Caspase activation in etoposide-treated fibroblasts is correlated to ERK phosphorylation and both events are blocked by polyamine depletion

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Abstract Activation of the extracellular signal-regulated kinases (ERKs) 1 and 2 is correlated to cell survival, but in some cases ERKs can act in signal transduction pathways leading to apoptosis. Treatment of mouse fibroblasts with 20 μM etoposide elicited a sustained phosphorylation of ERK 1/2, that increased until 24 h from the treatment in parallel with caspase activity. The inhibitor of ERK activation PD98059 abolished caspase activation, but caspase inhibition did not reduce ERK 1/2 phosphorylation, suggesting that ERK activation is placed upstream of caspases. Both ERK and caspase activation were blocked in cells depleted of polyamines by the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO). In etoposide-treated cells, DFMO also abolished phosphorylation of c-Jun NH_2-terminal kinases triggered by the drug. Polyamine replenishment with exogenous putrescine restored the ability of the cells to undergo caspase activation and ERK 1/2 phosphorylation in response to etoposide. Ornithine decarboxylase activity decreased after etoposide, indicating that DFMO exerts its effect by depleting cellular polyamines before induction of apoptosis. These results reveal a role for polyamines in the transduction of the death signal triggered by etoposide.

Key words: Apoptosis; Caspase; Extracellular signal-regulated kinase; c-Jun NH_2-terminal kinase; Polyamines; Ornithine decarboxylase

1. Introduction

Mitogen-activated protein kinases (MAPKs) are fundamental components of the signalling pathways transducing extracellular stimuli into a variety of cell responses [1]. The MAPK subtypes are preferentially regulated by diverse groups of extracellular stimuli. The classical MAPK cascade is activated in response to mitogenic signals that operate through different mechanisms, and results in the activation of the MAPK members ERK 1 and ERK 2 (extracellular signal-regulated kinase 1 and 2, also called p44 and p42 MAPK) through phosphorylation by the dual-specificity MAPK kinase MEK 1/2. Two other distinct MAPK cascades have been characterized, leading to the activation of JNKs (c-Jun N-terminal kinases, also called SAPKs, stress-activated protein kinases) and of p38 MAPKs respectively. These pathways, however are mainly activated by cytokines and environmental stress and are generally linked to induction of apoptosis [2]. ERK activation has an essential role in promoting cell cycle progression and ultimately, cell proliferation [1]. ERK activation has been generally correlated to cell survival [3,4]. However, growing evidence suggests that in some cases ERK can act in signal transduction pathways leading to apoptosis [5–8].

Another class of molecules correlated to both cell growth and cell death is represented by polyamines [9,10], and actually, evidence indicates a cross-talk between these organic polycations and the MAPK pathway [11–13]. Polyamines are absolutely required for cell proliferation, and the inhibition of their biosynthesis represents a pharmacological tool in cell growth control [9]. The role of polyamines in apoptosis is complex. Excessive polyamine levels directly triggers apoptosis [14,15], but polyamine depletion can sensitize or protect cells exposed to death triggers, depending on the pathway engaged by the death stimulus [16,17].

Apoptosis is characterized by the activation of the caspase proteases, that execute the death program [18]. Polyamine synthesis is necessary for caspase activation by the apoptosis-inducing drug etoposide in transformed mouse fibroblasts [16]. In order to study the mechanism(s) underlying the polyamine requirement for etoposide-induced apoptosis, we examined the role of polyamines in signal transduction pathways activated by etoposide. We found that etoposide elicited a sustained activation of ERK 1/2, whose phosphorylation was strongly correlated to caspase activation. Polyamine synthesis was found to be necessary for these events, since both ERK and caspase activation were blocked in cells depleted of

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; AcDEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; AcDEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; DFMO, α-difluoromethylornithine; NF-κB, nuclear factor-κB; ODC, ornithine decarboxylase

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polyamines by the ornithine decarboxylase (ODC) inhibitor α-difluoromethylornithine (DFMO)

2. Materials and methods

2.1. Materials and cells

Polyamines, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC), acetyl-Asp-Glu-Val-Asp-aldehyde (AcDEVD-CHO), etoposide and all other biochemical reagents were products of Sigma Chemical Company. Mouse monoclonal antibodies against caspase 3 and p53 were from Santa Cruz Biotechnology. Anti-ERK 1/2 and anti-phospho-ERK 1/2 as well as anti-phospho-JNKs and anti-phospho-p38 MAPK were from New England Biolabs. Horseradish peroxidase-conjugated anti-mouse IgG from Amersham was used as a secondary antibody.

Transformed mouse embryo fibroblasts were used. These cells were chosen because they allowed the comparison with the corresponding cells obtained from Gy mice that lack spermine being deficient in spermine synthase [19]. Fibroblasts from normal and Gy mice were derived from 14-day-old male embryos and immortalized by transfection with a plasmid expressing SV40 large and small T antigens, as described previously [19]. Cells were grown as described [16] in 10-cm culture dishes in Dulbecco's modified Eagle's medium containing pyruvate and pyridoxine (Gibco) supplemented with 2 mM glutamine, 10% fetal calf serum, 100 μg/ml each penicillin and streptomycin, at 37°C and 5% CO_2. In all the experiments, cells were cultured for 2 days and then treated with etoposide for the time indicated. To obtain polyamine depletion, the medium was added with 100 μM DFMO from the seeding. Cell viability was evaluated by trypan blue exclusion.

2.2. Caspase activity

The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate AcDEVD-AMC which represents a substrate for caspase 3 and other members of the caspase family. At the indicated time points, cells were washed in phosphate-buffered saline, harvested in 0.4 ml of lysis buffer [16] and subjected to two cycles of freeze–thawing. The lysates were centrifuged for 10 min at 28 000*g* at 4°C and the supernatant used to assay enzyme activity. 10 μl of extract were incubated for 15 min at 37°C in a final volume of 30 μl to determine caspase activity as described [20]. One unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per minute in the standard conditions described.

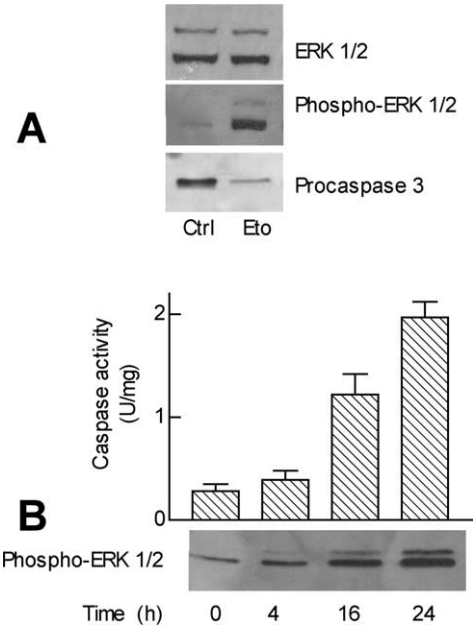

Fig. 1. Activation of caspase 3 in etoposide-treated fibroblasts is accompanied by the phosphorylation of ERK 1/2. A: Mouse fibroblasts were incubated for 24 h with 20 μM etoposide. Cell extracts (40 μg of proteins) were analyzed by Western blotting using specific antibodies for total ERK 1/2, or phosphorylated ERK, or caspase 3. B: Following treatment with 20 μM etoposide, the cells were harvested at the indicated time points for the determination of ERK 1/2 phosphorylation and caspase activity (mean ± S.E.M., $n = 3$).

2.3. Western blotting

The determination of ERK 1/2 levels and phosphorylation was performed as described [12]. Briefly, cells were scraped in 0.4 ml of lysis buffer and frozen/thawed twice. After centrifugation and denaturation of soluble proteins, cell proteins (40 μg) were fractionated by SDS–PAGE under reducing conditions (12% gel). After blotting, the membranes were incubated overnight at 4°C with the primary antibody anti-ERK 1/2 or anti-phospho-ERK 1/2 followed by incubation for 1 h with secondary antibody. Immunoreactive bands were visualized by chemiluminescence. The proteolytic processing of caspase 3 was detected in fibroblasts grown for 48 h and then exposed to etoposide for 24 h exactly as described [16]. Also, the p53 level was assayed by Western blotting and immunodetection [20].

2.4. Polyamine analysis and ODC assay

Cells were grown for 48 h, then were washed twice with phosphate-buffered saline, harvested in 0.4 ml of chilled 0.3 M perchloric acid and subjected to two cycles of freeze–thawing. After centrifugation at 12 000*g* for 5 min at 4°C, 0.3 ml of the clear supernatant was used for polyamine analysis, whereas the pellet was dissolved in 0.4 ml of 0.3 M NaOH for protein determination.

Polyamines were separated and quantified by high performance liquid chromatography after derivatization with dansyl chloride [15]. ODC activity was measured by estimation of the release of $^{14}CO_2$ from radiolabelled ornithine [12]. One unit catalyzes the decarboxylation of 1 nmol of ornithine per minute.

3. Results

3.1. Etoposide triggers ERK 1/2 phosphorylation

Etoposide is an inhibitor of topoisomerase II widely used in cancer chemotherapy that can induce apoptosis in a variety of cell types [21]. Similar to several other pharmacological or environmental stresses, etoposide-induced apoptosis is accompanied by activation of JNKs [22]. To investigate whether etoposide-induced apoptosis is also accompanied by changes in ERK activation, activated ERK 1 and ERK 2 were assayed by immunoblotting using an antibody that specifically recognizes the active, phosphorylated forms of both kinases. As seen in Fig. 1A, treatment of transformed embryo mouse fibroblasts with 20 μM etoposide for 24 h, caused a marked increase in the phosphorylation of ERK 1/2, as seen by the large increase in the bands corresponding to p44 (ERK 1) and p42 (ERK 2). In order to demonstrate uniform loading of gels and to exclude possible effects of etoposide on ERK expression, the blots were reprobed with an anti-ERK antibody that recognizes both ERK 1 and ERK 2. There was no change in the amount of either ERK 1 or ERK 2 indicating that the increase in the amount of activated ERKs did not result from change in the amount of the ERK proteins.

Under the same conditions, etoposide induced the processing of the effector procaspase 3 (Fig. 1A), that represents a crucial point in apoptosis [18]. The proteolytic processing of procaspases into active caspases determines the onset of their enzymatic activity, and actually, caspase activity increased over the time reaching a maximum after 24 h from etoposide treatment (Fig. 1B). The levels of phospho-ERK 1/2 also continued to increase over the time course of the experiment, accompanying the increased caspase activity. No further increase in caspase activity or ERK 1/2 phosphorylation was detected at later time points (not shown).

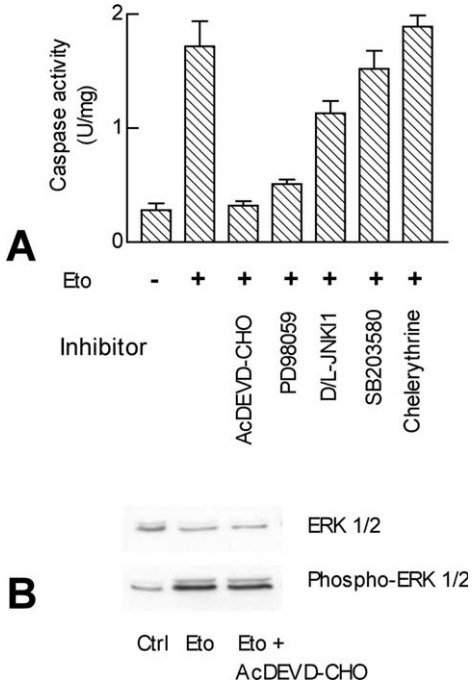

Fig. 2. Effect of caspase and kinase inhibitors on caspase activity in etoposide-treated fibroblasts. A: Mouse fibroblasts pretreated for 30 min with 200 μM AcDEVD-CHO (inhibitor of caspase 3 and other effector caspases), or PD98059 (50 μM), or 1 μM of both D- and L-JNK1, or SB203580 (10 μM), or chelerythrine (5 μM), were then incubated for 24 h with 20 μM etoposide. The cells were then collected to measure caspase activity digesting the peptide substrate Ac-DEVD-AMC. Data are means ± S.E.M. of three determinations. B: Fibroblasts were treated with 20 μM etoposide in the absence or presence of 200 μM AcDEVD-CHO for 24 h. Cells were harvested, and ERK phosphorylation was examined in extracts by Western blotting using anti-phospho-ERK 1/2 antibody.

The parallel time course suggested a correlation between caspase and ERK 1/2 activation. In order to assess a possible role of ERK 1/2 in the apoptotic response to etoposide and to determine whether it precedes or follows active caspases, inhibitors of ERK phosphorylation or caspase activity were used. Firstly, we tested the effect on etoposide-induced caspase activation of the specific MEK inhibitor PD98059 [23], which has been widely used to block ERK 1/2 activation. Fig. 2A shows that treatment of fibroblasts with 50 μM PD98059 given together with etoposide abolished the increase in caspase activity, the effect being similar to that obtained with 100 μM AcDEVD-CHO, an inhibitor of effector caspases 3, 6, and 7 [24]. On the other hand, AcDEVD-CHO treatment did not result in any reduction in the magnitude of ERK 1/2 activation, as assessed by the phospho-specific ERK 1/2 antibody (Fig. 2B). These results suggest that ERK 1/2 activation lies upstream of caspase activation. It is worth noting that the inhibition of p38 MAPK activity with SB203580 and of protein kinase C with chelerythrine did not protect the cells from caspase activation, whereas only a slight inhibition was obtained with 1 μM of both D and L isomers of the cell permeable JNK peptide inhibitor JNKI1 [25].

3.2. *Polyamine depletion inhibits ERK 1/2 phosphorylation and caspase activation in etoposide-treated fibroblasts*

Polyamine depletion obtained by the ODC inhibitor DFMO abolishes caspase activation triggered by etoposide in the current experimental model [16], establishing a necessary role for polyamines in etoposide-induced apoptosis. To assess if polyamine depletion can also interfere with the pathway resulting in ERK 1/2 phosphorylation or is placed downstream of this step, the fibroblasts were incubated for 2 days in the presence of 100 μM DFMO to deplete cellular polyamines, then were treated with etoposide. This pretreatment with DFMO by itself increased ERK 1/2 phosphorylation very slightly, but completely inhibited the strong effect of etoposide on ERK 1/2 activation (Fig. 3A). In accordance with the experiments reported in Fig. 2, inhibition of ERK 1/2 phosphorylation was coupled to inhibition of caspase 3 activation. Actually, in polyamine-depleted cells, etoposide treatment could not result in processing of procaspase 3. The inhibition of caspase activation in DFMO-treated cells was independent of etoposide dosage and was similar even when the drug concentration was five-fold higher than the usual one (not shown).

Lee et al. reported evidence for activation of the MAPK cascade in response to p53 expression [26]. Since etoposide-induced apoptosis may be related to p53, at least in some cells [20,27], and DFMO reportedly influences p53 level [28], the effect of polyamine depletion on p53 expression was studied. The cells were harvested at various times (2–24 h) after etopo-

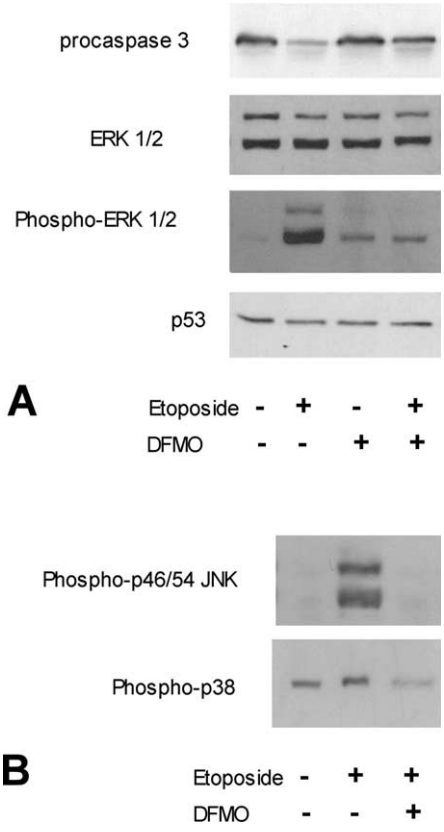

Fig. 3. Effect of polyamine depletion on caspase 3 activation, ERK and JNK and p38 phosphorylation, and p53 level in etoposide-treated fibroblasts. The cells were grown for 48 h after plating in the absence or presence of 100 μM DFMO, then were treated with 20 μM etoposide for further 24 h. Cells were harvested and cell extracts obtained. A: 40 μg of proteins in cell extracts were analyzed by Western blotting using specific antibodies for caspase 3, total ERK 1/2, phosphorylated ERK, or p53. B: The phosphorylation of JNK and p38 was examined by Western blotting using antibodies specific for the phosphorylated proteins.

side treatment, and p53 level was determined. The p53 protein was detectable even in control untreated cells, however neither etoposide nor DFMO elicited any significant effect on its expression at any time point (Fig. 3 shows p53 levels measured 6 h after etoposide).

DFMO also inhibited the phosphorylation of p46 and p54 JNK proteins as well as the slight increase in p38 MAPK phosphorylation that follows etoposide treatment (Fig. 3B).

In some experimental models, the induction of ODC that follows apoptotic treatments has been proposed to be actively involved in the activation of apoptosis [29,30]. However, in mouse fibroblasts etoposide did not induce ODC, whose activity was actually suppressed when the cells underwent apoptosis (Fig. 4). Furthermore, in preliminary experiments we observed that if DFMO was given together or immediately before etoposide, its inhibiting effect on both caspase and ERK activation was lost (not shown). These data indicate that the effect of DFMO is caused by depletion of cellular polyamines, which must be low at the beginning of the treatment with the inducer of apoptosis.

Fig. 5A shows the polyamine content in fibroblasts preincubated 2 days with DFMO, i.e. the polyamine level when etoposide is added. At the dosage used (100 μM), DFMO caused maximal reduction in putrescine and spermidine levels, and no further reduction was obtained with the higher concentrations of the drug generally used (5–10 mM). Putrescine decreased from 3.4 nmol/mg of protein to an undetectable level (less than 0.4 nmol/mg), while spermidine was decreased by about 80%. However, as it is characteristic for this drug, DFMO did not affect spermine content. This observation suggests that in the whole cell, spermine does not have a necessary role in the permissive effect of polyamines on ERK 1/2 phosphorylation that follows etoposide treatment. This conclusion was confirmed by the completely similar effects of etoposide on ERK 1/2 phosphorylation in normal fibroblasts and in the corresponding cells obtained from a Gy mouse that lack spermine, being deficient in spermine synthase [19] (not shown). Addition of 100 μM putrescine to DFMO-treated fibroblasts completely restored polyamine content, as well as

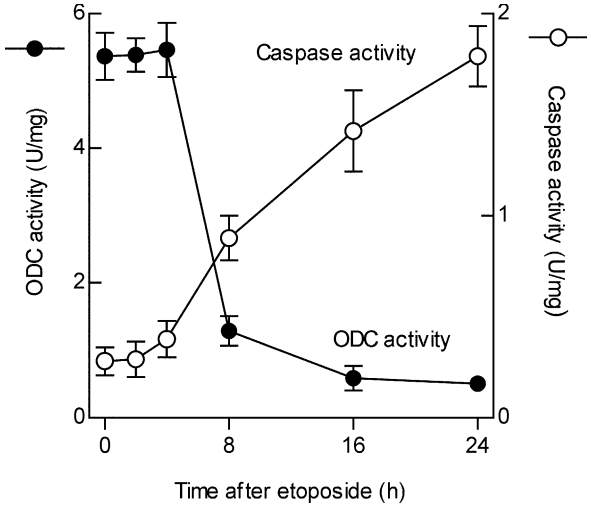

Fig. 4. Etoposide decreases ODC activity in mouse fibroblasts. The cells were treated with 20 μM etoposide. At the time indicated, the fibroblasts were harvested and cell extracts were assayed for ODC and caspase activities. Results are means ± the range obtained in two separate experiments.

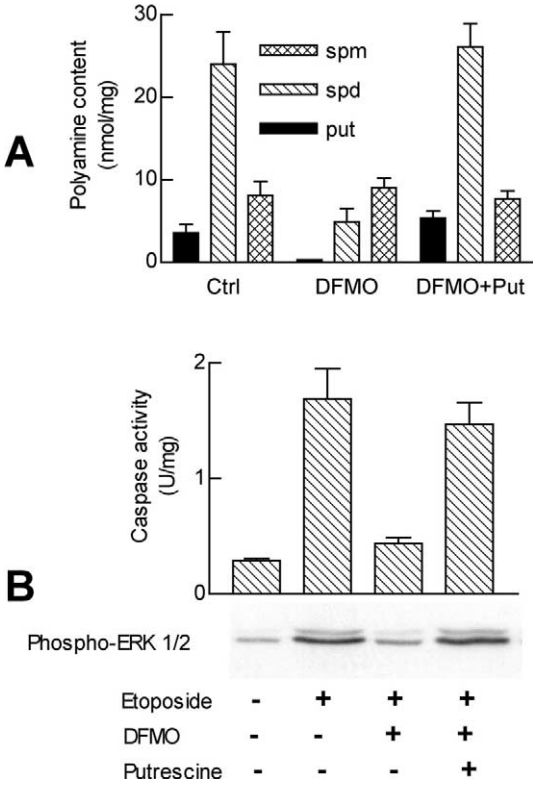

Fig. 5. Polyamine replenishment restores caspase activation and ERK phosphorylation in DFMO- and etoposide-treated fibroblasts. The cells were grown for 48 h after plating without any addition (Ctrl), or in presence of 100 μM DFMO, or in presence of 100 μM DFMO plus 100 μM putrescine (DFMO+Put), then (A) cells were harvested and polyamines were determined in acid extracts by high performance liquid chromatography (spm, spermine; spd, spermidine; put, putrescine); B: cells were treated with 20 μM etoposide for 24 h before the preparation of extracts for the determination of caspase activity and ERK phosphorylation. In both panels results are means ± S.E.M. of three determinations.

the ability of the cells to undergo caspase activation and ERK 1/2 phosphorylation in response to etoposide (Fig. 5B). In the absence of etoposide, replenishment of polyamine level by addition of putrescine to DFMO pretreated cells did not influence at all either ERK phosphorylation or caspase activity (not shown).

4. Discussion

MAPK cascades participate in diverse cellular functions such as cell growth, cell differentiation, and cell death. The ERK components of the MAPK family are involved in signals triggered by growth factors-stimulated receptor tyrosine kinases as well as by serpentine and cytokine receptors. All these signals generally lead to RAS activation that couples them to ERK activation [1,2].

The ERK pathway plays an essential role in promoting cell cycle progression and is generally involved in proliferative signaling and cell survival [1–4]. In contrast, JNKs and p38 MAPK, the other members of the MAPK family, are mainly activated by extracellular stress signals and involved in apoptosis [2,31]. Recently several reports have shown that the original model postulating that stress-activated pathway is related to apoptotic cell death, whereas the ERK pathway

protects the cells from apoptosis, is a simplistic one. A growing body of literature has demonstrated that ERK 1/2 activation does not necessarily deliver a survival signal, but itself can be correlated to induction of apoptosis in some contexts [5–8]. Actually, activation of all the MAPK pathways, including ERK, JNK and p38 pathways, may be present during apoptosis [2,31]. The stimulatory or inhibitory effect of these pathways depends on the cell type and the apoptotic trigger. Furthermore, even the timing of MAPK activation is thought to be important in the cell decision to die [5].

Previous studies have demonstrated an activation of JNK in response to etoposide [22], even if the actual role of such activation is not clear [32]. In mouse fibroblasts, etoposide induces phosphorylation of JNK enzymes, whereas the phosphorylation of p38 MAPK is hardly affected. However, in these cells, etoposide also triggers a sustained phosphorylation of both p42 and p44 ERK proteins that, on the basis of inhibitor studies shown in Fig. 2, has a predominant role in induction of apoptosis. ERK 1/2 phosphorylation is not a rapid and transient event, as in the case of the response to mitogenic stimuli, but it appears 2–4 h after the drug and continues to increase until 24 h. The ability of the MEK inhibitor PD98059 [23] to prevent the increase in caspase activity triggered by etoposide suggests that ERK 1/2 activation functions upstream of these proteases. This view is supported by the effect of the inhibitor of effector caspases AcDEVD-CHO [24], which blocks caspase activity without interfering with ERK 1/2 phosphorylation.

The molecular events linking the primary effect of etoposide, generally thought to be the interaction with topoisomerase II [21], with induction of apoptosis, represented by caspase activation triggered by cytochrome *c* exit from mitochondria [33], are largely unknown. We have shown that ERK 1/2 phosphorylation is one of these events. Also polyamines are important in the early phases of etoposide-induced apoptosis and actually etoposide cytotoxicity [34] and caspase activation [16] are attenuated in cells depleted of polyamines by pretreatment with the ODC inhibitor DFMO. Furthermore, ERK 1/2 phosphorylation is abrogated in polyamine-depleted cells suggesting that polyamines are required at a stage upstream ERK 1/2 activation that, in turn, precedes caspase activation.

As regards the possible mechanism of the inhibiting effect of polyamine depletion on etoposide-induced ERK activation and caspase activation, we have examined some possible targets. Firstly, the cytoprotective action of DFMO is not caused by interference with p53 expression, that in some cell systems can be influenced by etoposide [27], ERK [26], or DFMO [28]. Actually, in our experimental model, neither etoposide nor DFMO influence the p53 level (Fig. 3A). Possibly, the lack of p53 response may be brought about by the fact that the mouse fibroblasts used in our experiments were immortalized using a plasmid expressing SV40 large and small T antigens [19] that can interfere with p53 function [35]. Second, etoposide [36] and DFMO [37] influence polyamine transport, that in turn may affect the multidrug resistance (MDR) transporter [38], responsible of extrusion of cytotoxic drugs from the cell. However the inhibiting effect of DFMO was maintained even at very high concentration of etoposide, excluding an interference with MDR transporter. Third, since ODC activity is largely decreased after etoposide, a change in this enzyme is not involved in induction of apoptosis, and the effect of DFMO cannot be ascribed to an inhibition of polyamine accumulation that follows the treatment with the inducer of apoptosis [29]. Fourth, polyamine depletion can also induce nuclear factor-κB (NF-κB) activation [39], that in turn can influence polyamine metabolism [40] and protect the cells against apoptosis [39,41]. However, in preliminary experiments we found that in fibroblasts etoposide itself could cause a weak NF-κB activation that was hampered by DFMO (unpublished data).

Our results show that an early event triggered by etoposide and leading to phosphorylation of the MAPK members ERKs and JNKs is blocked in polyamine-depleted cells. Polyamines could be required for signal transduction leading to MAPK activation, or for the activity of topoisomerase II, that is important for etoposide action [42], or could act by affecting the cellular localization of members of the Bcl-2 family [43]. Another possible model that we hypothesize is correlated to the established role of polyamines in cell proliferation [9]. It is known that the sensitivity to etoposide cytotoxicity depends on the cell cycle [44] and is increased in transformed cells [45]. Actually, ERK activation is part of a proliferative pathway, while growth arrest is a characteristic of cytotoxic drugs. The generation of two conflicting signals by etoposide could lead to apoptosis as hypothesized in different experimental systems in which an imbalance between growth and arrest signals occurs [5,7]. On the other hand, polyamine-depleted cells are 'frozen' in a non-proliferative status, avoiding the engagement of pathways that can influence cell growth, such as the different MAPK pathways. As a consequence, there is no generation of conflicting signals, and the way leading to caspase activation is interrupted.

Finally, this experimental model suggests a need for caution when polyamines are chosen as chemotherapeutic target and polyamine synthesis inhibitors are used against cancer cells, since polyamine depletion can reduce the ability of chemotherapeutic drugs to trigger apoptosis.

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