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Polyamine Analogue Induction of Programmed Cell Death in Human Lung Tumor Cells

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Abstract

The naturally occurring polyamines putrescine, spermidine, and spermine are required for cell growth. Based on this requirement, several polyamine analogues that interfere with polyamine function and metabolism have been synthesized as antineoplastic agents. The symmetrically substituted $N^4, N^{12}$-bis(ethyl)spermine (BESpm), and unsymmetrically substituted $N^1$-ethyl-$N^{11}$-[cyclopropylmethyl]-4,8-diazaundecane (CPENSpm) have previously been shown to cause rapid cytotoxicity of NCI H157 cells, with concurrent high induction of the polyamine catabolic enzyme spermidine/spermine $N^1$-acyltransferase. However, the precise mechanism(s) of the cytotoxic action of the compounds is not known. We now demonstrate that treatment with either BESpm or CPENSpm results in morphological and biochemical changes consistent with the activation of programmed cell death pathways, and that the unsymmetrically substituted CPENSpm more rapidly activates the death program. These studies suggest that the cell type-specific cytotoxicity of these polyamine analogues may be a result of their ability to selectively activate the cell death pathway in sensitive phenotypes and indicate that the relationship between the structure of the polyamine analogues and the ability to induce programmed cell death should be investigated.

Introduction

The cellular requirement of polyamines for growth and differentiation has led to interest in the polyamine metabolic pathway as a target for antineoplastic therapy. One focus has been on polyamine analogues that are structurally similar to the naturally occurring polyamines, enter the cell through the specific polyamine transporter, and down-regulate polyamine bio-

synthesis, but cannot mimic the growth and survival functions of the natural polyamines (1–4). We originally described a cell-type specific cytotoxicity in human lung tumor lines to the $N^4, N^4'$-bis(ethyl) analogues of spermine in which the cellular response is associated with the ability of the analogues to induce the polyamine catabolic enzyme SSAT (1–3). Cytotoxicity occurs in those cell types in which SSAT is highly induced, while a cytostatic response occurs in cell types in which SSAT is not induced or is only moderately induced (1, 2, 5, 6). However, no data exist which directly link SSAT induction and cytotoxicity. Recently, we have synthesized several unsymmetrically substituted polyamine analogues as potential antitumor agents and potential inducers of SSAT (7). Testing of three of these new unsymmetrical polyamine analogues in both small cell and non-small cell lung carcinoma cell lines revealed similar cell-type-specific cytotoxicity and superinduction of SSAT as seen with the bis(ethyl)polyamine analogues (8).

We have now investigated activation of programmed cell death pathways during the cytotoxic response of NCI H157 cells to a representative bis(ethyl)polyamine analogue, BESpm, and a representative unsymmetrical polyamine analogue, CPENSpm (Fig. 1). Cell- or tissue-type-specific responses to drugs could be related to differential abilities of the drugs to activate the cell death program. Programmed cell death is a mechanism of cellular suicide in which the cell is an active participant in its own destruction (9–11). Specific signals activate a sequence of morphological and biochemical events which culminate in degradation of genomic DNA and subsequent cell death. The hypothesis that polyamines and/or polyamine analogues might regulate the cell death program is based partly on evidence that spermidine and spermine have been reported to stabilize chromatin (12, 13). Also, polyamine-depleted cells have been shown to undergo changes in chromatin and DNA structure (12, 13), and spermine can protect against programmed cell death in thymocytes (14). Additionally, the action of SSAT produces $N^1$-acetyl polyamine derivatives which are then oxidized by polyamine oxidase with the consequent production of hydrogen peroxide which has been postulated to activate programmed cell death (15).

The results demonstrate that both CPENSpm and BESpm initiate the cell death program and suggest that the ability to induce programmed cell death may be involved in the cell-type-specific cytotoxicity of these polyamine analogues. The observation of differential timing in the onset of cell death without significant differences in polyamine-related parameters suggests that the slight structural differences between CPENSpm and BESpm affect the ability of the analogues to initiate the cell death program. These studies demonstrate that the relationship
between the structure of the analogues and their ability to induce programmed cell death should be explored for potential therapeutic use of these or related polyamine analogues, as well as for greater understanding of the mechanisms involved in polyamine function and programmed cell death activation.

Materials and Methods

Compounds and Cell Culture. CPENSpm was synthesized as previously described (7). For all experiments, a concentrated CPENSpm solution (10 mM in water, stored at -20°C) was diluted with medium to the desired concentration. BESpm was prepared as previously reported (16) and kindly supplied by R. Bergeron (University of Florida, Gainesville, FL). For all experiments, a concentrated BESpm solution (either 1 or 10 mM in 0.1 mM HCl, stored at -20°C) was diluted with medium to the desired concentration. As a control for the BESpm vehicle, 0.1 mM HCl was diluted similarly in the untreated control. NCI H157 cells were maintained in RPMI 1640 medium supplemented with 9% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37°C in a 5% CO2 atmosphere.

Assessment of Morphology. Exponentially growing NCI H157 cells were incubated in the presence or absence of 10 μM CPENSpm or BESpm for 8 to 120 h in 2-well Lab-tek chamber slides (Nunc, Naperville, IL). At the desired time, the medium was aspirated, the plastic growth chamber was removed, the slides were rinsed in PBS, and then the slides were placed in methanol to fix the cells. Fixed cells were stained with 0.1 mg/ml Hoechst dye #33342 (Sigma Chemical Co., St. Louis, MO) and visualized by fluorescence microscopy using a ZEISS Axioskop microscope (ZEISS, Hanover, MD) with filter set 4879/02.

DNA Fragmentation Assays. Exponentially growing cells were plated at 2–7 × 10⁶ cells/75 cm² culture flask. After attachment, the medium was changed, and cells were incubated with or without drug for the desired exposure time. At harvest, medium and trypanized cells were combined, and cells were pelleted by centrifugation. For analysis of oligonucleosomal DNA fragmentation, DNA was isolated as previously described (17). Equivalent amounts of DNA (25–30 μg) were loaded into wells of a 1.8% agarose gel and separated by electrophoresis in TBE. For analysis of high molecular weight DNA fragmentation, the cell pellet was resuspended in 1% low melting point agarose and cast in a plug mold. Following digestion with proteinase K and RNase A, plugs were loaded to a 0.8% agarose gel, and DNA was separated by electrophoresis in 0.5× TBE for 12 to 14 h at 6 V/cm using program 6 of a PPI-200 field inversion timer (MJ Research, Watertown, MA).

Analysis of Intracellular Polyamine Pools, SSAT Activity, and ODC Activity. The polyamine content of treated and untreated cells was determined by precolumn dansylation and reversed phase high-performance liquid chromatography using 1,7-diaminoheptane as the internal standard (18). This method is sufficiently sensitive to detect >5 pmol of the individual polyamines and is generally reproducible with a variation of <15%. SSAT and ODC activities were measured using cellular extracts as reported previously (19, 20). SSAT activity is expressed as pmol N1-[14C]acetylspermidine formed/mg protein/min. Protein concentrations were determined according to the method of Bradford (21).

Results

The human non-small cell lung carcinoma cell line NCI H157 has previously been found to respond in a cytotoxic manner to treatment with either CPENSpm or BESpm (2, 3, 8). Therefore, this cell line was tested for evidence of programmed cell death activation following exposure to the analogues. NCI H157 cells were examined following treatment with 10 μM CPENSpm or 10 μM BESpm to determine whether morphological changes characteristic of activation of programmed cell death were present. These changes include nuclear condensation, chromatin aggregation, and fragmentation of the cell into membrane-bound apoptotic bodies (10). CPENSpm-treated cells exhibited detectable chromatin alterations 24 h after initial drug exposure. The number of CPENSpm-treated cells in which morphological changes were observed increased at 36 h, and by 48 h, ≥90% of the cell population exhibited morphological changes suggestive of programmed cell death (Fig. 2). Examination of BESpm-treated cells revealed that exposure to BESpm also resulted in similar programmed cell death-associated morphological changes, but at later time points than CPENSpm treatment. The earliest alterations were detected 48 h after BESpm exposure, and involvement of the majority of cells was not noted until 72 h (data not shown).

A critical event which occurs during programmed cell death is the fragmentation of genomic DNA to high molecular weight fragments of ≥50 kb (11). Field inversion gel electrophoresis was used to determine whether such fragmentation resulted from treatment of NCI H157 cells with CPENSpm or BESpm. High molecular weight DNA fragments were detected following treatment with either analogue, suggesting that both CPENSpm and BESpm were activating the death program in this cell line (Fig. 3). However, as with the morphological changes, the time sequence of induction of the programmed cell death differed between the analogues. DNA fragmentation was clearly evident 24 h after CPENSpm exposure (Fig. 3), but following BESpm exposure, DNA fragmentation was just detectable at 48 h and clearly evident only after 72 h (Fig. 3). No significant DNA fragmentation was detected in untreated control cells.

Fragmentation of DNA to oligonucleosomal sized fragments was also assessed following CPENSpm and BESpm treatment since such fragmentation, although not absolutely
required, can indicate the occurrence of programmed cell death (10, 11). CPENSpm exposure resulted in oligonucleosomal sized DNA fragmentation that was first detectable 60 h after initial drug exposure (Fig. 4). However, over the same time course, exposure to BESpm, unlike CPENSpm treatment, did not result in detectable oligonucleosomal DNA fragmentation (data not shown). Since high molecular weight DNA fragmentation resulting from BESpm treatment was detected later than that from CPENSpm (Fig. 3), BESpm-treated cells were also evaluated for oligonucleosomal DNA fragmentation at the later time points of 120 and 144 h after initial drug exposure, but still none could be detected (Fig. 4).

The observed differences between the two analogues, with respect to the timing of the activation and execution of the cell death program, led to a comparison of the effects of these
analogue functions on other polyamine-related parameters. These polyamine analogues have been shown previously to accumulate intracellularly, deplete intracellular polyamine pools, highly induce activity of the polyamine catabolic enzyme SSAT, and inhibit activity of the polyamine synthetic enzyme ODC (22, 23). Since the first changes indicative of programmed cell death activation, the morphological alterations observed following CPENSpm exposure, were detectable 24 h after drug treatment, several polyamine-associated parameters were assessed 24 h after exposure of the cells to 10 μM CPENSpm or 10 μM BESpm. Under those conditions, similar intracellular accumulation of CPENSpm and BESpm as well as similar depletion of the intracellular pools of the natural polyamines resulted (Table 1). The effects of these analogues on the polyamine regulatory enzymes SSAT and ODC were also similar under these conditions. Both CPENSpm and BESpm highly induced SSAT activity and inhibited ODC activity to ≤0.4% of control levels (Table 1).

Discussion

The aim of the current studies was to investigate the mechanism of two polyamine analogues that were designed to be structurally similar to but not functionally substitute for the natural polyamines. The initial goal behind the development of these analogues was to interfere with cellular regulatory mechanisms involving the cationic polyamines which are essential to cellular growth and development (22). Previous studies in human lung tumor lines demonstrated these analogues to be phenotype specific in their cytotoxic activity (1–3, 8). However, the precise mechanism underlying this specificity is not known.

The results presented here provide evidence that both CPENSpm, a representative of the unsymmetrically substituted polyamine analogues, and BESpm, one of the bis(ethyl)polyamine analogues, can induce programmed cell death in the non-small cell lung carcinoma cell line NCI H157. Morphological changes and high molecular weight DNA fragmentation characteristic of programmed cell death were observed following treatment with 10 μM CPENSpm for ≥24 h or 10 μM BESpm for ≥48 h. Oligonucleosomal DNA fragmentation was also demonstrated to result from CPENSpm, but not BESpm treatment. CPENSpm and BESpm accumulated intracellularly to the same degree, and treatment with either led to depletion of intracellular polyamine pools, induction of SSAT, and inhibition of ODC. Recently, we demonstrated the ability of CPENSpm to initiate programmed cell death in human breast cancer cell lines (23). However, those results indicated that although growth inhibition was observed, CPENSpm was cytotoxic to only a subpopulation of cells since the overall cell number continued to increase throughout the treatment. This is in contrast to results in the lung tumor line where the entire population is ultimately killed by treatment with either CPENSpm or BESpm. CPENSpm activation of programmed cell death in the NCI H157 cells was more rapid than that previously observed in the breast cancer cell lines where the earliest detection was after 72 h (23).

These data suggest that the cell-type-specific cytotoxicity as demonstrated among the human lung tumor phenotypes by these polyamine analogues may be related to their ability to induce programmed cell death. It is important to try to understand exactly how the cell death program could be activated by these analogues, since such a cell-type-specific action can provide the basis for therapeutic selectivity. One detectable difference between the action of these two analogues was that the onset of programmed cell death occurred more quickly following CPENSpm treatment. The observed difference in the timing of onset of cell death is consistent with previous data which indicated a greater sensitivity of NCI H157 cells to CPENSpm than to BESpm after 96 h of drug exposure (8). Although it is possible that more detailed evaluation of the initial 24 h of drug exposure could reveal differences not detected at 24 h, the earlier onset of programmed cell death in the CPENSpm-treated cells does not currently appear to result from differential effects on polyamine metabolism or polyamine pools between CPENSpm and BESpm. Rather, the results suggest that the direct effects of the compounds are different, resulting both in the earlier onset of CPENSpm-induced programmed cell death and the ability of CPENSpm but not BESpm to induce DNA ladder formation.

These data suggest that the slight structural differences between CPENSpm and BESpm may be responsible for the differential timing in the initiation of the cell death program. This could be related to the DNA-binding properties of the analogues since direct interaction of polyamine analogues with DNA and consequent structural changes have been postulated as one site of action (24, 25), and the natural polyamines are thought to bind DNA and affect gene expression (26). Analogue treatment could affect polyamine-controlled functions directly through analogue/DNA interactions as well as indirectly.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Polyamines (nmol/mg protein)</th>
<th>SSAT activity (pmol/mg protein/min)</th>
<th>ODC activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Put' Spd Spm CPENSpm BESpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3.62 9.12 8.30 ND ND</td>
<td>44 ± 1.11</td>
<td>100</td>
</tr>
<tr>
<td>CPENSpm</td>
<td>0.54 0.00 0.60 48.36 ND</td>
<td>39,876 ± 1.495</td>
<td>0.4</td>
</tr>
<tr>
<td>BESpm</td>
<td>0.00 0.00 0.58 ND</td>
<td>46,679 ± 3.015</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Values represent the means of duplicate determinations.

*Values for SSAT and ODC enzyme activities represent the means of triplicate determinations ± SD.

*Put', putrescine; Spd, spermidine; Spm, spermine; ND, not detected.*

Materials and Methods

Intracellular polyamine levels, SSAT activity, and ODC activity were determined as described in "Materials and Methods" following incubation of NCI H157 cells for 24 h in the presence or absence of 10 μM CPENSpm or BESpm.

Table 1 Comparison of effects of 10 μM CPENSpm or BESpm on polyamines, SSAT activity, and ODC activity in NCI H157 cells

Intracellular polyamine levels, SSAT activity, and ODC activity were determined as described in "Materials and Methods" following incubation of NCI H157 cells for 24 h in the presence or absence of 10 μM CPENSpm or BESpm.
through decreasing DNA/polyamine interactions as the analogue accumulates intracellularly and the natural polyamines are depleted. BESpm may more closely mimic natural polyamines with respect to DNA binding, and disruptions in function may manifest more slowly and result in slower onset of cell death. Differences in analogue/DNA binding could also affect the accessibility of DNA to endonucleases which are responsible for the oligonucleosomal type of DNA fragmentation observed to result from CPENSpm, but not BESpm treatment. Investigation of analogue effects on expression of genes that have been associated with programmed cell death should also be useful in understanding how these compounds are acting. The cell-type specificity of cell death induction could be related to a difference in importance of expression of polyamine-regulated genes to the normal cellular functioning of various cell types. Additional studies of structure/activity relationships of these and others of the related polyamine analogues will be necessary to explore which structural elements are important for programmed cell death induction and execution.

It is also currently not clear what role, if any, the induction of SSAT plays in the activation of programmed cell death by CPENSpm and BESpm. CPENSpm induction of programmed cell death in NCI H157 cells was accompanied by high induction of SSAT; however, in the breast cancer cell lines, SSAT induction was observed in only one of the five instances where programmed cell death initiation was demonstrated (23). Additionally, there are polyamine analogues that are cytotoxic without inducing SSAT (27). It is currently not known whether the cell death program is also initiated by these agents.

These studies indicate that the potential use of polyamine analogues to exploit programmed cell death pathways for therapeutic use should be explored. Greater understanding of the actions of these polyamine analogues to cause death may also ultimately lead to elucidation of precisely why polyamines are required for normal cellular survival and why interruption of these functions leads to death in some cell types and merely cessation of growth in others. Additional detailed studies of the structure/activity relationship between these and other polyamine analogues could possibly lead to the design of polyamine analogues which specifically target cell death pathways.

References


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