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cAMP increasing agents attenuate the generation of apoptosis by etoposide in promonocytic leukemia cells

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SUMMARY

Treatment of U-937 promonocytic cells with the DNA topoisomerase II inhibitor etoposide rapidly caused death by apoptosis, as determined by changes in chromatin structure, production of DNA breaks, nucleosome-sized DNA degradation, decrease in mitochondrial membrane potential and phosphatidyl serine translocation in the plasma membrane, and at the same time induced intracellular acidification. Both the execution of the apoptotic process and the intracellular acidification were reduced by the addition of forskolin plus theophylline or other cAMP increasing agents. These agents also attenuated the induction of apoptosis by camptothecin, heat-shock, cadmium chloride and X-radiation. Although etoposide slightly increased the production of reactive oxygen intermediates, this increase was not prevented by forskolin plus theophylline, and the addition of antioxidant agents failed to inhibit apoptosis. Etoposide caused a great increase in NF-κB binding activity, which was not prevented by forskolin plus theophylline, while AP-1 binding was little affected by the topoisomerase inhibitor. The treatments did not significantly alter the levels of Bcl-2 and Bax. By contrast, the expression of c-*myc*, which was very high in untreated U-937 cells and only partially inhibited by etoposide, was rapidly and almost totally abolished by the cAMP increasing agents. Finally, it was observed that etoposide caused a transient dephosphorylation of retinoblastoma (Rb), which was associated with cleavage of poly(ADP-ribose) polymerase (PARP). Both Rb dephosphorylation and PARP cleavage were inhibited by forskolin plus theophylline. The inhibition of Rb (type I) phosphatase and ICE/CED-3-like protease activities, and the abrogation of c-*myc* expression, are mechanisms which could explain the anti-apoptotic action of cAMP increasing agents in myeloid cells.

Key words: cAMP, Etoposide, Apoptosis, U-937 promonocytic cells

INTRODUCTION

Several DNA topoisomerase inhibitors, such as the DNA intercalators amsacrine, doxorubicin and mitoxantrone, and the non-intercalators etoposide and teniposide, are clinically useful antitumour drugs. The primary action of these agents is to stabilize the cleavable DNA-topoisomerase II covalent complexes, preventing subsequent DNA religation and producing, as a consequence, topoisomerase-linked DNA breaks (D'Arpa and Liu, 1989). Although this primary breakage is reverted upon drug removal, the toxic effect is persistent, eventually leading to cell death with characteristics of apoptosis (Bertrand et al., 1993; Pérez et al., 1997). Since topoisomerase inhibitors and other chemotherapeutic drugs cause cytoreduction by provoking apoptosis (Mesner et al., 1997), it is of great importance to investigate the mechanisms that regulate this process and the factors that could potentiate or decrease its efficacy.

The capacity of cAMP increasing agents to modulate apoptosis has been investigated in different cell types, often with conflicting results. For instance, while some reports indicated that cAMP causes apoptosis or potentiates its induction by other agents in thymocytes and T lymphocytes (Kizaki et al., 1990; McConkey et al., 1993), other studies indicated that cAMP prevents apoptosis in T lymphocytes and T cell hybridomas (Lee et al., 1993; Goetzl et al., 1995). In a similar manner, cAMP increasing agents have been reported both to potentiate (Lannotte et al., 1991; Lotem and Sachs, 1994) and to prevent (Watabe et al., 1996) the generation of apoptosis in myeloid cell lines. Whatever the case, the mechanisms by which cAMP modulates apoptosis are poorly known.

In the present work we analyze the capacity of cAMP increasing agents to modulate the toxicity of the DNA topoisomerase II inhibitor etoposide in U-937 promonocytic cells. These cells possess two characteristics important for the regulation of apoptosis which are frequent in leukemia cells, namely an elevated expression of c-*myc* and the lack of

functional p53 (Sugimoto et al., 1992). In addition we investigate several factors which, according to the current knowledge, are involved in the regulation of the apoptotic process.

MATERIALS AND METHODS

Cell culture and treatments

The human myeloid leukemia cells U-937 (promonocytic) and HL-60 (promyelocytic) used were mycoplasma-free. The cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum and 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2 atmosphere at 37°C. Etoposide, camptothecin, N^6,2′-0-dibutyryl 3′:5′-cyclic monophosphate (dibutyryl cAMP), isoproterenol, forskolin, theophylline, N-acetyl-L-cysteine (NAC), reduced glutathione (GSH), thioproline and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) were obtained from Sigma Química, Madrid, Spain; cadmium chloride and sodium butyrate from Merck, Darmstad, FRG; and TNF-α from ImmunoKontact, Frankfurt am Main, FRG. 1,25-dihydroxyvitamin D_3 was a generous gift of Dr M. Uskokovic (Hoffman-LaRoche, Nutley, NJ, USA). Etoposide, camptothecin, TPA and NAC were dissolved in dimethyl sulfoxide at 20 mM, 10 mM, 1.5 mM and 3 M, respectively; forskolin and 1,25-dihydroxyvitamin D_3 in absolute ethanol at 10 mM and 10 μM, respectively; and dibutyryl cAMP, GSH and thioproline in RPMI at 10 mM, 0.5 M and 0.22 M, respectively. All these solutions were stored at −20°C. Sodium butyrate was freshly prepared in RPMI at 100 mM, and isoproterenol, theophylline and cadmium chloride in distilled water at 10, 100 and 100 mM, respectively. For treatments, the drugs were applied to the cultures at the desired final concentrations. For heat shock, the cultures were placed in a bath at 43.5°C for 30 minutes. For recovery after treatments, the cells were collected by centrifugation, washed once (in the case of heat shock or X-radiation) or three times (in the case of etoposide, camptothecin and cadmium chloride) with pre-warmed (37°C) RPMI medium, and then cultured under standard conditions. As controls, cells were subjected to the same manipulations as treated cells, but in the absence of the drugs.

Determination of apoptosis

To analyze changes in chromatin structure, cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), resuspended in PBS and mounted on glass slides. After fixation in cold absolute methanol, the cells were stained for 20 minutes at room temperature with 4,6-diamino-2-phenylindole (DAPI) (Serva, Heidelberg, FRG) at a concentration of 1 μg/ml in PBS, and examined by fluorescence microscopy. Cells with fragmented chromatin (‘apoptotic bodies’) were considered to be apoptotic.

Production of DNA strand breaks with free 3′-OH ends was quantified using the TDT-mediated dUTP-X nick end labeling (TUNEL) technique. For this purpose, an In Situ Cell Death Detection kit containing fluorescein-dUTP (Boehringer Mannheim, Barcelona, Spain) was employed in combination with flow cytometry (using an EPICS XL flow cytometer, Coulter, Fl, USA), according to the instructions described by the manufacturer. The reliability of the method was confirmed by fluorescence microscopy.

Nucleosome-sized DNA degradation was assessed by agarose gel electrophoresis, as previously described (Pérez et al., 1994).

Reduction in mitochondrial membrane potential was measured essentially as described by Zanzami et al. (1995). Cells were collected, washed with PBS and incubated for 30 minutes at 37°C in PBS containing 100 ng/ml rhodamine 123 (Sigma Química). After washing, the cells were resuspended in PBS and the fluorescence measured by flow cytometry.

To measure phosphatidyl serine translocation from the inner to the outer layer of the plasma membrane, cells were collected by centrifugation, washed with PBS and incubated for 15 minutes at 37°C in a buffer consisting of 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.4, containing 0.8 mg/ml FITC-conjugated chicken annexin V (kindly supplied by Dr J. Turnay, Universidad Complutense, Madrid, Spain). After washing twice with the same buffer, the fluorescence was measured by flow cytometry. The reliability of the method was confirmed by fluorescence microscopy.

Measurement of intracellular ionic variations

The intracellular pH was measured by flow cytometry after loading the cells with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxy-methyl ester (BCECF/AM, Boehringer Mannheim) and calibration with nigericin (Sigma Química), as described by Pérez-Sala et al. (1995). The intracellular free calcium concentration was measured by fluorimetry after loading the cells with FURA 2-AM (Sigma Química), as described by Lennon et al. (1992).

Measurement of intracellular reactive oxygen intermediates

Dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes, INC, Eugene, OR, USA) and dihydroethidium (Sigma Química) were used to measure intracellular hydrogen peroxide and anion superoxide by means of flow cytometry, as described by Hockenbery et al. (1993) and Zanzami et al. (1995), respectively.

Immunoblot assays

Cell lysis, electrophoretic separation, blotting onto nitrocellulose and immunological detection of proteins were carried out as previously described (Vilaboa et al., 1995), with the following modifications: for analysis of retinablastoma (Rb), 6.5% SDS-polyacrylamide maxi-gels; for analysis of poly(ADP-ribose) polymerase (PARP), 10% mini-gels (using a Mini-PROTEAN II, Bio-Rad Laboratories, SA, Madrid, Spain); and for analysis of all other proteins, 12% mini-gels. The antibodies used were: mouse anti-human Bcl-2 mAb and rabbit anti-human Bax pAb (Santa Cruz Biotechnology Inc.); 9E10 mouse anti-human Myc mAb (kindly supplied by Dr C. Calés, Universidad Autónoma, Madrid, Spain); mouse anti-human Rb mAb (Pharmyngen, San Diego, CA, USA); mouse anti-chicken β-tubulin mAb (Amersham International, Little Chalfont, UK); and rabbit anti-human PARP pAb (Boehringer Mannheim).

RNA blot assays and gel retardation assays

The whole procedures were as previously described (Pérez et al., 1997). The probe used for RNA blot assays was the 1.5 kb human c-*myc*-specific *Cla*I-*Eco*RI fragment of pMC413rc plasmid (Dalla Favera et al., 1982).

RESULTS

Effect of etoposide and cAMP increasing agents

The administration of 6 μM etoposide induced apoptosis in U-937 cells, which could already be detected after 3 hours of treatment. The fraction of apoptotic cells was reduced by the presence of cAMP increasing agents, namely 25 μM forskolin plus 1 mM theophylline (F/T), 1 μM isoproterenol plus 1 mM theophylline (I/T), and 1 mM dbcAMP (Fig. 1). The maximum reduction was provided by F/T and I/T, but I/T was itself toxic (16% apoptotic cells after 12 hours). For this reason, F/T was selected for further experiments. The attenuation of apoptosis by cAMP was corroborated in HL-60 promyelocytic cells (results not shown), indicating that it is not a cell line-specific phenomenon.

Although the attenuation of apoptosis by cAMP was still evident after 48 hours of treatment (Fig. 1), treatments longer than 24 hours with cAMP increasing agents caused alterations, such a cell growth inhibition and occasional expression of differentiation markers (results not shown), which could themselves interfere with apoptosis. For this reason, experiments were carried out in cells that had been treated for 6-24 hours with etoposide, alone or in combination with F/T, and then allowed to recover in the absence of all drugs. A representative result is shown in Fig. 2. The frequency of apoptotic cells in cultures treated with etoposide plus F/T always increased abruptly upon drug removal, approaching the frequency obtained in cultures treated with etoposide alone. This suggests that the increase in intracellular cAMP levels prevents the execution of the apoptotic process, but does not promote the survival of etoposide-treated cells.

To further investigate this problem, U-937 cells were pulse-treated for 1 hour with etoposide alone, which suffices to generate primary topoisomerase-mediated DNA breakage (Pérez et al., 1997), and then allowed to recover either in the absence or the presence of F/T. As shown in Fig. 3, under these conditions F/T attenuated apoptosis, as indicated by changes in chromatin structure, generation of DNA strand breaks with free 3′-OH ends, and nucleosome-sized DNA fragmentation, as well as by extranuclear events such as phosphatydylserine translocation in the plasma membrane and reduction of mitochondrial membrane potential. By contrast, the attenuation was not observed when cells were pulse-treated with etoposide plus F/T, and then allowed to recover in the absence of all drugs (Fig. 4). Taken together, these results indicate that the protective action of cAMP is due to direct interference with the apoptotic process rather than with the generation of topoisomerase-associated DNA breaks.

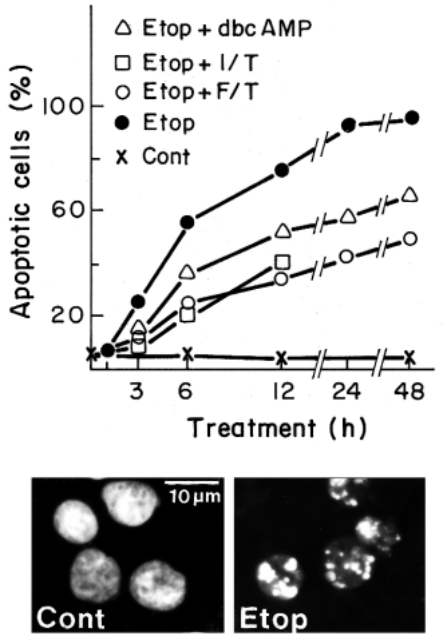

Fig. 1. Frequency of apoptotic U-937 cells, measured by chromatin fragmentation (i.e. formation of apoptotic bodies) after different times of treatment with 6 μM etoposide (Etop), either alone or in combination with 1 mM dibutyryl cAMP (dbcAMP), 1 μM isoproterenol plus 1 mM theophylline (I/T), or 25 μM forskolin plus 1 mM theophylline (F/T). Cont, untreated cells. All data are from one of at least three different experiments with similar results. An example of etoposide-treated cells exhibiting apoptotic bodies is shown in the photograph.

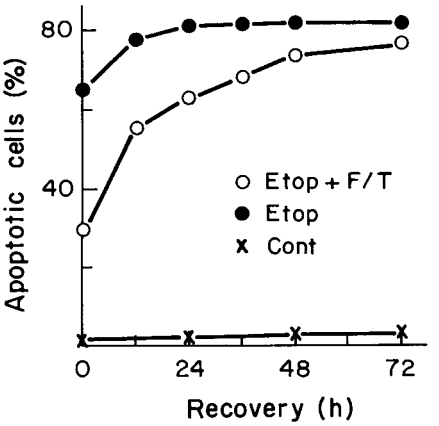

Fig. 2. Frequency of apoptotic cells during recovery from a 12 hour treatment with etoposide, alone or in combination with F/T. All other conditions were as in Fig. 1.

Fig. 5 shows that the induction of apoptosis by etoposide was accompanied by intracellular acidification in a fraction of cells (pH 6.6-6.8, in contrast to a normal value of 7.4), and that this fraction was reduced when F/T was present during the recovery period. By contrast, the treatments did not significantly alter the intracellular free Ca^{2+} concentration (approximately 40 nM: data not shown), a result that confirms earlier observations in myeloid cells (Lennon et al., 1992).

Effect of other agents

To determine whether cAMP could also inhibit the induction of apoptosis by agents other than etoposide, cells were treated with the topoisomerase I inhibitor camptothecin (1 hour at 0.5 μM), heat (30 minutes at 43.5°C), cadmium chloride (2 hours at 125 μM), or X-radiation (20 Gy), and then allowed to recover either in the absence or presence of F/T. The results in Table 1 indicate that the cAMP increasing agents also attenuated the generation of apoptosis by these cytotoxic agents.

Oxidative stress

It was suggested that the induction of apoptosis by cytotoxic drugs in myeloid cells might be mediated by the production of reactive oxygen intermediate(s) (ROI) production (McGowan et al., 1996). For this reason, we measured ROI production in cells pulse-treated with etoposide and allowed to recover in the absence or presence of F/T, as well as the capacity of antioxidant agents to prevent apoptosis. In all cases, the pro-oxidant agent TNF-α was used as a control. Some of the results obtained are shown in Fig. 6. Etoposide caused a weak increase (0.5- to 1-fold) in the intracellular H_2O_2 (Fig. 6) and O_2^- (result not shown) levels at hours 1-6 of recovery, which affected the whole cell population. However, such an increase was not prevented and was even slightly potentiated by F/T. In addition, the antioxidant agents NAC, GSH and thioproline, which reduced the generation of apoptosis by TNF-α, failed to attenuate its production by etoposide, as revealed by alterations

Table 1. Frequency of apoptotic cells (%) in cultures pulse-treated with different cytotoxic agents and allowed to recover in the absence or presence of F/T

	Recovery time (hours)			
Agent	3	6	12	24
None	–	3±4	–	3±1
*Camptothecin	32±4	52±6	–	–
Camptothecin+F/T	17±3	28±3	–	–
†X-rays	–	–	45±4	60±7
X-rays+F/T	–	–	20±3	25±2
‡Heat shock	–	42±4	53±4	–
Heat shock+F/T	–	29±3	36±3	–
¶$CdCl_2$	–	–	28±2	43±4
$CdCl_2$+F/T	–	–	18±3	24±3

All results are the mean±s.d. of at least three determinations. Approximately 500 cells per point were scored in each determination
*1 hour at 1 μM.
†20 Gy.
‡30 minutes at 43.5°C.
¶2 hours at 125 μM.

Table 2. Frequency of apoptotic cells (%) in cultures treated with etoposide or TNFα, either in the absence or the presence of antioxidant agents

	Treatment or recovery time (hours)			
Agent	3	6	12	24
None	–	2±1	–	3±1
*Etop	19±3	46±6	–	–
Etop+ NAC	17±2	48±4	–	–
Etop+GSH	19±2	49±3	–	–
Etop+Thiop.	20±3	46±4	–	–
†TNFα	–	–	14±2	30±4
TNFα+NAC	–	–	5±1	13±1
TNFα+GSH	–	–	5±2	12±2
TNFα+Thiop.	–	–	6±1	14±3

All results are the mean±s.d. of at least three determinations. Approximately 500 cells per point were scored in each determination.
*Etoposide (6 μM) was applied for 1 hour. The antioxidants (15 mM NAC, 5 mM GSH, 2 mM thioproline) were applied 2 hours before etoposide, and maintained during the treatment, washing and recovery periods.
†TNFα (500 U/ml) was applied as a continuous treatment. The antioxidants (same concentrations as above) were applied 2 hours before TNFα, and maintained during the treatment.

chromatin structure (Table 2) and other apoptotic markers (results not shown).

AP-1 and NF-κB binding

It was suggested that the induction of apoptosis by etoposide or teniposide in leukemia cells was accompanied and probably regulated by a transient stimulation of AP-1 and NF-κB binding activities (Kim and Beck, 1994; Bessho et al., 1994). For this reason, we measured AP-1 and NF-κB binding in U-937 cells pulse-treated with etoposide and allowed to recover

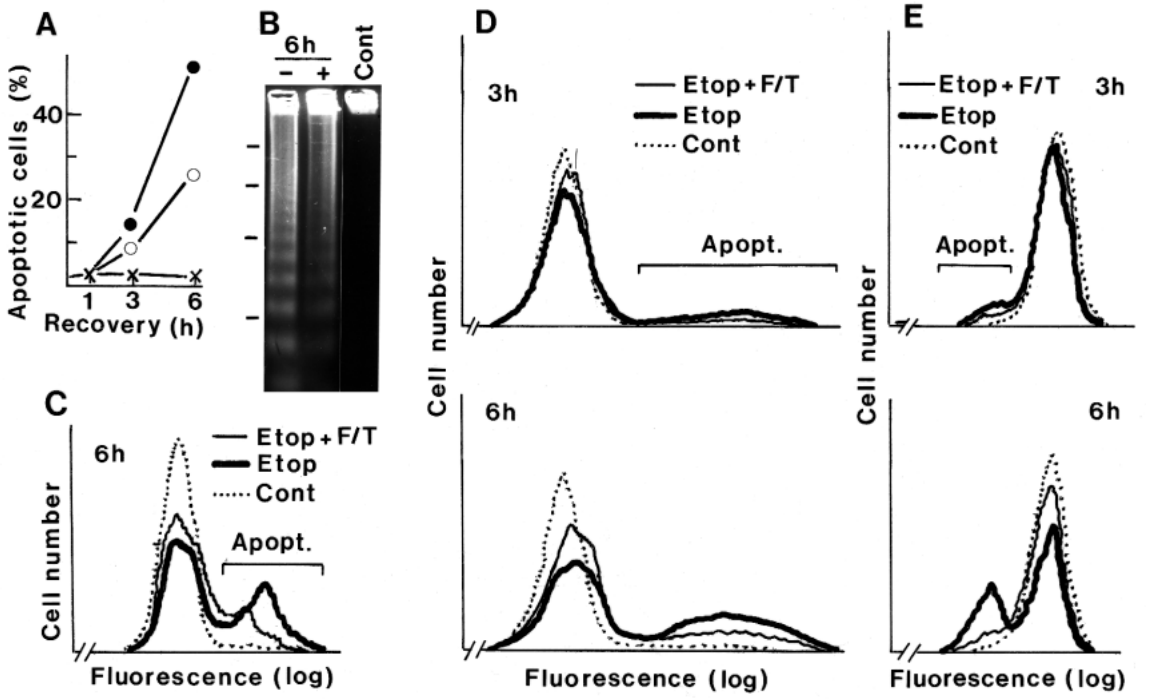

Fig. 3. Induction of apoptosis in U-937 cells pulse-treated for 1 hour with etoposide alone, and allowed to recover for the indicated time periods in the absence or the presence of F/T. (A) Frequency of cells exhibiting apoptotic bodies. (B) DNA fragmentation, in the absence (–) or the presence (+) of F/T, determined by agarose gel electrophoresis. The horizontal lines at the margins indicate the position of simultaneously run markers (from top to bottom: 3.76, 1.93, 1.26 and 0.7 kb). (C) Production of DNA breaks with free 3′-OH ends, determined by the TUNEL technique in combination with flow cytometry. (D) Phosphatidyl serine translocation in the plasma membrane, detemined by FITC-annexin V binding in combination with flow cytometry. (E) Changes in mitochondrial membrane potential, determined by rhodamine 123 incorporation in combination with flow cytometry. The areas corresponding to apoptotic cells are indicated in C-E. All data are from one of at least two determinations with similar results. All other conditions and abbreviations were as in Fig. 1.

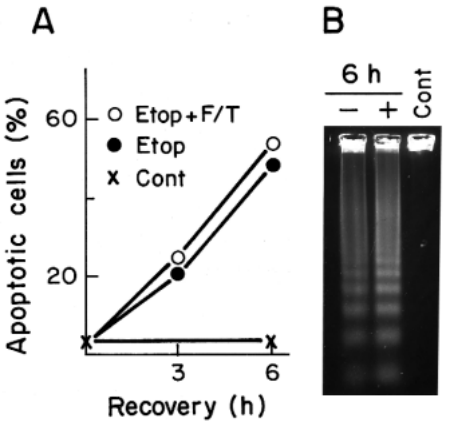

Fig. 4. Induction of apoptosis in U-937 cells pulse-treated for 1 hour with etoposide, alone or together with F/T, and allowed to recover for the indicated time periods in the absence of all drugs. (A) Frequency of cells exhibiting apoptotic bodies. (B) DNA fragmentation, in the absence (–) or the presence (+) of F/T. All other conditions were as in Fig. 1.

either in the absence or presence of F/T. The results are shown in Fig. 7. The etoposide treatment caused a great stimulation of NF-κB binding, which was already detectable at hour 0 of recovery and maintained at later times. The increase in NF-κB binding was little affected by the presence of F/T. Etoposide only caused a slight increase (maximum onefold) in AP-1 binding at 0-1 hours of recovery, which was greatly potentiated by the presence of F/T.

Bcl-2, *bax* and c-*myc* expression

It was reported that an elevated *bcl*-2 expression, or more probably an elevated ratio of Bcl-2 to Bax, may protect the cells from apoptosis, while an elevated c-*myc* expression may facilitate this process (for reviews, see Reed, 1994; Harrington et al., 1994; Hale et al., 1996). For these reasons, we wanted to analyze *bcl*-2, *bax* and c-*myc* expression in cells pulse-treated with etoposide and allowed to recover in the absence

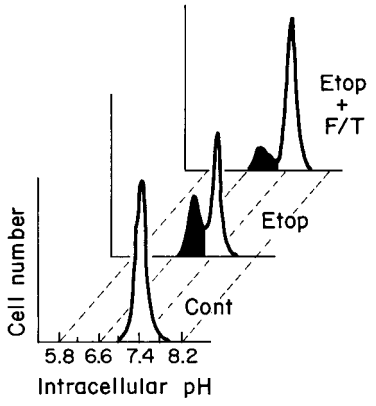

Fig. 5. Changes in intracellular pH in U-937 cells pulse-treated with etoposide and allowed to recover for 6 hours in the absence or presence of F/T. The cells were loaded with BCECF-AM and their distribution according to their intracellular pH was determined by flow cytometry. The data are from one of three experiments with similar results.

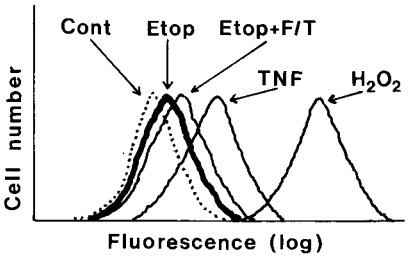

Fig. 6. Changes in peroxide levels. The intracellular peroxide levels were measured by H_2DCFDA fluorescence in combination with flow cytometry in U-937 cells pulse-treated with etoposide and allowed to recover for 6 hours in the absence (Etop) or presence of F/T (Etop+F/T), in cells treated for 6 hours with 500 U/ml TNFα (TNF), and in cells treated for 2 minutes with H_2O_2.

or the presence of F/T. Our results are shown in Fig. 8. It was observed that the levels of Bcl-2 and Bax were little affected by etoposide and F/T (Fig. 8A). By contrast, the expression of c-*myc* (measured at the protein and RNA levels), which was very high in untreated cells and only partially decreased by etoposide, was almost totally abolished by the presence of F/T during the recovery period (Fig. 8A,B).

The apparent correlation between abrogation of c-*myc* expression and attenuation of apoptosis could be corroborated using 1,25-dihydroxyvitamin D_3. After a 12-hour treatment with 100 nM 1,25-dihydroxyvitamin D_3, c-*myc* expression was reduced to less than 5% and the capacity of etoposide to induce apoptosis decreased to 55%. Other experimental approaches, such as the use of specific antisense oligonucleotides, only partially reduced c-*myc* expression, and provoked cell growth inhibition and expression of differentiation markers (results not shown).

Rb phosphorylation and PARP cleavage

It was recently reported that etoposide and other cytotoxic drugs stimulate Rb (type I) phosphatase activity, which may trigger the activation of ICE/CED-3-like proteases and all subsequent events of apoptosis (Dou et al., 1995; Morana et al., 1996). Hence, we wanted to analyze the status of Rb phosphorylation and ICE/CED-3 protease activity (measured by PARP cleavage) during recovery from etoposide pulse-treatment, either in the absence or presence of F/T. It was found that etoposide caused a transient dephosphorylation of Rb, detectable at 3 hours of recovery (Fig. 9A), and also induced PARP cleavage, detectable at 3 and 6 hours of recovery (Fig. 9B). The level of hypophosphorylated Rb was greatly reduced and the cleavage of PARP abrogated, when F/T was present during the recovery period (Fig. 9A,B).

DISCUSSION

Our results demonstrate that cAMP increasing agents reduce the toxicity of the topoisomerase II inhibitor etoposide and other chemical or physical insults in U-937 human myeloid leukemia cells by interfering with the process of apoptosis. It was reported that cAMP was able to ‘rescue’ neurons from apoptosis, i.e. to promote their survival when cultured in the absence of nerve growth factor (Deckwerth and Johnson, 1993). In this particular case the cAMP increasing agents

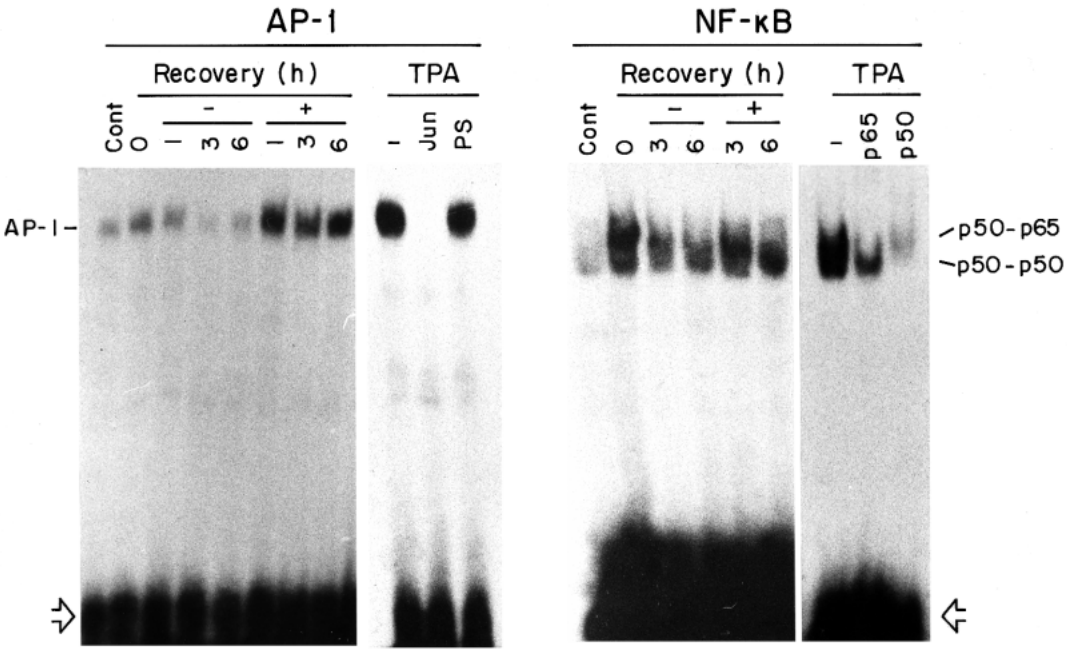

Fig. 7. Changes in AP-1 and NF-κB binding activities. Nuclear extracts from untreated U-937 cells (Cont) and cells pulse-treated with etoposide and allowed to recover in the absence (–) or in the presence (+) of F/T were used for gel retardation assays. As a control of the specificity of binding, extracts from cells treated for 6 hours with 15 nM TPA were assayed in the absence of antibodies (–), in the presence of either anti-Jun antibody (Jun) or preimmune serum (PS) in the case of AP-1, and in the presence of anti-p65 (p65) or anti-p50 (p50) antibodies in the case of NF-κB. The binding reactions were totally abolished by incubation with 50-fold excess of specific unlabeled probes (results not shown). The positions of free oligoprobe (arrows) and oligoprobe-protein complexes are indicated at the margins. The data are from one of two experiments with similar results.

replaced the trophic factor in provoking neuronal differentiation (Rydel and Greene, 1988). By contrast, in etoposide-treated cells cAMP seems only to prevent or delay the execution of the apoptotic process, and not to suppress the commitment to death. This result could be expected, since the cAMP increasing agents did not apparently affect the primary action of etoposide, namely the production of topoisomerase-mediated DNA damage. Similar conclusions were obtained using etoposide in combination with other anti-apoptotic agents in HL-60 cells (Bertrand et al., 1993), or etoposide in *bcl*-2-transfected mouse L929 fibroblasts (Gardner et al., 1997).

The induction of apoptosis by etoposide was associated with intracellular acidification, but not with changes in Ca^{2+} concentration. This fits with the hypothesis that DNA cleavage in myeloid cells undergoing apoptosis is carried out by pH-dependent endonucleases (Barry et al., 1993). The cAMP increasing agents, which reduced the frequency of apoptotic cells, also reduced the number of cells with low pH. A possible explanation is that cAMP increases the intracellular pH by activating the Na^+/H^+ antiporter, as has been demonstrated in murine macrophages (Kong et al., 1989), thus counteracting the etoposide-induced acidification. Whatever the case, F/T not only reduced DNA fragmentation but also inhibited extranuclear events such as phosphatidyl serine translocation and the drop of mitochondrial membrane potential. This indicates that cAMP increasing agents inhibit the process of apoptosis as a whole, affecting regulatory steps prior to endonuclease activation.

Although our results indicated that etoposide slightly increases the intracellular ROI levels, they do not sustain the hypothesis that ROI production may be the trigger for apoptosis in etoposide-treated myeloid cells, as earlier proposed (McGowan et al., 1996). In fact, ROI production was not prevented by F/T, which attenuated apoptosis, and antioxidant agents failed to inhibit the generation of apoptosis by etoposide. A similar conclusion was recently reached with

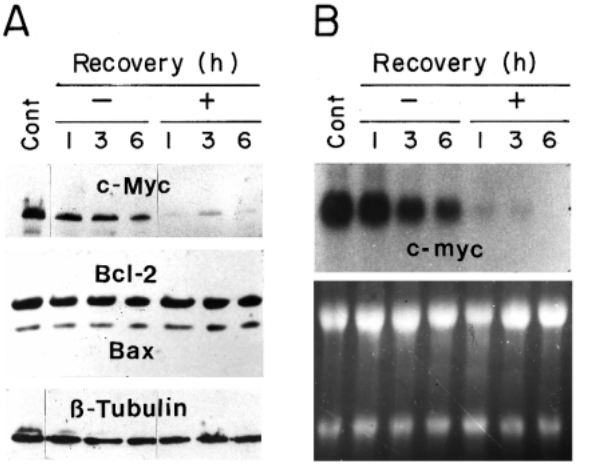

Fig. 8. Modulation by etoposide and F/T of *bcl*-2, *bax* and c-*myc* expression. U-937 cells were pulse-treated with etoposide and allowed to recover for the indicated time-periods in the absence (–) or the presence (+) of F/T. (A) protein samples (10 μg per lane) obtained from untreated (Cont) and treated cells were assayed by immunoblot, using antibodies that specifically recognized the human Bcl-2, Bax and c-Myc proteins. As a control, the same blots were assayed with β-tubulin. The approximate molecular mass of these proteins is: 67 kDa for c-Myc, 26 kDa for Bcl-2, 21 kDa for Bax and 51 kDa for β-tubulin. The data show one of three experiments with similar results. (B) Total RNA samples (15 μg per lane) were assayed by northern blot using a ^{32}P-labeled human c-*myc*-specific cDNA probe. The approximate size of c-Myc RNA is 2.4 kb. Ethidium bromide staining of ribosomal RNAs in the gel is shown at the bottom, as a control of sample loading. All data are from one of two experiments with similar results.

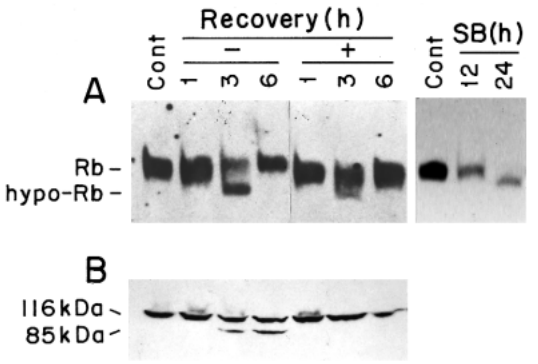

Fig. 9. Changes in Rb phosphorylation and PARP integrity. U-937 cells were pulse-treated with etoposide and allowed to recover in the absence (–) or the presence (+) of F/T. (A) Status of Rb phosphorylation (hypo-Rb: hypophosphorylated Rb). As a control, cells were treated for 12 and 24 hours with 0.75 mM sodium butyrate (SB), an agent that decreases Rb expression and phosphorylation (Buquet-Fagot et al., 1996). The molecular mass of Rb ranges from 115 to 120 kDa. (B) PARP cleavage. The positions of the intact protein (116 kDa) and the larger fragment resulting from cleavage (85 kDa) are indicated. All data are from one of two experiments with similar results.

murine L929 fibroblasts, in which antioxidants failed to prevent the etoposide-provoked apoptosis, although this agent caused ROI production (Gardner et al., 1997). Moreover, this conclusion seems to agree with the inability of etoposide to alter the Bcl-2 levels. In fact, it was reported that pro-oxidant agents such as TNF-α and ionizing radiation reduced *bcl*-2 expression in U-937 and HL-60 cells (Chen et al., 1995).

Activation of NF-κB binding was the earliest observed event in response to etoposide treatment, confirming that this transcription factor probably mediates the triggering of apoptosis by etoposide (Bessho et al., 1994). NF-κB may not, however, mediate the anti-apoptotic action of the cAMP increasing agents, since the binding was not affected by F/T. The role of AP-1 seems to be more complex. While some reports indicated that this transcription factor is strictly required for apoptosis in myeloid leukemia cells (Sawai et al., 1995; for a review see also Hale et al., 1996), in the present work AP-1 binding was only marginally increased by etoposide. Moreover, heat shock induces apoptosis in U-937 cells in the total absence of c-*jun* expression and AP-1 activation (Vilaboa et al., 1997, and our unpublished results). Hence, it appears that the behaviour of AP-1 largely depends on the inducer used, a problem which is under investigation. Interestingly, recent results indicate that AP-1 may even provide protection against some pro-apoptotic stimuli (for a review see Karin et al., 1997). This is important since F/T, which reduced apoptosis, overincreased AP-1 binding activity in U-937 cells.

Our observation that etoposide caused Rb dephosphorylation and PARP cleavage agrees with the hypothesis that Rb (type I) phosphatase activation (Dou et al., 1995), or an inbalance between Rb phosphatases and kinases (Morana et al., 1996), could be the trigger for ICE/CED-3-like protease activities and all subsequent apoptotic events in drug-treated myeloid cells. In addition, our results showed that both Rb dephosphorylation and PARP cleavage were inhibited by F/T, which attenuated apoptosis. In this respect, the action of F/T is similar to that of typical serine/threonine phosphatase inhibitors, such as okadaic acid and caliculin A, which also inhibited Rb dephosphorylation, PARP cleavage and apoptosis in etoposide-treated myeloid cells (Morana et al., 1996, and references therein). Hence, inhibition of phosphatase I activation could be a way by which cAMP increasing agents prevent apoptosis. Actually, it has been demonstrated that cAMP-dependent protein kinases inactivate phosphatase I by different mechanisms (for a review see Shenolikar, 1994).

It was indicated that the efficacy of etoposide and other chemotherapeutic drugs to kill tumor cells by apoptosis directly correlates with the level of endogenous c-*myc* expression (Bertrand et al., 1991). In addition, the abrogation of endogenous c-Myc expression by antisense oligonucleotides or other procedures decreased the susceptibility to apoptosis of different cell types (Shi et al., 1992; Miner et al., 1993; Jänicke et al., 1994). Hence, it seems probable that the abrogation of c-*myc* expression by cAMP may render the U-937 cells more resistant to the generation of apoptosis by etoposide and other cytotoxic agents. Alternatively, c-*myc* inhibition might merely be an early manifestation of broader alterations, such as the cessation of the proliferation program and/or the trigger of the differentiation program, which decrease the susceptibility to apoptosis (Cotter et al., 1994). Actually, this oncoprotein plays a pivotal role in the control of proliferation and differentiation of myeloid cells. This possibility remains under investigation.

In summary, the inhibition of posphatase I and ICE/CED-3-protease activities and the abrogation of c-*myc* expression are mechanisms which, according to current knowledge, could explain the anti-apoptotic action of cAMP in myeloid cells. A recent report indicated that pre-treatment of U-937 cells with cAMP increasing agents prevented the induction of apoptosis by bufalin, a drug which decreases the intracellular cAMP levels (Watabe et al., 1997). In our experience, pulse treatments with etoposide, heat or cadmium failed to decrease the cAMP levels (Vilaboa et al., 1995; and results not shown). The control of apoptosis is still poorly known, however, and cAMP is a pleiotropic molecule with multiple effects. Hence, additional regulatory mechanisms cannot be excluded.

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