Changes in polyamine catabolism in HL-60 human promyelogenous leukaemic cells in response to etoposide-induced apoptosis

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The topoisomerase II inhibitor etoposide induced apoptosis in HL-60 cells within 4 h of exposure to the drug, as measured by changes in morphology, DNA fragmentation and cytotoxicity assays. Etoposide-induced apoptosis was accompanied by an increase in polyamine efflux from the cells and a decrease in total polyamine content during the first 24 h of exposure to the drug. Although both enzyme activities increased slightly, there were no significant changes in spermidine/spermine N^1-acetyltransferase activity or polyamine oxidase activity. After longer exposures (48–72 h), significant induction of spermidine/spermine N^1-acetyltransferase activity and loss of polyamine content occurred. These results suggest that polyamine oxidation and the resultant hydrogen peroxide produced may be associated with the initiation of apoptosis, while induction of the acetyltransferase and overall loss of intracellular polyamines may be involved in the final, possibly necrotic, stages of cell death.

Key words: cancer, cell death, polyamine efflux, polyamine oxidase, spermidine/spermine N^1-acetyltransferase.

INTRODUCTION

The polyamines spermidine and spermine, and their diamine precursor putrescine, form a subgroup of the family of biogenic amines that are distributed ubiquitously across all species [1]. They are positively charged at physiological pH, and can therefore interact with negatively charged macromolecules, such as DNA and RNA. They are important in terms of cell proliferation as, in the presence of inhibitors of polyamine biosynthesis and in the absence of a source of exogenous polyamines, cells fail to grow. In contrast, cells that are dividing rapidly have high concentrations of intracellular polyamines [2]. In addition, malignant cells have been found to have raised intracellular polyamine concentrations [3,4]. These observations have led to these amines being considered essential for cell proliferation and differentiation [5]. Of the enzymes involved in polyamine metabolism, the rate-limiting enzyme, ornithine decarboxylase, is arguably the most studied, and is one of the early genes to respond to a growth-inducing stimulus [6]. In addition, it has been shown that, in NIH 3T3 cells, overexpression of ornithine decarboxylase will induce cell transformation [7].

While increases in polyamine biosynthesis have been associated with cell growth, increases in polyamine breakdown have, by corollary, been linked to growth inhibition and cell death. Early studies showed that the polyamine content of normal cells approaching confluence decreased [8]. This was later shown to be the result of efflux of polyamines from the cells, mainly as the N^1-acetylspermidine derivative [9]. Inhibition of cell growth was then associated with induction of the catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT), and Xiao and Casero [10] suggested that superinduction (a greater than 100-fold increase within 24 h) of SSAT is necessary for cell-type-specific cytotoxicity of certain polyamine analogues. This hypothesis is supported by the work of Porter and co-workers [11], who showed that, in melanoma cells, the greater the induction of SSAT, the greater the growth inhibition, and vice versa. More recent studies have linked the induction of SSAT by some novel polyamine analogues with the altruistic form of cell death known as apoptosis, whereby individual cells can be lost from a tissue or culture without causing inflammation or damage to surrounding cells [12].

Apoptosis is the opposite of mitosis, and maintains normal tissue homoeostasis. It can be induced by both endogenous and exogenous stimuli, including several anti-cancer agents and elevated calcium concentrations, both of which can also induce SSAT [13,14].

The link between polyamine metabolism and cell death is not clear, and so the present study set out to investigate the temporal relationship between polyamine catabolism and the induction of apoptosis. A well characterized cell culture model of apoptosis, namely etoposide induction of apoptosis in HL-60 promyelogenous leukaemic cells, has been used, and the early and late responses of these cells to apoptotic cell death have been characterized in terms of changes in polyamine metabolism. This work differs from previous studies [10–12] which have used alterations in polyamine metabolism to induce cell death, in that here changes in polyamine content and enzymology brought about by initiation and progression of apoptotic cell death have been determined.

EXPERIMENTAL

Materials

Acetyl-CoA, etoposide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Cell Death ELISA was from Boehringer Mannheim G.m.b.H. (Mannheim, Germany), and cell culture plastics and RPMI 1640 growth medium were from Life Technologies (Paisley, Scotland, U.K.) and Greiner Labortechnik Ltd. (Dursley, U.K.). The HL-60 (human promyelocytic leukaemia) cell line was kindly donated by Dr Keith Stewart (Department of Medicine and Therapeutics, University

Abbreviations used: BrdU, bromodeoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAO, polyamine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase.

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of Aberdeen). [^{3}H]Acetyl-CoA (1.2 Ci/mmol) was obtained from Dupont, NEN Division (Dreiech, Germany).

Cell culture methods

The HL-60 cell line was grown in RFC_{10} medium [RPMI 1640 medium supplemented with 10 % (v/v) fetal calf serum] at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2 in a Gallenkamp incubator. Due to the susceptibility of HL-60 cells to differentiate spontaneously beyond the promyelocytic stage with time in culture or at high seeding density, cells were routinely passaged every 3–4 days at a ratio of 1:4 to maintain a low culture density [$(1–5) \times 10^5$ cells/ml], and were only used for a maximum of 6 weeks, after which time a fresh supply was recovered from storage at −135 °C [15].

Analytical methods

Total cellular protein content was determined using a modification of the method described by Lowry et al. [16]. Standards were prepared from a stock solution of BSA (500 μg/ml) by serial dilution with 0.3 M NaOH to give standards within the range 0–250 μg/ml BSA.

Cells for polyamine analysis were harvested in perchloric acid as described previously [17], and the acid fraction containing the polyamines was stored at −20 °C until analysis by HPLC. Polyamines and their monoacetyl derivatives were separated and quantified by the HPLC method of Seiler and Knodgen [18], as modified by Wallace et al. [17].

The SSAT assay was performed as described by Wallace and Evans [19]. For each treatment, 3×2 replicate 10 cm-diam. plates were used. Results are expressed as pmol of N^1-acetylspermidine formed/min per mg of protein.

Polyamine efflux from HL-60 cells was measured using the radiolabel tracer technique devised by Wallace and Keir [8] and modified by Wallace and Mackarel [20]. Cells were labelled with [^{3}H]putrescine (1 mCi/ml; 37 MBq/ml) in the presence of 1 mM aminoguanidine for 36 h. The cells were washed twice in serum-free medium (RPMI 1640), resuspended in serum-containing medium (RFC_{10}) and allowed to grow for a further 12 h. The cells were again washed twice in serum-free medium and plated out for experimentation in RFC_{10} containing 1 mM aminoguanidine. The polyamine content of fetal calf serum when analysed by HPLC was found to be below the limit of detection. Drug was added and, at the appropriate time thereafter, cells were harvested and the radioactive polyamines in the cells and medium were determined by liquid scintillation spectrometry. The cell pellet was solubilized and protein content analysed by the Lowry method [16]. Results are expressed as d.p.m./mg of protein.

The production of hydrogen peroxide from the oxidation of N^1-acetylspermine by polyamine oxidase (PAO) was used as a measure of PAO activity. The H_2O_2 produced reacts with homovanillic acid to form a fluorescent product which can be detected by fluorescence spectroscopy and quantified using H_2O_2 standards by the method of Suzuki et al. [21].

Cytotoxicity was quantified by the method of Mossman [22], which was modified by Denizot and Lang [23] and was adapted for use in suspension cells. Briefly, HL-60 cells were plated on to a 24-well tissue culture plate and allowed to grow for 48 h. Cells were then exposed to the appropriate treatment and, at the desired time, 100 μl of a 5 mg/ml sterile solution of MTT in RPMI was added to the cells, which were then incubated for 4 h at 37 °C in a humidified incubator (Gallenkamp) containing 5 % CO_2 and 95 % air. If the cells are viable, they will metabolize MTT using the mitochondrial enzyme succinate dehydrogenase, reducing it to a formazan salt which can be dissolved in acidified propan-2-ol and detected by absorbance at 570 and 690 nm.

Cellular DNA fragmentation ELISA

This assay uses an ELISA kit from Boehringer Mannheim (cat. no. 1585 045) which contains two mouse monoclonal antibodies, directed against DNA and bromodeoxyuridine (BrdU), thus allowing the specific detection and quantification of BrdU-labelled DNA fragments in the cytoplasm of apoptotic cells. Cells were grown for 48 h either in the presence or in the absence of drug. During the final 12 h of growth, the cells were labelled with 10 μM BrdU as per kit instructions. The cells were pelleted by centrifugation at 800 $\boldsymbol{g}_{av}$ in an MSE bench centrifuge and resuspended in fresh medium at a concentration of 1×10^6 cells/10 ml. A 100 μl aliquot (1×10^4 cells) of this cell suspension was dispensed into a 96-well sterile microtitre plate, and 100 μl of drug at 2× the desired final concentration was added. Controls included were medium only and cells plus medium. Triton X-100 (0.25 %, v/v) in complete PBS was used as a positive control, and measures the endogenous endonuclease activity of the cells, since, once lysed, the calcium and magnesium in the medium activate the cell's own endonuclease. After the appropriate incubation time, the microtitre plate was centrifuged in an MSE Mistral 3000i bench centrifuge for 5 min at 250 $\boldsymbol{g}_{av}$, and 100 μl of supernatant was removed and either analysed immediately or stored at −20 °C overnight until analysis. A 100 μl portion of lysis buffer was added and the samples were incubated at room temperature for 30 min. The microtitre plate was centrifuged as before and 100 μl of lysate was used to quantify the extent of DNA fragmentation or was stored overnight at −20 °C until analysis as per kit instructions.

Cell cycle analysis and the percentage apoptotic cells (A_0) was determined by flow cytometry using 1×10^6 cells by the method of Vindelov et al. [24].

RESULTS

The effects of etoposide on the growth of HL-60 cells were measured over a range of concentrations (Figure 1). At concentrations of 5 μM and above, etoposide was cytotoxic at all

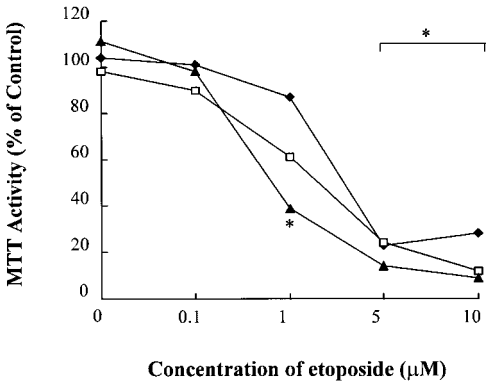

Figure 1 Cytotoxic effect of etoposide on HL-60 cells

HL-60 cells were seeded at a density of 6.8×10^4 cells/ml and grown for 48 h in RFC_{10} medium. Cells were transferred to 24-well plates and exposed to etoposide or DMSO (0.2 %, v/v) for the appropriate time (◆, 24 h; □, 48 h; ▲, 72 h). The MTT assay was carried out as described in the Experimental section, and values are means ± S.D. of three separate experiments with three replicates per experiment. Results were analysed by ANOVA with Dunnett's post test (*$P < 0.01$).

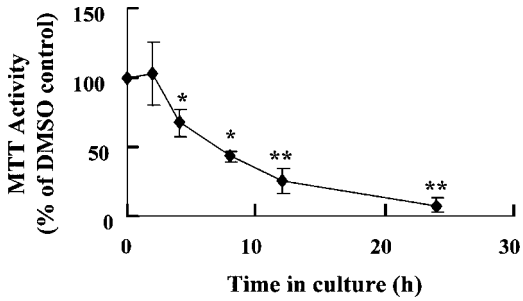

Figure 2 Cytotoxicity of 10 μM etoposide in HL-60 cells

HL-60 cells were grown on 24-well plates for 48 h prior to the addition of 10 μM etoposide. Cells were exposed to the drug for the appropriate times and the viable cell number was determined by MTT assay. Results are expressed as means ± S.D. ($n = 3$, with three replicates per experiment). Individual time points were compared with controls by Student's *t*-test (*$P <$ 0.05; **$P < 0.01$).

exposure times tested. At lower concentrations there was evidence of inhibition of growth, but only after 48 h of exposure. IC_{50} values were calculated to be in the range 1–4 μM. Protein content and cell number followed a similar pattern (results not shown). As 10 μM etoposide was cytotoxic, this concentration was chosen for further investigation. The time course of the cytotoxic effect with this concentration of etoposide was rapid, with significant decreases in MTT activity being observed as early as 4 h after exposure to the drug (Figure 2). Cell number was significantly decreased 12 h after etoposide addition ($P < 0.05$; results not shown), yet cell viability, as measured by Trypan Blue exclusion, only decreased significantly after 24 h of exposure (Figure 3). Etoposide significantly inhibited both DNA and protein synthesis, but had little effect on RNA synthesis; however, the possibility that the uptake of thymidine or amino acids was also influenced cannot be ruled out (Table 1).

Analyses of polyamine catabolic enzymes showed there were no significant differences in either SSAT or PAO activity following exposures to etoposide of up to 24 h, although the trend was for an increase in activity in the presence of the drug (Table 2). Polyamine efflux, however, did increase significantly ($P <$ 0.01) after a 24 h treatment (Table 2). Total polyamine content was maintained for the first 12 h of etoposide treatment. However, this had decreased to approx. 50 % of control value after 24 h (results not shown).

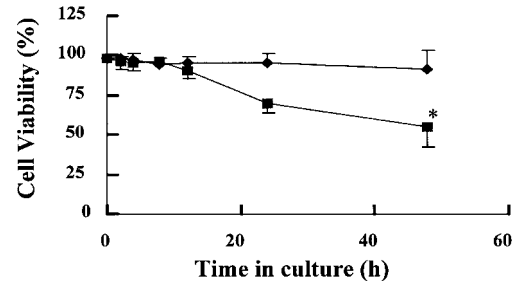

Figure 3 Effect of 10 μM etoposide on cell viability

HL-60 cells were seeded at 6.8×10^4 cells/ml and grown for 48 h on 5 cm diam. plates prior to drug addition. Cell viability was determined by the Trypan Blue exclusion method. Results are means ± S.D. of three separate experiments with three replicates per experiment, and were analysed by Student's *t*-test (*$P < 0.01$). ◆, DMSO (0.2%); ■, 10 μM etoposide.

Table 1 Effect of 10 μM etoposide on macromolecular synthesis in HL-60 cells

HL-60 cells were grown on 24-well plates for 48 h prior to the addition of etoposide or DMSO (0.2%, v/v). Cells were pulse-labelled with [^{3}H]thymidine (0.0185 MBq/ml) for 1 h to measure DNA synthesis, with ^{14}C-labelled amino acid mix (0.925 MBq/ml) for 4 h to measure protein synthesis and with [^{3}H]uracil (0.0085 MBq/ml) for 1 h to measure RNA synthesis. Protein content was determined by the method of Lowry et al. [16]. Values represent the incorporation of label into acid-insoluble material and are means ± S.E.M. of three separate experiments with three replicates per experiment for DNA and RNA synthesis (*$P < 0.01$ compared with control), and median with range ($n = 2$ with three replicates per experiment) for protein synthesis.

Synthesis of:	Time (h)	Control	Etoposide (10 μM)
DNA (10^{-3} × d.p.m./min per mg of protein)	0	43.9 ± 2.2	–
	12	72.3 ± 4.6	0.1 ± 0.08*
	24	25.3 ± 2.7	5.1 ± 1.7*
RNA (10^{-2} × d.p.m./min per mg of protein)	0	1.43 ± 0.04	–
	12	1.23 ± 0.18	1.20 ± 0.52
	24	1.29 ± 0.73	1.72 ± 0.50
Protein (10^{-3} × d.p.m./min per mg of protein)	0	11.7 ± 2.6	–
	12	12.8 ± 0.4	1.5 ± 0.2
	24	12.2 ± 1.6	4.2 ± 0.5

Table 2 Effect of 10 μM etoposide on polyamine catabolism: short time course

HL-60 cells were exposed to etoposide or DMSO (0.2%, v/v) for the appropriate times after an initial growth period of 48 h. Activities of SSAT and PAO, and polyamine content and efflux, were measured as described in the Experimental section. Values are means ± S.D. ($n = 3$, with three replicates per experiment); *$P < 0.01$ compared with control.

Polyamine catabolism	Time (h)	Control	Etoposide
SSAT activity (pmol/min per mg of protein)	0	2.7 ± 0.4	–
	12	4.4 ± 2.2	5.2 ± 2.0
	24	6.2 ± 3.5	9.5 ± 2.7
PAO activity (pmol/min per mg of protein)	0	5.2 ± 1.2	–
	12	2.8 ± 1.0	4.2 ± 1.2
	24	1.8 ± 0.7	8.4 ± 2.8
Polyamine efflux (10^{-4} × d.p.m./mg of protein)	0	6.9 ± 2.4	–
	12	20.5 ± 12.2	33.1 ± 15.3
	24	21.5 ± 12.2	111.2 ± 13.5*
Total polyamine content (nmol/mg of protein)	0	26.6 ± 2.8	–
	12	22.8 ± 3.2	27.4 ± 3.5
	24	27.1 ± 2.8	13.2 ± 0.7*

DNA fragmentation, as measured by the Boehringer DNA ELISA, showed an increase from 4 h onwards (Figure 4a). A similar pattern was observed using flow cytometric analysis (Figure 4b). Fragmentation as assessed by gel electrophoresis was found to be a less sensitive measure of apoptosis, as the characteristic ladder was only detected after 8 h of exposure to the drug (results not shown).

Longer exposures (up to 72 h) to etoposide produced significant increases in SSAT and PAO activity, further increases in polyamine efflux and significant polyamine depletion (Table 3).

DISCUSSION

In agreement with previous studies, etoposide induced cell death in HL-60 cells [25,26]. This death was identified as apoptosis, and not necrosis, by a number of characteristic features, including DNA fragmentation and cell morphological studies. DNA

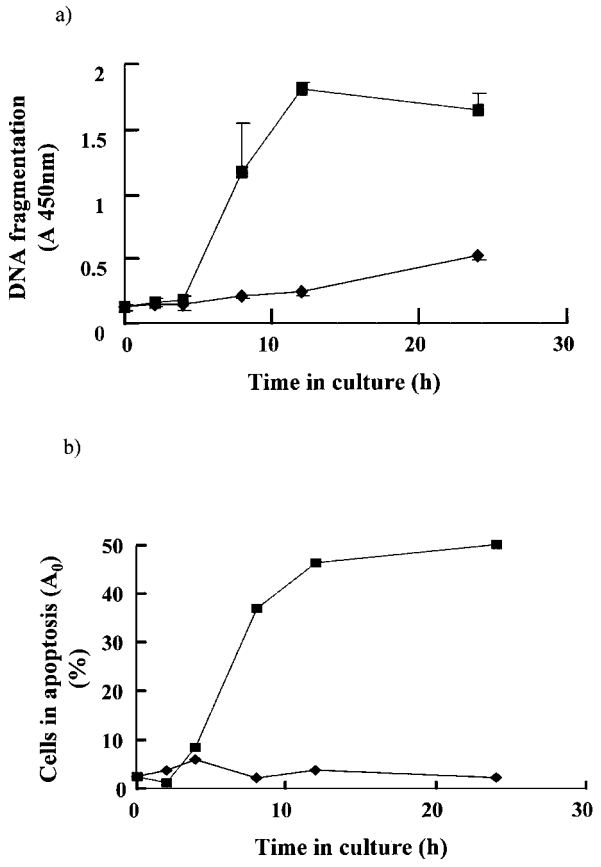

Figure 4 Time course of the effect of 10 μM etoposide on HL-60 cells

HL-60 cells were grown for 48 h in RFC_{10} medium prior to the addition of etoposide. For DNA fragmentation (**a**), cells were labelled with BrdU for 12 h and the extent of DNA fragmentation was assayed by ELISA. Results are median and range ($n = 2$, with three replicates per experiment). For flow cytometry (**b**), cells were fixed and analysed as described in the Experimental section [24]. Values are means ($n = 2$). ◆, DMSO (0.2%); ■, 10 μM etoposide.

Table 3 Effect of 10 μM etoposide on polyamine catabolism: long time course

HL-60 cells were exposed to etoposide or DMSO (0.2%, v/v) for 48 and 72 h after an initial growth period of 48 h. SSAT and PAO activities, and polyamine content and efflux, were measured as described in the Experimental section. Values are median and range ($n = 2$, with three replicates per experiment), except for those for polyamine efflux, which are means ± SD ($n = 3$, with three replicates per experiment; $^*P < 0.01$ compared with control).

Polyamine catabolism	Time (h)	Control	Etoposide
SSAT activity (pmol/min per mg of protein)	48	2.68 ± 0.72	15.12 ± 2.30
	72	1.18 ± 2.36	8.29 ± 3.96
PAO activity (pmol/min per mg of protein)	48	3.0 ± 1.2	15.8 ± 1.2
	72	1.6 ± 0.7	14.5 ± 4.9
Polyamine efflux (10^{-4} × d.p.m./mg of protein)	48	3.17 ± 1.85	185.5 ± 26.9*
	72	4.08 ± 0.11	296.3 ± 121.0*
Total polyamine content (nmol/mg of protein)	48	28.7 ± 6.5	9.5 ± 0.3
	72	29.5 ± 8.2	10.1 ± 2.5

disruption was observed from as early as 4 h after exposure to the drug (Figures 4a and 4b). The typical morphological features of apoptosis, including membrane blebbing and condensation of chromatin, were noted from 2 h onwards, with cell vacuolation occurring from about 8–12 h post-treatment (results not shown). Thus the death seen in this model at early times was clearly established as apoptosis.

Since increases in SSAT activity have been associated with both decreases in cell growth [10–12,17,27] and a cytotoxic response [28], it has been suggested that superinduction of SSAT could be causal in terms of cell death. However, to date this has only been demonstrated in a specific cell type, namely human non-small-cell lung cancer cells (NCI H157) [29]. The majority of studies have, however, used agents that disrupt polyamine metabolism itself as the signal inducing cell death. In the present study we have employed a different approach, taking the view that, if polyamines are involved in apoptosis, then agents that are known to induce apoptotic cell death will, by necessity, affect polyamine metabolism. Although there is clear evidence of apoptosis, the cell death was not, in the first instance, accompanied by changes in polyamine acetylation or oxidation (Table 2). There was a significant increase in polyamine efflux into the extracellular medium which could explain the decrease in total polyamine content seen at 24 h. Differential induction of SSAT has been reported in other systems. In human small-cell lung cancer cells, there was no induction of SSAT and no cytotoxicity in response to the polyamine analogue bis(ethyl) spermine, while in the related human non-small-cell lung cancer cells, superinduction of SSAT was observed after exposure to bis(ethyl)spermine [10]. The opposite response has also been noted, in that 1,12-dimethylspermine, another polyamine analogue, can induce SSAT activity significantly without exhibiting any cytotoxicity [30].

It has been suggested that the catabolism of polyamines by PAO could be an important initiating reaction in cytotoxicity, since the hydrogen peroxide produced stoichiometrically from the catabolism of spermine or spermidine can induce apoptosis [31,32]. Indeed, it has recently been shown that hydrogen peroxide can also induce SSAT [31]. Thus with increased PAO activity and increased SSAT activity there may effectively be a cell-death-generating cycle, with increased PAO activity producing hydrogen peroxide, which then induces SSAT activity, which produces further substrates for PAO, and so on. The significant increases in PAO and SSAT activities observed at later times are consistent with this hypothesis. Thus the relationship between cell death and polyamine catabolism is complex.

One interesting observation was the maintenance of viability, as measured by Trypan Blue exclusion (Figure 3). This is another feature of apoptosis, whereby the cell membrane becomes impermeable to vital dyes due to the activation of calcium-dependent tissue transglutaminase [33]. The cross-linking of membrane proteins through the activity of tissue transglutaminase prevents leakage of the cell contents and thus prevents the inflammatory response typical of necrosis. In the absence of phagocytosis, apoptosis is essentially complete when the cell becomes permeable to vital dyes. Thus in the case of HL-60 cells, apoptosis can be said to be complete by 24 h, by which time cell viability had decreased (Figure 3). Therefore the significant changes occurring in polyamine catabolic enzymes are at later times when apoptosis, and possibly necrosis, is ongoing. Morphological analysis of the cells at later times did reveal typical signs of necrosis, such as cell and mitochondrial swelling (results not shown), as well as apoptosis. Interestingly, the cross-linking of the cell membrane did not prevent polyamine efflux, supporting the hypothesis of a carrier protein for polyamine export.

In summary, alterations in polyamine catabolism are not required for the initiation of the apoptotic process, at least in

human leukaemic cells induced to undergo apoptosis by the topoisomerase II inhibitor etoposide. Increased polyamine catabolic activity does, however, seem to be associated with the continuation of cytotoxicity, and may be an essential part of that process.

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