Excess putrescine accumulation inhibits the formation of modified eukaryotic initiation factor 5A (eIF-5A) and induces apoptosis

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DH23A cells, an α-difluoromethylornithine-resistant variant of the parental hepatoma tissue culture cells, express high levels of stable ornithine decarboxylase. Aberrantly high expression of ornithine decarboxylase results in a large accumulation of endogenous putrescine and increased apoptosis in DH23A cells when α-difluoromethylornithine is removed from the culture. Treatment of DH23A cells with exogenous putrescine in the presence of α-difluoromethylornithine mimics the effect of drug removal, suggesting that putrescine is a causative agent or trigger of apoptosis. Accumulation of excess intracellular putrescine inhibits the formation of hypusine *in vivo*, a reaction that proceeds by the transfer of the butylamine moiety of spermidine to a lysine residue in eukaryotic initiation factor 5A (eIF-5A). Treatment of DH23A cells with diaminoheptane, a competitive inhibitor of the post-translational modification of eIF-5A, causes both the suppression of eIF-5A modification *in vivo* and induction of apoptosis. These data support the hypothesis that rapid degradation of ornithine decarboxylase is a protective mechanism to avoid cell toxicity from putrescine accumulation. Further, these data suggest that suppression of modified eIF-5A formation is one mechanism by which cells may be induced to undergo apoptosis.

INTRODUCTION

The polyamines, spermidine and spermine, and putrescine, the diamine precursor for spermidine synthesis, are abundant and ubiquitous polycations in eukaryotic cells. Although specific functions for the polyamines have often been difficult to define, they have been shown to be necessary for cell growth [1,2]. Generally, cells and tissues undergoing periods of rapid proliferation are characterized by high polyamine contents which then fall as cells become quiescent [3]. Conversely, severe depletion of polyamines reduces growth in mammalian cells [2]. The control of cellular polyamine contents may therefore be a major component in cell growth regulation.

Regulation of cellular polyamine contents in eukaryotic cells is a complex multilevel process with control points at the level of biosynthesis, catabolism, uptake and excretion. Ornithine decarboxylase (ODC), the first and usually rate-limiting enzyme in polyamine biosynthesis, is a key component in the regulation of cellular polyamine contents. Regulation of ODC itself is a complex combination of control at the level of transcription [4,5], mRNA stability [6,7], translation [8–11] and in particular protein degradation (reviewed in [12]). This multilevel regulation of ODC has been thought to allow a rapid increase in the enzyme and corresponding cellular polyamine contents in response to mitogenic stimuli. Morris [13] has suggested that rapid degradation of ODC is necessary to prevent accumulation of toxic levels of polyamines.

Polyamine overaccumulation has been shown to be toxic both in whole organisms [3] and *in vitro* [14–18]. The toxicity of some potential polyamine metabolites, particularly polyamine-oxidation products, has been well established [19,20]; however, there is some evidence that the amines themselves may be the toxic agents, although the mechanism is not clear. In mammalian cells *in vitro*, excess spermidine [17] or spermine [18] accumulation can be toxic even under conditions where known polyamine-oxidation pathways have been blocked, suggesting that the amines themselves can cause cell death. Toxicity from excess putrescine accumulation in *Neurospora crassa* [21] and *Anacystis nidulans* [22] has also been documented. Work in our laboratory and others has shown that excess putrescine accumulation by uptake from the medium and/or by synthesis as the result of overproduction of ODC is toxic in mammalian cells *in vitro* as well [14–16]. Selection of hepatoma tissue culture (HTC) cells in α-difluoromethylornithine (DFMO), an inhibitor of ODC, has produced a cell line, DH23A/b, that produces aberrantly high levels of very stable ODC [23] and overaccumulates putrescine in the absence of DFMO [16]. Overaccumulation of putrescine in these cells results in cell death [14,16]; however, the mechanism by which putrescine causes cell death is not known. These studies were designed to elucidate the mechanism by which putrescine may cause cell death.

EXPERIMENTAL

Cell culture and drug treatments

HTC cells were obtained from Dr. Peter McCann (Marion Merrell Dow Pharmaceutical Co., Cincinnati, OH, U.S.A.). DH23A cells, a variant of HTC cells selected for their resistance to growth inhibition in DFMO, were donated by Dr. John L. A. Mitchell (Northern Illinois University, DeKalb, IL, U.S.A.). Their development and characterization have been described by Mitchell et al. [23]. HTC and DH23A cells were grown as described [16]. DH23b cells are a later selection variant of DH23A cells, also donated by Dr. Mitchell, and grown in medium similar to that used for DH23A cells as described [24]. No differences were found between DH23A and DH23b cells in our hands or by Dr. Mitchell (personal communication). Cells were incubated at 37 °C and diluted into new medium every 3–4

Abbreviations used: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; DAH, diaminoheptane; eIF-5A, eukaryotic initiation factor 5A; HTC cells, hepatoma tissue culture cells.

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days. DH23A/b cells were maintained in medium containing 10 mM DFMO unless otherwise noted.

Drugs and chemicals were added directly to the growth medium before the addition of the cells. DFMO was a gift from Marion Merrell Dow. Putrescine hydrochloride, diaminoheptane (DAH) and ornithine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Determination of cell number and viability

Cell number for cells growing in suspension was determined by averaging at least four counts using a haemocytometer. When cells were grown attached, they were removed from the monolayer by treatment with trypsin (approx. 1500 units/ml; Calbiochem, San Diego, CA, U.S.A.)/EDTA (0.7 mM) and counted using a Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.). Viability was measured by the ability of the cells to metabolize diacetylfluorescein using the method of Freshney [25] as described in [16]. A minimum of 1000 cells was scored for each measurement.

Measurement of polyamine and protein content

Acid-soluble polyamines were extracted and cellular protein precipitated as previously described [16]. Polyamines were eluted sequentially from an HPLC column, derivatized and detected as discrete peaks using the method of Seiler and Knodgen [26]. Peaks were identified and quantified by comparison with commercially available compounds. Acid-precipitable proteins were quantified using the BCA protein assay kit from Pierce (Rockford, IL, U.S.A.) as previously described [16].

Determination of cell morphology

Samples for light microscopy were removed directly from the suspension culture or prepared from attached cultures. In the attached cultures, samples were prepared by pooling the supernatant medium, the trypsin-treated cell suspension and an additional wash of the tissue culture plate. Slides for light microscopy were prepared by placing 10000 or 20000 cells into the cytospin cup and pelleting the cells on to the slide by centrifugation using a cytospin (Shandon, Pittsburgh, PA, U.S.A.) at 600 rev./min for 2 min at low acceleration. Cells were fixed on to the slides by immersion in 100 % ethanol for 1 min. The slides were stained using the DiffQuick staining kit (Baxter Diagnostics, McGraw Park, IL, U.S.A.) according to the manufacturer's directions and scored. At least 500 cells were scored for each treatment for each day, and the frequencies of apoptotic and mitotic cells were expressed as a percentage of the total cells counted.

Electron-microscopy samples were prepared as described in [27]; 1 μm sections were stained with uranyl acetate/lead citrate and photographed using an electron microscope (Philips CM12S; Philips Electronics, Mahwah, NJ, U.S.A.).

Modification of eukaryotic initiation factor 5A (eIF-5A) in intact cells

Inhibition of eIF-5A modification *in vivo* was analysed by incubating an aliquot of the cells with 2 μCi/ml terminal methylenes [1,8-^{3}H]spermidine (DuPont–NEN, Boston, MA, U.S.A.) for a 24 h period. At specified times, cells were harvested by centrifugation (500 ***g*** for 5 min) and washed twice in PBS, pH 7.4. The amount of modified eIF-5A produced was measured by eluting radiolabelled hypusine from an HPLC column after precipitation and hydrolysis of total cell protein as described [28]. Spermidine specific radioactivity was calculated by counting the column eluate fractions containing spermidine and comparing them with cellular spermidine content.

Measurement of unmodified eIF-5A

eIF-5A was partially purified for analysis using a modification of the procedure of Park [29] as described in Tome and Gerner [24]. Cellular unmodified eIF-5A content was measured by quantifying the incorporation of [1,8-^{3}H]spermidine into the unmodified precursor *in vitro* using a modification of the conditions in Wolff et al. [30] as described in Tome and Gerner [24]. Amounts were normalized for cellular protein content. Inhibition of eIF-5A modification was measured using similar conditions except that 28 nM eIF-5A partially purified from HTC cells by the method of Park [29] modified as described in Tome and Gerner [24] was used as the substrate and the specific inhibitor was added to the reaction mixture before the addition of the enzyme. Sample incubation time was decreased to 6 h. Incorporation of [^{3}H]putrescine into eIF-5A was tested in a similar reaction using 6.6 μM [^{3}H]putrescine (20 μCi) (DuPont–NEN) instead of [1,8-^{3}H]spermidine.

Northern-blot analysis

RNA was isolated from 2×10^6 cells harvested under the specified conditions (see Figure legends); the 20 μg of RNA/lane was separated by size and blotted using standard protocols as described [24]. The blots were probed and analysed as previously described [24].

RESULTS

Effects of altered ODC activity on maintenance of exponential cell growth

HTC cells, the parental cell line, contain moderate cellular polyamine concentrations and show the fastest growth rate (Figure 1). In the presence of DFMO, which suppresses ODC activity, DH23A cells grow exponentially, albeit at a much lower rate than HTC cells. Under these conditions, putrescine is not detectable, spermidine is 10–20 % of that seen in the parental HTC cells and spermine is similar to that in HTC cells (results not shown; [16]).

DH23A cells, grown in the absence of DFMO (conditions in which the overexpressed ODC is no longer inhibited), show a very different growth pattern. The cells initially begin to increase in number and then enter a period of cytostasis; this is followed by a loss of viable cells. Concomitant with the period of cytostasis and cell loss is a very large increase in cellular putrescine content which remains elevated. Cellular spermidine is initially similar to that seen in the parental cells, then tends to decrease slightly after continuous culture in the absence of DFMO (see days 7–9).

Chronically elevated endogenous putrescine, caused by overaccumulation from medium containing DFMO and putrescine, produces the same growth pattern as in DH23A cells in the absence of DFMO. Cells treated with exogenous putrescine accumulate putrescine and enter cytostasis more quickly than those in cultures where putrescine accumulates through endogenous production. Overaccumulation of putrescine from an exogenous source also correlates with loss of viable cells. Treatment of cells with 1 mM ornithine in the absence of DFMO causes the production of the highest concentrations of putrescine and the greatest reduction in cell number. The addition of exogenous 1 mM ornithine in the presence of DFMO does not

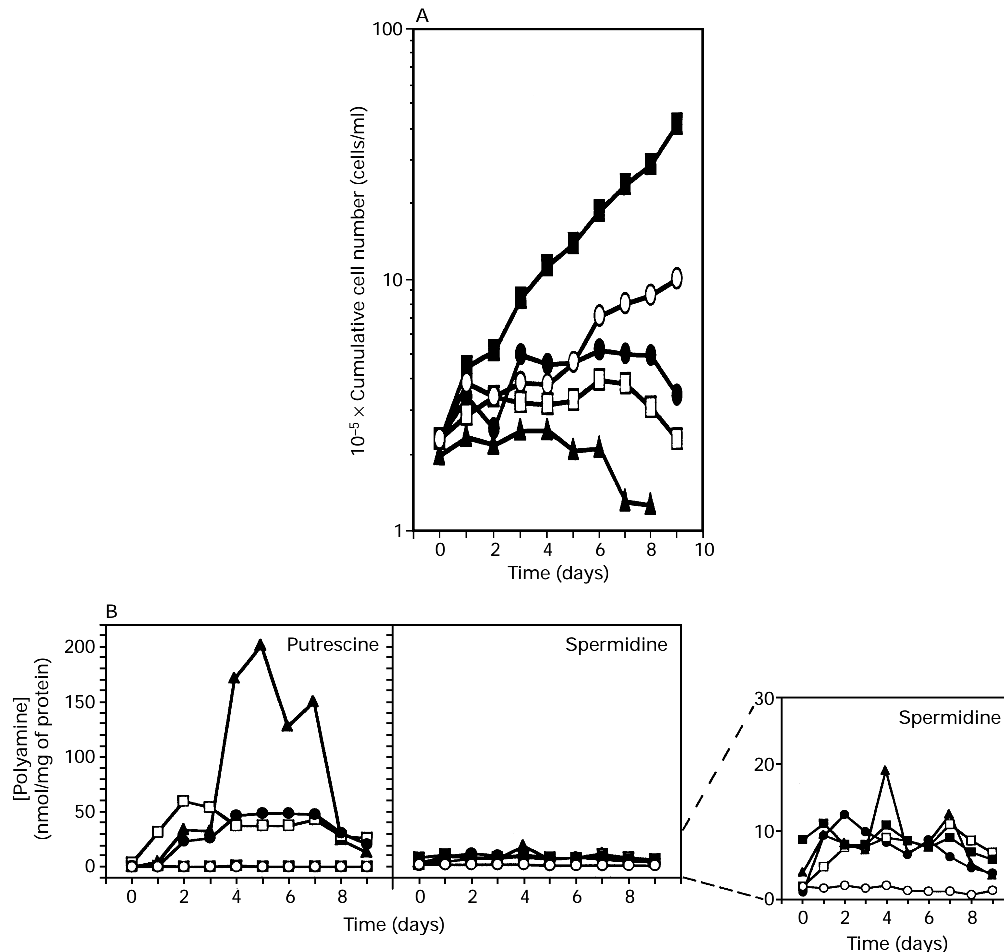

Figure 1 Effect of putrescine accumulation on growth and polyamine profile in DH23A cells in the presence or absence of DFMO compared with that in HTC cells

(**A**) Cell number and (**B**) polyamine profile in DH23A cells in the absence of DFMO (●), the absence of DFMO + 1 mM ornithine (▲), the presence of 10 mM DFMO (○) or the presence of 10 mM DFMO + 1 mM putrescine (□) compared with that in HTC cells (■). Cells were diluted into new medium with the above treatments on day 0 and transferred to fresh medium on days 3 and 6 in the continued presence or absence of the drugs. Cell number data have been corrected for viability (range 76–96%) and dilution. Data shown are for one representative experiment which was replicated. Parts of this Figure have previously been published in Tome et al. [16].

affect either growth kinetics or polyamine content in DFMO-treated cells (results not shown).

Effects of altered polyamine profile on cell morphology and mitosis

Alterations in cell growth characteristics correlate with differences in mitotic and apoptotic frequency. A comparison of morphological features seen in HTC cells and DH23A cells in the presence of 10 mM DFMO with those in DH23A cells cultured in the absence of DFMO for 10 days shows an increase in the number of cells that appear to be apoptotic in the DH23A minus DFMO cell cultures (Figure 2). Cell shrinkage, chromatin condensation, fragmentation of the nuclei and formation of apparently apoptotic bodies are visible at the light-microscopic level. Cells that show the hallmarks of classic apoptotic morphology [margination of the chromatin at the nuclear periphery (Figure 2E) and formation of double-membrane-bound nuclear fragments (Figure 2D)] can be seen at higher magnification. The number of cells that appear to be apoptotic is low ($< 2\%$) in HTC cultures and fairly low ($< 10\%$) in DH23A cells cultured in the presence of 10 mM DFMO. After removal from DFMO, apoptotic frequency is initially low in the DH23A cells; however, the number of apoptotic cells increases sharply when the culture has been maintained in the absence of DFMO for 5–6 days and continues to rise (Figure 3A). Increases in the number of apoptotic cells correlate with a decrease in viable cells. The addition of exogenous putrescine mimics the effect of endogenous putrescine production on cell growth kinetics, endogenous putrescine and apoptosis except that these all occur earlier in the time course. Throughout the time course, as the apoptotic frequency increases, as judged by morphological parameters, there is no evidence for internucleosomal cleavage of DNA (results not shown).

Differences in cell number observed in the HTC cells compared with DH23A cells in the presence of DFMO correlate with alterations in mitotic frequency (Figure 3B). HTC cells exhibit a higher rate of mitosis than DH23A cells in the presence of the drug for most points during the time course. Mitotic frequency in DH23A cells minus DFMO is in general intermediate between that of HTC and DH23A cells plus DFMO, suggesting that the decrease in cell number observed in DH23A cell cultures in the

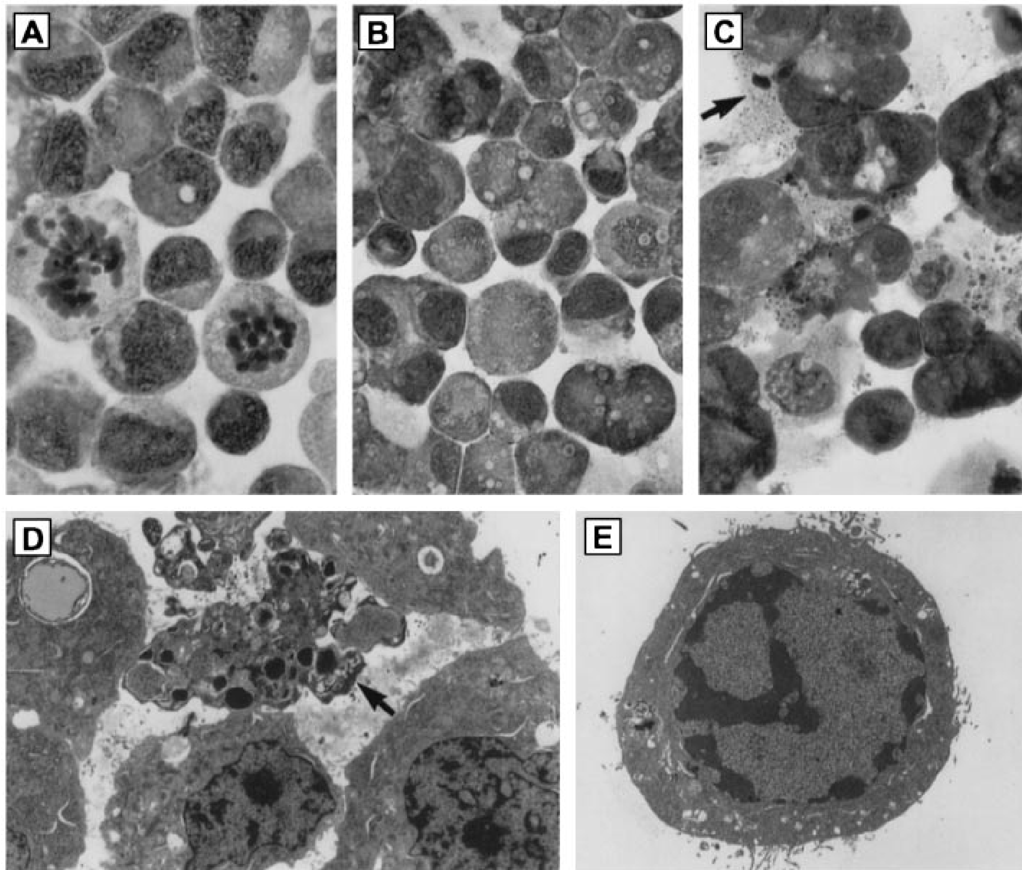

Figure 2 Comparison of cell morphology after 10 days continuous culture in the presence or absence of DFMO

(**A**) HTC cells (light micrograph; 100 ×, oil); (**B**) DH23A cells in the presence of DFMO (light micrograph; 100 ×, oil); (**C**) DH23A cells in the absence of DFMO (light micrograph; 100 ×, oil); (**D**) and (**E**) DH23A cells in the absence of DFMO (electron micrograph magnification: **D** approx. 7500 × ; **E** approx. 10 000 × ; staining: uranyl acetate/lead citrate). Apoptotic cells are indicated by arrows.

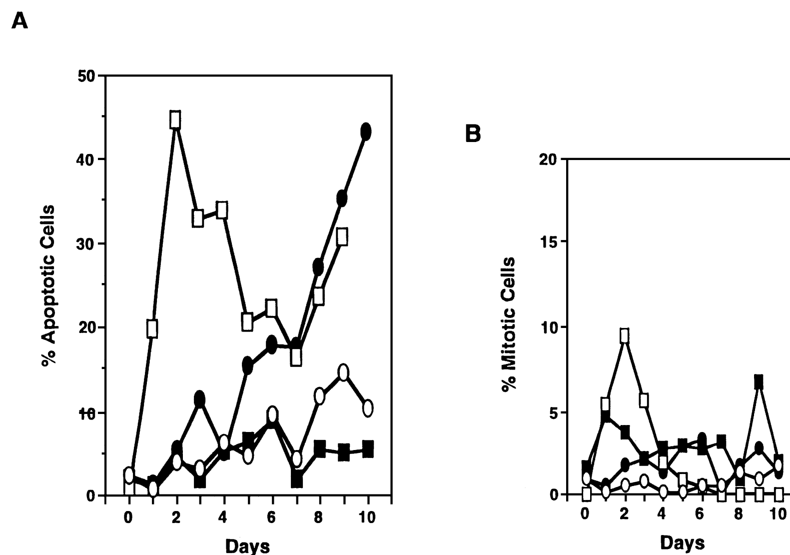

Figure 3 Mitotic frequency and apoptotic frequency in cell cultures maintained in exponential growth

(**A**) Apoptotic frequency and (**B**) mitotic frequency in HTC cells (■), DH23A cells in the presence of 10 mM DFMO (○), DH23A cells in the absence of DFMO (●) or DH23A cells in the presence of 10 mM DFMO plus 1 mM exogenous putrescine (□). Cells were diluted into medium with the above treatments on day 0 and transferred to new medium on days 3, 6 and 9 in the continued presence or absence of the drugs. Data shown are for one representative experiment which was replicated.

Table 1 Comparison of eIF-5A modification by spermidine *in vitro* in the presence of putrescine or DAH

Addition	Modified eIF-5A (% of control)
Control (no addition)	100
1 mM Putrescine	45
2 mM Putrescine	31
1 μM DAH	84
10 μM DAH	59
25 μM DAH	30

absence of the drug is primarily due to alterations in apoptotic frequency. In the presence of DFMO and 1 mM putrescine, mitotic frequency in DH23b cell cultures initially appears to increase but tapers off to zero by 7 days. An increased number of aberrant mitoses are also seen in cultures containing exogenous putrescine when compared with control cells (0.2 % in DH23b+DFMO compared with 9.5 % in DH23b+DFMO+1 mM putrescine on day 2). The initial increase in mitoses observed during this treatment may partially reflect the increase in aberrant mitoses.

Effect of increased putrescine and DAH on eIF-5A modification

Post-translational modification of eIF-5A by spermidine has been shown to be essential for viability in yeast [31] and is inhibited by compounds with chemical structures related to spermidine [32,33]. To test the hypothesis that excess putrescine inhibits the formation of modified eIF-5A, the effect of putrescine addition on eIF-5A modification *in vitro* and *in vivo* was measured. As shown in Table 1, *in vitro*, putrescine addition inhibits the formation of modified eIF-5A, in a similar way to DAH, a competitive inhibitor of the modification reaction *in vitro* [33]. Incorporation of radiolabelled putrescine into eIF-5A in a similar experiment was not detected.

In vivo, putrescine also inhibits the formation of modified eIF-5A (Figure 4). Both exogenous addition and endogenous production of putrescine suppress eIF-5A formation. This is particularly apparent on day 2 when the concentration of spermidine is similar in DH23b cells in the absence of DFMO and in the presence of DFMO plus exogenous putrescine (Figure 4C); however, endogenous putrescine content is radically different (Figure 4B) and the formation of modified eIF-5A is suppressed in the presence of putrescine. Further, as putrescine increases over time after the removal of DFMO, formation of modified eIF-5A is suppressed. Accumulation of DAH in the cells (Figure 4D) also suppresses the formation of modified eIF-5A. The specific radioactivity of spermidine is similar in the samples containing DFMO alone and the DH23b cells in the presence of DFMO plus DAH; however, the rate of formation of modified eIF-5A is suppressed as the cells take up DAH (compare days 3 and 6).

Effect of DAH on cell growth and apoptosis

Figure 5 shows the effect of exogenous DAH on cell growth kinetics, apoptosis and mitosis in DH23A cells in the presence of DFMO. Increased apoptosis is seen in cultures approx. 4 days after the addition of exogenous DAH (Figure 5A). Mitosis in the presence and absence of DAH is relatively similar (except for at day 4) (Figure 5D). On day 4, most mitoses in cells treated with DFMO are in metaphase and some appear aberrant, suggesting

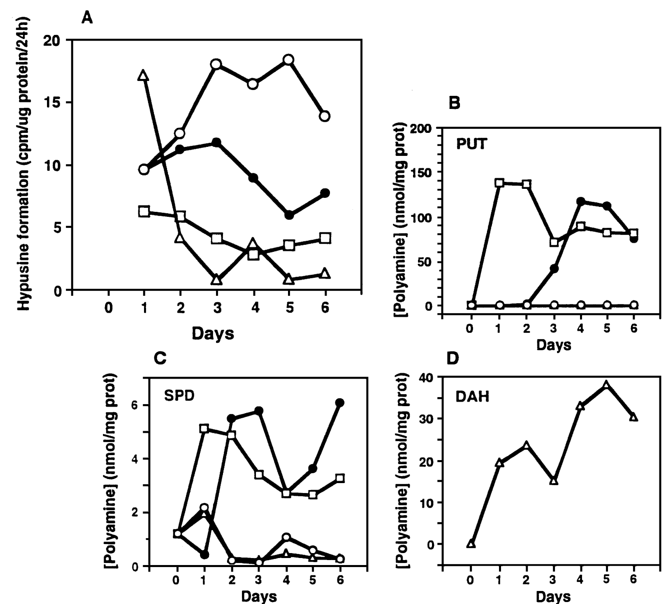

Figure 4 Effect of putrescine or DAH on the modification of eIF-5A *in vivo*

(**A**) Synthesis of hypusine per 24 h period in DH23b cells in the absence of DFMO (●), in the presence of 10 mM DFMO (○), in the presence of 10 mM DFMO+1 mM putrescine (□) or in the presence of 10 mM DFMO+10 μM DAH (△). The corresponding accumulation of intracellular putrescine (**B**), intracellular spermidine (**C**) and DAH (**D**) is shown. Cells were diluted into new medium with the corresponding treatments at day 0 and the stock transferred to new medium on day 3 in the continued presence or absence of the drugs. For each measurement an aliquot was removed from the stock and incubated separately in the presence of [1,8-^{3}H]spermidine for 24 h. This is a representative experiment which was replicated.

the possibility of mitotic arrest [34]; however, because this is a one-point peak, further investigation is needed to clarify this point. These rates are reflected in the growth kinetics; treatment with DAH is shown to inhibit the increase in cell number (Figure 5C).

Effect of increased putrescine or DAH on accumulation of unmodified eIF-5A

If both putrescine and DAH suppress the formation of modified eIF-5A by acting as competitive inhibitors of the addition of the butylamine moiety of spermidine to the newly synthesized eIF-5A, then unmodified eIF-5A should accumulate. A comparison of the cellular unmodified eIF-5A contents (Table 2) shows that unmodified eIF-5A accumulates in the presence of DAH. However, in the presence of increased putrescine, achieved either by exogenous addition or endogenous formation, no accumulation of unmodified eIF-5A is detected. Sampling on day 9 verified that apoptotic frequency was greater in the presence of putrescine or DAH than in cultures grown in DFMO alone.

Effect of increased putrescine on eIF-5A transcription

eIF-5A mRNA content is not altered by the presence of elevated putrescine levels (Figure 6). Although the concentration of eIF-5A mRNA in DH23b cells fluctuates slightly over the time course, no suppression or elevation of eIF-5A mRNA levels occurs in correlation with the increased endogenous putrescine (compare Figure 6A with Figure 6B).

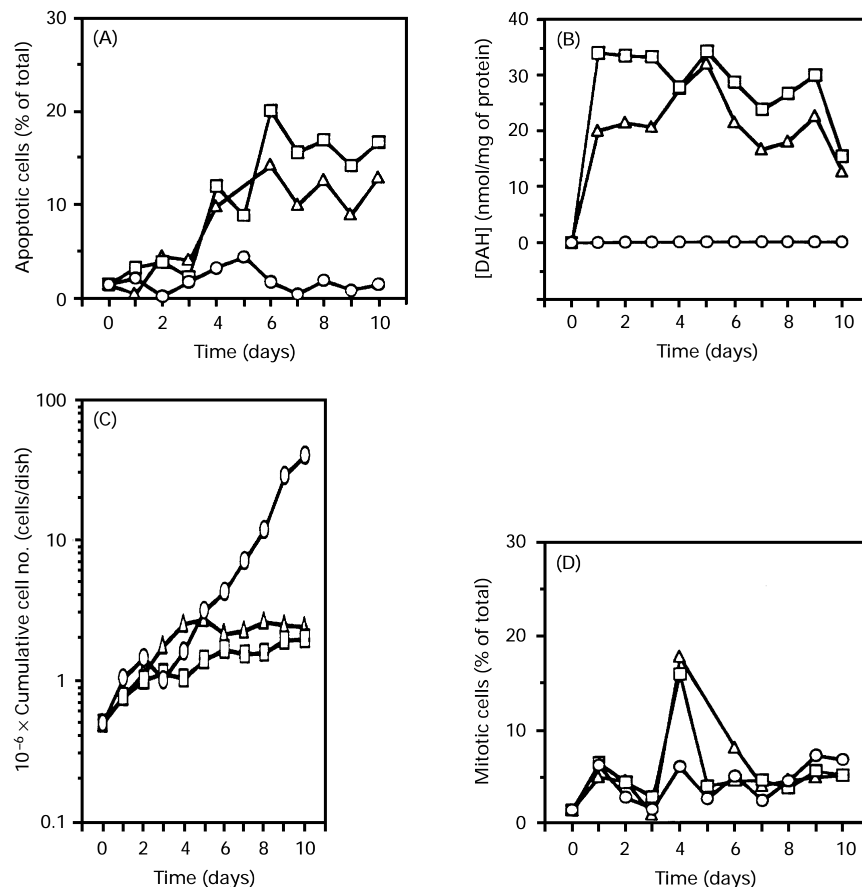

Figure 5 Effect of exogenous DAH addition on cell growth and apoptosis

(**A**) Frequency of apoptotic cells in DH23b cell cultures grown in the presence of 10 mM DFMO alone (○) or in 10 mM DFMO + 25 μM (△) or 100 μM (□) DAH. (**B**) shows the corresponding endogenous DAH accumulation, (**C**) the cumulative cell number and (**D**) the mitotic frequency in the same cell cultures. Cells were diluted into new medium with the above treatments on day 0 and transferred to new medium on days 3, 6 and 9 in the continued presence of drugs. Cell number data have been corrected for viability (range 86–99%) and dilution.

Table 2 Comparison of unmodified eIF-5A accumulation in DH23b cells with or without DFMO and in the presence of exogenous putrescine or DAH

n.d. denotes measurements below the detection limits.

Treatment	Unmodified eIF-5A (fmol/μg of protein)	Putrescine (nmol/mg of protein)	DAH (nmol/mg of protein)
Day 0			
+ DFMO	13.7	n.d.	n.d.
Day 6			
+ DFMO	41.1	n.d.	n.d.
− DFMO	< 1	81.3	n.d.
+ DFMO + 1 mM putrescine	< 1	75.5	n.d.
+ DFMO + 10 μM DAH	30.7	n.d.	30.2

DISCUSSION

Moderate levels of cellular polyamines promote optimal cell growth. Apparent growth rates in cell culture are a reflection of both increased cell number due to mitosis and decreased cell number due to cell loss or death. A comparison of mitotic and apoptotic frequencies in HTC cells and DH23A cells in the presence of DFMO suggests that the difference in cell growth between the cell types is primarily due to alterations in mitosis. Reduced cellular polyamine levels result in reduced mitotic frequency. These data fit with observations that polyamines are necessary for the G1/S transition [34a,35] or the progression through S phase [36,37]. Decreased polyamine concentrations would then be expected to decrease mitoses, as seen here and in other systems [36,38].

Down-regulation of cellular polyamines appears to be necessary not only for cell growth but also for survival. The culture of DH23A cells in the absence of DFMO results in loss of viable cells (Figure 1). Concomitant with the loss of viable cells is a large increase in intracellular putrescine. This decrease in cell number also correlates with a dramatic increase in the number of apoptotic cells seen in these cultures, but little alteration in mitotic frequency, suggesting that the increased putrescine, which is partly due to chronically high ODC activity, alters cell growth primarily by inducing the cells to undergo apoptosis.

Polyamines have been implicated in apoptosis induced by several mechanisms. In several instances, they appear to play a protective role in the induction of apoptosis. Spermidine and spermine have been found to prevent apoptosis in thymocytes by preventing endonuclease activation [39,40] and have been proposed as protective agents during DNA damage by singlet oxygen because of their quenching effect [41]. In addition,

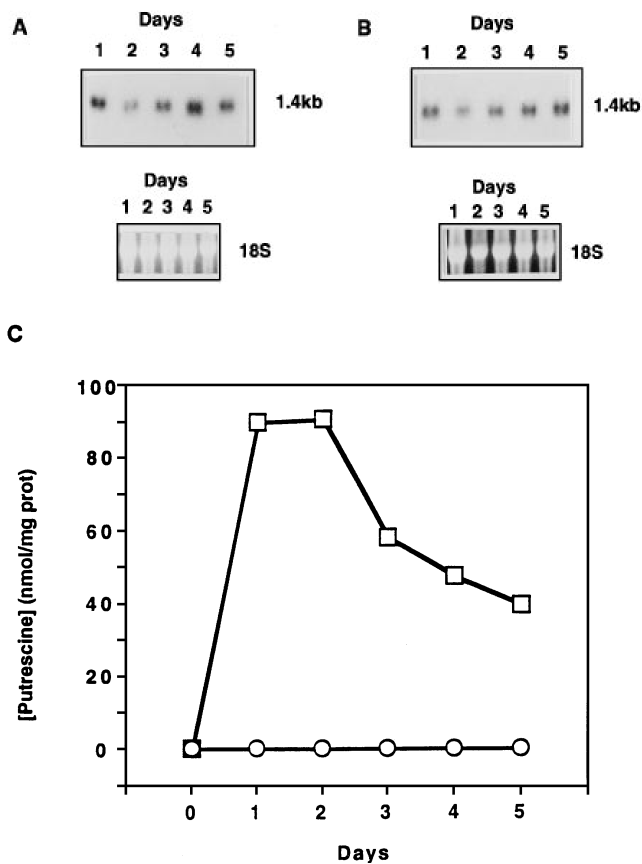

Figure 6 Effect of excess putrescine on eIF-5A mRNA

(**A**) Level of eIF-5A mRNA in DH23b cells on days 1–5 in culture in the presence of 10 mM DFMO; (**B**) eIF-5A mRNA levels in DH23b cells in the presence of 10 mM DFMO+1 mM putrescine on days 1–5. Ethidium bromide staining is provided as a loading control. Cells were diluted into new medium with the above treatments on day 0 and transferred to new medium with similar treatments on day 4. (**C**) Intracellular putrescine accumulation in cells from the corresponding cultures: 10 mM DFMO (○) or 10 mM DFMO+1 mM putrescine (□).

spermine, and to a lesser extent spermidine, at low concentrations have been shown to protect against neuronal damage in cerebral ischaemia under certain conditions [42] and to decrease nerve cell loss during the normal loss of neurons in newborn rats [43]. Polyamines have also been implicated in apoptosis as inducers or enhancers. Packham and Cleveland [44] have shown that Myc-induced apoptosis in interleukin 3-dependent murine myeloid cells after interleukin 3 withdrawal can be partially traced to induction of ODC. Recently, McCloskey et al. [45] reported that a polyamine analogue can induce apoptosis in human breast cancer cell lines.

Excess putrescine accumulation triggers apoptosis in DH23A/b cells. Cytostasis or cytotoxicity attributed to excess putrescine has been found in other systems. Treatment of human fibroblasts with exogenous putrescine prevented phytohaemagglutin-stimulated proliferation [46]. Toxicity attributable to excess putrescine has also been observed in *N. crassa* [21] and *A. nidulans* [22]. In *N. crassa* excessive putrescine accumulation correlates with decreased intracellular K^+ [21], whereas in *A. nidulans* putrescine toxicity is apparently due to cessation of protein synthesis [22]. Excess putrescine accumulation has also been reported to correlate with apoptosis in mouse myeloma cells [15]. Down-regulation of cellular polyamines by some combination of decreased synthesis and uptake and increased export appears to be necessary for survival.

The mechanism by which putrescine overaccumulation induces apoptosis appears to involve suppression of hypusine formation in eIF-5A. Our data show that putrescine overaccumulation reduces the amount of modified eIF-5A in DH23A/b cells. The mechanism by which putrescine suppresses formation of modified eIF-5A is unclear. Our data suggest that putrescine acts post-transcriptionally to reduce eIF-5A levels. Both modified and unmodified eIF-5A contents are reduced in DH23A cells growing in the absence of DFMO, although levels of eIF-5A mRNA are not suppressed by excess putrescine.

Support for the hypothesis that inhibition of modified eIF-5A formation may be sufficient to induce apoptosis comes from the results for DAH-treated cells. Treatment of DH23A/b cells with DAH, an *in vitro* competitive inhibitor of eIF-5A hypusinylation [33], in the presence of DFMO to suppress putrescine levels causes suppression of the formation of modified eIF-5A, with a concomitant increase in apoptosis. DAH may have additional intracellular effects. Because of the non-specific nature of many of the polyamine–macromolecular interactions in the cell, it is possible that, as with many of the polyamines or polyamine analogues, DAH replaces other polyamines in certain cases [47–49]. DAH may partially substitute for the natural amines, particularly when first added to DFMO-treated spermidine-depleted cells, temporarily stimulating such cellular processes as growth or the increased synthesis of unmodified eIF-5A reflected on day 1 (Figure 4). Although we have not ruled out other intracellular DAH effects contributing to apoptosis, the effect on eIF-5A modification may be a critical one.

Post-translational modification of eIF-5A by spermidine and subsequent hydroxylation to form hypusine in mature eIF-5A has been shown to be necessary for growth and viability in yeast [50–52] and proliferation in a number of mammalian systems [33,53–56]. The cytostasis seen under conditions of severely limited polyamines in mammalian cells may also be due to lack of modified eIF-5A because the substrate for the modification reaction, spermidine, is reduced to critical levels [49,57,58]. Re-addition of spermidine to the cells in these studies reduced the unmodified eIF-5A and reversed the cytostasis [49,57,58]. The precise function of eIF-5A and its role in proliferation is unclear. Work on HIV has shown that host cell eIF-5A is necessary for Rev function during the replication of HIV using host cell machinery [59,60]. Recently, Hanauske-Abel et al. [61] found that, in cells treated with an inhibitor of eIF-5A modification, the mRNA for methionine adenosyltransferase disappears from the polysomes, which may affect *de novo* nucleotide synthesis. DH23A cells, after removal of DFMO, also show a decrease in the *S*-adenosylmethionine pool [16] concomitant with the suppression of modified eIF-5A formation, suggesting that this may be one consequence of excess putrescine accumulation.

The addition of mitogenic stimuli after the suppression of modified eIF-5A formation by putrescine or DAH (a proliferation block) may induce DH23A/b cells to undergo apoptosis because the cells receive conflicting signals. DH23A/b cells, after the removal of DFMO, may be unable to proliferate because of a lack of modified eIF-5A (or *S*-adenosylmethionine); however, by changing the medium, new mitogenic stimuli and the polyamines themselves continue to provide a growth stimulus. The cells may then undergo apoptosis. This conflicting signal hypothesis has been suggested as an explanation for p53-induced apoptosis [62]. In the presence of p53, cells with damaged DNA undergo apoptosis if a growth signal is provided; however, in the absence of p53 the same damaged cells proliferate on receiving a growth signal. p53 apparently induces apoptosis by providing a

proliferation block in the presence of damaged DNA. When cells receive both a signal to stop proliferation (by p53) and a mitogenic signal, apoptosis occurs. A similar type of signal conflict could be responsible for triggering apoptosis in the DH23A/b cells.

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