Induction of Programmed Cell Death in Human Breast Cancer Cells by an Unsymmetrically Alkylated Polyamine Analogue

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Abstract

The need for antineoplastic compounds with novel mechanisms of action is great. One such agent is the recently synthesized polyamine analogue N'-ethyl-N'-((cyclopropyl)methyl)-4,8-diazaundecane (CPENSpm). Exposure of hormone-dependent and -independent human breast cancer cells to 0.1-10 μM CPENSpm led to both growth inhibition and induction of programmed cell death. Fragmentation of DNA to high molecular weight fragments and oligonucleosomal-sized fragments, both characteristic of programmed cell death, was determined to be time and concentration dependent. Depletion of natural polyamine pools and accumulation of the analogue was also demonstrated. These data provide the first evidence that a polyamine analogue induces programmed cell death.

Introduction

The polyamine metabolic pathway is a potential chemotherapeutic target because polyamines are essential for cellular growth and differentiation (1, 2). We have reported previously that N,N'-bis(ethyl)spermine inhibits growth and depletes intracellular polyamine pools in several breast cancer cell lines (3). Polyamine analogues that are unsymmetrically alkylated but structurally similar to the bis(ethyl)polyamines have been synthesized as potential antitumor agents (4, 5). We have now investigated the ability of one of these analogues, CPENSpm,3 to deplete intracellular polyamines, inhibit growth, and activate programmed cell death pathways in human breast cancer cell lines. Programmed cell death is an active, energy-dependent process in which the cell is an active participant in its own destruction (6, 7). Initiation by specific signals activates a cascade of biochemical and morphological events that results in the irreversible degradation of genomic DNA and subsequent elimination of the cell. Fragmentation of genomic DNA is considered the committed step of programmed cell death (8), and any compound that affects DNA structure could potentially be involved in the activation or inhibition of this pathway. A relationship between polyamines and programmed cell death, therefore suggested since spermine and spermine have been reported to stabilize chromatin (9, 10), polyamine-depleted cells have been shown to undergo changes in chromatin and DNA structure (9, 10), and spermine can protect against programmed cell death in thymocytes (11).

Programmed cell death pathways are of specific interest as a therapeutic target in breast cancer. Previously, we have demonstrated that hormone-dependent MCF7 human breast cancer cells, growing in vivo, undergo programmed cell death upon estrogen withdrawal (12). Furthermore, even hormone-independent breast cancer cells retain the ability to undergo programmed cell death in response to diverse agents including fluoropyrimidines (13) and paclitaxel.4 The evidence presented here suggests that the polyamine analogue CPENSpm, in addition to significantly reducing the growth of both hormone-dependent and hormone-independent breast cancer cells, can initiate the cell death program.

Materials and Methods

Compounds, Cell Lines, and Culture Conditions. CPENSpm was synthesized as described previously (4). For all experiments, a concentrated solution (10 mM in water, stored at -20°C) was diluted with medium to the desired concentration. MDA-MB-468 and ZR-75-1 cells were maintained in an improved minimal essential medium supplemented with 5% fetal bovine serum (Biofluids, Rockville, MD) and 2 mM glutamine. MCF7, T47D, MDA-MB-231, and Hs578t cells were maintained in DMEM supplemented with 5% fetal bovine serum and 2 mM glutamine. Cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere and passaged every 5 days. Mycoplasma testing was routinely negative.

Growth Inhibition Assay. Exponentially growing cells were plated in triplicate at 1-5 × 10⁴ cells/cm² in 24-well plates. After attachment, medium was changed, and cells were incubated in the absence or presence of at least six CPENSpm concentrations. After 120 h, the cells were detatched by trypsinization and counted using a Coulter Counter. Initial experiments demonstrated that cell numbers determined using a Coulter counter were equivalent to numbers of trypan blue excluding cells (viable cells) determined by cell counting using a hemocytometer. IC₅₀ values were determined from plots of the percentage of untreated control cell number versus the logarithm of the drug concentration. All experiments were carried out at least twice, and values reported are mean ± SD of individual points from all experiments.

DNA Fragmentation Assays. Exponentially growing cells were plated at 1-5 × 10⁴ cells/cm². After attachment, the medium was changed, and cells were incubated with or without drug for the desired exposure time. At harvest, medium and trypsinized cells were combined, and cells were pelleted by centrifugation. For analysis of oligonucleosomal DNA fragmentation, DNA was isolated as described previously (13). Equivalent amounts of DNA (15-20 μg) were loaded into wells of a 1.6% agarose gel and electrophoresed 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 electrophoresed in 0.5X TBE for 12-14 h at 6 V/cm using program 6 of a PPI-200 field inversion timer (MJ Research, Watertown, MA).

Analysis of Intracellular Polyamine Pools and SSAT Activity. The polyamine content of treated and untreated cells was determined by precolumn dalylation and reversed-phase HPLC using 1,7-diaminoheptane as the internal standard (14). SSAT activity was measured using cellular extracts as reported previously (15). Enzyme activity is expressed as pmol N₁-[¹⁴C]ace-

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3 The abbreviations used are: CPENSpm, N'-ethyl-N'-((cyclopropyl)methyl)-4,8-diazaundecane; SSAT, spermidine/spermine N₁-acetyltransferase; IC₅₀, concentration required to inhibit cell growth 50%; TBE, 8.9 mM Tris, 89 mM boric acid, and 2 mM EDTA.
Results

Inhibition of Growth of Human Breast Cancer Cell Lines by CPENSpm. The sensitivity of six breast cancer cell lines to CPENSpm was assessed by measurement of growth inhibition resulting from continuous exposure to 0.1–30 μM CPENSpm. The concentration dependence of growth inhibition in a representative hormone-dependent cell line (MCF7) and hormone-independent cell line (MDA-MB-468) is shown in Fig. 1. All cell lines exhibited concentration-dependent growth inhibition from 0.1 to 10 μM CPENSpm, with growth inhibition of 75 to 98% resulting from exposure to 10 μM CPENSpm. The IC₅₀s for CPENSpm ranged from 0.2 to 1.3 μM, and there was no correlation between the estrogen receptor status of the cell lines and sensitivity to CPENSpm (Table 1).

Induction of Programmed Cell Death by CPENSpm. The significant growth inhibitory activity of CPENSpm led us to investigate whether part of this effect was a result of programmed cell death induction. Fragmentation of genomic DNA to high molecular weight (≥50 kb) fragments is characteristic of the occurrence of programmed cell death and may represent the committed step of the process (8). Field inversion gel electrophoresis was used to assess whether 96 h exposure to CPENSpm resulted in high molecular weight DNA fragmentation (Fig. 2A). CPENSpm concentrations ≥5 μM for MCF7 cells and ≥2 μM for MDA-MB-468 cells were sufficient to induce DNA fragmentation in a concentration-dependent manner. DNA fragmentation was not detected in untreated control cells of either cell line. Similar concentration-dependent high molecular weight DNA fragmentation was also detected in T47D, ZR-75-1, MDA-231, and Hs578t cells (data not shown).

Table 1 Sensitivity of breast cancer cell lines to CPENSpm

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Estrogen receptor status</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>+</td>
<td>1.1</td>
</tr>
<tr>
<td>T47D</td>
<td>+</td>
<td>0.4</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>+</td>
<td>1.1</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>−</td>
<td>0.6</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>−</td>
<td>1.3</td>
</tr>
<tr>
<td>Hs578t</td>
<td>−</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Northern Blot Analysis. Total cellular RNA was isolated by the method of Chomczynski and Sacchi (17). Northern blot analysis was carried out as described previously (3) using the full-length human SSAT cDNA clone AP3/F7 as a probe (3, 18).
POLYAMINE ANALOGUE-INDUCED CELL DEATH

Table 2. Effects of CPENSpm on polyamine pools, SSAT activity, and SSAT mRNA expression in MDA-MB-468 cells

Following incubation of exponentially growing MDA-MB-468 cells in the presence or absence of 10 μM CPENSpm for 24 h, intracellular polyamine levels and SSAT activity were determined as described in "Materials and Methods." Values are averages ± SD of ≥6 determinations. SSAT mRNA expression was determined by Northern analysis, and the value reported was determined by densitometric analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Polyamines (nmol/mg protein)</th>
<th>SSAT activity (pmol/mg protein/min)</th>
<th>SSAT RNA expression (fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Put</td>
<td>Spd</td>
<td>Spm</td>
</tr>
<tr>
<td>Control</td>
<td>2.0 ± 0.4</td>
<td>7.2 ± 2.0</td>
<td>21.1 ± 5.8</td>
</tr>
<tr>
<td>CPENSpm</td>
<td>1.4 ± 0.5</td>
<td>1.9 ± 0.7</td>
<td>8.3 ± 3.4</td>
</tr>
</tbody>
</table>

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

The above data suggested that programmed cell death pathways had been activated. Because fragmentation of DNA into oligonucleosomal-sized fragments is also associated with programmed cell death in many cell systems, DNA isolated from CPENSpm-treated cells was examined for evidence of such fragmentation. The hormone-independent MDA-MB-468 cell line was chosen as the main focus of these studies because we have previously characterized fluoropyrimidine, paclitaxel, and epidermal growth factor induction of programmed cell death in this cell line (13, 19). MCF7 cells were included as a representative hormone-dependent cell line. Oligonucleosomal DNA fragmentation was detected in both MCF7 and MDA-MB-468 cells after exposure to 10 μM CPENSpm for 96 h but not in untreated control cells (Fig. 2B). Induction of MDA-MB-468 DNA fragmentation was both time and concentration dependent. Detectable oligonucleosomal fragmentation resulted from 72 h exposure to 5 or 10 μM CPENSpm, while CPENSpm concentrations as low as 1 μM were sufficient to induce detectable fragmentation after 96 h (data not shown).

**Effects of CPENSpm on Intracellular Polyamines, SSAT Activity, and SSAT mRNA Expression.** The detection of DNA fragmentation in these breast cancer cell lines suggested that CPENSpm activity was cytotoxic to at least a portion of the cell population rather than merely antiproliferative. As exposure to polyamine analogues can result in polyamine depletion (3, 20, 21), it was of interest to determine whether polyamine depletion could be involved in the observed CPENSpm-induced DNA fragmentation. Exposure of MDA-MB-468 cells to 10 μM CPENSpm for 24 h resulted in a significant decrease in the natural polyamines and intracellular accumulation of the analogue (Table 2). Polyamine depletion can result from increased activity of SSAT, and induction of SSAT activity by symmetrically substituted polyamine analogues has been correlated with the cytotoxicity of these compounds in non-small cell lung cancer and melanoma cell lines (20, 21). Thus, the effect of CPENSpm on SSAT activity was examined. SSAT activity was highly induced (~300-fold induction over control) in MDA-MB-468 cells after 24 h exposure to 10 μM CPENSpm (Table 2), and this induction was associated with a 4-fold increase in SSAT mRNA expression (Table 2). SSAT activity was not significantly induced in any of the other five breast cancer cell lines tested (data not shown).

**Discussion**

The search for better cancer chemotherapeutic agents has included agents that target polyamine pathways because polyamines are essential for tumor cell growth (1, 2). N,N'-bis(ethyl) analogues of spermine have been of particular interest, and one of the members of this family, N,N,N',N'-tetraethylsilver, is now in Phase 1 clinical trials. Unsymmetrically substituted polyamine analogues that are structurally similar to the bis(ethyl)polyamine analogues have been synthesized recently for evaluation as potential antitumor agents (4, 5). The studies presented here demonstrate that one of these unsymmetrically alkylated analogues, CPENSpm, has significant growth-inhibitory effects in human breast cancer cells and provide the first evidence that polyamine analogue treatment can induce programmed cell death. High molecular weight DNA fragmentation, which may represent the committed step of this cell death process, resulted from continuous exposure of six breast cancer cell lines to CPENSpm. Oligonucleosomal-sized DNA fragmentation, which is associated with programmed cell death in many cell systems, was also observed. Intracellular depletion of natural polyamine pools and accumulation of the analogue were both associated with CPENSpm treatment. No relationship was found between the steroid hormone receptor status of the cells and the effects of CPENSpm because programmed cell death was detected in both hormone-dependent and hormone-independent cell lines and sensitivity to CPENSpm was similar for all cell lines. This suggests that CPENSpm has potential for use even against hormone-independent breast cancers.

It is currently not known how polyamine analogues exert their effects, but several possible mechanisms are consistent with this demonstrated ability to induce programmed cell death. Polyamines have been shown to interact with and stabilize DNA and direct interaction of polyamine analogues with DNA, and consequent structural changes have been postulated as one site of action (22, 23). The accumulation of CPENSpm and the depletion of the natural polyamines by down-regulation of the biosynthetic enzymes and the up-regulation of the catabolic pathway produce a natural polyamine-depleted environment that could favor the analogue/DNA interaction. Such analogue/DNA interactions may result in greater accessibility and susceptibility of DNA to the fragmentation that is an essential part of programmed cell death. Similarly, loss of natural polyamine/DNA interactions, which could interfere with normal gene expression and cellular function, may result and trigger cell death as depletion of natural polyamines occurs concurrently or subsequently to accumulation of the analogue. These possibilities are consistent with reports that spermine can protect against programmed cell death in thymocytes (11), and polyamine-depleted cells can undergo changes in chromatin structure (9, 10).

Recent reports have detailed complex regulation of polyamine transport to maintain intracellular polyamine content at levels that are sufficient for, but not toxic to, normal cellular function (24, 25). This suggests another mechanism by which polyamine analogues could exert cytotoxic effects. Interference with regulatory mechanisms of polyamine transport could result in the accumulation of the analogue to a level that results in disruption of the balance of positive and negative charges normally maintained intracellularly and could lead to cell death.

The up-regulation of the polyamine catabolic enzyme SSAT by polyamine analogues provides another potential link to programmed cell death induction. The action of SSAT produces N-acetyl polyamine derivatives, which are then oxidized by polyamine oxidase with the consequent production of hydrogen peroxide. It has been postulated that, in the developing embryo, oxidation of polyamines by polyamine derivatives, which are then oxidized by polyamine oxidase, with the consequent production of hydrogen peroxide, which activates programmed cell death (26). However, it is unlikely that CPENSpm cytotoxicity is solely dependent on SSAT induction be-
cause preliminary evidence indicates that of the breast cancer cell lines tested, MDA-MB-468 is the only one in which SSAT activity is significantly induced.

Additional studies are needed to dissect the mechanisms by which CPENSpm acts to produce both antiproliferative and cytotoxic effects. However, these studies indicate that the relationship between polyamines, polyamine analogues, and cell death pathways should be explored. Additionally, the combination of CPENSpm or other polyamine analogues with other agents that act through programmed cell death induction should be considered.

Acknowledgments

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References

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