 1. The effect of BHA (200 µM), cysteamine (1 mM), and PDTC (40 µM) on apoptosis induced by (a) UV irradiation and H_2O_2 and (b) by several diverse acting drugs. Apoptosis was assessed morphologically on cytospin preparations. Results are means ± *SE* values from three independent experiments.

cubation with similar doses of DEDTC or DMDTC had the same effect. Similar results were obtained with UV-irradiated human promonocytic U937 cells incubated with PDTC (data not shown), indicating that the observed effect of PDTC is not restricted to the promyelocytic HL-60 line. We have found that using concentrations of 40 µM PDTC and greater has a cytostatic effect on HL-60 cells, and by 24 hr these cells are apoptotic. Higher concentrations of both BHA (200 µM) and Cysteamine (1 mM) compared to PDTC were required for an inhibitory effect to be observed (Figs. 2b,c). Concentrations above those indicated for these inhibitors proved toxic very quickly (<2 hr).

Dithiocarbamates inhibit apoptosis induced by a range of diverse acting compounds

Whereas cytospin preparations of UV-irradiated HL-60 cells showed nuclear and cellular fragmentation typical for apoptosis (Fig. 3), irradiated HL-60 cells incubated with 40 µM PDTC showed reduced nuclear fragmentation, and cells stayed intact for more than 3 hr after irradiation. Similarly, PDTC prevented drug-induced cell death for up to 8 hr (data not shown). To further

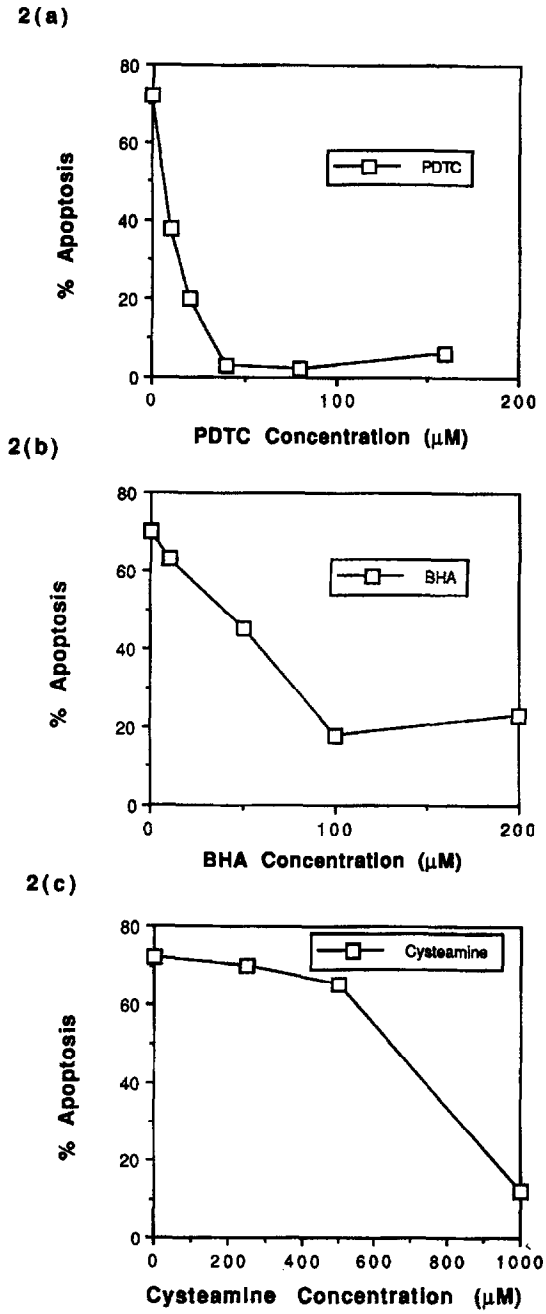

Fig. 2. Dose response inhibition of apoptosis by (a) PDTC, (b) BHA, and (c) cysteamine. The most effective concentration for PDTC was between 40–160 μM, for BHA 200 μM, and for cysteamine 1 mM. Concentrations above those indicated for each agent proved toxic over a very short time period (< 2 hr).

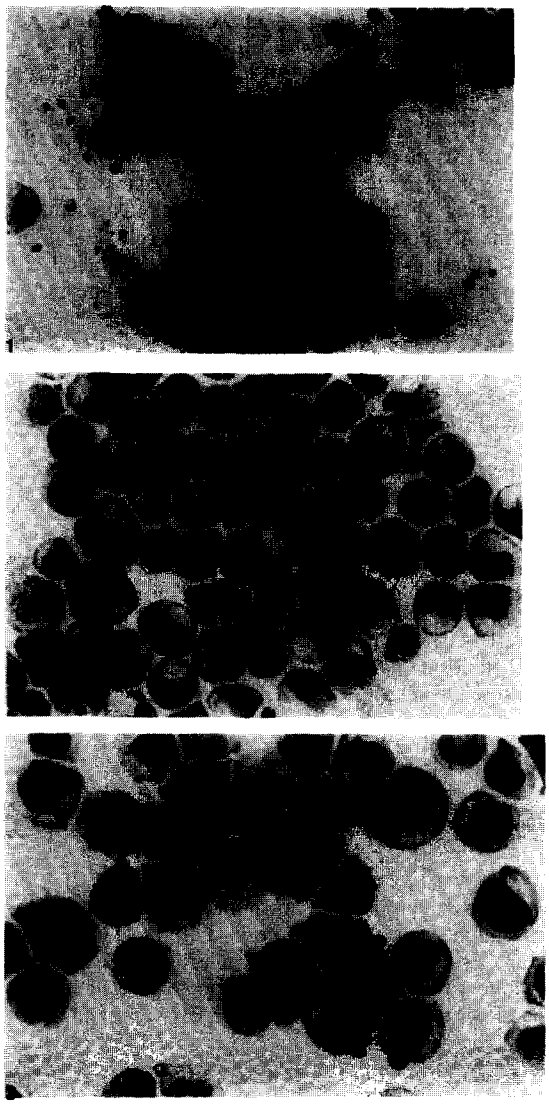

Fig. 3. (1) Morphological features of U.V.-irradiated HL-60 cells, cultured for 3 hr. Most cells show the typical characteristics of apoptosis: decreased cell volume, nuclear fragmentation, and cellular break-up into apoptotic bodies. (2) Compare this to UV-irradiated HL-60 cells cultured in the presence 40 μM PDTC. Cells show reduced shrinkage and inhibition of nuclear fragmentation. Apoptotic body formation is also absent. (3) Normal untreated HL-60 cells.

investigate the involvement of oxygen radicals during apoptosis, we looked at peroxide radical production using the fluorescent probe DCFH/DA. UV and H_2O_2 produced peroxide radicals (Fig. 4a), whereas apoptosis induced by the other drugs did not stimulate radical production (Fig. 4b), suggesting that perhaps another radical is more important or, alternatively, the drugs were killing independently of oxidant production. PDTC did not prevent peroxide production, and so may instead be acting as a scavenger. These possibilities may explain why PDTC was the most effective inhibitor against a wide range of different acting agents, as this dithiocarbamate is known to have a wide range of actions [42–48] compared to our other inhibitors.

PDTC and BHA inhibit DNA nicking in UV-irradiated HL-60 cells

Previous observations showed that the inhibition of UV-induced apoptosis in HL-60 cells by Zn^{2+} ions was characterized by reduced nuclear fragmentation, as assessed morphologically, and reduced DNA nicking, as shown by *in situ* labelling of DNA-strand breaks (TUNEL-assay) [21]. These morphological observations showed that PDTC prevented nuclear fragmentation in UV-irradiated HL-60 cells, so we further examined the

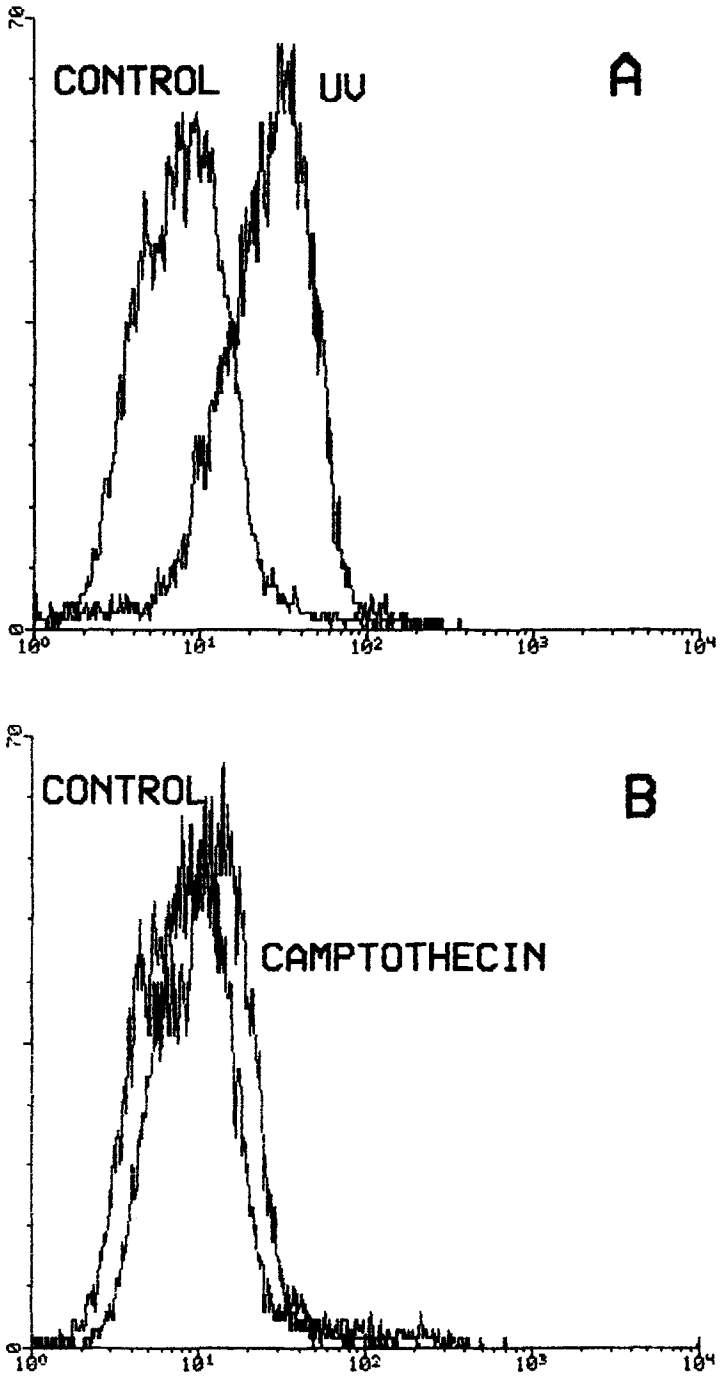

Fig. 4. (a) Peroxide levels in UV-irradiated HL-60 cells. The generation of intracellular peroxide was measured 30 minutes after treatment. (b) Peroxide levels in camptothecin-treated (10 μg/ml) HL-60 cells. There is no detectable peroxide production even after 4 hr and, at this time point, apoptosis is clearly visible on cytospin samples. Actinomycin-D (10 μg/ml), etoposide (50 μg/ml), and melphalan (50 μg/ml) also failed to produce any detectable peroxide (data not shown). In all cases peroxide levels were measured at intervals of 15 min to ensure proper detection (log scale).

effect of antioxidants on DNA fragmentation. UV-irradiated HL-60 cells were subjected to a TUNEL-assay to quantify the amount of DNA nicking occurring in the presence and absence of PDTC or BHA. As is clear from the fluorescence flow cytometry data obtained (Fig. 5), both PDTC and BHA were able to reduce the amount of DNA-strand nicking up to 3 hr after UV-irradiation, but clearly PDTC was more effective.

Effect of antioxidants on endonuclease activity

To test whether the inhibition of DNA nicking was due to direct action of PDTC on the endonuclease involved in apoptosis at a nuclear level, we compared its action with Zn^{2+} ions, a known inhibitor of endogenous endonucleases involved in apoptosis [21]. Incubation of isolated nuclei of untreated HL-60 cells under low salt

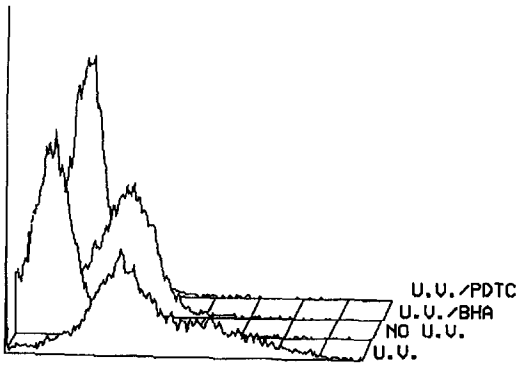

Fig. 5. Fluorescence flow cytometry data of HL-60 obtained by *in situ* nick-end labelling of DNA for: non-irradiated cells; UV-irradiated cells, in the presence or absence of 40 μM PDTC; or in presence or absence of 200 μM BHA. Both BHA and PDTC reduce the amount of DNA nicking, particularly PDTC (log scale.

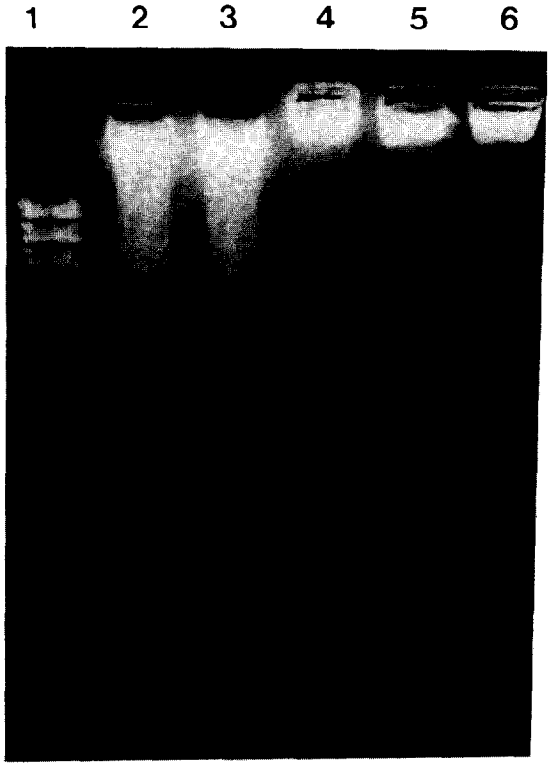

Fig. 6. Photograph of 1.5% agarose gel electrophoresis of DNA isolated from nuclei incubated under low salt conditions to activate the endogenous endonuclease. Phi X174 RF/HaeIII marker DNA (lane 1). Nuclei incubated for 6 hr in the absence of PDTC (lane 2). The characteristic ladder pattern for internucleosomal degradation is visible. Nuclei were incubated under similar conditions, in presence of 40 μM PDTC. PDTC fails to inhibit the ladder formation (lane 3). Nuclei incubated in the presence of 150 mM NaCl and 40 μM PDTC. PDTC does not induce activation of the endonuclease (lane 4). Nuclei incubated in the presence of 1 mM $ZnCl_2$. The ladder pattern is absent, indicating inhibition of the endonuclease by $ZnCl_2$ (lane 5). Control lane, loaded with DNA isolated from normal untreated cells. No DNA ladder is visible (lane 6).

concentrations is known to activate the endonuclease, resulting in the formation of a DNA-ladder pattern, a hallmark of apoptosis [34]. We have previously shown that $ZnCl_2$ can inhibit DNA fragmentation under these conditions [21, 34]. DNA-gel electrophoresis of DNA isolated from the nuclei after incubation in low salt conditions showed that whereas the addition of $ZnCl_2$ prevented the formation of DNA ladders, 40 μM PDTC failed to inhibit the endonuclease. This concentration of PDTC did not induce DNA-ladder formation by itself, as was demonstrated by incubating the nuclei in buffer with high salt concentration in the presence of PDTC (Fig. 6). These results indicate that the inhibitory effect of PDTC on nuclear fragmentation seen in apoptotic HL-60 cells is independent of a direct inhibitory action on the endogenous endonuclease thought to be involved in the DNA fragmentation during apoptosis.

PDTC prevents cell volume shrinkage and formation of apoptotic bodies

To further quantify the effect of PDTC on the morphology of cells undergoing apoptosis, we used flow cytometry analysis of the light scatter properties of UV-irradiated HL-60 cells. When incubated for 3 hr in the absence of PDTC, a sharp increase in the number of cells with low forward and sidescatter, typical for the presence of apoptotic bodies, is visible (Fig. 7a). In contrast, this peak was absent in UV-irradiated HL-60 cells protected by 40 μM PDTC (Fig. 7B). This reduction in apoptotic body formation was visible up to 6 hr after irradiation (Fig. 7c). Incubation of non-irradiated control cells with 40 μM PDTC for similar time periods had no significant effect on forward and side scatter (data not shown). Untreated control cells after 6 hr showed no changes in cell size or volume (Fig. 7d). A similar protective effect was seen with DEDTC and DMDTC.

DISCUSSION

Our results indicate that antioxidants are potential inhibitors of apoptosis. Although both BHA and PDTC are considered antioxidants, their capacity to interfere with the apoptotic process differs strikingly. Whereas addition of PDTC is able to block apoptosis induced by a wide variety of stimuli (i.e., UV irradiation, H_2O_2, actinomycin-D, camptothecin, melphalan, or etoposide-treatment), pretreatment with BHA or cysteamine only leads to a significant reduction of apoptosis induced with UV irradiation or H_2O_2 treatment. This restricted spectrum of the oxygen-radical scavenger BHA does not seem limited to our system. BHA has previously been shown to inhibit the cytotoxic effects of TNF in L929 and WEHI 164 mouse fibrosarcoma cells [16]. In contrast, it failed to inhibit oxidative damage caused to plasmid and cellular DNA by gliotoxin [36].

Although PDTC is regarded as an antioxidant, little is known about the exact biochemical working mechanism of DTCs. Antioxidant mechanisms are suggested to be involved in inhibition of the NF-κB transcription factor by DTCs [26, 27]. Recent work by Bessho *et al.* [37] using PDTC on HL-60 cells has shown it inhibits etoposide-induced cell death and concomitantly blocks NF-κB activation. They suggest that this transcription factor may be a common mediator of apoptosis. Schreck *et al.* [38] propose that reactive oxygen species may be used as messengers to activate NF-κB. This could explain how PDTC inhibits NF-κB and subsequently apoptosis by

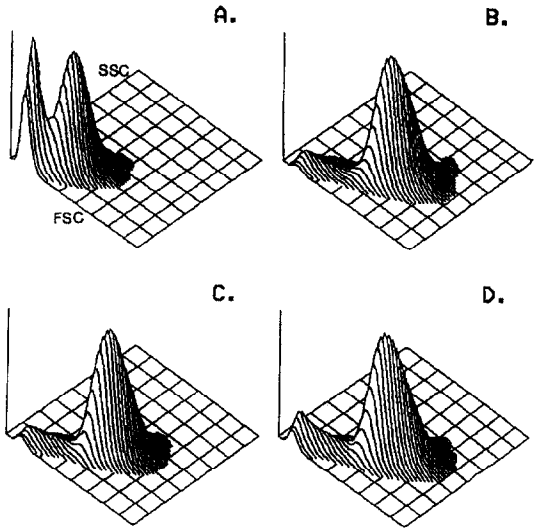

Fig. 7. Light scatter properties of HL-60 cells (A) 3 hr after UV irradiation. Notice the large population with low forward and sidescatter, typical for the presence of apoptotic bodies. This population is absent in (B), where UV-ed cells have been cultured in the presence of 40 μM PDTC. (C) This inhibition of cell volume shrinkage and of apoptotic body formation persisted up to 6 hr. Non-irradiated cells treated with 40 μM PDTC show a scatter profile typical for healthy cells (data not shown). Untreated control cells after 6 hr show no change in cell size or volume (D).

interfering with this oxygen radical signal. However, an NF-κB-related inhibition of apoptosis by DTCs seems unlikely in our case, as we have found that the NF-κB inhibitor herbimicine A was unable to reduce UV-induced apoptosis in HL-60 cells (data not shown). Furthermore, both staurosporine, an inhibitor of NF-κB, and okadeic acid, an inducer of NF-κB, have been shown to inhibit apoptosis-related phenomena [39, 40]. Some circumstantial evidence suggests that DTCs might interfere with enzyme activity by direct cross-linking of proteins [41]. Other biological effects include reduced plasma-membrane fluidity in T-cells derived from DEDTC-treated HIV-infected patients [42], enhanced mitogenic response of PHA-stimulated human T-cells [43], histological changes in lymphoid organs of mice [44], restoration of impaired T-cell responses in aged Balb/c-mice [45], and increased cytokine production during bone marrow recovery [46]. No effect was seen on the expression of different oncogenes known to modulate apoptosis. Indeed, with fluorescence flow cytometry, no changes were seen in the protein levels of either c-Abl and Bcl-2, known suppressors of apoptosis [12, 13], or of c-Myc, an enhancer of apoptosis [47]. As we have previously shown that UV- and drug-induced apoptosis in HL-60 cells can occur in the absence of *de novo* mRNA or protein synthesis [20], we can exclude the possibility that the inhibition of apoptosis seen in presence of DTCs depends on a specific inhibition of these activities.

In our hands PDTC shows the same spectrum of apoptosis inhibition as Zn^{2+} ions. Previous studies have shown that Zn^{2+} ions are an inhibitor of DNA fragmentation associated with apoptosis induced by topoisomerase inhibitors, camptothecin and etoposide [17] or UV [21, 10]. In addition, Zn^{2+} ions were able to inhibit glucocorticoid-induced apoptosis in thymocytes [48]. Observations in our laboratory indicate that this inhibition is caused by direct inactivation of a calcium/magnesium-independent endogenous endonuclease involved in apoptosis [33]. PDTC, however, is unable to reduce DNA fragmentation by directly inhibiting the endonuclease activity in isolated nuclei from normal HL-60 cells subjected to buffers with low salt concentration. These results seems to indicate that the mechanism by which PDTC prevents apoptosis blocks the apoptosis pathway at an earlier stage than the activation of the endonuclease(s). Alternatively, PDTC might need to be metabolized in the cytoplasm with subsequent inhibition of the endonuclease by one of its metabolites. This latter scenario would go undetected under the conditions used, as PDTC was added directly to isolated nuclei in the absence of cytoplasm.

One reason why DTCs prove such potent inhibitors of apoptosis might be the fact that DTCs interfere with more than one component of the antioxidant pathway. Firstly, DTCs seem to posses a glutathione-peroxidase (GSH-PX)-like activity on their own and, hence, catalyse the reduction of H_2O_2 to water, supplementing the intracellular GSH-PX and catalase pathways [49]. Secondly, the metal chelating properties of the sulfhydryl moieties make DTCs a rather nonspecific inhibitor of several metal-containing enzymes, including superoxide dismutase (SOD) [50]. Although at first sight this may look contradictory with the role of DTCs as antioxidants, it should be kept in mind that inhibition of SOD activity will reduce the formation of H_2O_2 from superoxide anion, O_2^-. H_2O_2 can then form the very reactive hydroxyl radical, OH*, by the metal-catalyzed Fenton reaction. Hence, reduced turnover of O_2^- into H_2O_2 indirectly prevents the formation of a more aggressive radical. Furthermore, it gives the GSH-PX and catalase-mediated pathways a better chance of dealing with reduction of H_2O_2 formed, again decreasing the rate at which this latter can form the more damaging hydroxyl radical.

Thirdly, again because of their metal-chelating capacity, DTCs are potent inhibitors of the Fe^{2+} and Cu^{3+} catalyzed hydroxyl radical formation, again protecting the cell from this damaging radical. A role for Fe ions in the oxygen radical mediated induction of apoptosis is further supported by the fact that the iron chelator phenanthroline is capable of inhibiting UV- and H_2O_2-induced apoptosis, albeit at higher concentrations than DTCs (data not shown). This could suggest that peroxide-generated hydroxyl radicals are important in UV- and H_2O_2-mediated apoptosis, as these are the only two cytotoxic agents to produce peroxide radicals, and subsequently the only ones to be inhibited by phenanthroline. The other cytotoxic agents used in this study were not affected by phenanthroline, and appear to act independently of oxidant production.

We are currently investigating the effects of DTCs on cytokine production, as part of their effectiveness may be due to their ability to increase cytokine levels [43] and in this way inhibit cell death. Although further experiments are needed to elucidate the exact mechanism by which dithiocarbamates are able to inhibit apoptosis, our results support recent evidence that oxidative stress plays a role in apoptosis induced by a wide range of agents [16]. Hence, these observations suggest that some of the therapeutic actions of dithiocarbamates is due to their capacity to protect cells against cytotoxic stimuli

leading to cell death by interfering with the oxygen radical cascade.

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