Spermine causes caspase activation in leukaemia cells

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Abstract Exposure of several leukaemia cell types to the polyamine spermine triggered caspase activation. In HL60 cells, the onset of caspase activity correlated with the accumulation of spermine, and was accompanied by the processing of the caspase-3 precursor and the digestion of the substrate proteins PARP and gelsolin. Spermine also induced the accumulation of cytochrome *c* in the cytosol. Caspase activation triggered by spermine was not blocked by antioxidants or inhibition of polyamine oxidase. The deregulation of polyamine uptake strongly sensitised the cells to spermine-induced caspase activation. These data show that an excessive intracellular level of spermine triggers caspase activation that is not mediated by oxidative mechanisms, and suggest a model where elevated free cytosolic polyamines may act as transducers of a death message.

Key words: Apoptosis; Caspase; Cytochrome *c*; Polyamine; Spermine

1. Introduction

The control of the intracellular polyamine pool is a highly regulated process that involves the modulation of the synthesis and activity of the key enzymes responsible for polyamine synthesis and interconversion as well as the regulation of polyamine transport [1]. In fact, substantial levels of polyamines are required for normal cell growth, but excess polyamines elicit toxic effects [2,3]. It has been known for many years that polyamine oxidation by plasma amine oxidase may cause cytotoxicity [4]. But more recently, oxidation-independent mechanisms for polyamine toxicity have been found [5,6], and several data indicate that high levels of polyamines or their analogues can kill cells by inducing apoptosis [6–10].

Apoptosis, consisting in a regulated process of cell death, is characterised by several biochemical events, but the central point of the execution phase is represented by the activation of caspase proteases [11]. Caspases are a family of cysteine proteases responsible for many, if not all, of the characteristics of apoptotic cell death. Caspases are synthesised as inactive precursors, which can be activated by proteolytic cleavage, and receptor-linked events and/or mitochondria are involved in this process [11].

To date, there is no information about the effect of polyamines on caspase activity. In this paper it is reported that cell exposure to spermine triggers caspase activation, indicating a precise mechanism responsible for polyamine cytotoxicity. The presented data also suggest a possible involvement of polyamines in the transduction of a death message.

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Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amido-4-methylcoumarin; DFMO, DL-α-difluoromethylornitine; L1210-DR, DFMO-resistant L1210 cells; MDL 72527, *N*,*N*′-bis(2,3-butadienyl)-1,4-butanediamine; NAC, *N*-acetylcysteine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; PARP, poly(ADP-ribose) polymerase; SSAT, spermidine/spermine N^1-acetyltransferase; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

2. Materials and methods

2.1. Materials and cells

Spermine and other polyamines, Ac-DEVD-AMC, anti-gelsolin antibody and all other biochemicals were products of Sigma. Mouse monoclonal antibodies against caspase-3 and PARP were from Transduction Laboratories and anti-cytochrome *c* was obtained from Pharmingen. Horseradish peroxidase-conjugated anti-mouse IgG from Amersham was used as secondary antibody.

HL60, U937 and Jurkat human leukaemia cells were set up at 4×10^5 cells/ml in RPMI 1640 medium supplemented with 10% foetal calf serum (20% in the case of HL60) and 100 U/ml of both penicillin and streptomycin. L1210-DR cells, an ODC-overproducing cell line, were maintained as previously described [12] in the presence of DFMO, and seeded at 4×10^5 cells/ml for experiments. Hypo-osmotic medium was prepared exactly as described by Poulin et al. [6,13] by omitting NaCl from the RPMI 1640 formulation. In the course of the experiments, the media always contained 1 mM aminoguanidine, in order to inhibit serum amine oxidase activity [6]. Cell death was evaluated by trypan blue exclusion.

2.2. Determination of caspase activity

At the end of the experiment, the cells (4×10^5 in 1 ml of medium) were collected, washed in phosphate-buffered saline, and homogenised in 0.1 ml of chilled lysis buffer. A detailed description of sample preparation and caspase assay is given elsewhere [14]. The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate Ac-DEVD-AMC during a 15 min incubation at 37°C. One unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per minute. Since the sequence DEVD represents a substrate for caspase-3 and other members of the caspase family [15], this activity will be referred to as caspase activity.

2.3. Cleavage of caspase-3 and its substrates

At the end of the incubations, the cells ($0.5–1\times10^7$) were collected, washed once in phosphate-buffered saline, suspended in lysis buffer (150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P40, 1 mM NaF, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml each of aprotinin, leupeptin and pepstatin) and sonicated in ice for 5 min. The homogenates were centrifuged at $28\,000\times g$ for 10 min and, after the determination of protein concentration, the supernatants were diluted in loading buffer and boiled for 3 min. Aliquots corresponding to 50 μg protein of each sample were analysed by SDS-PAGE on a polyacrylamide gel (12% for caspase-3 or 7.5% for PARP or gelsolin). Standard protein markers were used for molecular weight calibration. After blotting, the nitrocellulose membrane was blocked for 1 h with 5% non-fat milk, washed with Tris-buffered saline, and probed for 1 h with specific primary antibody. After a further wash, the membrane was incubated 30 min with the secondary antibody. Immunoreactive bands were visualised with an enhanced chemoluminescence kit (Amersham).

2.4. Determination of cytochrome c release in cytosol

The release of cytochrome *c* from mitochondria was examined in cytosolic extracts obtained by subcellular fractionation. Briefly, the

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cells (20×10^7) were suspended in ice-chilled buffer A [16] and broken by 30 strokes with a Teflon pestle in a Pyrex homogeniser. After centrifugation at $750\times g$ for 10 min, the resultant supernatant was centrifuged at $10\,000\times g$ for 15 min, and the supernatant recentrifuged for 1 h at $100\,000\times g$ to obtain a cytosolic fraction [17]. The protein concentration was determined and, after boiling in loading buffer, 20 μg protein of each sample was loaded onto a 15% polyacrylamide gel and separated by SDS-PAGE. The content of cytochrome *c* was determined by Western blotting followed by immunodetection and chemoluminescence visualisation as described for caspase-3 analysis, except that the time of incubation with the primary antibody was 30 min.

2.5. Polyamine analysis

The cells (8×10^5 in 2 ml of medium) were collected at the end of the experiment and washed three times with 20-ml aliquots of ice-cold phosphate-buffered saline. The resulting pellet was resuspended in 0.4 ml of chilled 0.3 M perchloric acid and subjected to two cycles of freeze-thawing. After centrifugation at $12\,000\times g$ for 5 min, 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. Spermine and other polyamines were separated and quantified by HPLC after derivatisation with dansyl chloride [18].

3. Results

3.1. High doses of spermine activate caspase-3

To investigate the involvement of caspase enzymes in the cytotoxic action of polyamines, HL60 cells were exposed for 24 h to increasing concentrations of spermine in the medium. As shown in Fig. 1, caspase activity was dose-dependently activated by spermine at concentrations higher than 1 mM. Spermine was much more efficient than spermidine in activating caspase activity, whereas putrescine did not elicit significant effects up to 5 mM. The onset of caspase activity paralleled the increase in spermine content of the cells (Fig. 1B).

Activation of the members of the caspase family involves proteolytic cleavage of precursor proenzyme into smaller subunits [11]. Total cell lysates were collected after 24 h of spermine or staurosporine treatment and subjected to Western blotting analysis (Fig. 1C). Staurosporine was used as a positive control, since it rapidly induces apoptosis in all cell types. The activation of caspase-3, as indicated by the processing of the full-length p32 procaspase-3, was observed in either staurosporine- and spermine-treated cells, and was accompanied by the cleavage of substrate proteins PARP and gelsolin.

In some cases, the oxidation of polyamines or their analogues has been implicated in induction of apoptosis [7,9,19], but evidence also exists that polyamines may exert cytotoxic effects not mediated by their oxidation byproducts [5,6]. In order to test the involvement of oxidative mechanisms in spermine-induced caspase activation, HL60 cells were treated with spermine alone or together with *N*-acetylcysteine or trolox, a vitamin E analogue. These antioxidants, which have been shown to inhibit apoptosis induced by oxidative stress [20,21], did not affect the action of spermine (Fig. 2). Since H_2O_2 is generated by the action of PAO [22], the effect of MDL 72527, a potent irreversible inhibitor of PAO [23], was investigated. MDL 72527 had no effect alone, but dose-dependently increased the activation of caspase activity by spermine, in agreement with the data of Brunton et al. [5], who reported an exacerbation of spermine toxicity in kidney cells by MDL 72527. It is worth noting that 1 mM aminoguanidine was always added to the media, to prevent spermine degradation by serum amine oxidase. If aminoguanidine was omitted, caspase activation by spermine was reduced by 30–60% (not shown). Altogether, these data show that oxidative mechanisms are not involved in spermine-induced apoptosis.

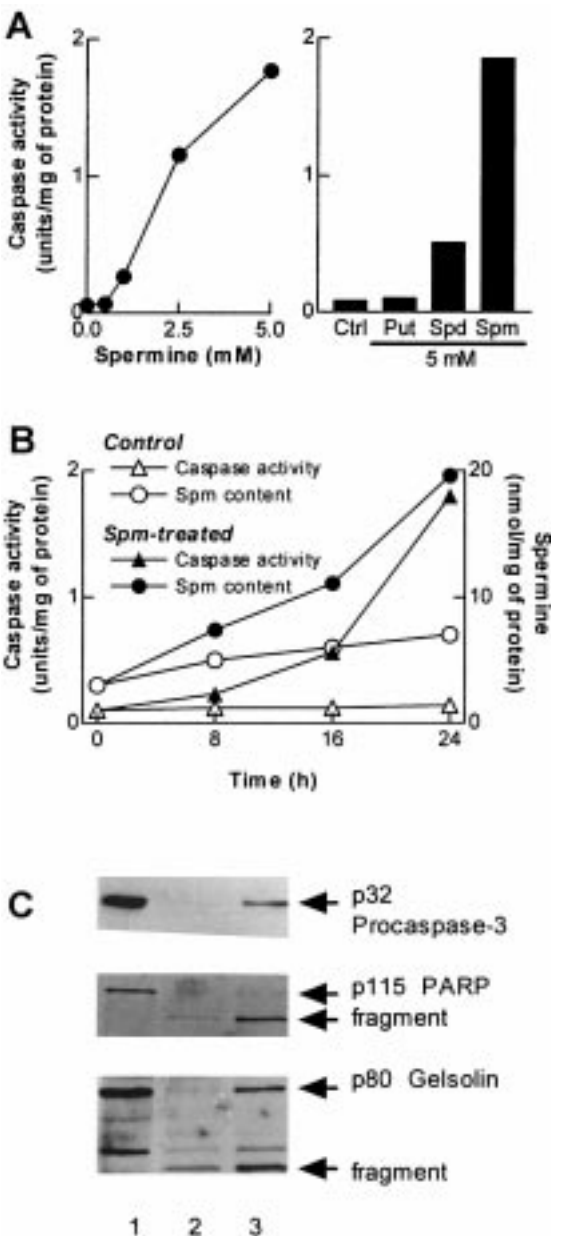

Fig. 1. Spermine triggers activation of caspase-3 in HL60 leukaemia cells. A: The cells were incubated for 24 h in the presence of the indicated concentration of spermine (Spm) or other polyamines spermidine (Spd) or putrescine (Put), afterwards caspase activity was measured. B: The cells were incubated for the indicated time in the absence (control) or presence (Spm-treated) of 5 mM spermine before the assays of spermine levels and caspase activity. C: The cleavage of procaspase-3, PARP and gelsolin was determined in cells incubated for 24 h with none (control, lane 1), 1 μM staurosporine (lane 2) or 5 mM spermine (lane 3). All the panels represent results obtained in a typical experiment representative of more (two to four).

3.2. Spermine causes cytochrome c release in cytosol

Previous studies have shown that accumulation of cytochrome *c* in the cytosol occurs in response to several apoptotic stimuli [16,24]. In order to examine whether spermine induced the release of cytochrome *c* from mitochondria, HL60 cells

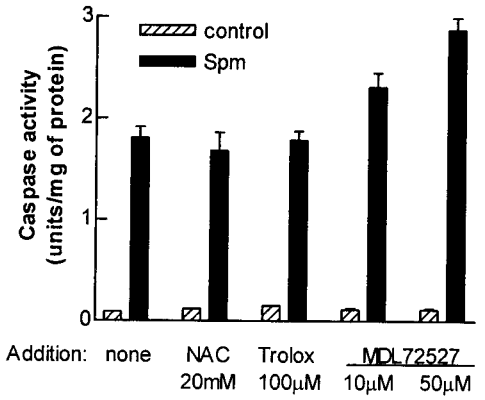

Fig. 2. Effect of antioxidants and inhibition of polyamine oxidase on spermine-induced caspase activation. HL60 cells were incubated for 24 h in the presence or absence of 5 mM spermine (Spm) together with the indicated treatments. 20 mM *N*-acetylcysteine (NAC) and 100 μM trolox were added at the same time as spermine. In the case of MDL 72527, the cells were preincubated for 16 h with the indicated concentrations of the PAO inhibitor before spermine treatment. The data are means of results obtained in two separate experiments, whose range is shown.

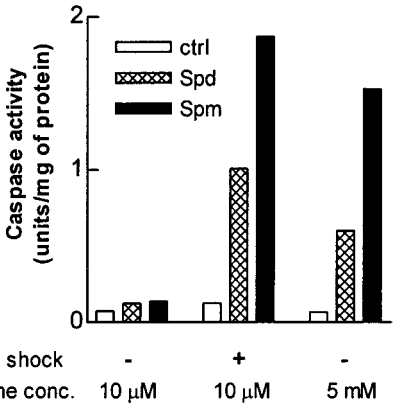

Fig. 4. Effect of the deregulation of polyamine uptake on caspase activity. L1210-DR cells kept in the presence of DFMO were incubated for 24 h under normo- or hypo-osmotic conditions. Thereafter the indicated concentration of spermidine (Spd) or spermine (Spm) was added to the medium and the incubation was continued for a further 8 h before the determination of caspase activity. The figure reports data obtained in one experiment representative of three.

were exposed for 20 h to spermine or etoposide, a classical inducer of apoptosis. The cytosolic S-100 fractions were then isolated and analysed for the content of cytochrome *c*. Fig. 3 shows that cytosolic cytochrome *c* levels were increased following either spermine or etoposide. It should be noted that these treatments similarly induced caspase activity in HL60 cells as well as in U937 and Jurkat leukaemia cells (Fig. 3, bottom).

3.3. Deregulation of polyamine uptake potentiates the caspase-activating effect of spermine

A stringent control of polyamine pool size is operative in eukaryotic cells, and increased levels of polyamines block their uptake. However, the uptake of polyamines may be deregulated in L1210-DR cells. In this ODC-overproducing cell line, the feedback inhibition of polyamine transport is lost after hypo-osmotic shock, with resulting accumulation when exogenous polyamines are provided [13]. L1210-DR cells were hypo-osmotically stressed for 24 h, afterwards 10 μM spermine or spermidine was added to the medium for 8 h. Polyamine addition had a dramatic effect on caspase activity and again spermine was the most powerful activator (Fig. 4). In the absence of hypo-osmotic shock, the usual 5 mM concentration of polyamines was required to activate caspase to a comparable extent. It is worth noting that the time required to reach maximal caspase activation by polyamines was shorter in L1210-DR cells (8 h) than in other cell lines, such as HL60, U937 or Jurkat (24 h) (not shown).

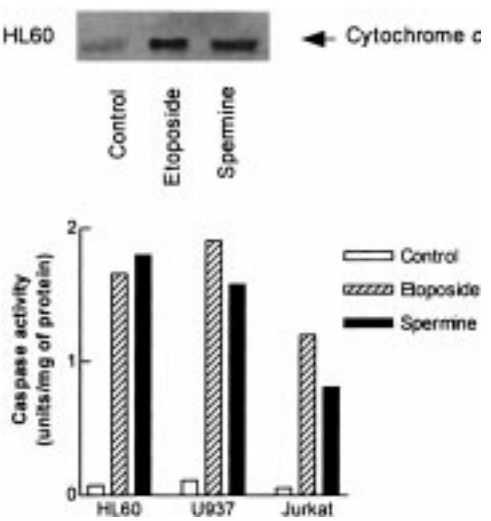

Fig. 3. Spermine causes cytochrome *c* accumulation in the cytosol. HL60 and other leukaemia cells were incubated without any treatment (control), or with 10 μM etoposide or 5 mM spermine. The content of cytochrome *c* in the cytosolic fraction of HL60 cells (top) was determined after 20 h of treatment. Caspase activity in leukaemia cell lines (bottom) was measured following 24 h of incubation. The depicted data were obtained in one experiment representative of two.

4. Discussion

The present data clearly show that polyamines trigger caspase activation in leukaemia cells. Spermine is the stronger activator of caspase activity, and causes the processing of the p32 precursor of caspase-3 into the active enzyme and the consequent digestion of PARP and gelsolin, two proteins recognised as substrates of caspase-3 [25,26].

A prolonged exposure to high doses of exogenous spermine is required to trigger caspase activity, in agreement with previous reports on spermine accumulation and cytotoxicity [5,27,28]. The levels of free polyamines within the cell are tightly regulated by means of several homeostatic mechanisms. The entered polyamines are sequestered in intracellular binding sites [3,27], furthermore, the synthesis of SSAT is rapidly induced by polyamines, causing their acetylation and excretion [29]. However, the main mechanism that limits the accumulation of exogenously provided polyamines is the feedback inhibition of their transport [30–32]. This form of regulation may be partially circumvented by using high doses of polyamines (in the millimolar range), as we did, since in this case polyamines may partly enter by diffusion [27]. In this way, within 24 h the cellular spermine content increases more than four times, and caspase activity is largely induced

(Fig. 1B). The high doses of spermine required to trigger caspase activation, however, could raise doubts on whether the effect of spermine is a specific one, or an unspecific pharmacological response. In order to answer this question, a well known model of deregulation of polyamine uptake, represented by hypo-osmotically stressed L1210-DR cells, was used [13]. In this system, the feedback inhibition of polyamine uptake is lost, and intracellular spermidine levels are increased 2–3 times after exposure to low concentrations of exogenous polyamine [6,13], causing the morphological changes typical of apoptosis [6]. In this model, the dose of polyamines required to induce caspase activity was reduced by 500-fold, indicating that it is the entry of polyamines, and not their extracellular level, that triggers caspase activation. All these data, together with the observation that caspase activation by spermine is not inhibited by antioxidants, aminoguanidine or a PAO inhibitor, support the hypothesis that an elevated polyamine level per se, and not their oxidation, is responsible for polyamine toxicity [5,6,33], and indicate a precise mechanism, i.e. the activation of caspase-3 and, possibly, other members of the caspase family.

It is worth noting that spermine causes cytochrome *c* accumulation in the cytosolic fraction (Fig. 3). The translocation of cytochrome *c* from mitochondria into the cytosol is triggered by many, but not all, inducers of apoptosis [16,17,24], and represents an important step in caspase activation [34,35]. Spermine efficiently binds to mitochondria [36], and a direct effect on the release of cytochrome *c* could be hypothesised. Actually, several data from our laboratory support this hypothesis, and work is in progress.

In conclusion, an excess of polyamines, and particularly of spermine, is able to trigger caspase activation. This finding reveals a direct mechanism of polyamine-induced toxicity, and can also contribute to explain previous data [6–10], possibly including the mechanism of c-*myc*-induced apoptosis, in which polyamines appear to be involved [7]. Furthermore, the capability of spermine to cause the activation of caspase-3 suggests a possible role of polyamines in the activation of the death programme. In fact, spermine is present in high amounts in all eukaryotic cells, sequestered in intracellular binding sites [3,37]. The accumulation of free spermine resulting from insults to anionic structures like DNA or membranes could act as a cellular sensor of damage, transducing a death signal.

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