Role of peroxide and superoxide anion during tumour cell apoptosis

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Abstract Apoptosis or programmed cell death was induced in the human promyelocytic leukemia cell line HL-60 by UV irradiation or treatment with cytotoxic drugs (etoposide, camptothecin, melphalan or chlorambucil). These treatments caused a rapid increase in intracellular peroxide levels. Preincubation of HL-60 cells with the hydrogen peroxide-scavenging enzyme catalase (500 U/ml) inhibited apoptosis due to UV irradiation or low concentrations of camptothecin, etoposide or melphalan, but did not protect against higher concentrations. In contrast, superoxide anion levels in the cells remained unchanged upon treatment with cytotoxic drugs, while UV irradiation led to a transient doubling in superoxide levels. Exogenous superoxide dismutase (400 U/ml) provided modest protection against UV irradiation and had no effect on cytotoxic drug-induced apoptosis. The results suggest that both hydrogen peroxide and superoxide anion may be involved in the induction of apoptosis by UV irradiation. On the other hand, while exposure to cytotoxic drugs induces a large increase in intracellular peroxide levels, catalase is able to protect the cells from apoptosis only when low concentrations of these compounds are used, thus indicating the involvement of other factors in this process, particularly at higher drug concentrations.

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Key words: Apoptosis; Peroxide; Superoxide anion; Catalase; Superoxide dismutase

1. Introduction

Apoptosis or programmed cell death is a highly regulated form of cell death. Morphologically it is characterised by chromatin condensation, cell shrinkage and membrane blebbing. Within the cell there occurs a carefully orchestrated cascade of enzymatic activities leading to protein and nucleic acid degradation. Although many components of the apoptosis jigsaw puzzle are continually being uncovered and put in place, many of the initial signals which are important in the induction of apoptosis, particularly by cytotoxic compounds, are still not fully understood.

There is a wide variety of conditions which induce cells to die by apoptosis. These include physiological conditions, such as the binding of tumor necrosis factor to its receptor [1] or crosslinking of the Fas receptor [2]. The ability of a range of cytotoxic agents to kill cells in a controlled manner, i.e. by apoptosis, has been exploited in the chemotherapeutic treatment of many cancers [3–9].

In order that the process of apoptosis may be manipulated in the treatment of various diseases such as cancer and AIDS, much effort has been expended in the search for a common signal which mediates the induction of this pathway. During recent years a large body of evidence has accumulated to suggest that oxidative stress may play a role as a common mediator of apoptosis [10,11]. For example, many chemotherapeutic agents induce apoptosis while at the same time inducing intracellular production of reactive oxygen intermediates (ROI). On the other hand, antioxidant molecules or molecules which enhance the endogenous antioxidant defence systems of the cells can inhibit apoptosis to varying extents [12–14].

Whether ROI production is the primary trigger for apoptosis in these cells or a contributory factor is not clear. However, in many instances the induction of oxidative stress is crucial to the process as seen from the protective effects afforded by antioxidant compounds [12,14]. Yet there remains many questions to be answered regarding the role played by oxidative stress in apoptosis. One of these questions is which ROIs are most important during apoptosis?

The term ROI is a collective one that includes free radicals such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and also some nonradical derivatives of oxygen, such as hydrogen peroxide (H_2O_2). Both superoxide and hydrogen peroxide are relatively unreactive compared with hydroxyl radicals which can cause damage to most biological molecules, including DNA, proteins and lipid membranes [15]. Superoxide may be important in apoptosis as it reacts with nitric oxide ($NO^{\cdot}$) to yield peroxynitrite, which has been found to be toxic to HL-60 cells [16].

The cytotoxic agents used in this study were chosen for their usefulness in anticancer treatments [3–9]. Firstly, camptothecin and etoposide are topoisomerase I and II inhibitors, respectively. They inhibit these enzymes by stabilization of the cleavable complex between the enzyme and DNA and lead to the formation of protein-bound DNA breaks. It is thought that cytotoxicity occurs because the cells are prevented from completing the S-phase or undergoing mitosis. The other apoptosis-inducing agents used, melphalan and chlorambucil, are alkylating agents. DNA is generally thought to be the principle molecular target of alkylating agents [17,18] leading to an increased the rate of depurination. It is likely that the damage caused to the cell by alkylating agents triggers the cell to engage the apoptotic pathway. However, there is evidence that there are other mechanisms involved [19–21]. The induction of oxidative stress is one potential candidate particularly since antioxidant compounds can inhibit apoptosis by topoisomerase inhibitors [12] and the toxicity of melphalan is highly dependent on the glutathione content [20,21].

The majority of work performed to date has involved the use of non-specific antioxidant compounds to inhibit apopto-

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Abbreviations: DCFH/DA, 2′,7′-dichlorofluorescin diacetate; ROI, reactive oxygen intermediate

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sis. Alternatively workers have used end-point measurements such as lipid peroxidation or measurement of protein oxidation to demonstrate an involvement of oxidative stress. In this paper we investigate the relative importance of hydrogen peroxide and superoxide anions in the initiation of apoptosis by a number of anticancer agents. Firstly, the fluorescent probes, 2′,7′-dichlorofluorescin diacetate (DCFH/DA) and hydroethidine were employed to evaluate intracellular production of peroxides and superoxide anion, respectively, upon induction of apoptosis. Secondly, we tested the ability of catalase and superoxide dismutase, which are specific for the degradation of hydrogen peroxide and of superoxide, respectively, to inhibit apoptosis.

2. Materials and methods

2.1. Cell culture

The human promyelocytic HL-60 leukemic cell line [22] was used. Cells were cultured in RPMI-1640 medium (Gibco, UK) supplemented with 10% FCS (Biochrom KG, Germany) and 2 mM glutamine. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.2. Treatment of cells with cytotoxic agents

HL-60 cells (5×10^5/ml) were seeded in 24-well plates (Nunc, Roskilde, Denmark) and exposed to various concentrations of cytotoxic agents: camptothecin (0.05–0.5 μg/ml), etoposide (5–20 μg/ml), melphalan (20–60 μg/ml) and chlorambucil (80–100 μg/ml). Stock solutions of these compounds were made in dimethylsulfoxide (DMSO) and diluted by at least 1 in 1000 to ensure solvent levels not greater than 0.1%. Alternatively cells were exposed from below to a 302 nm UV transilluminator source at a distance of 2.5 cm for 15–60 s at room temperature. Cells were then incubated at 37°C and assessed for apoptosis or ROI levels at the indicated times. In some experiments cells were incubated with either catalase (Bovine liver, EC 1.11.1.6, Sigma) (500 U/ml) or superoxide dismutase (Bovine erythrocyte, EC 1.15.1.1, Sigma) (400 U/ml) for 10 min prior to treatment with cytotoxic agents.

2.3. Cell viability and morphology

Cell number was assessed using a Neubauer haemocytometer, and viability was determined by the ability of cells to exclude trypan blue. Cell morphology was evaluated by staining cytocentrifuge preparations with Rapi-Diff II (Paramount reagents Ltd., UK). Apoptotic cells were identified as described previously [23].

2.4. DNA isolation and electrophoresis

DNA was isolated by centrifuging cells at $200\times g$ for 5 min at room temperature. Cell pellets (containing 5×10^5 cells) were resuspended in 20 ml of lysis buffer which contained 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, and 0.8% (w/v) sodium lauryl sarcosinate. RNAse A (10 μl of 1 mg/ml prepared in 0.1 M sodium acetate and 0.3 mM EDTA, pH 4.8) was added. The samples were incubated overnight at 37°C. Then 10 ml of proteinase K (20 mg/ml in distilled water) was added and the samples were heated at 50°C for 1.5 h. Prior to loading onto agarose gels 5 ml of loading buffer (10 mM EDTA, 0.25% (w/v) bromophenol blue and 50% (w/v) glycerol) was added to each sample. DNA was electrophoresed in 1.5% agarose gels at 55V for 3–4 h in buffer containing 2 mM EDTA, 89 mM Tris-HCl and 89 mM boric acid, pH 8.0. Gels were cast in apparatus supplied by CBS Scientific Co. (CA, USA).

2.5. Measurement of intracellular peroxide levels

Peroxide levels were assessed using the method of [24] as previously described [12]. Briefly cells (5×10^5/ml) were incubated with 5 μM DCFH/DA (Molecular Probes), made as a 10 mM stock in DMSO, for 1 h at 37°C. Cells were treated with apoptosis-inducing agents either before, after or during the loading period (depending on the time point at which ROI measurement was to be made). Peroxide levels were measured using a Becton Dickinson FACScan flow cytometer with excitation and emission settings of 488 and 530 nm, respectively.

2.6. Measurement of intracellular superoxide anion

Superoxide anion levels were measured using an adaptation of the method employed by [25]. We used the dye hydroethidine (Molecular Probes) which is oxidised by superoxide anion within the cell to produce ethidium bromide, which fluoresces when it intercalates into DNA. Briefly, cells (5×10^5/ml) were incubated with 10 μM hydroethidine (Molecular Probes) (made as a 10 mM stock in DMSO) for 15 min at 37°C. Cells were treated with apoptosis-inducing agents either before, after or during the loading period (depending on the time point at which ROI measurement was to be made). Intracellular superoxide anion levels were assessed by measuring the fluorescence due to ethidium bromide (i.e. FL2) using a Becton Dickinson FACScan flow cytometer with excitation and emission settings of 488 and 600 nm, respectively.

3. Results

3.1. Peroxide and superoxide production upon UV irradiation

Oxidative stress has been implicated in apoptosis in a number of systems. In the present study HL-60 cells were treated with UV irradiation or a number of cytotoxic drugs. Exposure of HL-60 cells to UV irradiation (15–60 s) induced the morphological changes typical of apoptosis after 4 h (Fig. 1). Similar results were obtained upon exposure to camptothecin (0.05–0.5 μg/ml), etoposide (5–20 μg/ml), melphalan (20–60 μg/ml) or chlorambucil (80–100 μg/ml) (data not shown).

Treatment of the cells with UV irradiation also caused a time-dependent increase in intracellular peroxide production as demonstrated by an increase in fluorescence due to oxidation of DCFH-DA (Fig. 2A). In addition, there was a small, transient increase in intracellular superoxide levels which was monitored by an increase in fluorescence due to the oxidation of hydroethidine (Fig. 2B). Both of these ROIs were induced

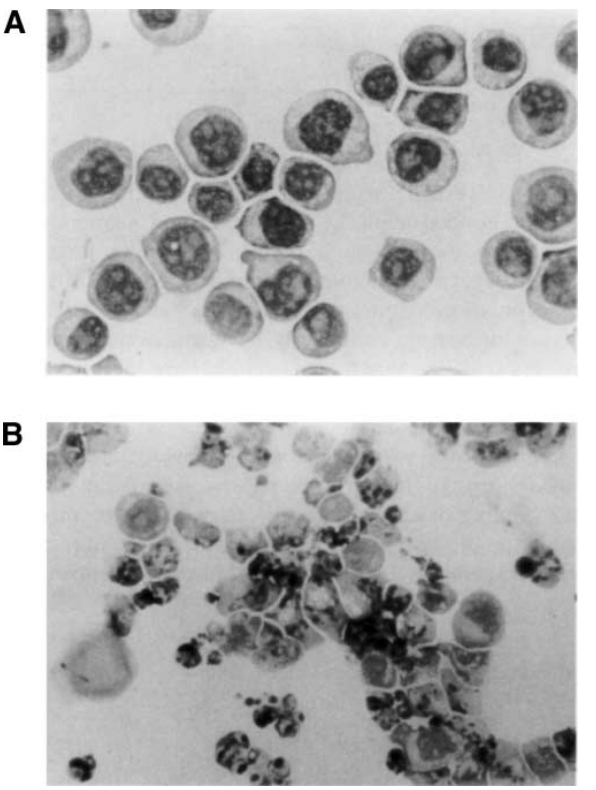

Fig. 1. Morphological features of (A) untreated HL-60 cells or (B) cells exposed to UV irradiation for 15 s.

rapidly prior to any other noticeable changes, indicating their candidacy as signals for initiation of apoptosis.

3.2. Protection by catalase against UV irradiation

In order to ascertain whether these ROIs are important for the induction of apoptosis by UV irradiation we decided to evaluate the ability of ROI-scavenging enzymes to protect the cells from apoptosis. Firstly, the hydrogen peroxide-scavenging enzyme, catalase was used. This enzyme converts two molecules of hydrogen peroxide into two molecules of water and one of oxygen. Catalase was found to cause an increased rate of clearance of peroxide from UV irradiated cells (Fig. 3). As the enzyme is not cell-permeable (in contrast to hydrogen peroxide) this is probably due to a removal of hydrogen peroxide from the culture medium which would promote the exit of hydrogen peroxide from the cellular cytoplasm to establish equilibrium [26].

In accord with this finding, catalase conferred protection on HL-60 cells against UV-induced apoptosis as assessed either by morphological criteria (Fig. 4A) or by the degree of DNA laddering produced by the cells (Fig. 4B). In contrast, superoxide dismutase, which catalyzes the dismutation of superoxide into hydrogen peroxide, was found to confer only slight

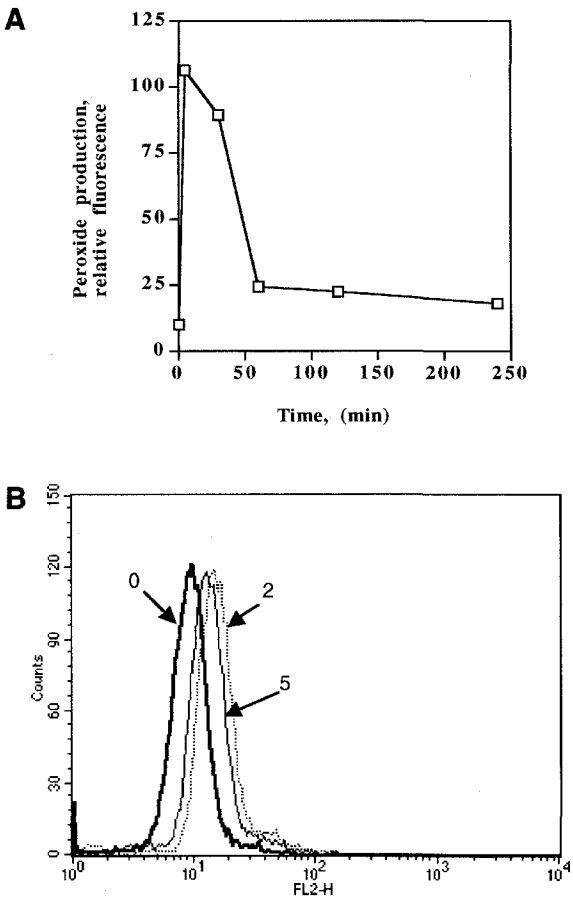

Fig. 2. A: Time course of peroxide levels in HL-60 cells treated with UV irradiation. HL-60 cells were UV-irradiated for 60 s, and intracellular peroxide levels were detected by measuring the relative FL1 values due to oxidation of DCFH/DA. B: Superoxide production in cells irradiated with UV for 60 s. Superoxide levels were detected by the relative FL2 values due to oxidation of hydroethidine. Measurements were made at 0 (untreated cells), 2 or 5 min after UV irradiation as indicated.

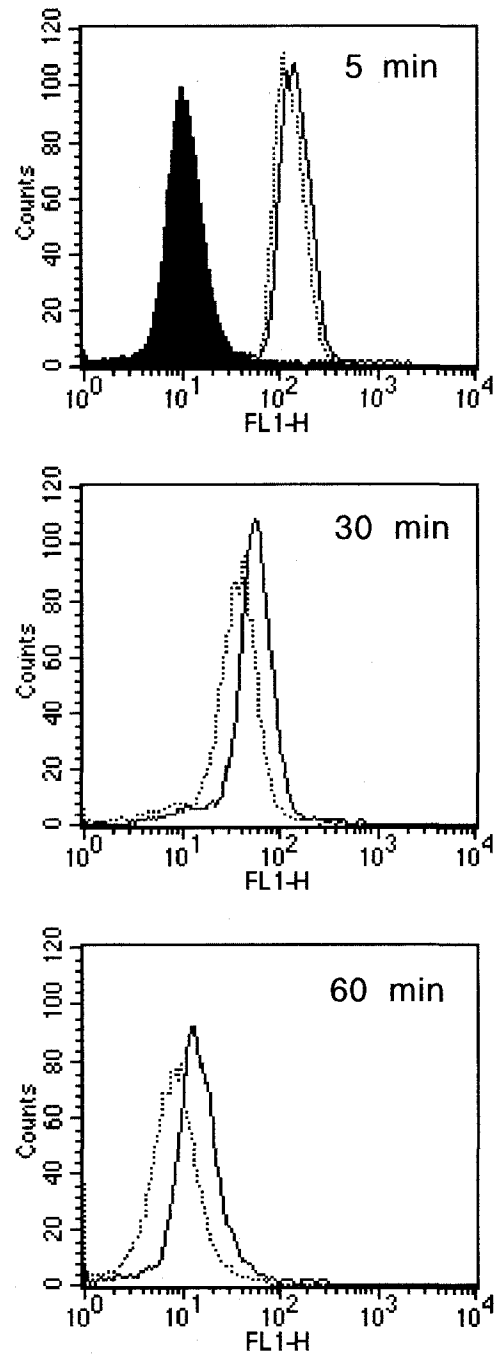

Fig. 3. Catalase increases clearance rate of peroxides from UV irradiated cells. HL-60 cells were either untreated (shaded trace; omitted from the second and third plots for the sake of clarity), or UV-irradiated for 60 s in the absence (solid line) or presence (broken line) of catalase (500 U/ml). Peroxide levels (FL1 values) were measured at various times thereafter as indicated. Peroxide levels in cells treated with catalase (500 U/ml) were superimposable on untreated cells and these traces were omitted for clarity.

protection on the cells against UV-induced apoptosis (Fig. 5). This degree of protection was not significant.

3.3. ROI involvement during cytotoxic drug-induced apoposis

Treatment of HL-60 cells with cytotoxic drugs also caused an increase in intracellular peroxide levels (Fig. 6). This increase was time and concentration-dependent (Fig. 6 and data

not shown). The increase was rapid with peroxide levels decreasing gradually, particularly in the case of camptothecin and etoposide, whereas levels remained relatively high in the case of melphalan and chlorambucil. In contrast to UV irradiation, the use of cytotoxic drugs, camptothecin and etoposide, did not produce any increase in intracellular levels of superoxide (Fig. 7) up to 60 min. In fact there was a slight decrease in levels of this ROI. There was no alteration in superoxide levels in cells treated with melphalan (40 μg/ml) or chlorambucil (100 μg/ml) up to 10 min (data not shown).

Catalase, provided partial inhibition against the induction of apoptosis by cytotoxic drugs (Fig. 8). Protection was more evident at low concentrations camptothecin (0.05 μg/ml), etoposide (5 μg/ml) or melphalan (40 μg/ml) while there was no protection from chlorambucil-induced apoptosis. On the other hand, superoxide dismutase did not have any protective effects under these conditions (data not shown).

4. Discussion

The present study demonstrates the involvement of hydrogen peroxide in the induction of apoptosis by UV irradiation,

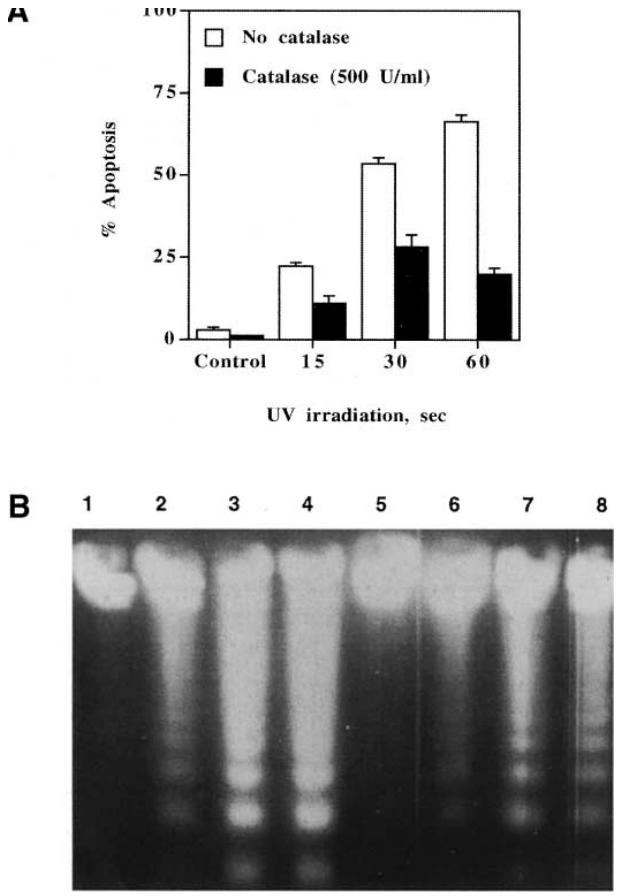

Fig. 4. Inhibition of UV-induced apoptosis by catalase (500 U/ml). HL-60 cells were UV-irradiated for the indicated lengths of time in the presence or absence of catalase (500 U/ml). A: After 4 h the % apoptosis was determined by morphological means from cytospin preparations of the cells. B: The degree of DNA laddering was determined. HL-60 cells were UV-irradiated for 0 (lanes 1 and 5), 15 s (lanes 2 and 6), 30 s (lanes 3 and 7) or 60 s (lanes 4 and 8) in the presence (lanes 5 to 8) or absence (lanes 1 to 4) of catalase (500 U/ml).

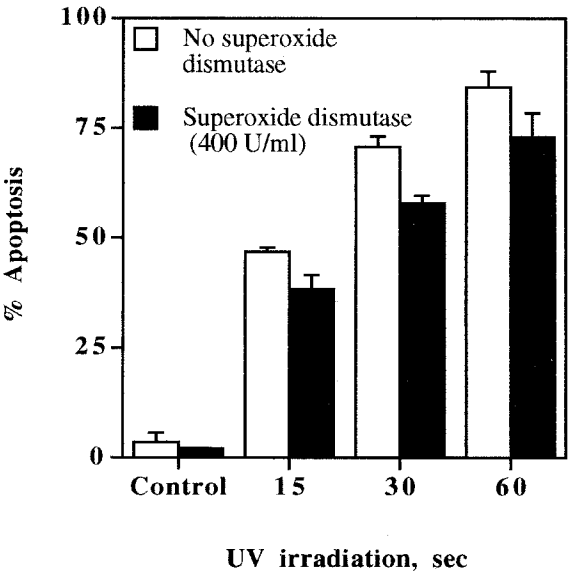

Fig. 5. Lack of inhibition of UV-induced apoptosis by superoxide dismutase. HL-60 cells were UV-irradiated for the indicated lengths of time in the presence or absence of superoxide dismutase (400 U/ml). After 4 h the % apoptosis was determined by morphological means from cytospin preparations of the cells.

while the involvement of superoxide may be by an indirect mechanism. For example, it may act as an intermediate in the formation of hydrogen peroxide or of hydroxyl radicals. The involvement of hydrogen peroxide is seen both by the large rapid increase in intracellular peroxide levels as determined by fluorometric methods, and further by the protective effect of exogenous catalase. The results obtained are in agreement with a number of other workers who have observed that catalase acts as an anti-apoptotic factor in conditioned medium to block density-dependent apoptosis [27] and that it delays the induction of apoptosis by dexamethasone in murine thymocytes [28].

In contrast, intracellular superoxide anion levels increased only slightly and transiently in UV-irradiated cells. Exogenous superoxide dismutase provided only slight protection. However the notion that this ROI is involved in apoptosis cannot by completely ruled out. For example, it may be an important intermediate in the generation of increased levels of either hydrogen peroxide (due to the activity of intracellular superoxide dismutases). Indeed the transient nature of superoxide anion accumulation during apoptosis may result from a high rate of superoxide dismutase activity within the cell. Moreover hydroxyl radicals can be generated from superoxide anions by the metal-catalysed Fenton or Haber-Weiss reactions. It has been suggested that superoxide production serves as an early signal, rather than a toxic agent, to mediate apoptosis, as injection of Cu/Zn superoxide dismutase into sympathetic neurons delayed apoptosis due to deprivation of nerve growth factor [29] and addition of nerve growth factor back to the culture medium after the period of peak ROI generation prevented apoptosis.

Treatment of the cells with various cytotoxic drugs induced a large and rapid increase in the intracellular levels of peroxides. Interestingly, catalase is capable of protecting the cells from apoptosis induced by low concentrations of these com-

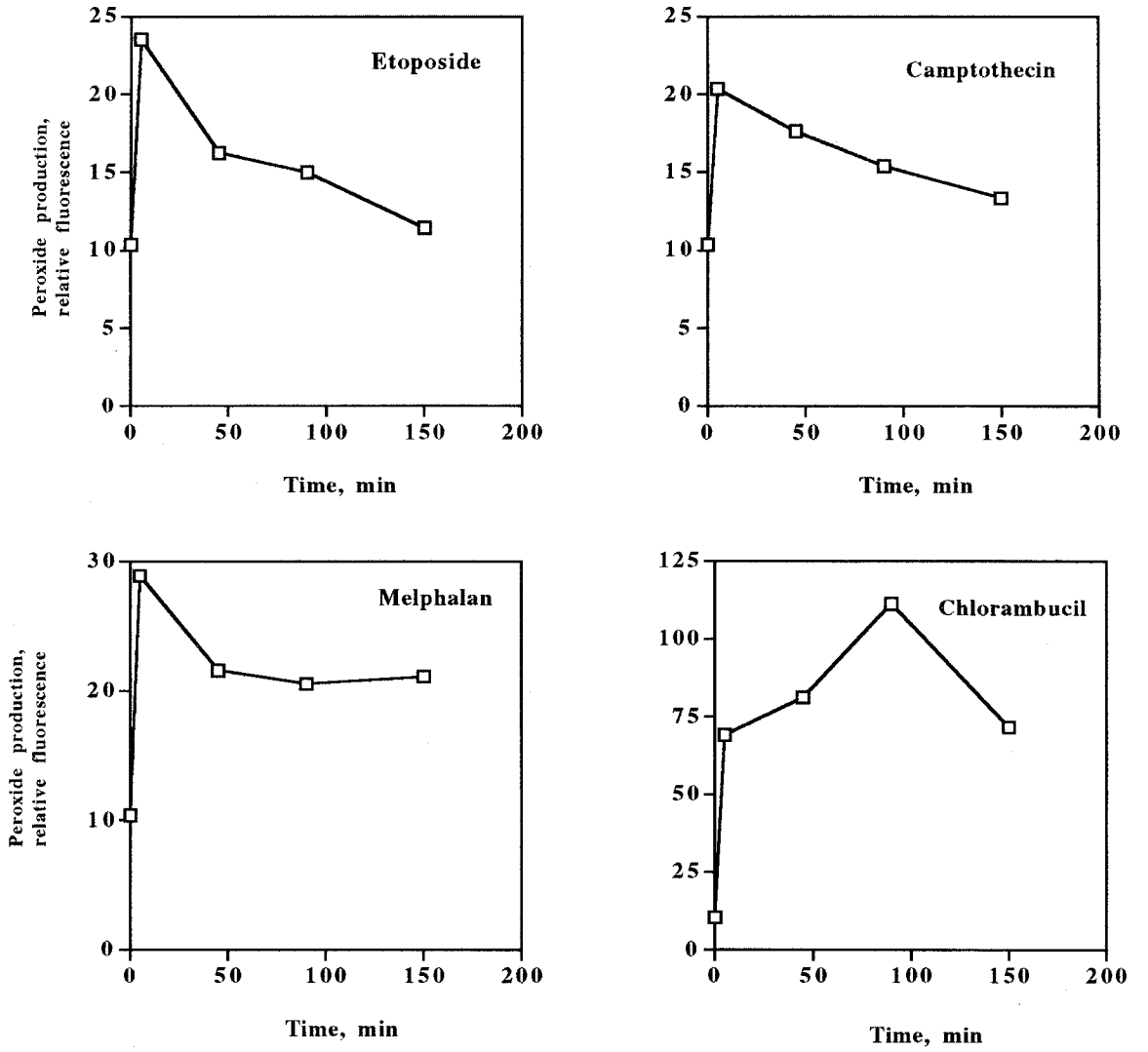

Fig. 6. Time course of peroxide levels in HL-60 cells treated with cytotoxic drugs. Cells were treated with the following concentrations of cytotoxic agents: etoposide, 10 μg/ml; camptothecin, 0.1 μg/ml; melphalan, 60 μg/ml; chlorambucil, 100 μg/ml. Intracellular peroxide levels were detected by measuring the relative FL1 values due to oxidation of DCFH/DA.

pounds while failing to protect against higher concentrations. One possible explanation for this is that hydrogen peroxide is not the main peroxide produced by these treatments. Furthermore the results indicate a mixed action of cytotoxic drugs in inducing apoptosis, of which the induction of oxidative stress is a contributory factor. Another interesting observation is that the time course of peroxide production in cells treated with either topoisomerase inhibitors or alkylating agents was markedly different. Both classes of drugs led to a rapid increase in intracellular peroxide levels, but in the case of topoisomerase inhibitors this decreased to basal levels over a period of about 2 h while remaining at high levels for at least 2.5 h after treatment with alkylating agents. In contrast, even though UV irradiation led to higher levels of peroxide, these had reduced to basal levels by 1 h. The greater sensitivity of UV and topoisomerase inhibitor-induced apoptosis to catalase inhibition may reflect the more transient nature of peroxide generation by these compounds and suggests that the peroxide produced may be hydrogen peroxide. In contrast to peroxide, superoxide anions were not elevated during the induction of apoptosis by cytotoxic compounds. This suggests either a lack

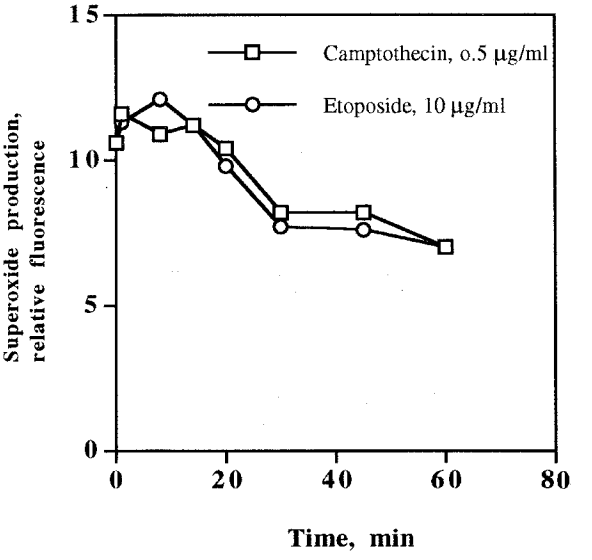

Fig. 7. Lack of superoxide production by cytotoxic agents. Cells were treated with the following concentrations of cytotoxic agents: etoposide, 10 μg/ml; camptothecin, 0.5 μg/ml.

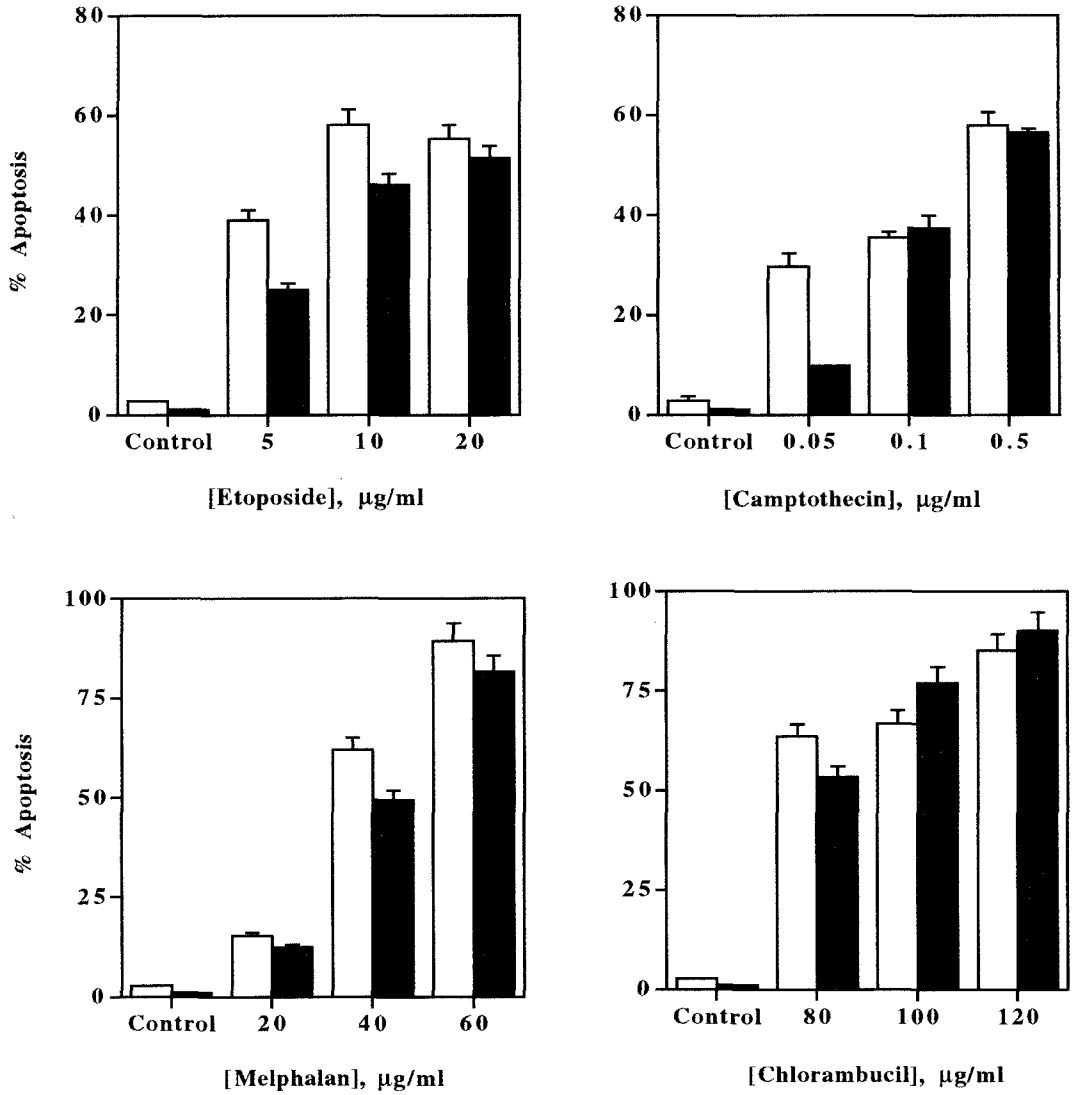

Fig. 8. Inhibition by catalase (500 U/ml) of the induction of apoptosis by cytotoxic agents. HL-60 cells were incubated with the indicated concentrations of apoptosis-inducing drugs in the presence (shaded bars) or absence (open bars) of catalase (500 U/ml). After 4 h the % apoptosis was determined by morphological means from cytospin preparations of the cells.

of involvement or their rapid removal by cellular superoxide dismutases.

Although we demonstrate here a minimal involvement of hydrogen peroxide and superoxide anion in drug-induced apoptosis, we have previously shown that a number of antioxidant compounds can protect HL-60 cells from both cytotoxic drug or UV-induced apoptosis [12]. These agents, i.e. butylated hydroxyanisole (BHA), cysteamine or particularly pyrrolidinedithiocarbamate (PDTC) give better protection than either catalase or superoxide dismutase. This is likely to be at least partly due to the necessity for hydrogen peroxide and superoxide anion to diffuse out of the cells in order to allow protection while the antioxidant molecules used are taken up by cells and thus have access to the intracellular compartments where ROIs are produced. It is also likely to be at least partly due to the multiple biological effects of antioxidant compounds, e.g. PDTC (which has metal-chelating properties and ability to react with sulfhydryl groups on other molecules) compared with the specific nature of the ROI-scavenging enzymes used in the present study. It has also been found that increased intracellular glutathione (part of the cell's antioxidant defence mechanism) can decrease the sensitivity of cells to melphalan [20,21].

The source of peroxide production in cells treated with cytotoxic drugs is not known. There is evidence for the direct production of free radicals by toxic foreign compounds, e.g. etoposide can be metabolized to free-radical derivatives which may contribute to its cytotoxicity [30]. It has been postulated that cytotoxic drugs can generate elevated level of ROIs through interference with mitochondrial function as this is a main site of free-radical production in cells. However, we have shown previously that collapse of the mitochondrial transmembrane potential is a late rather than an early event in apoptosis by cytotoxic drugs such as camptothecin [31].

Under normal circumstances, the major source of free radicals in cells is electron leakage from electron transport chains associated with mitochondrial membranes and the endoplasmic reticulum, to generate superoxide anions [32]. The nuclear membrane also contains electron transport systems which may produce free radicals. Under physiological conditions super-

oxide is readily converted to hydrogen peroxide by superoxide dismutase. The enzymes NADPH oxidase and xanthine oxidase are also two well known sources of superoxide generation [33,34]. Although superoxide does not appear to be crucial for apoptosis we have previously shown that inhibition of NADPH oxidase by diphenyliodinium can protect HL-60 cells from apoptosis by UV irradiation, hydrogen peroxide or actinomycin D [31] suggesting that at least one of the potential sources of ROIs under these conditions is NADPH oxidase. Superoxide anion produced in this way may be rapidly converted to hydrogen peroxide by superoxide dismutase. Its importance in the induction of apoptosis by UV and cytotoxic drugs may also arise from the production of hydroxyl radicals. These can be generated by the metal-catalysed Fenton reaction or through interaction with superoxide in the Haber-Weiss reaction.

It should be remembered that hydrogen peroxide is not an oxygen free radical and is relatively unreactive. It may, however, act as a metabolic signal, possibly by oxidizing specific protein thiol groups and triggering intracellular events leading to apoptosis. For example it can cause activation of the nuclear regulatory protein NFkB [35,31]. Other transcription factors, such as fos and myc [36], are also sensitive to redox regulation and thus may act as signalling molecules in the apoptosis process.

Overall the results obtained suggest that induction of apoptosis by UV irradiation proceeds through a ROI-dependent pathway, where hydrogen peroxide is an important intermediate presumably being converted to the more toxic hydroxyl radical. On the other hand, while the induction of apoptosis by cytotoxic drugs does appear to be mediatied by an ROI-dependent pathway, the results presented here indicate a mixed action of these agents in inducing apoptosis. It is clear that antioxidants can protect cells under these conditions [12,20,21]. However, the limited protective effects of catalase suggests that at high concentrations of drugs there are other effects of these drugs apart from inducing oxidative stress, i.e. damage to proteins and to DNA, which the use of catalase alone cannot overcome. However, it should be noted that antioxidant molecules (with the exception of PDTC, which as well as being an 'antioxidant', may inhibit the apoptotic process itself [37]) are only moderately protective against cytotoxic drugs relative to their ability to inhibit UV or hydrogen peroxide-induced apoptosis [12].

In conclusion, the results are interesting in terms of our understanding of the mechanism of UV and cytotoxic drug-induced apoptosis in leukemic cells. They imply that hydrogen peroxide is particularly important in UV-induced cell death and that the duration of elevated peroxide levels may be important in determining whether the cells are responsive to protection by catalase.

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