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A role for metals and free radicals in the induction of apoptosis in thymocytes

James T. Wolfe, David Ross**, Gerald M. Cohen*

MRC Toxicology Unit, Hodgkin Building, University of Leicester, P.O. Box 138, Lancaster Road, Leicester, LE1 9HN, UK

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Abstract Recent reports have implicated a possible but undefined role for reactive oxygen species in the induction and mediation of apoptosis. In the present study, the role of free radicals and metal ions in apoptosis induced in rat thymocytes by dexamethasone and etoposide was examined. Copper chelators, but not iron specific chelators, inhibited apoptosis induced by both these stimuli. Several antioxidants also possessed potent inhibitory effects. We therefore propose that diverse agents may induce apoptosis in thymocytes by a common mechanism involving a copper mediated Fenton reaction, generating site specific hydroxyl radicals, possibly as a result of activation of the redox sensitive transcription factor NF-κB.

Key words: Thymocyte apoptosis; Copper; Free radicals; NF-κB; Rat

1. Introduction

Apoptosis is a form of cell death characterized by cell shrinkage, membrane blebbing and DNA fragmentation [1]. It can be induced by a variety of stimuli such as ionizing radiation, glucocorticoids, chemotherapeutic agents and lymphokine deprivation [1,2]. Recently there has been an immense amount of interest in characterizing mechanisms underlying apoptosis because of the potential applications to treating diseases such as cancer, autoimmune disease and AIDS [2,3]. Although the signals which induce apoptosis in various systems vary markedly, the morphological features of apoptosis are conserved in diverse cell types [1,4]. This suggests a possible convergence of metabolic events which then results in activation of the cellular machinery responsible for apoptosis. Despite the identification of genes responsible for both apoptosis and its suppression [2,5], the critical biochemical events which result in apoptosis remain unclear.

Oxidative stress has been suggested to play a role as a common mediator of apoptosis [6] and recent independent observations in diverse systems support a role for oxidative mechanisms in the induction of apoptosis [7–12]. Aerobic organisms generate reactive oxygen species during normal metabolism and have consequently developed many antioxidant systems for purposes of cellular protection [13,14]. The hypothesis that such species may be utilized by cells to induce apoptosis is supported by the observations that oxidants or treatments which generate reactive oxygen species, such as hydrogen peroxide [7], *t*-butyl hydroperoxide [15], radiation [16] and tumor necrosis factor [17] induce apoptosis, while the antioxidants *N*-acetylcysteine [8,11], butylated hydroxyanisole [12], vitamin E analogs [18] and glutathione peroxidase [8] inhibit apoptosis to varying extents dependent on the biological system. In addition, the proto-oncogene bcl-2, which inhibits many forms of apoptotic cell death, has been suggested to mediate an antioxidant pathway to prevent apoptosis [8,9].

There has not been a systematic study of the potential role of free radicals in apoptosis in a single well-defined system. It remains unclear if radicals are critical mediators of apoptosis, and if so the species involved, their mechanism and sites of generation and the process responsible for activation of the apoptotic machinery. In order to address these problems, we have examined the effects of metal chelators and inhibitors of radical mediated processes on dexamethasone and etoposide induced apoptosis in rat thymocytes, a well characterized model system for the study of apoptosis [19,20]. We demonstrate a key role for copper in the induction of apoptosis in rat thymocytes, most probably mediated by free radicals generated via a Fenton reaction.

2. Materials and methods

2.1. Reagents

Trolox, 1,7- and 4,7-phenanthroline, *N,N'*-diphenyl-1,4-phenylenediamine (DPDD) and 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO) were obtained from Aldrich Chemical Co. Ltd., Dorset, UK. 1,2-Diethyl-3 hydroxypyridin-4-one (CP-94) was a gift from Dr. R.C. Hider, Kings College, London, UK. Unless otherwise stated all other chemicals were purchased from Sigma Chemical Co., Dorset, UK.

2.2. Preparation and treatment of rat thymocytes

Male Fischer 344 rats (65–85 g), bred at the University of Leicester and allowed food and water ad libitum, were sacrificed with an overdose of Sagatal (pentobarbitone, 60 mg/kg i.p.) (May & Baker Ltd, Dublin, Ireland). Thymocytes were isolated as previously described [21] and diluted to a final density of 20×10^6 cells/ml with RPMI 1640 (Gibco, Paisley, UK) gassed with 95% O_2/5% CO_2 and supplemented with 10% fetal bovine serum (Gibco). Isolated thymocytes were incubated for 4 h either alone (as controls), with etoposide (10 μM) or dexamethasone (0.1 μM) at 37°C in a humidified 95% air/5% CO_2 atmosphere in a final volume of 0.5 ml in 24-well plates.

Antioxidants/chelators, where used, were added in nontoxic concentrations (as assessed by propidium iodide exclusion), 10 min prior to the addition of dexamethasone or etoposide unless stated. *N*-Acetylcysteine was dissolved in PBS and neutralized before addition. Other compounds were dissolved in either ultrapure (18 MΩ) water (EDTA,

*Corresponding author. Fax: (44) (533) 525 616.

**On sabbatical assignment from the School of Pharmacy, University of Colorado, Health Sciences Center, Denver, CO 80262, USA.

Abbreviations: Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl; PBN, α-phenyl-*tert*-butylnitrone; BHA, butylated hydroxyanisole; DMPO, 5,5'-dimethyl-1-pyrroline-*N*-oxide; PDTC, pyrrolidine dithiocarbamate; CP-94, 1,2-diethyl-3-hydroxypyridin-4-one; DPDD, *N,N'*-diphenyl-1,4-phenylenediamine; Detapac, diethylenetriaminepentaacetic acid.

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EGTA, Detapac, desferrioxamine, CP-94, PDTC, Tempol, DMPO and ascorbate) ethanol (neocuproine, 1,10-phenanthroline and its analogs, BHA, vitamin E succinate and PBN) or DMSO (Trolox and DPPD). The volume of vehicle added was normally 1% v/v but was reduced to 0.5% v/v in the case of DMSO. Controls using vehicle alone were performed in each experiment. All chelators were weighed out using non-metal spatulas.

2.3. Quantitation of apoptosis by flow cytometry

Subsequent to treatment, thymocytes were incubated with Hoechst 33342 and propidium iodide to quantify the percentage of apoptotic cells, essentially as previously described [22], except that analysis was performed using the Becton Dickinson FACS Vantage flow cytometer. The cells with high blue fluorescence due to increased Hoechst 33342 intensity have previously been shown to be apoptotic based on a number of criteria including decreased size, ultrastructure and the presence of DNA ladders [22,23].

2.4. Agarose gel electrophoresis

After incubation at 37°C, 2×10^6 cells/lane were analysed for the presence of oligonucleosomal fragmentation on 1.8% agarose gels as described [24].

2.5. Statistical analysis

Data were taken from experiments performed on separate days using different animals. Data were analyzed either by a one way analysis of variance followed by a post-hoc Dunnett's *t*-test for comparison of multiple groups to a single control or in some cases by a paired Student's *t*-test. The significance level was set at $P \leq 0.05$.

3. Results

3.1. Effects of iron and copper chelators on apoptosis

Apoptosis was induced in rat thymocytes by both the glucocorticoid, dexamethasone [1,2,4] and the DNA topoisomerase II inhibitor, etoposide (VP-16) [25] and the ability of both metal chelators and antioxidants to modulate apoptosis by these agents was examined. The thiocarbamate PDTC, which has both metal chelating and antioxidant properties [26], caused a concentration dependent inhibition of both dexamethasone and etoposide induced apoptosis (Fig. 1A). In order to examine the role of metals in more detail, the effects of other chelators were then examined. 1,10-Phenanthroline, which is used to chelate both iron and copper [27], also effectively inhibited apoptosis induced by both etoposide and dexamethasone (Fig. 1B). In contrast, the non-chelating analogs of phenanthroline, 1,7-phenanthroline (100 μM) and 4,7-phenanthroline (100 μM) [28] had relatively little or no effect on either dexamethasone or etoposide induced apoptosis (Table 1), suggesting that the effects observed were due to the metal chelating properties of the phenanthroline molecule. These results suggested the involvement of either iron or copper in the induction of apoptosis in thymocytes.

The membrane permeable iron III chelators, CP-94 [29] and desferrioxamine [29] had no effect on apoptosis (Fig. 1C and D). Similar results were obtained when the preincubation period of desferrioxamine with cells was extended from 10 min to 2 h prior to dexamethasone or etoposide addition. Similarly ferrozine, a compound used to chelate iron II ions [30], had little effect on etoposide or dexamethasone induced apoptosis (Table 1). In marked contrast to the iron chelators, neocuproine, a phenanthroline with a high affinity for copper ions [28,31], was a potent inhibitor of both etoposide and dexamethasone induced apoptosis (Fig. 1E). The effects of EDTA, EGTA and Detapac were also examined in this system but these compounds had little effect on apoptosis induced by either stimuli (Table 1), probably due to their limited membrane permeability under physiological conditions [29]. The effects of 1,10-phenanthroline and neocuproine suggest an important role for copper but not iron in the induction of apoptosis in thymocytes.

3.2. Inhibition of apoptosis by radical scavengers

The ability of traces of soluble iron or copper to form highly reactive hydroxyl radicals (•OH) by a metal catalyzed Fenton reaction is well known [32,33]. We therefore examined the effects of the nitroxide spin probe, Tempol, and the spin trap PBN, which are both membrane-permeable free radical scavengers [34–36]. Tempol effectively inhibited both etoposide and dexamethasone induced apoptosis to control levels (Fig. 1F) supporting a role for free radicals in apoptosis. Pretreatment of cells with PBN also markedly inhibited apoptosis (Fig. 1G). The involvement of free radicals was also supported by the modest inhibition of both etoposide and dexamethasone induced apoptosis by the antioxidant BHA and the spin trap DMPO. DMPO (50 mM) inhibited both dexamethasone (32.9 ± 2.0% to 24.3 ± 2.2%, $n = 4$, $P \leq 0.05$) and etoposide (46.7 ± 4.5% to 41.6 ± 4.1%, $n = 4$, $P \leq 0.05$) induced apoptosis. BHA (0.1mM) also inhibited dexamethasone (33.8 ± 2.9% to 25.9 ± 3.5%, $n = 5$, $P \leq 0.05$) and etoposide (49.7 ± 4.5% to 43.2 ± 3.4%, $n = 4$, $P \leq 0.05$) induced apoptosis. Several other antioxidants, including ascorbate and Trolox, did not markedly inhibit apoptosis induced by either stimuli (Table 1). These results indicated that the antioxidants and chelators used in Table 1 exhibited little or no inhibitory effects on apoptosis compared to those obtained with PDTC, 1,10-phenanthroline, neocuprine, Tempol or PBN (Fig. 1).

Table 1
Effects of antioxidants and chelators on apoptosis

Addition[a]	Concentration	Inhibition[b]	
		Dexamethasone[c]	Etoposide[c]
Vitamin E succinate	25 μM	1.8	4.0
	50 μM	6.2	5.3
N,N'-diphenyl-1,4-phenylenediamine (DPPD)	100 μM	2.3	2.8
Ascorbic acid	100 μM	+0.4	1.5
N-Acetylcysteine	10 mM	1.4	+0.9
	30 mM	3.8	5.2
Trolox	100 μM	+0.1	6.0
EDTA	1 mM	3.7	2.3
EGTA	1 mM	4.6	4.2
Detapac	1 mM	0.2	4.2
Ferrozine	100 μM	1.8	1.4
1,7-Phenanthroline	100 μM	4.2	7.2
4,7-Phenanthroline	100 μM	4.1	2.8

A concentration range of each compound was examined in order to establish a non-toxic concentration. The results represent either single values or means of two determinations.
Thymocytes were incubated for 10 min at 37°C prior to a further incubation of 4 h in the presence of either dexamethasone (0.1 μM) or etoposide (10 μM). Apoptosis was then assessed by flow cytometry.
Values shown represent the decrease in the total percentage of cells undergoing apoptosis induced by either dexamethasone or etoposide. Values preceeded by a plus sign showed an increase in apoptosis.
% apoptosis induced by dexamethasone and etoposide were 34.2 ± 2.2 and 49.7 ± 6.6 (Means ± S.E., $n = 8$) respectively.

3.3. Copper but not iron chelators inhibit internucleosomal cleavage of DNA

Cleavage of DNA into nucleosomal fragments of 180–200 base pairs or multiples thereof has often been considered as the biochemical hallmark of apoptosis [37]. Although more recent studies have suggested that DNA is initially cleaved into large fragments of 200–300 and 30–50 kilobase pairs in length [25,38], possibly representing rosettes and loops in higher order chromatin structure [39], the recognition of nucleosomal ladders is still a useful marker of apoptosis, particularly in thymocytes. A small amount of internucleosomal cleavage was observed in control cells (Fig. 2A and B, lane 1) consistent with a small amount of control apoptosis (Fig. 1). Following incubation for 4 h with dexamethasone (Fig. 2A, lane 2) or etoposide (Fig. 2B, lane 2) a marked increase in internucleosomal cleavage was observed in agreement with many previous studies [25,37]. Co-incubation of thymocytes in the presence of dexamethasone or etoposide in the presence of the copper chelators, neocuproine, 1,10-phenanthroline and PDTC, prevented this increase (Figs. 2A and B, lanes 3–5, respectively) consistent with the observed inhibition of apoptosis as assessed by flow cytometry (Fig. 1). Similarly, Tempol, the free radical scavenger, markedly inhibited dexamethasone (Fig. 2A, lane 6) and etoposide (Fig. 2B, lane 6) induced internucleosomal cleavage in agreement with its ability to inhibit apoptosis (Fig. 1). In contrast, the iron chelators, CP-94 and desferrioxamine, did not inhibit dexamethasone or etoposide induced increases in internucleosomal cleavage (Figs. 2A and B, lanes 7 and 8, respectively) consistent with their inability to inhibit apoptosis as assessed by flow cytometry (Fig. 1). BHA appeared to cause a slight decrease in DNA laddering while *N*-acetylcysteine had little effect (Fig. 2A and B, lanes 10 and 9 respectively). Thus compounds which inhibited apoptosis also inhibited internucleosomal cleavage of DNA, while those compounds which had little effect on apoptosis similarly did not inhibit internucleosomal DNA fragmentation.

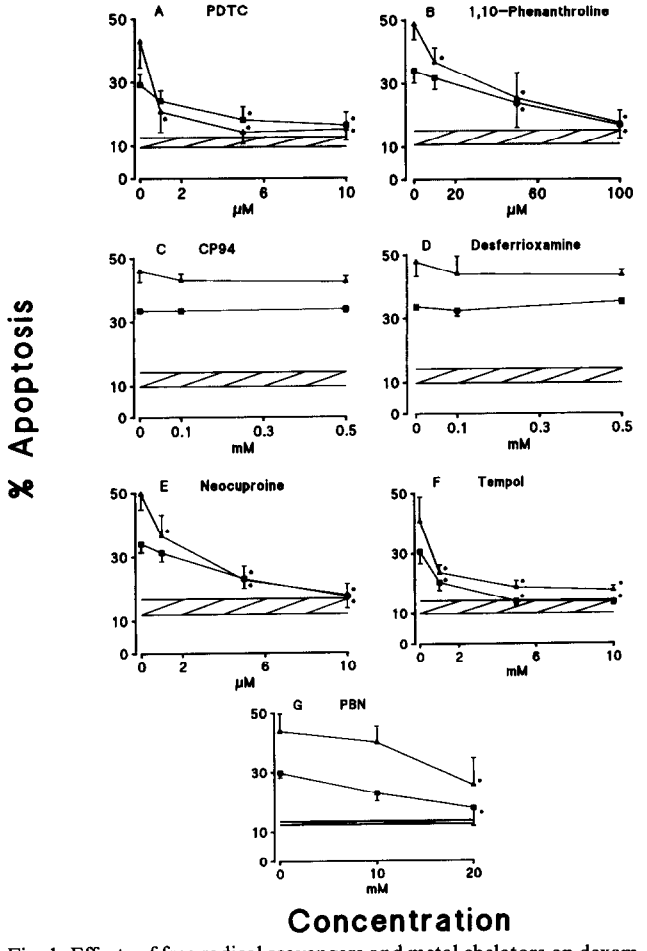

Fig. 1. Effects of free radical scavengers and metal chelators on dexamethasone and etoposide induced apoptosis. Thymocytes were incubated for 4 h with either dexamethasone (0.1 μM) (■) or etoposide (10 μM) (△) and then apoptosis assessed by flow cytometry. Thymocytes were incubated in the presence of the indicated concentrations of various compounds and the results represent the mean ±S.E.M. of at least three determinations. The shaded area represents the control level of apoptosis without any added inducing agent. ‹notes significantly different to the value obtained with either dexamethasone or etoposide alone, $P < 0.05$.

4. Discussion

Our data provide strong evidence for the involvement of metals and free radicals in the induction of apoptosis in rat thymocytes following treatment with two agents, dexamethasone and etoposide, with different mechanisms of action. The observations that both metal chelators and radical scavengers inhibited dexamethasone and etoposide induced apoptosis, suggested that hydroxyl radicals, generated via an iron or copper-mediated Fenton reaction, may play a role in the induction of apoptosis. It has previously been shown that 1,10-phenanthroline and the hydroxyl radical scavenger, DMSO, inhibited apoptosis in porcine aortic endothelial cells via a mechanism presumed to involve blocking of iron-mediated Fenton chemistry [10].

4.1. Copper but not iron is involved in the induction of apoptosis

One of the most interesting aspects of our data was the lack of effect of either the ferric ion chelators, CP-94 and desferrioxamine [29], or the ferrous ion chelator, ferrozine [30], together with the observation that both 1,10-phenanthroline and neocuproine were potent inhibitors of both dexamethasone and etoposide induced apoptosis (Figs. 1 and 2). 1,10-Phenanthroline inhibits both copper and iron-mediated Fenton reactions but neocuproine only blocks the copper-mediated reaction [27]. Thus our data supports a critical role for copper ions in dexamethasone and etoposide induced apoptosis. However, we cannot totally exclude the possibility of the involvement of other metals, as metal chelators are not totally specific for a single metal, although the binding constants for different metals vary considerably. 1,10-Phenanthroline and even neocuproine, which is used as an analytical reagent for the detection of copper [40], will also bind zinc, but with a binding constant three to four orders of magnitude lower than copper [28,31]. Paradoxically, chelation of intracellular zinc has been reported to induce apoptosis in rat thymocytes [41]. Thus data obtained in this study together with the relative specificity of neocuproine for copper strongly supports the suggestion that this is the metal involved in activation of apoptosis in thymocytes.

4.2. Involvement of free radicals in dexamethasone and etoposide induced apoptosis

A marked inhibition of both dexamethasone and etoposide induced apoptosis was obtained using the spin probe Tempol

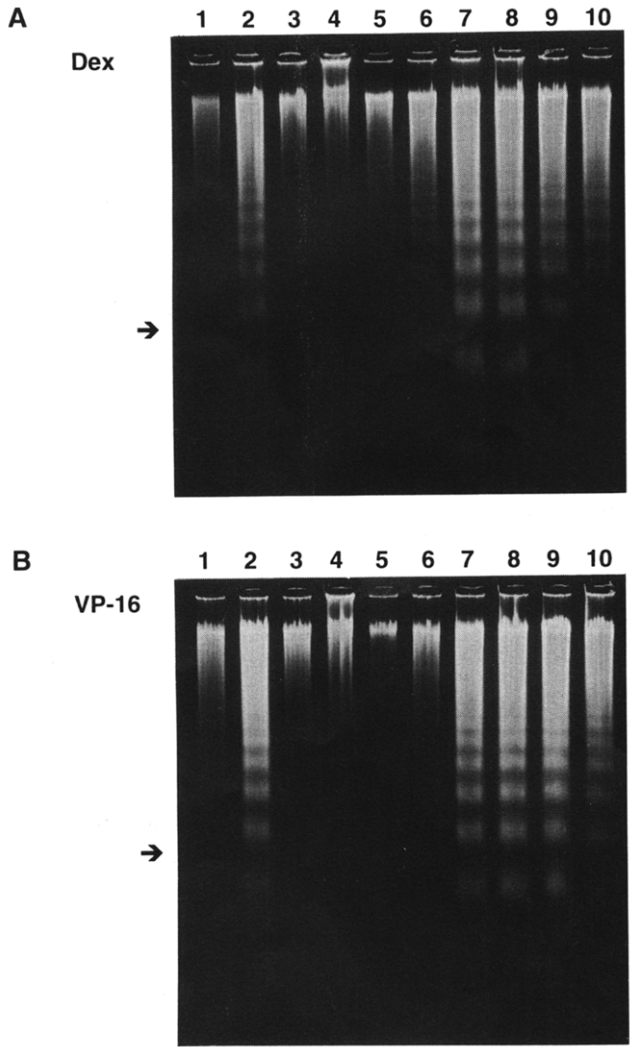

Fig. 2. Inhibition of internucleosomal cleavage by copper but not iron chelators. Thymocytes were incubated for 4 h, (A) with dexamethasone (0.1 μM), or (B) with etoposide (10 μM) in the presence or absence of various compounds. Thymocytes were incubated either alone (lane 1) or with dexamethasone or etoposide either alone (lane 2) or in the presence of neocuproine (10 μM) (lane 3), 1,10-phenanthroline (100 μM) (lane 4), PDTC (10 μM) (lane 5), Tempol (10 μM) (lane 6), CP-94 (500 μM) (lane 7), desferrioxamine (500 μM) (lane 8), *N*-acetylcysteine (30 mM) (lane 9) and BHA (100 μM) (lane 10). The cells were examined for DNA laddering by agarose gel electrophoresis. The distance migrated by the 564 base pair standard is marked.

and the spin trap PBN, supporting a role for free radicals in apoptosis. This role was also supported by the modest inhibitory effects of the spin trap DMPO and the antioxidant BHA. The concentrations of Tempol used were similar to those used to protect against X-ray induced cytotoxicity and hydrogen peroxide-mediated mutagenicity [42,43], whilst those of PBN and DMPO were similar to those used in ESR spin trapping experiments [44,45]. The spin traps and probes used are nonspecific free radical scavengers and are not specific for oxygen radicals [34,44,46,47]. It is feasible therefore that radicals other than the hydroxyl radical may be involved in the induction of apoptosis. The greater efficacy of PBN and Tempol compared to DMPO to inhibit apoptosis may be a result of their increased cellular penetration due to increased lipophilicity. The octanol/water partition coefficients for DMPO, PBN and Tempol are 0.03, 11.4 and 5.3, respectively [34–36]. Nitroxides, such as Tempol, also demonstrate superoxide dismutase like activity and under certain conditions can oxidize ferrous ions complexed to DNA, thereby preventing metal mediated reduction of hydrogen peroxide to hydroxyl radicals at a critical site [48]. Tempol also oxidizes ferrous ions chelated to ADP and blocks iron-ADP dependent lipid peroxidation [35]. The effects of Tempol observed in our study may therefore reflect more than one mechanism of action, although the interaction of nitroxides with complexed ferrous ions is markedly slower ($k \cong 40$ $M^{-1} \cdot s^{-1}$) [48] than with radicals ($k \cong 10^7$–10^8 $M^{-1} \cdot s^{-1}$) [34,46]. The antioxidant BHA and the spin trap DMPO caused only a modest inhibition of apoptosis whilst several other antioxidants had little or no inhibitory effects (Table 1). Their lack of inhibition may be due to an inability to achieve adequate concentrations at the sites of free radical generation. Alternatively, since some compounds such as *N*-acetylcysteine have been shown to effectively inhibit apoptosis in other systems [8,11], their inactivity in our study may reflect a varying susceptibility of different apoptotic systems to inhibition. Previous independent observations have documented the ability of free radical scavengers to inhibit apoptosis to varying degrees in diverse systems [8,11,12,18]. These data are consistent with our results and therefore suggest that a role for free radicals in apoptosis may not be limited to thymocytes.

4.3. Potential mechanisms of apoptosis

It is possible that the inhibitory metal chelators and antioxidants used in our study exert their effects independently. Whether the requirement for metals reflects the involvement of specific metalloenzymes or metal-dependent transcription factors requires further investigation. Our findings that both metal chelators and radical scavengers inhibited dexamethasone and etoposide induced apoptosis, however, suggested the involvement of a copper mediated Fenton reaction. Copper is an efficient mediator of this reaction, generating highly reactive hydroxyl radicals, which can cause oxidative damage to proteins, lipids and site specific damage in DNA [27,32,49–52]. A copper mediated Fenton reaction also requires the presence of hydrogen peroxide and it is not known whether addition of etoposide or dexamethasone to thymocytes stimulates peroxide production.

A major objection to a role for oxygen radicals in apoptosis concerns the mechanism whereby the specificity of the apoptotic process could be maintained with such indiscriminate reactants. Although not all radicals are reactive, many, such as $^{\bullet}OH$, react rapidly with biological macromolecules. Metals such as copper II are relatively insoluble and remain in solution only by chelation with cellular components [32,33]. As the metal is localized at a specific site, any highly reactive free radicals formed will attack close to their site of generation resulting in localized damage [32,33]. This mechanism would explain how reactive radicals may react in a controlled manner to activate cellular machinery leading to apoptosis. One such attractive possibility is a metal and radical dependent transcriptional activation leading to apoptosis. It is interesting that 1,10-phenanthroline blocks the binding of an activated glucocorticoid-receptor complex to DNA [53] and these authors suggested that the activated complex was a metalloprotein. Oxidative stress is

known to activate the transcription factor NF-κB and antioxidants can inhibit this process [54,55]. Dithiocarbamates are known inhibitors of NF-κB activation and the most potent inhibitor identified in a recent study was PDTC [26], which in our hands markedly inhibited both dexamethasone and etoposide induced apoptosis. These data suggest that NF-κB may be a critical modulator of apoptosis and Sikora et al. [56] have recently shown that in thymocytes both AP-1 and NF-κB are activated during apoptosis induced by heat shock and dexamethasone. Activation of NF-κB as a mechanism underlying apoptosis in thymocytes and the role of copper and free radicals in this process are currently under investigation.

In summary, our data demonstrate a critical role for metals, specifically copper, and free radicals in the induction of apoptosis from diverse stimuli in rat thymocytes. We suggest that copper-mediated Fenton reactions generate site specific free radicals, such as hydroxyl radicals, leading to apoptosis possibly as a result of activation of the transcription factor, NF-κB.

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