RESEARCH COMMUNICATION

Induction of apoptosis by excessive polyamine accumulation in ornithine decarboxylase-overproducing L1210 cells

Richard POULIN*,†, Georges PELLETIER* and Anthony E. PEGG†

*Department of Physiology, Laboratory of Molecular Endocrinology, Laval University Medical Research Center, 2705 Laurier Blvd., Ste. Foy, Quebec, Canada G1V 4G2, and †Departments of Cellular and Molecular Physiology and Pharmacology, The Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA 17033, U.S.A.

Deregulation of polyamine transport in L1210 cells over-expressing ornithine decarboxylase leads to a lethal accumulation of spermidine. We now provide evidence that over-accumulation of natural and synthetic polyamines, but not putrescine, rapidly induces apoptosis, as shown by hyper-

condensation of peripheral chromatin and internucleosomal cleavage, followed by nuclear fragmentation. Polyamine oxidation is not responsible for the apoptosis observed. Thus, abnormally high polyamine pools could be an important physiological trigger of apoptosis.

INTRODUCTION

While polyamines are required for cell growth in eukaryotes, polyamine pools show only modest fluctuations during the cell cycle [1,2]. This stability results from the ability of polyamines to repress the synthesis and enhance the degradation of ornithine and *S*-adenosylmethionine decarboxylases, as well as to induce spermidine/spermine *N*¹-acetyltransferase, which limits the rate of polyamine degradation and excretion [1,3,4]. Moreover, polyamine transport is strongly repressed by internalized polyamines, thus rapidly limiting the expansion of the polyamine pool [1,5].

A narrow control of polyamine pool size could be required if excessive polyamine levels were cytotoxic [2,6]. H₂O₂ and amino-aldehydes resulting from polyamine degradation by copper-containing serum amine oxidase (SAO) are well-known causes of polyamine toxicity [3,7]. Nevertheless, polyamine over-accumulation can kill mammalian cells in the apparent absence of oxidation [8–10]. In variant L1210 mouse leukaemia cells (D-R) selected for the amplification of ornithine decarboxylase (ODC), feedback inhibition of spermidine transport is lost after a hypo-osmotic shock, with resulting over-accumulation when exogenous substrate is provided [10]. This deregulation of polyamine uptake is most likely due to massive ODC accumulation caused by hypo-osmotic stress [11], which depletes antizyme, the putative repressor of polyamine transport in mammalian cells [12,13]. Spermidine over-accumulation kills about 80 % of hypotonically shocked D-R cells within 24 h in the virtual absence of polyamine oxidation [10]. Both spermidine and the non-oxidizable homologue, 1-methylspermidine [14], are similarly lethal [10], unlike putrescine, which plays an osmoprotective role [15].

The oxidation-independent cytotoxicity of excessive polyamine levels is still unexplained. In this study, we have taken advantage of the capacity of hypo-osmotic stress to de-repress polyamine uptake in D-R cells to investigate the mechanism responsible for

the lethality of spermidine over-accumulation. This report demonstrates that excessive polyamine accumulation induces apoptosis as defined by both morphological and biochemical criteria and independently from their oxidation.

EXPERIMENTAL

Reagents

N,N'-bis(2,3-butadienyl)-1,4-butane-diamine (MDL 72527) and DL- α -difluoromethylornithine (DFMO) were generously provided by the Marion Merrell Dow Research Institute (Cincinnati, OH, U.S.A.). 1-Methylspermidine and 1,12-dimethylspermine [14] were gifts from Dr. J. K. Coward (Department of Medicinal Chemistry, University of Michigan, Ann Arbor, MI, U.S.A.).

Cell culture and hypo-osmotic shock experiments

D-R cells were routinely maintained as suspension cultures in the presence of 10 mM DFMO in RPMI 1640 medium containing 10 % (v/v) Nu-Serum (Collaborative Research, Lexington, MA, U.S.A.) and supplemented as described [10]. Media used for hypotonic shock were prepared by deleting NaCl from the RPMI 1640 medium formulation, and hypo-osmotic stress was imposed exactly as previously described [10,15]. Aminoguanidine (1 mM) was added to all media to prevent polyamine degradation by SAO [3]. Polyamines were determined by ion-pair reverse-phase HPLC with fluorimetric post-column detection with *o*-phthalaldehyde as described [16], except that the methanol concentration in buffer B was raised to 20 % (v/v).

Transmission electron microscopy

Cells were harvested by low-speed centrifugation (2000 *g* for 90 s), rinsed once with serum- and amino-acid-free RPMI 1640

Abbreviations used: SAO, copper-containing serum amine oxidase; ODC, ornithine decarboxylase; MDL 72527, *N,N'*-bis(2,3-butadienyl)-1,4-butane-diamine; DFMO, DL- α -difluoromethylornithine; PAO, FAD-dependent polyamine oxidase.

† To whom correspondence should be addressed.

glucose/salts solution at the same osmolality as the experimental medium, and fixed for 4 h in 2.5 % (v/v) glutaraldehyde. After rinsing twice with 0.1 M sodium phosphate/150 mM sucrose (pH 7.4), cell pellets were fixed for 2 h with 1 % (w/v) OsO_4 , rinsed three times with de-ionized water and successively dehydrated in 50 % (10 min), 70 % (10 min), 90 % (15 min) and 100 % (3×20 min) EtOH (v/v), followed by propylene oxide (2×15 min). Cells were then embedded in Araldite 502/propylene oxide (1:1, v/v), and encapsulated by baking at 37 °C (24 h), 45 °C (24 h) and 60 °C (48 h). Thin sections were cut, stained with uranyl acetate and lead citrate, and viewed in a JEOL 100S electron microscope.

DNA fragmentation analysis

DNA extraction and analysis by agarose-gel electrophoresis were performed as described [17]. Cytoplasmic content in mono- and oligo-nucleosomal particles was determined using the Cell Death Detection enzyme-linked immunosorbent assay kit (Boehringer Mannheim, Indianapolis, IN, U.S.A.), which makes use of two mouse monoclonal antibodies directed against DNA and histones respectively, in a peroxidase-linked sandwich-enzyme immunoassay with photometric detection (A_{405}) using 2,2'-azino-di-(3-ethylbenzthiazolinesulphonate) as a substrate.

RESULTS

An early addition of spermidine (10 μM) to hypo-osmotically stressed D-R cells progressively increased spermidine content about 1.8-fold over a 48 h time course, whereas a delayed addition ($t = 24$ h) led to a 2.7-fold increase in this parameter within only 4 h (results not shown), as previously reported [10]. Ultrastructural damage caused by spermidine over-accumulation was examined by transmission electron microscopy. In the absence of spermidine, nuclei from D-R cells kept under isotonic conditions (Figure 1a) were round to ovoid in shape and exhibited a granular aspect with regions of moderately condensed chromatin, much in contrast with the extensively convoluted contour and strongly euchromatic character of nuclei in hypotonically shocked cells (Figure 1b). The morphology of mitochondria and other organelles was remarkably preserved throughout a 48 h incubation at 150 mosmol/kg, except for the incidence of vacuolation (Figure 1b and Figures 1d–1f). On the other hand, spermidine addition had a dramatic effect on nuclear morphology in hypotonically shocked D-R cells, without ostensibly affecting other structures. Whereas spermidine addition at the time of osmotic shock led to moderate chromatin condensation in the form of dispersed clumps of granular chromatin (Figure 1c), delaying polyamine treatment markedly increased the degree of chromatin condensation (Figures 1d–1f). Within 4 h after delayed polyamine addition, electron-opaque masses of heterochromatin budded at the nuclear periphery, with clear central areas of nucleoplasm (Figures 1d–1f). After 24 h, most spermidine-treated cells exhibited various degrees of peripheral chromatin condensation as well as nuclear fragmentation into membrane-limited, uniformly condensed masses of heterochromatin (Figure 1f).

Ultrastructural changes induced by spermidine over-accumulation, i.e. marginal chromatin condensation followed by nuclear fragmentation with conservation of the fine structure of other organelles, and their association with cell shrinkage, are the morphological hallmarks of apoptosis [18,19]. Since DNA cleavage into mono- or oligo-nucleosome-sized fragments is often

Figure 1 Ultrastructural effects of hypo-osmotic stress and spermidine accumulation in D-R cells

Cells were incubated for a total period of 48 h under normo-osmotic (325 mosmol/kg) (a) or hypo-osmotic conditions (150 mosmol/kg) (b–f), in the absence of exogenous polyamines (a,b), or in the presence of spermidine added at time zero (c) or 24 h after osmotic shock (d–f). Panels (d), (e) and (f) illustrate typical stages in nuclear disintegration observed 4, 12 and 24 h respectively after a delayed ($t = 24$ h) spermidine addition. Bar, 1 μm ; vac, vacuoles; mt, mitochondria.

diagnostic of apoptosis [18,19], we next examined the effect of spermidine over-accumulation on DNA integrity. Hypo-osmotic stress alone induced a brief wave of internucleosomal DNA fragmentation which peaked at about 36 h and had nearly subsided 48 h after shock (Figure 2, lanes 6 and 9). Addition of spermidine at the onset of osmotic shock increased the intensity of this fragmentation, but did not affect its duration (Figure 2, lanes 7 and 10). On the other hand, delaying spermidine supplementation strongly enhanced DNA fragmentation (Figure 2, lanes 8 and 11). As shown in Figure 3, DNA degradation into oligonucleosomal particles was abruptly activated between 2 and 4 h after spermidine addition, and remained at a near plateau thereafter. A slight decline in cytoplasmic nucleosome content noted toward the end of incubation could result from the loss of membrane integrity previously observed 24 h after polyamine addition [10].

Figure 2 Time-course of induction of DNA fragmentation by spermidine in hypo-osmotically stressed D-R cells

Cells were incubated for 24 h [lanes 1 and 2], 25.5 h [lanes 3–5], 36 h [lanes 6–8] or 48 h [lanes 9–11] at 150 mosmol/kg. Spermidine (10 μ M) was added at the time indicated after hypo-osmotic shock. Left lane, DNA size markers (in kDa).

Figure 3 Time-course of accumulation of cytoplasmic oligonucleosomal fragments induced by excessive spermidine uptake in hypo-osmotically stressed D-R cells

Twenty-four hours after osmotic shock, medium was supplemented with 10 μ M spermidine (●) or no amine (○) ($t = 0$) and cultures were then harvested at the indicated time for photometric assay of cytoplasmic oligonucleosomal particles by ELISA (cf. Materials and methods section). Data represent the means $\pm$ S.D. of determinations from triplicate cultures in a representative experiment.

H₂O₂ production by polyamine oxidation has been proposed to mediate apoptosis in the inner cell mass during blastulation [7]. Previous analyses have nevertheless shown that 1-methylspermidine, which mimics spermidine functions while being resistant to attack by SAO and FAD-dependent polyamine oxidase (PAO) [14], was as potent as spermidine in killing hypo-osmotically stressed D-R cells [10]. As shown in Figure 4 (lanes 3–6) and Table 1, intense internucleosomal fragmentation was induced by delayed, equimolar additions of spermidine and spermine, as well as of two metabolically resistant analogues, 1-methylspermidine and 1,12-dimethylspermine. As expected from

Figure 4 Effect of putrescine and polyamine over-accumulation on DNA integrity and lack of effect of PAO inhibition on spermidine-induced DNA cleavage

Twenty-four hours after osmotic shock, cultures were supplemented with 10 μ M of either putrescine (Put, lane 2), spermidine (Spd, lanes 3 and 8), spermine (Spm, lane 4), 1-methylspermidine (MeSpd, lane 5) or 1,12-dimethylspermine [(Me)₂Spm, lane 6], or received no amine addition (lane 1). Lanes 7 and 8: MDL 72527 (10 μ M) was added at the time of osmotic shock and cells were supplemented (lane 8) or not (lane 7) with 10 μ M spermidine 24 h later. Cells were harvested 48 h after osmotic shock for DNA extraction and analysis. Left lane, DNA size markers (in kDa).

its osmoprotective function [15], putrescine did not induce significant DNA cleavage (Figure 4, lane 2 and Table 1). Moreover, spermidine-induced enhancement of internucleosomal

Table 1 Effect of exogenous natural and synthetic polyamines on cytosolic content in oligonucleosomal particles in D-R cells

D-R cells were incubated for 48 h at 325 or 150 mosmol/kg without amine addition (control), or in the presence of 10 μ M of the indicated compound for the last 24 h, before determination of cytoplasmic content in oligonucleosomal fragments as described in the Experimental section. Results are presented as the mean $\pm$ S.D. of determinations of A_{405} for triplicate cell cultures from a representative experiment. *Significantly different ($P < 0.05$) from the value for hypo-osmotically stressed cells incubated under amine-free conditions.

Osmolality (mosmol/kg of H ₂ O)	Addition	Cytosolic oligonucleosomal fragments (A_{405})
325	None	0.29 $\pm$ 0.18
150	None	0.20 $\pm$ 0.08
150	+ Spermidine	1.04 $\pm$ 0.13*
150	+ Putrescine	0.30 $\pm$ 0.10
150	+ Spermine	0.81 $\pm$ 0.14*
150	+ 1-Methylspermidine	0.73 $\pm$ 0.05*
150	+ 1,12-Dimethylspermine	0.65 $\pm$ 0.20*

fragmentation could not be prevented by the PAO inhibitor MDL 72527 [3] (Figure 4, lanes 7 and 8). Apoptosis induced by spermidine thus clearly resulted from its excessive accumulation *per se*, and was not due to by-products of its oxidation.

DISCUSSION

Apoptosis is the major form of cell death observed during embryogenesis, in the regulation of the immune and reproductive systems and in the cytotoxic action of several chemotherapeutic agents [18,19]. Activation of specific genes (e.g. *p53*, *c-myc*) and/or cysteine proteases is involved in many forms of apoptosis [17,18,20] whereas the proto-oncogene *bcl-2* almost uniformly antagonizes this process [21]. Other factors such as Ca²⁺ and ceramide may also play key roles in the transduction of apoptotic stimuli [19,21]. The present data provide the first example that polyamines can rapidly become natural inducers of apoptosis when their intracellular concentration exceeds a critical threshold, and without a requirement for prior oxidation, as shown with polyamine analogues and polyamine oxidase inhibitors.

Polyamines are well known to induce aggregation and condensation of naked DNA and chromatin, to favour transition of B- to A- or Z-DNA and to induce bending between specific sequences [for review see [22]]. Thus, rapid chromatin condensation observed upon polyamine over-accumulation might result from excessive nuclear loading by these compounds, thus causing the aggregation of unfolded or linker histone-depleted nucleosome arrays [23]. This would imply that DNA charge neutralization due to intracellular polyamine binding is normally maintained well below some critical threshold. A preferential cytoplasmic compartmentalization of polyamines in interphase cells is in fact supported by biochemical and cytological evidence [24,25]. Thus, a sharp increase in the free rather than total polyamine concentration could be critical for the activation of apoptosis.

Alternatively, polyamines might trigger apoptosis through an indirect pathway rapidly converging to the same events activated by other apoptotic stimuli. In support of this, high concentrations of exogenous spermine (10⁻⁴–10⁻³ M), and to a much lesser degree, spermidine, can prevent DNA fragmentation in glucocorticoid-treated thymocytes [26]. It is noteworthy that internucleosomal DNA degradation by a constitutive Ca²⁺/Mg²⁺-dependent endonuclease is inhibited by spermine, but not by spermidine or putrescine [27]. In fact, the effect of polyamines on nuclease activity depends on the enzyme considered, ionic strength, pH and DNA conformation [22]. It is at present

difficult to predict the ultimate effect of elevated polyamine levels on apoptotic endonucleolytic events without more knowledge of the enzyme(s) and pathway involved.

The present data strongly support the hypothesis that the potent homeostatic mechanisms that limit the polyamine pool size in eukaryotic cells act to prevent the toxicity of these compounds [2,6], which are shown here to act as triggers for apoptosis. Interestingly, ODC overexpression resulting from its trans-activation by *c-myc* plays an active role in apoptosis caused by cytokine deprivation in interleukin-3-dependent cells [20]. Thus, in addition to derepressed polyamine uptake, excessive rates of polyamine biosynthesis may promote apoptosis under some physiological circumstances.

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