Reactive oxygen intermediate(s) (ROI): Common mediator(s) of poly(ADP-ribose)polymerase (PARP) cleavage and apoptosis

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Abstract HL-60 acute myeloblastic and U937 monoblastoid leukaemic cell lines both cleave poly(ADP-ribose)polymerase (PARP), at the onset of apoptosis, in response to a wide range of cytotoxic agents. This appears to be a common feature of leukaemic cell apoptosis. However, in the chronic myelogenous leukaemic (CML) derived cell line, K562, no such cleavage was detectable. This correlated with previous findings that this cell line is particularly resistant to apoptosis induced by cytotoxic agents. Proteolytic cleavage of PARP and the subsequent progression to apoptosis was inhibited by two protease inhibitors NEM and IOD. As both PARP cleavage and DNA fragmentation appeared closely linked in these cell lines, anti-oxidants (previously shown to be effective inhibitors of DNA fragmentation and apoptosis) were also demonstrated to prevent PARP cleavage. These results combine to suggest that ROI may mediate PARP cleavage, DNA fragmentation and the eventual apoptosis of these cells following cytotoxic insult.

Key words: Reactive oxygen intermediates; Apoptosis; Poly(ADP-ribose)polymerase

1. Introduction

Apoptosis is a regulated process, allowing the selective elimination of cells in an orderly fashion [1,2]. It occurs both during normal development [3,4] and as a result of a diverse range of cytotoxic insults [5,6]. There is also compelling evidence implicating apoptotic cell death as a factor during AIDS, human neurodegenerative diseases, autoimmunity and oncogenesis [7,8].

However, the biochemistry and molecular control of apoptosis remain to be delineated. The biochemical event most intensively studied has been endonuclease activity [9,10]. The recent discovery of a family of cysteine proteases, closely related to *Ced-3*, has identified another biochemical participant of apoptosis [11,12]. These proteases include ICE, CPP-32/Yama/apopain, ICH1/Nedd-2, TX/ICH-2, MCH-2, -3 and -4 and MIH-1, each of which induce apoptosis once expressed [13]. They exist as inactive precursors and remain so until activated by a specific processing event [13,14]. Several of these proteases activate or inactivate one another and so a cascade effect involving one or more has been envisaged [13]. These proteases cleave a number of substrates which may contribute to eventual cell death. These include the 70 kDa protein component of small nuclear ribonucleoprotein U1, lamin B1, α fodrin, topoisomerase 1, β-actin and poly-(ADP-ribose)polymerase or PARP [13,14].

The protease CPP-32 has been directly linked to apoptosis [15,16]. In addition, this protease together with MCH-3 resembles *Ced-3* most closely in sequence homology and substrate specificity [17]. Both of these proteases specifically cleave the DNA repair enzyme PARP as an early apoptotic event. PARP cleavage has been linked to endonuclease activation, with PARP demonstrated to negatively regulate endonuclease activity [19]. On cleavage of PARP, this block is removed and the endonuclease re-activated during apoptosis [19]. PARP itself functions as a molecular nick sensor [20]. Cleavage of PARP results in decreased enzyme activity which precludes binding of the enzyme to the damaged site and thus compromises the response to DNA damage [20,21].

Previously in our laboratory, we have demonstrated the rapid production of peroxide during apoptosis in HL-60 and U937 leukaemic cell lines [22]. Furthermore, a number of anti-oxidant compounds prevented DNA fragmentation and apoptosis in these cell lines [23,24]. As mentioned above, PARP cleavage has been linked to endonuclease activity and DNA fragmentation. Therefore, we investigated PARP cleavage in HL-60, U937 and K562 cell lines with particular emphasis being given to the role of ROI during PARP cleavage. In conjunction with earlier reports, the cleavage of PARP, DNA fragmentation and subsequent apoptosis were common events on addition of cytotoxic agents. To establish further that PARP cleavage and DNA fragmentation follow the same common pathway to apoptosis, protease inhibitors were tested against both PARP cleavage and DNA fragmentation. Given the protection observed with anti-oxidants against DNA fragmentation and apoptosis in these cells [22–24], we screened a range of anti-oxidants for the ability to prevent PARP cleavage. Our results indicate that both of these events (PARP cleavage and DNA fragmentation) occur following ROI production and suggest that ROI may act as common mediators, for PARP cleavage, DNA fragmentation and the eventual apoptosis of these leukaemic cells.

2. Materials and methods

2.1. Cell culture conditions

The human HL-60 acute myeloblastic leukaemic cell line, the U937 monoblastoid cell line and K562, a CML derived cell line, were used in the present study [25–27]. Cells were cultured in RPMI 1640 medium (Gibco, UK), supplemented with 10% FCS (Biochom KG, Germany) and 1% penicillin/streptomycin (Gibco, UK). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.2. 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 preparations

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Abbreviations: IOD, iodoacetamide; NEM, *N*-ethylmaleimide; PARP, poly(ADP-ribose)polymerase; ROI, reactive oxygen intermediate(s).

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with Rapi-Diff II (Paramount Reagents Ltd, UK). Apoptotic cells were identified by examining the changes in forward and side scatter using a FACScan flow cytometer (Becton-Dickinson, USA) equipped with Lysis II software [23].

2.3. UV- and drug-induced apoptosis

Apoptosis was induced in HL-60 and U937 cells by exposure to UV irradiation as previously described [23]. 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 10 min at room temperature. Cells were then returned to 37°C and assessed for apoptosis at appropriate time periods. Actinomycin D (Act-D., 5 μg/ml), camptothecin (Camp., 5 μg/ml), etoposide (Etop., 25 μg/ml) were added. These agents were supplied by Sigma Chemical Co. (St. Louis) and stock solutions were prepared in DMSO. Chlorambucil (Chlor., 50 μM) and melphalan (Melp., 50 μM) were also used and 0.1 M stocks were prepared in DMSO. Hydrogen peroxide (0.5 mM) was also added to induce apoptosis and this was obtained from BDH Chemicals. As a positive control for PARP cleavage, Jurkat T-cells were treated with 1 μg/ml anti-Fas antibody for 2 h (anti-Fas CH-11 IgM, Medical and Biological Laboratories, USA). Anti-oxidants were prepared as previously described [22–24] and the ones chosen were zinc chloride, 1 M in 0.15 saline, pyrrolidine dithiocarbamate (PDTC), 1 M in distilled water, butylated hydroxyanisole (BHA) and phenanthroline (Phen.), 1 M in ethanol, 2,2,6,6-tetramethylpiperidinoxyl (Tempo.) and 3β-doxyl-5α-cholestane (Dox.), 1 M in DMSO.

2.4. DNA isolation and electrophoresis

DNA was isolated and treated in the manner described previously [28]. Briefly, cells were pelleted by centrifugation at 200×*g* for 5 min at room temperature. The pellets were re-suspended in 20 μl lysis buffer and 10 μl RNase A and then incubated at 37°C for 18 h. Proteinase K (10 μl) was then added and samples incubated at 50°C for 2 h. DNA electrophoresis was carried out for 4 h at 55 V as previously described [23,28].

2.5. 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. [29]. Briefly, cells were fixed in 1% paraformaldehyde and stored overnight at −20°C. After washing in PBS, cells were re-suspended in 50 μl of reaction mixture. This mixture was incubated at 37°C for 30 min. Cells were then washed in PBS and re-suspended in 100 μl of staining buffer. Cells were subsequently 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 [24]. Bio-16-dUTP and the TdT-enzyme were obtained from Boehringer Mannheim. All other chemicals were obtained from Sigma.

2.6. Immunoblotting for PARP cleavage products

The analysis of PARP cleavage was performed using the method previously described by Kaufmann et al. [18]. Briefly, 1×10^6 cells were used per sample; these were centrifuged and washed in PBS. Next 40 μl of sample buffer (containing 50 mM Tris, pH 6.8, 6 M urea, 6% β-mercaptoethanol, 0.003% bromophenol blue and 3% SDS) was added and the samples sonicated. These were then heated at 80°C for 5 min and samples subsequently loaded and run on a 10% SDS-PAGE gel for 1 h at 140 V. After electrophoretic transfer of the separated polypeptides to nitrocellulose, the membrane was probed with an antibody Anti-F2 (generously provided by Dr. G. deMurcia, Centre National de la Recherche Scientifique, Strasbourg Cedex, France) which is a rabbit polyserum raised against a synthetic peptide corresponding to the second zinc finger of human PARP. Detection of bound antibody was performed using an enhanced chemiluminescent blotting detection system (ECL, Amersham Corp., UK) which was then visualised by exposure to auto-radiographic film.

2.7. Measurement of intracellular peroxides

Peroxide levels were assessed using the method of Hockenbery et al. [30]. Briefly, cells (5×10^5/ml) were loaded with 5 μM DCFH/DA (Molecular Probes, UK, dissolved at 2000× in DMSO) for 1 h at 37°C prior to cytotoxic insult. The apoptosis-inducing agents used were UV irradiation, H_2O_2, Act-D., Camp., Etop., Chlor. and Melp. Peroxide levels were measured using a Becton Dickinson FACScan flow cytometer. An increase in FL1-H fluorescence correlates with increased peroxide production.

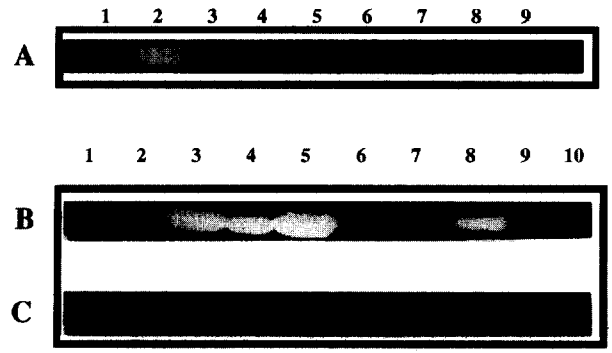

Fig. 1. Production of the 29 kDa PARP cleavage product in (A) HL-60 cells that were (1) UV treated 30 min, (2) UV treated 2 h, (3) Act-D. 30 min, (4) Act-D. 2 h, (5) Camp. 30 min, (6) Camp. 2 h, (7) untreated 30 min, (8) untreated 2 h, (9) Jurkat cells treated with 1 μg/ml anti-Fas antibody for 2 h. The time points 30 min and 2 h refer to the incubation times, following cytotoxic addition, at which PARP cleavage was determined. (B) HL-60 cells lanes (1–5) and U937 cells lanes (6–10) at 2 h: (1) untreated, (2) Chlor., (3) Etop., (4) H_2O_2, (5) Melp., (6) Chlor., (7) Etop., (8) H_2O_2, (9) Melp., (10) untreated, (C) K562 cells at 2 h (1) UV, (2) Act-D., (3) Camp., (4) Etop., (5) H_2O_2, (6) Chlor., (7) Melp., (8) untreated, (9) Jurkat cells treated with 1 μg/ml anti-Fas antibody (10) untreated Jurkat cells.

3. Results

3.1. PARP cleavage during leukaemic cell apoptosis

PARP cleavage has been associated with the onset of apoptosis in several model systems [15,16,18]. In the present study, using three leukaemic cell lines, the formation of the 29 kDa PARP cleavage product was demonstrated to follow cytotoxic insult (Fig. 1A,B). The cytotoxic agents used were UV irradiation, actinomycin D, camptothecin, etoposide, chlorambucil, melphalan and hydrogen peroxide. These agents were used at concentrations known to produce apoptosis (50–60%) in these cells after a 4 h incubation. The formation of the 29 kDa PARP fragment mirrors the decay of the intact enzyme (116 kDa) and therefore only this band is shown for the purposes of clarity. Time course experiments established the 2 h time point as the earliest time at which PARP cleavage was visible with these cytotoxic agents (data not shown). Similar results were obtained using either human HL-60 acute myeloblastic cells or U937 monocytes.

3.2. DNA fragmentation and PARP cleavage

From Fig. 1C, it is clear that none of the cytotoxic agents were capable of cleaving PARP in the K562 human CML cell line. This cell line exhibits a marked resistance to apoptosis when subjected to cytotoxic insult with no DNA fragmentation evident [31].

As mentioned above, these agents at the concentrations used, induce apoptosis to a similar degree. Therefore, between 2 and 4 h after cytotoxic insult, an increasing percentage of apoptosis was visible morphologically, by DNA gel electrophoresis and by in situ nick end-labelling (Fig. 2A–C). However, in all cases, these cytotoxic agents first induced PARP cleavage then DNA fragmentation. This confirmed the order

of these two events in these leukaemic cells. Figs. 1 and 2 illustrate that the proteolytic cleavage of PARP occurs prior to endonuclease activation and before any morphological signs of apoptosis are evident. It also suggests that both events are closely associated, with cleavage resulting in fragmentation.

3.3. Protease involvement during apoptosis

The ability of CTLs and NK cells to kill target cells using proteases was an early indication of the importance of these enzymes during apoptosis [13]. To distinguish the specific involvement of these cysteine proteases, above any other, during apoptosis, researchers have compiled an inhibitor profile [15,16]. A number of inhibitors can block the cleavage site of PARP, thus preventing PARP cleavage and the subsequent apoptosis. More recently, through the use of specific inhibitors, CPP-32 was identified as the protease responsible for PARP cleavage [16]. Therefore, using two of these protease inhibitors, NEM and IOD, effective inhibition of UV-induced PARP cleavage, DNA fragmentation and subsequent apoptosis was achieved (Fig. 3A,B). These results indicated the importance of these cysteine proteases during DNA fragmentation and apoptosis.

3.4. ROI, PARP cleavage and apoptosis

The protection afforded by anti-oxidants against leukaemic cell apoptosis and the apparent link between PARP cleavage and apoptosis prompted an investigation of the role of ROI during PARP cleavage. In conjunction with this, anti-oxidant addition, *bcl-2* or *CrmA* expression provided similar protection against TNF-α-induced apoptosis [32]. This suggested that *CrmA* (and possibly other protease inhibitors) may act in a similar fashion to *bcl-2* and other anti-oxidants in order to prevent cell death.

We utilised the following anti-oxidants, previously shown to inhibit apoptosis in our cell lines [22–24], zinc chloride, PDTC, Dox., Tempo., BHA, and Phen. Peroxide production was detected as early as 10–15 min in HL-60 cells and this was evident with all of the cytotoxic agents used in this study (Fig. 4A). From Fig. 4B, the protection afforded by these anti-oxidants against PARP cleavage is obvious. The extensive correlation between PARP cleavage and subsequent apoptosis was once again evident as these inhibitors were effective against both the proteolytic cleavage of PARP and endonuclease activation.

The results from Fig. 4 identify ROI production as the earliest detectable change in leukaemic cells undergoing apoptosis. ROI production led to PARP cleavage then DNA fragmentation and apoptosis, all of these events were prevented using anti-oxidants. This is in agreement with the most recent results from our laboratory which suggest ROI production may mediate leukaemic apoptosis following cytotoxic insult [22].

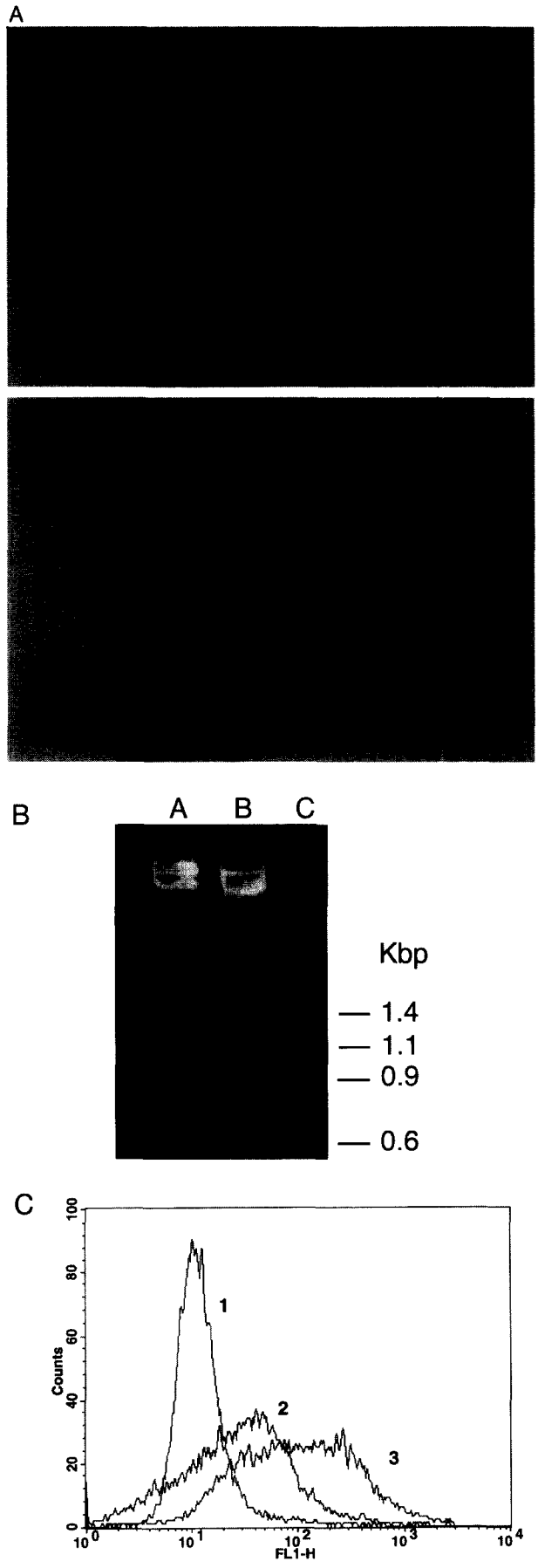

Fig. 2. (A) Morphology of: (1) untreated HL-60 cells and (2) Camp. treated cells, both taken after 3 h in culture. (B) DNA gel electrophoresis of: (lane A) untreated HL-60 3 h, (lane B) Camp. treated 2 h, (lane C) Camp. treated 3 h. (C) Tunel method on: (1) untreated HL-60 cells 3 h, (2) Camp. treated 2 h, (3) Camp. treated 3 h.

4. Discussion

The biochemical machinery required to initiate the programme for cell death is gradually being realised. Previous efforts were directed at the identification of the enzyme(s) responsible for the DNA fragmentation associated with apoptosis [2,9]. However, more recent results have shifted the emphasis from nuclear to cytoplasmic based components of the cell death process [13,14]. Early evidence suggesting the involvement of proteases during apoptosis arose from the realisation that CTLs and NK cells could kill using proteases. In conjunction with this, the results with *ced-3* related mammalian proteases suggest that the cytoplasm may contain critical components of the apoptotic cascade [13]. This has been clearly established in cell free systems [33,34].

In this study, we have demonstrated PARP cleavage as a common factor in response to cytotoxic insult in both HL-60 and U937 cells. This cleavage occurred prior to any other of the associated features of apoptosis such as DNA fragmentation and morphological deterioration. However, in the CML derived K562 cell line, previously shown to be extremely resistant to cytotoxic insult [31], no PARP cleavage was detectable with any of the cytotoxic agents screened. These agents also failed to produce DNA fragmentation in this cell line [31]. This shows the close correlation between PARP cleavage and subsequent endonuclease activation. This correlation has been linked to the inhibitory effect of PARP activity on endonuclease activity [19]. Once PARP is cleaved, this block is removed and the endonuclease is re-activated under these circumstances [19].

The specific involvement of these cysteine proteases has been established during apoptosis [15,16]. These experiments were required to distinguish these particular proteases from others such as proteinase K, fragmentin or chymotrypsin which will also induce apoptosis [35]. The use of specific inhibitors helped to identify the cysteine proteases as central components of the apoptotic process [15,16]. During the course of our work, effective inhibition of PARP cleavage and the resultant apoptosis were achieved using the protease inhibitors, NEM and IOD.

Recently, we have proposed ROI as a common mediator of apoptosis following cytotoxic insult to these leukaemic cells [22]. In this present study, both PARP cleavage and endonuclease activity appeared as closely associated events. As a result, we hypothesised that ROI may mediate both PARP cleavage and DNA fragmentation. In several earlier instances, we have successfully inhibited apoptosis using a range of antioxidants [22–24]. The involvement of reactive oxygen intermediates (ROI) was further implicated during apoptosis when it was observed that H_2O_2 production followed cytotoxic insult. This H_2O_2 production was detectable as early as 10–15 min following cytotoxic insult.

As a continuation of this earlier work, the present study demonstrated that peroxide production preceded PARP cleavage and furthermore, a number of anti-oxidants successfully prevented the cleavage of this enzyme. This suggested that peroxide was a common mediator of several of the biochemical events leading to apoptosis. In agreement with this, direct addition of hydrogen peroxide also induced PARP cleavage and apoptosis in these cells. Peroxide production has also

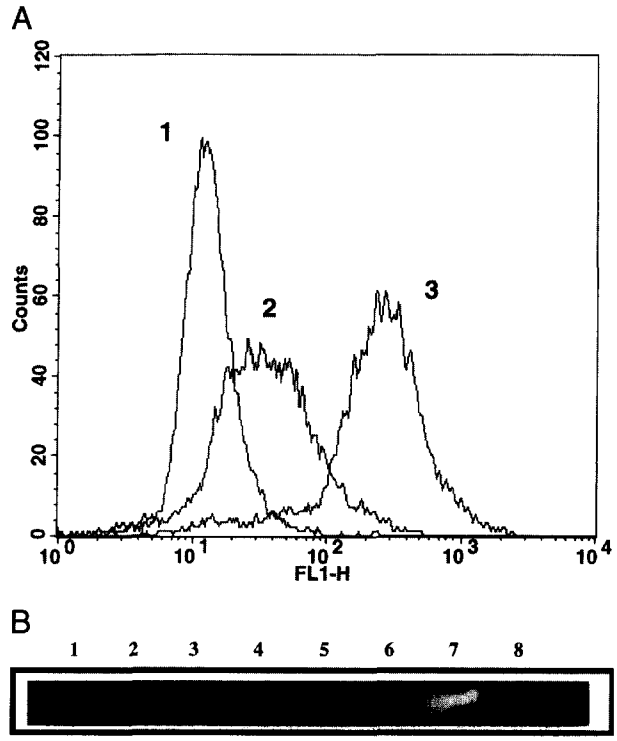

Fig. 4. (A) Peroxide production, which correlates with an increase in FL1-H fluorescence, was assessed 15 min following cytotoxic insult. (1) Untreated, (2) Act-D. treated, (3) UV treated. The other cytotoxic agents also produced peroxide to varying degrees (data not shown). (B) Inhibition of UV-induced PARP cleavage by a variety of anti-oxidants at 2 h. (1) Dox. 1 mM, (2) Zn 1 mM, (3) PDTC 40 μM, (4) Tempo. 1 mM, (5) BHA 200 μM, (6) Phen. 1 mM, (7) UV 10 min, (8) untreated HL-60 cells.

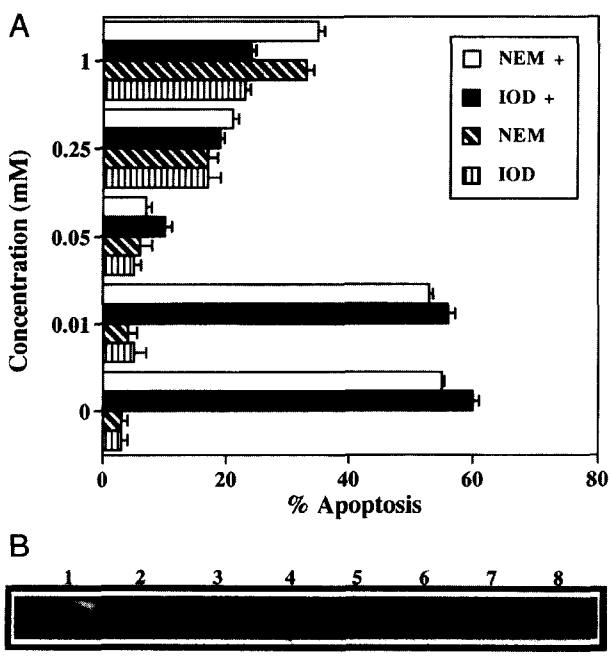

Fig. 3. (A) Dose-response inhibition of UV-induced apoptosis using NEM and IOD. Cells were either UV treated plus inhibitors (NEM +/IOD +) or treated with inhibitors alone (NEM/IOD). Apoptosis was assessed at 3 h. (B) Dose-dependent inhibition of UV-induced PARP cleavage in HL-60 cells by IOD lanes (2–4) and NEM lanes (5–7). (1) UV (positive control), (2) UV+250 μM IOD, (3) UV+50 μM IOD, (4) UV+10 μM IOD, (5) UV+250 μM NEM, (6) UV+50 μM NEM, (7) UV+10 μM NEM, (8) untreated control cells.

been recently reported during dexamethasone-induced apoptosis in thymocytes suggesting that oxygen radical production due to cytotoxic insult may be quite a common occurrence [36]. A similar report demonstrated anti-oxidant disruption in Jurkat cells and thymocytes during apoptosis [37]. In many model systems, oxidative stress has been linked to perturbation of the mitochondrial membrane which is a recognised site for oxygen radical generation [38]. To the contrary, we have found that mitochondrial membrane depolarisation does not appear to be a potential source of ROI in these cell lines [22].

Regardless of the growing number of reports, the relationship between oxidative stress and proteases has received scant attention. A report indirectly linking oxidative stress and proteases arose from the work of Talley and colleagues [32]. They demonstrated that TNF-α-induced apoptosis involved ROI formation which was effectively blocked by addition of an anti-oxidant (NAC) or by the overexpression of *bcl-2* or *crmA*. This may suggest that *bcl-2* and *crmA* were functioning as anti-oxidants. More direct evidence by Grune and co-workers [39] demonstrated that oxidatively modified proteins were more prone to proteolytic degradation and so this may explain the protection observed with anti-oxidants against PARP cleavage in our experiments. In line with this, ceramide-induced PARP cleavage was prevented using Bcl-2. Ceramide can provoke ROI production and so this inhibitory effect may be a direct result of the anti-oxidant ability of Bcl-2.

In the present study, the overall results established the early involvement of proteases during leukaemic cell apoptosis and provide further evidence that the cleavage of PARP is a common event following cytotoxic insult. We demonstrated a close association between PARP cleavage and subsequent endonuclease activation and suggest a common mediator, ROI, exists in these cells. ROI induced a number of the biochemical events detected, including PARP cleavage and DNA fragmentation. We have also recently reported the activation of the redox sensitive transcription factor NF-κB, by ROI, during apoptosis [22]. Furthermore, anti-oxidants prevented both NF-κB activation and the subsequent apoptosis in HL-60 leukaemic cells. Early detection of peroxide in these cell lines also suggest that anti-oxidants may act at the onset of the process and so these ROI and/or the sources of this ROI production may represent sites for therapeutic intervention and manipulation of cell death [22,38].

The discovery of cytoplasmic components of a process that was largely focused on nuclear events suggests that crucial pieces of the apoptotic puzzle are finally being realised. In addition, the observation that a growing number of cell lines experience changes in cellular redox status during the onset of apoptosis, may suggest a more general role for ROI in the regulation of apoptosis.

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