Constitutive nuclear NF κ B/rel DNA-binding activity of rat thymocytes is increased by stimuli that promote apoptosis, but not inhibited by pyrrolidine dithiocarbamate

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Rat thymocytes spontaneously undergo apoptotic death in cell culture, and are also sensitive to the induction of apoptosis by various stimuli. We show that unstimulated thymocytes constitutively express a p50-containing nuclear factor κ B (NF κ B)/rel DNA-binding activity in their nuclei. When the cells were fractionated by density-gradient centrifugation this activity was found to be most pronounced in immature CD4⁺8⁺ thymocytes, the cell population that undergoes selection by apoptosis *in vivo* and that is most sensitive to external inducers of apoptosis *in vitro*. The intensity of the NF κ B/rel protein–DNA complex was significantly enhanced 30 min after exposing thymocytes to methylprednisolone or etoposide, two agents well known to induce apoptosis in these cells. Expression

of this DNA-binding activity therefore correlates with the subsequent occurrence of apoptosis. By analogy to other systems, it has been suggested that antioxidants such as pyrrolidine dithiocarbamate (PDTC) inhibit thymocyte apoptosis by preventing the activation of an NF κ B/rel transcription factor. However, we have found that etoposide induces a very similar enhancement of the NF κ B/rel DNA-binding activity in the presence or absence of PDTC, despite a pronounced inhibition of apoptotic DNA fragmentation in the former situation. Dithiocarbamates therefore do not exert their anti-apoptotic activity in thymocytes by inhibiting the activation of this transcription factor.

INTRODUCTION

Although apoptotic cell death is widely believed to be essential for the development and survival of multicellular organisms, the molecular mechanisms underlying this phenomenon are poorly understood. The activation of proteases related to interleukin 1- β converting enzyme is thought to be an early event in apoptosis in many (if not all) cells [1,2], although the critical protein substrates for these enzymes are unknown. Various antioxidants have also been observed to delay or inhibit apoptotic cell death in model systems as diverse as neuronal cells deprived of nerve growth factor, fibroblasts exposed to tumour necrosis factor- α (TNF- α), or T-cell hybridomas stimulated through their T-cell receptor [3–7]. The biochemical explanation for this antioxidant effect, and the way in which it interacts with proteolysis, is also unclear.

We and others have recently shown that antioxidants delay the apoptosis that occurs after thymocytes are exposed to glucocorticoid hormones, agents that elevate intracellular Ca²⁺ or the topoisomerase II inhibitor etoposide [8,9]. Thymocyte apoptosis is also variably sensitive to inhibition of protein synthesis; for example the spontaneous apoptosis of cells that occurs in culture is unaffected by the presence of cycloheximide, while the increased rate of apoptosis occurring in response to glucocorticoid hormones or etoposide is strongly inhibited by the same compound [10–12]. Although alterations in the transcription and translation of a conserved subset of genes are not common events in the activation of all forms of apoptosis, in specific situations such as etoposide-induced thymocyte apoptosis they may be essential.

The biological activities of several transcription factors are regulated both at the level of nuclear translocation and DNA

binding by alterations in intracellular redox and/or proteolysis, and it has been suggested that changes of this type may be involved in the activation of glucocorticoid and etoposide-induced thymocyte apoptosis [9]. Proteins in the nuclear factor κ B (NF κ B)/rel family of transcription factors share a conserved amino acid domain involved in both DNA binding and protein–protein dimerization [13]. A cytoplasmic form of the NF κ B/rel transcription factor complexed with an inhibitor of nuclear translocation (I κ B) has been extensively characterized in many mammalian cell lines [13]. Destruction of I κ B by the concerted action of kinases and proteases is promoted by various stimuli including oxidative stress [14–17], thereby liberating the transcription factor and allowing its migration into the nucleus. In contrast, the DNA binding of both NF κ B/rel and another well-characterized transcription factor AP-1 depends on the presence of reduced cysteine residues, and is consequently inhibited by thiol oxidation [18–21]. Pyrrolidine dithiocarbamate (PDTC) is one of the most potent inhibitors of NF κ B/rel activation described [22,23], while Bessho et al. [24] and Wolfe et al. [9] have both shown that the same compound is an effective inhibitor of thymocyte apoptosis. As PDTC possesses both metal-chelating and radical-scavenging properties, it has been concluded that it exerts these cellular effects by acting as an antioxidant [9,22]. We have recently reported that thymocytes experience an oxidative stress at an early stage of apoptosis [8], which by analogy with other systems may be sufficient to permit nuclear translocation of NF κ B/rel. Some preliminary indication of NF κ B/rel activation following exposure of thymocytes to dexamethasone has already been obtained [25], prompting us to investigate whether this event was redox-sensitive. We now report that immature CD4⁺CD8⁺ rat thymocytes constitutively express a NF κ B/rel DNA-binding activity in their nuclei.

Abbreviations used: DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; MPS, 6- α -methylprednisolone; NF κ B, nuclear factor κ B; NP-40, Nonidet P-40; PDTC, pyrrolidine dithiocarbamate; PE, phycoerythrin; TNF- α , tumour necrosis factor- α .

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Although an increase in this activity occurs within 30 min of exposing cells to glucocorticoids or etoposide, it is not blocked by PDTC and is therefore unlikely to be a critical redox-sensitive event in the activation of thymocyte apoptosis.

MATERIALS AND METHODS

Materials

The following suppliers were used: Amersham (ECL Western blotting detection reagents and T4 polynucleotide kinase), Bio-Rad (acrylamide and minigel system), DuPont NEN ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$), Pharmacia (Percoll and poly[dI-dC]), Pierce [goat anti-(rabbit IgG) peroxidase-conjugated antibody and BCA protein assay kit], Santa Cruz Biotechnology (oligonucleotide probes and anti-NF κ B/rel antibodies), Sartorius (nitrocellulose membrane), Serotec [fluorescein isothiocyanate (FITC)-conjugated mouse anti-(rat CD8) and phycoerythrin (PE)-conjugated mouse anti-(rat CD4) monoclonal antibodies], and Sigma [etoposide and 6- α -methylprednisolone (MPS)].

Thymocyte culture and extraction

Suspensions of rat thymocytes were prepared and cultured as described previously [8]. At the end of each experiment thymocytes were collected by centrifugation and processed to obtain extracts of cytoplasmic and nuclear proteins [26]. Briefly, the cells were homogenized on ice in a hypotonic buffer [10 mM Hepes, 20 mM KCl, 1 mM MgCl₂, pH 8.0, with 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM 1,10-phenanthroline and 100 μ M leupeptin] containing 0.5% Nonidet P-40 (NP-40). The homogenates were left for 20 min on ice, and then centrifuged at 3000 *g* for 15 min at 4 °C to pellet nuclei. The supernatant (cytoplasmic extract) was collected and aliquots frozen at -80 °C. The nuclear pellet was washed once in hypotonic buffer and then resuspended in a high-salt buffer (20 mM Hepes, 400 mM NaCl, 1 mM MgCl₂, pH 8.0, with 0.5 mM DTT, 1 mM PMSF, 1 mM 1,10-phenanthroline and 100 μ M leupeptin) containing 0.1% NP-40. Each sample was vortexed vigorously, placed on ice for 30 min, and then centrifuged at 15000 *g* for 30 min at 4 °C to pellet debris. The supernatant (nuclear extract) was again collected, divided into aliquots and frozen at -80 °C.

Percoll fractionation

Discontinuous Percoll gradients were prepared and used at 4 °C as described by Slater et al. [8]. Five different cell fractions are obtained by this method (designated fractions 1–5); our previous analysis has shown that cells in fraction 4 are pre-apoptotic and those in fraction 5 apoptotic [8]. The cells in each fraction were washed once in PBS, pH 7.4, and then either extracted as described above or phenotyped by flow cytometry (see below).

Flow cytometry

Thymocytes (either unfractionated or recovered from a Percoll gradient) were washed in PBS, pH 7.4, and then resuspended in the same buffer supplemented with 1% BSA and 0.1% NaN₃. PE-conjugated mouse anti-(rat CD4) (1:5 dilution) and FITC-conjugated mouse anti-(rat CD8) (1:40 dilution) monoclonal antibodies were added, and the cells incubated on ice for 30 min. After two washes, 1×10^6 cells were analysed by flow cytometry on a Becton Dickinson FACScan. Following excitation at 488 nm, green fluorescent CD8⁺ cells (emission 530 nm) and red fluorescent CD4⁺ cells (emission 585 nm) were identified and counted using appropriate gating. PE- and FITC-conjugated

mouse IgGs were used as negative controls for each antibody. Results are expressed as a percentage of total thymocytes with a surface phenotype of CD4⁻8⁻, CD4⁺8⁺, CD4⁺8⁻ or CD4⁻8⁺.

Electrophoretic mobility shift assay (EMSA)

NF κ B/rel consensus (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and mutant (5'-AGT TGA GGC GAC TTT CCC AGG C-3') oligonucleotides were end-labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using T4 polynucleotide kinase. Nuclear extracts (typically 4–5 μ g of protein) were preincubated for 10 min at room temperature in binding buffer [60 mM Hepes, 150 mM NaCl, 15 mM MgCl₂, pH 8.0, with 3 mM EDTA, 3 mM PMSF, 3 mM DTT and 35% (w/v) glycerol] containing 20 μ g/ml poly[dI-dC], before ^{32}P -labelled oligonucleotides were added and the incubation continued for a further 30 min. Each extract was then subjected to EMSA in a non-denaturing 4% acrylamide gel buffered with 50 mM Tris/HCl, 380 mM glycine and 2 mM EDTA, pH 8.6. Autoradiography was conducted after fixing and drying each gel.

Western blotting

Samples (25 μ g of protein per lane) were electrophoresed through an SDS/12% polyacrylamide minigel under reducing conditions, and then transferred to a nitrocellulose membrane in 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid, pH 11.0, with 20% (v/v) methanol. Each nitrocellulose membrane was blocked in a Tris/Tween/BSA buffer (50 mM Tris, 500 mM NaCl, 1% BSA and 0.05% Tween-20, pH 7.5) containing 5% (w/v) milk powder, incubated for 1 h with the primary antibody (1:1000 dilution in Tris/Tween/BSA buffer), washed and then incubated for 1 h with an anti-(rabbit IgG) peroxidase-conjugated secondary antibody (1:40000 dilution in Tris/Tween/BSA buffer). Protein-antibody complexes were subsequently detected using the ECL system (Amersham). Primary antibodies (obtained from Santa Cruz) were rabbit polyclonals raised against peptide fragments corresponding to (a) a 15-amino-acid sequence in the nuclear localization region of human NF κ B p50, (b) amino acids 3–19 in the N-terminal region of human NF κ B p65 (rel A), or (c) amino acids 540–558 in the C-terminus of murine rel B.

Other methods

Thymocyte DNA fragmentation was quantified using the diphenylamine reagent [27]. Protein concentrations were determined using the BCA protein assay kit (Pierce).

RESULTS

To investigate the status of the NF κ B/rel protein family in normal thymocytes, cell suspensions prepared from thymus of 6-week-old rats were directly extracted without further treatment to yield cytoplasmic (low-salt) and nuclear (high-salt) fractions. When protein in the nuclear extract was subjected to an EMSA with an oligonucleotide probe containing the consensus NF κ B/rel DNA-binding element, a DNA-protein complex was consistently resolved (Figure 1a). The intensity of the gel shift was greatly reduced when the experiments were repeated with a probe containing a single base pair change in the DNA-binding site (Figure 1a), suggesting that a specific protein-DNA interaction involving a NF κ B/rel family member(s) was being observed. DNA binding of the labelled probe was competed with by the addition of an excess of unlabelled oligonucleotide (Figure 1b) while, as expected, addition of increasing amounts of nuclear protein into the assay enhanced the intensity of the specific gel-

Figure 1 Identification of a constitutive NF κ B/rel DNA-binding activity in nuclear extracts from unstimulated rat thymocytes

(a) Nuclear extracts (3 μ g of protein per sample) were prepared either directly from unstimulated thymocytes (lanes 1 and 2) or after incubating the cells for 30 min with 500 nM MPS (lanes 3 and 4). EMSA were then performed with a consensus (lanes 1 and 3) or mutated (lanes 2 and 4) NF κ B/rel-specific oligonucleotide probe. (b) Nuclear extracts (4 μ g of protein per sample) from unstimulated thymocytes were preincubated for 10 min with increasing concentrations of the unlabelled consensus oligonucleotide probe, and then subjected to EMSA. (c) Nuclear extracts from unstimulated thymocytes (1–30 μ g of protein per sample) were analysed in an EMSA. On each autoradiograph, a filled arrow indicates the specific NF κ B/rel gel-shifted complex, * marks a non-specific complex and an open arrow is unbound probe.

Table 1 Surface CD4 and CD8 antigen phenotypes of rat thymocytes recovered from a Percoll gradient

Thymocytes were layered on to a discontinuous Percoll density gradient (3 ml each of 95%, 80%, 70%, 65%, 60% and 55% Percoll), and then separated by centrifugation. Cells at each interface were collected, washed and the surface antigen phenotype determined by flow cytometry (10^6 cells counted per fraction). Results show percentages of total cells in each category.

Percoll fraction	Thymocyte phenotype (% of total population)			
	CD4 ⁻ 8 ⁻	CD4 ⁺ 8 ⁺	CD4 ⁺ 8 ⁻	CD4 ⁻ 8 ⁺
1	4.1	58.5	22.9	14.5
2	0.4	90.3	4.3	5.0
3	0.4	96.2	1.3	2.1
4	0.6	98.2	0.1	1.1
5	16.8	79.1	1.8	2.3
Unseparated	1.3	89.5	5.0	4.2

shifted band, but did not reveal the presence of any other DNA-binding proteins (Figure 1c).

Thymocyte suspensions comprise a heterogeneous population of cells with precursor CD4⁻8⁻ cells; immature CD4⁺8⁺ thymocytes and mature CD4⁺8⁻ or CD4⁺8⁺ thymocytes each being present [28]. The immature CD4⁺8⁺ thymocyte population is also heterogeneous, as these cells are actively undergoing

Figure 2 Nuclear NF κ B/rel DNA-binding activity in different subpopulations of thymocytes

(a) Unstimulated rat thymocytes were centrifuged through a discontinuous Percoll density gradient. Nuclear extracts prepared from cells in fractions 1–4 were analysed by EMSA (4 μ g of protein per sample). (b) Thymocytes were incubated with 25 μ M etoposide for 25 min, and then prepared as in (a). EMSA with nuclear extracts of cells recovered from Percoll fractions 2–4 are shown (4 μ g of protein per sample). The migration position of the specific NF κ B/oligonucleotide complex is the same as in Figure 1.

positive selection and therefore constitutively contain a small number of both pre-apoptotic and apoptotic cells. In order to determine the status of the NF κ B/rel DNA-binding activity in the different subpopulations, thymocytes were centrifuged through a discontinuous Percoll density gradient. Antibody phenotyping of the different fractions revealed that the cells in fractions 3 and 4 contained more than 96% CD4⁺8⁺ cells (Table 1), while our previous experiments have shown that the heavier cells recovered in fraction 4 are enriched in pre-apoptotic thymocytes by virtue of their slightly higher DNA fragmentation, reduced volume and lower glutathione content [8]. EMSA results with nuclear extracts from thymocytes in fractions 1, 2, 3 and 4 are shown in Figure 2(a). The specific NF κ B/rel DNA-binding activity is seen in each fraction, although the intensity is greatest in the purest populations of normal (fraction 3) and pre-apoptotic (fraction 4) CD4⁺8⁺ thymocytes. This experiment was repeated three times with similar results, although the apparent difference between normal (fraction 3) and pre-apoptotic (fraction 4) nuclear extracts in Figure 2(a) was not consistently seen. We conclude that immature thymocytes contain a constitutive NF κ B/rel DNA-binding activity in their nuclei, and that the intensity of this activity is not markedly altered when the cells enter a pre-apoptotic state. The thymocytes used in this Percoll

Figure 3 Distribution of (a) NF κ B p50, (b) NF κ B p65 and (c) rel B proteins in cytoplasmic (low-salt; LS) and nuclear (high-salt; HS) fractions of unstimulated thymocytes

Cells were sequentially extracted in a low-salt and then a high-salt buffer, and each extract (25 μ g of protein per lane) analysed by the Western blotting method. An arrow marks the specific protein band on each gel, while to the left the migration positions of molecular-mass standards are indicated.

fractionation were unstimulated, and therefore insufficient apoptotic cells were recovered in fraction 5 for analysis.

Five mammalian members of the NF κ B/rel protein family are currently known, and various homodimeric and heterodimeric combinations of these polypeptides are reported to form active DNA-binding entities [13,29]. Antibodies raised against conserved peptide fragments of human (p50 and p65) or mouse (rel B) NF κ B/rel proteins were therefore used in an attempt to identify the polypeptide components of the thymocyte activity. Preincubation of a rat thymocyte nuclear extract with an anti-(human p50) antibody prior to an EMSA retarded the electrophoretic migration of a fraction of the specific protein–DNA complex, although the effect was inefficient (results not shown). In contrast, both the anti-(human p65) and anti-(mouse rel B) antibodies were completely without effect in our EMSAs. Western blotting was also used to directly assay for the presence of the various NF κ B/rel polypeptides (Figure 3). NF κ B p65 (rel A) and rel B were both detected in the cytoplasmic extracts, while NF κ B p50 and rel B were present in the nuclear fractions. A protein cross-reacting with the anti-p65 antibody was also variably observed in the nuclear extract, although its migration was slightly faster than cytoplasmic p65 (Figure 3b). This may therefore represent either a proteolytically degraded form of the cytoplasmic protein or another smaller protein sharing a cross-reactive epitope. With reference to previously published work [13,29], the constitutive NF κ B/rel DNA-binding activity present in the nuclei of rat thymocytes probably comprises homodimers of p50 or a heterodimer of p50 and rel B. However, in the absence of definitive results with the antibodies in the EMSA it is not possible to discriminate between these possibilities.

To determine whether the intracellular distribution of these three proteins varied with thymocyte development, the Percoll gradient fractions described above were also subject to analysis

Figure 4 An induction of the constitutive NF κ B/rel DNA-binding activity occurs when thymocytes are incubated with etoposide or MPS

Cells were left unstimulated (lane 1) or incubated with 25 μ M etoposide (lane 2) or 500 M MPS (lane 3) for 30 min. Nuclear extracts were then prepared and analysed by EMSA (5 μ g of protein per assay). Symbols designating the different protein/oligonucleotide complexes are described in Figure 1.

by Western blot (results not shown). p65 was confined to the cytoplasmic extract and p50 to the nuclear extract of each cell population. In contrast, rel B was principally found in both cytoplasmic and nuclear extracts of the cells recovered in fractions 1 and 2, suggesting that it is only expressed in mature single positive thymocytes. As the most intense gel-shifted protein–DNA complex was seen in nuclear fractions prepared from immature double-positive thymocytes (fractions 3 and 4; Figure 2a), a homodimeric p50 complex is probably the active DNA-binding protein detected in these cells.

It is well established that incubation of rat thymocytes with various agents, including Ca^{2+} ionophores, glucocorticoid hormones and topoisomerase II inhibitors (e.g. etoposide), induces apoptotic cell death within 4–8 h [10,11,27]. Inhibition of protein synthesis is known to block apoptosis in both the glucocorticoid and etoposide systems [10–12], suggesting that alterations in gene transcription occur prior to cell death. Proteins belonging to the NF κ B/rel family are plausible candidates for the inducible transcription factor(s) mediating these changes, and we therefore investigated whether their DNA-binding activity was enhanced during apoptosis. In EMSA the activity of the constitutive NF κ B/rel protein–DNA complex of unstimulated thymocytes was constant during a 2 h incubation (results not shown). However, the intensity of the same gel shift was consistently increased 30 min after adding either MPS or etoposide. Results from a representative experiment are shown in Figure 4. In time-course experiments this increase in DNA binding was first seen after 20 min and persisted until at least 60 min after the addition of etoposide (Figure 5a). The induced NF κ B/rel gel shift migrated identically to the constitutive activity already described, while in antibody super-shift experiments a weak positive result was again only obtained with the anti-p50 antibody (results not shown). The MPS- and etoposide-induced NF κ B/rel gel shift is therefore probably the same p50-containing NF κ B complex present in unstimulated thymocytes. When thymocytes exposed to etoposide for 25 min were fractionated by Percoll density-gradient centrifugation, an intense gel-shifted band was seen in cell fractions 2, 3 and 4 (Figure 2b). This indicates that the enhanced NF κ B/rel DNA-binding activity of unfractionated thymocytes exposed to etoposide is not restricted to the nuclei of one specific thymocyte subpopulation.

Figure 5 PDTC inhibits apoptotic DNA fragmentation but has no detectable effect on the NF κ B/rel DNA-binding activity of thymocytes

Cells were incubated for various times with 25 μ M etoposide in the absence (a) or presence (b) of 20 μ M PDTC, and 4 μ g of protein from a nuclear extract of each sample was then analysed in an EMSA. Inset: A sample of cells from the same experiment were cultured for 5 h and DNA fragmentation quantified with diphenylamine. Symbols designating the different protein/oligonucleotide complexes on the gels are described in Figure 1.

When thymocytes were co-incubated with PDTC and an inducer of apoptosis, a significant inhibition of apoptotic DNA fragmentation could be consistently observed several hours later (results not shown; [9,24]). To investigate whether this inhibition was associated with reduced NF κ B/rel DNA-binding activity, thymocytes were incubated with etoposide in the presence or absence of 25 μ M PDTC and nuclear extracts prepared at various times (Figure 5). Etoposide treatment alone gave a strong enhancement of the specific NF κ B/rel protein–DNA complex, which was not detectably altered in the presence of PDTC. However, when DNA fragmentation was determined in a sample of the cells 5 h later, PDTC was seen to exert a strong inhibitory effect on the apoptotic response (Figure 5, inset). The direct addition of either PDTC or its oxidized analogue disulphiram was also found to have no effect on formation of the constitutive protein–DNA complex in an EMSA (results not shown). It is therefore unlikely that the inhibitory action of PDTC on thymocyte apoptosis observed in this and other studies is mediated via an effect on the DNA binding of an NF κ B/rel transcription factor.

DISCUSSION

The results presented in this paper differ from a previous report that rat thymocytes do not contain a constitutive NF κ B/rel DNA-binding activity [25]. The reasons for this discrepancy are unclear. Constitutive nuclear NF κ B/rel proteins have been recorded in macrophages, B-cells, splenocytes and thymocytes [30–33]. For example, Weih et al. [30] and Lernbecher et al. [31,32] have both described a constitutive NF κ B/rel activity in murine thymocytes, consisting either of p50 homodimers or p50–52/rel B heterodimers. The constitutive NF κ B/rel tran-

scription factor in monocytes also involves p50 homodimers, which unlike lipopolysaccharide-inducible p50–p65 heterodimers are unaffected when cells are treated with PDTC [33]. Our findings are clearly in broad accord with these results, and we conclude that immature CD4⁺8⁺ rat thymocytes have a constitutive p50 NF κ B-like DNA-binding activity in their nuclei.

Thymocytes maintained under conventional cell culture conditions are known to slowly undergo apoptosis, such that approx. 30–35 % of the cells are dead after 24 h in culture. This presumably correlates with the situation *in vivo*, where more than 90 % of immature CD4⁺8⁺ thymocytes undergo programmed cell death by positive selection [28,34]. It is possible that the constitutive NF κ B/rel DNA-binding activity present in the nuclei of these cells is of functional significance for this process. Promotion or repression of the transcription of genes facilitating thymic selection (and thereby indirectly apoptosis induced by exposure to various other reagents) may continuously occur, while other primary cells lacking such a constitutive NF κ B/rel activity would be predicted to be much more refractory to apoptotic stimuli. In contrast to the potent transactivating properties of p65-containing NF κ B transcription factors, it is interesting to note that p50 NF κ B homodimers have been shown to have the potential to down-regulate the transcription of some genes [35]. Recent evidence suggests that transcriptional repression rather than activation is actually the critical event in glucocorticoid-induced lymphocyte apoptosis [36]. NF κ B/rel-dependent repression of the transcription of specific genes may therefore be an important contributing factor in sensitizing thymocytes to apoptosis.

After treating thymocytes with etoposide or MPS the intensity of the constitutive NF κ B/rel gel shift was increased. Sikora et al. [25] also described induction of an NF κ B/rel DNA-binding activity after stimulation of rat thymocytes with glucocorticoids or heat shock. Activation of this family of transcription factors thus appears to be an early and reproducible event in the onset of apoptosis in these cells. Our result with etoposide indicates that the DNA damage occurring after inhibition of topoisomerase II somehow promotes an increase in the nuclear DNA-binding activity of NF κ B/rel (although it is unclear whether this is due to an increased nuclear accumulation or an enhanced activity of pre-existing nuclear protein). A similar activation of NF κ B/rel DNA binding has recently been observed in lymphocyte and monocyte cell lines exposed to a photosensitizing agent that specifically generates single-strand DNA breaks [37]. DNA-damaging agents in general are known to promote the increased synthesis and nuclear accumulation of the p53 protein, while the p53 gene itself is transcriptionally up-regulated by NF κ B/rel [38,39]. As the ability of etoposide to induce thymocyte apoptosis is reduced when cells are prepared from animals deleted of their p53 genes [40], it is possible that the apparent interaction between DNA damage, increased nuclear p53 and subsequent apoptosis in part requires the transcriptional activity of NF κ B/rel.

In agreement with previous studies the dithiocarbamate PDTC was found to be a potent inhibitor of thymocyte apoptosis [9,24], although it was without effect on either the constitutive or etoposide-induced p50 NF κ B/rel-like DNA-binding activity in these cells. This is consistent with the findings of Frankenberger et al. that PDTC inhibits the lipopolysaccharide-induced formation of active p65-containing NF κ B transcription factors in human monocytes, but has no effect on the constitutive DNA-binding activity of p50–p50 homodimers also present in the same cells [33]. Dithiocarbamates have further been reported to inhibit TNF- α cytotoxicity in murine fibroblasts without a corresponding effect on cytokine-induced NF κ B/rel DNA binding [41]. Despite their frequent use as antioxidants, dithiocarbamates also possess well-characterized pro-oxidant effects at a cellular level

[42,43]. In agreement with this, we have recently found a significant increase in the glutathione disulphide content of thymocytes 90 min after adding PDTC [43a]. Dithiocarbamates inhibit various metal-dependent enzymes (such as superoxide dismutase and several classes of cytochrome *P*-450 [44,45]), and can be metabolized to other biologically active products including carbon disulphide and an *S*-methylthiocarbamate sulfoxide [46,47]. As inhibition of NF κ B/rel activation clearly cannot explain the anti-apoptotic activity of PDTC in thymocytes, we suggest that direct inhibition of a specific metal-dependent enzyme or secondary formation of mixed protein-glutathione disulphides is instead responsible for its prevention of apoptosis in these cells. It remains to be determined whether other more conventional antioxidants are similarly inactive in preventing an increase of NF κ B/rel DNA-binding activity after thymocytes are exposed to stimuli that induce apoptosis.

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