Sensitivity to Polyamine-induced Growth Arrest Correlates with Antizyme Induction in Prostate Carcinoma Cells

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Abstract

High local polyamine concentrations may suppress cell growth of primary prostate carcinomas. When cell growth assays were conducted in defined serum-free medium, spermine inhibited the growth of poorly metastatic rat prostate carcinoma cells, causing cell cycle arrest in the G1 phase. In contrast, highly metastatic prostate carcinoma cells were resistant to the growth inhibitory activity of spermine. Ornithine decarboxylase antizyme levels, measured by Western blotting, were elevated 1–2 h after spermine treatment of spermine-sensitive cells but not spermine-resistant cells. Spermine uptake was similar in both the sensitive and resistant cell lines. These results suggest that failure to induce antizyme correlates with spermine resistance in prostate carcinoma.

Introduction

We previously purified an endogenous growth inhibitor from human prostate and identified it as spermine, a polyamine secreted in high concentrations by the prostatic ductal epithelium (1). Spermine was effective in inhibiting prostate cancer cell growth both in vitro and in vivo. This work suggested that spermine might act as an endogenous repressor of prostate carcinoma cell growth. In this study, we investigated whether certain prostate carcinoma cells might lose their sensitivity to spermine as they progress to the metastatic state. Our results suggest that highly metastatic prostate carcinoma cells may become spermine resistant and consequently grow more easily in the spermine-rich microenvironment. Resistant cells failed to undergo growth arrest or apoptosis after spermine treatment.

Elevated intracellular spermine levels generally up-regulate levels of ODC (2) antizyme (2). We consequently investigated whether antizyme levels were altered in the spermine-resistant prostate cancer cells. Our results imply that tumor progression in prostate carcinoma involves faulty antizyme regulation resulting in spermine-resistant cells that can grow preferentially in the spermine-rich prosthetic environment.

Materials and Methods

Cell Culture. Cell lines derived from the Dunning rat prostate carcinoma were obtained from Dr. John Isaacs, Johns Hopkins University, Baltimore, MD (AT2.1, AT3.1, MatLyLu) and Dr. Michael Freeman, Children’s Hospital, Boston, MA (NbE, FB2) and maintained as described previously (3, 4).

Cell Proliferation Assays. Cells were plated at a density of 6 × 10^4 cells/well in a 96-well plate (Corning Costar, Cambridge, MA). After 24 h, the cells were incubated in serum-free medium containing 10 μM spermine along with 10 nM [3H]spermine (Amersham Pharmacia, Piscataway, NJ) for the indicated times. Cells were then washed twice in PBS, lysed with 1 N NaOH, and counted in a liquid scintillation counter.

Immunoblotting. Antiantizyme antibodies were obtained from Dr. John Mitchell, Northwestern University, Chicago, IL (5) and from Dr. Philip Coffino, University of California, San Francisco, CA (6). Similar results were obtained with both antibodies.

Antizyme Levels. Antizyme levels were measured by Western blotting. Antiantizyme antibodies were obtained from Dr. John Isaacs, Johns Hopkins University, Baltimore, MD (5). Cells were lysed in SDS-PAGE sample buffer and run on 10% SDS-PAGE gels. After transfer to nitrocellulose, the blot was blocked with 5% milk, incubated with antiantizyme antibodies, and washed with stripping buffer. The blot was then incubated with secondary antibodies coupled to alkaline phosphatase. The blot was visualized using an alkaline phosphatase substrate kit (Perkin-Elmer, Boston, MA). Films were then scanned and quantitated.

Results

Differential Responses of Prostate Carcinoma Cell Lines to Spermine. We previously showed that spermine treatment could cause a reduction in cell growth and viability in human and rat prostate carcinoma cell lines (1). These studies were performed in serum-supplemented medium and were potentially complicated by the action of serum amino oxidases, which can catabolize spermine to toxic byproducts, including acrolein and hydrogen peroxide. To demonstrate that spermine could directly inhibit prostate cancer cell lines, we used a defined serum-free medium containing insulin, transferrin, sodium selenite, and recombinant epidermal growth factor (Intergen, Purchase, NY), 5 μg/ml bovine insulin (Sigma), 5 μg/ml transferrin (Sigma), and 5 ng/ml sodium selenite (Sigma). Some of the cultures received varying concentrations of spermine tetrahydrochloride (Sigma) or Taxol (Sigma). Seventy-two h later, the cells were trypsinized and counted with a Coulter particle counter (Coulter Electronics Inc., Hialeah, FL). Cell viability was determined by staining with 0.2% trypan blue (Sigma) and examining immediately under bright-field microscopy.

Cell Cycle Analysis. Single cell suspensions were prepared by trypsinization and resuspended in Krishan’s reagent (0.05 mg/ml propidium iodide-0.1% sodium citrate-0.02 mg/ml RNase A-0.3% NP-40, pH 8.3). Cell nuclei were then analyzed for DNA content using a Becton Dickinson FACScan cell sorter.

Annexin V Staining. Cells were cultured on glass chamber slides (Nunc, Naperville, IL) that had been coated overnight with 1% gelatin (Difco Laboratories, Detroit, MI) solution in PBS. After cells were washed once with PBS, they were incubated with 0.5 μg/ml fluorescein-conjugated Annexin V (Clontech Laboratories, Inc., Palo Alto, CA) for 15 min at room temperature in the dark. Cells were washed with PBS and fixed with 2% formaldehyde before visualization. Cells were observed under fluorescent illumination using a filter set with absorption at 485 nm and emission at 510 nm.

Spermine Transport. AT2.1 and AT3.1 cells were plated at a density of 10^4 cells/well in a 96-well plate (Corning Costar, Cambridge, MA). After 24 h, the cells were incubated in serum-free medium containing 10 μM spermine for 0–8 h and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, 0.25% deoxycholic acid, 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml Aprotinin, 1.0 μg/ml Leupeptin, 1.0 μg/ml Pepstatin, 1.0 mM Na3VO4, and 1.0 mM dl-DTT. Twenty μg of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 10% milk, followed by 1-h incubations with primary and secondary (donkey antirabbit antibody conjugated to horseradish peroxidase; Amersham) antibodies and finally developed using the Amersham ECL Western blotting detection kit.
overnight exposure to 10 mM metastatic prostate tumor cell lines, including Nbe (IC50 treatment. Cells were exposed to 10 mM spermine resistance developing as metastatic potential increases. As shown in Fig. 1a, the AT2.1 and AT3.1 cells are equally sensitive to inhibition by Taxol, a reagent that disrupts microtubule formation (7).

**Cell Growth Arrest in Spermine-treated Prostate Carcinoma Cells.** The decrease in cell number observed after spermine treatment of spermine-sensitive prostate carcinoma cells could result from an inhibition of cell growth, an induction of cell death, or a combination of these two mechanisms. To determine whether spermine-treated cells undergo cell cycle arrest, we used fluorescence-activated cell sorting to analyze the DNA content of AT2.1 and AT3.1 cells grown in the presence or absence of spermine. In untreated, unsynchronized AT2.1 cells, 50.6% of the cell population was in the G1 phase, with the remaining cells in the S, G2, or M phase (Fig. 2). In contrast, after overnight exposure to 10 mM spermine, the proportion of cells in the G1 phase increased to 70.1%, indicating that spermine-treated cells undergo G1 growth arrest (Fig. 2a). As expected, the percentage of spermine-resistant AT3.1 cells in the G1 phase was not substantially altered after spermine treatment (Fig. 2b).

**Differential Regulation of ODC-Antizyme in Spermine-treated Prostate Carcinoma Cells.** ODC antizyme (antizyme) is rapidly up-regulated after cellular exposure to elevated polyamine levels (8). To determine whether the growth inhibitory effects of spermine on Dunning rat carcinoma cell lines correlates with the induction of antizyme, we examined the levels of antizyme protein at various time points after spermine treatment of spermine-sensitive and spermine-resistant prostate carcinoma cell lines. Within 1 h after spermine treatment, the 23-kDa isofrom of antizyme could be detected by Western blot analysis in the spermine-sensitive AT2.1 cells (Fig. 3A). In AT3.1 cells, however, no significant spermine-induced increase in antizyme levels could be detected (Fig. 3A). Thus, spermine resistance in highly metastatic prostate carcinoma cells correlates with the failure to induce antizyme subsequent to spermine treatment.

One possible explanation for the failure of AT3.1 cells to induce antizyme in response to spermine treatment would be provided if these cells have a defect in spermine transport. To test this hypothesis, we measured spermine uptake in AT2.1 and AT3.1 cells. As shown in Fig. 3B, spermine uptake is not significantly different between these two cell lines over a time period sufficient to induce substantial antizyme levels in the spermine-sensitive AT2.1 cells.

**Evidence for Apoptosis in Spermine-treated Prostate Carcinoma Cells.** Antizyme recently has been demonstrated to cause cell death when overexpressed in certain cell lines such as ras-transfected NIH3T3 cells (9). We therefore investigated whether spermine treatment of prostate carcinoma cells led to apoptosis in spermine-sensitive and spermine-resistant prostate cancer cells. Our results showed signs of apoptosis, including nuclear condensation revealed by electron microscopy and membrane blebbing in the spermine-treated AT2.1 cells but not in the resistant AT3.1 cells (not shown). We did not, however, detect caspase activation in these cells (not shown). One indication of apoptosis is the translocation of phosphatidyl serine from the inner leaflet of the plasma membrane to the outer leaflet where it can be visualized after binding to FITC-conjugated Annexin V (10, 11). As shown in Fig. 4, Annexin V binds to AT2.1 cells within 1 h after treatment, and the staining intensifies over the next 8 h. In contrast, no significant Annexin V staining was observed with the spermine-resistant AT3.1 cells. Taken together, our findings indicate that spermine-resistant cells fail to undergo either cell cycle arrest or...
apoptosis after spermine treatment. Such resistant cells might be expected to have an in vivo growth advantage in the spermine-rich prostatic microenvironment.

Discussion

We showed previously that spermine could inhibit prostate cancer cell growth in vitro and in vivo and hypothesized that spermine may act as an endogenous inhibitor of prostate cancer cell growth in the primary site in vivo. Because some late-stage prostate cancers do acquire the ability to grow more rapidly in the prostate, we decided to investigate whether there was a class of prostate carcinoma cells that was resistant to spermine inhibition. We now demonstrate a differential sensitivity to spermine in prostate cancer cell lines. Aggressive, highly metastatic prostate cancer cell lines are relatively insensitive to the inhibitory effects of spermine, whereas other cell lines are highly sensitive, showing both inhibition of cell proliferation and apoptosis after spermine treatment.

It previously has been suggested that the cytotoxic effects of spermine are mediated by reactive oxygen species produced by the action of amine oxidases present in serum (12, 13). To determine whether this was true for prostate carcinoma cells, we cultured the cells in defined, serum-free medium that supported proliferation of both spermine-sensitive and spermine-resistant cell lines. Under these conditions, spermine-sensitive cells exposed to 10 mM spermine showed cell cycle arrest with subsequent cell death, suggesting that serum-derived enzymes do not account for the spermine-induced cell growth inhibition.

The spermine-sensitive prostate carcinoma cells showed several responses to spermine at a relatively low (10 μM) concentration. These included accumulation of cells in the G1 phase of the cell cycle and loss of viability characterized by markers of the apoptotic pathway including membrane blebbing, nuclear condensation, and translocation of Annexin V to the outer plasma membrane. We were unable to associate this apoptotic pathway with caspase activity. Caspase-independent apoptosis has been reported previously for cells with ubiquitin-independent protein degradation (14) and in BAX-induced cell death (15). In contrast to the spermine-sensitive cells, the highly metastatic, spermine-resistant cells showed no cell cycle inhibition and no loss of viability when treated for the same time periods with the same concentrations of spermine. These results suggest that cells can develop resistance to the inhibitory effects of spermine as they progress toward the metastatic phenotype. If this was to occur in vivo, such spermine-resistant cells would be expected to have a growth advantage in the spermine-rich prostatic environment (16).

A potential mechanism for the development of spermine resistance may be found in the differential induction of the protein ODC-antizyme (antizyme) in spermine-sensitive and spermine-resistant cells. Antizyme is rapidly up-regulated in many cells in response to elevated spermine levels (2). After up-regulation, antizyme binds to the polyamine-synthetic enzyme ODC (17, 18) and facilitates its degradation by the 26S proteasome (19). When overexpressed, antizyme inhibits cell growth and reduces viability both in vitro and in vivo (9, 20). It is interesting, therefore, that our results show that antizyme levels are up-regulated in spermine-sensitive but not in spermine-resistant prostate carcinoma cells.

Our results suggest that failure to up-regulate antizyme after sperm-
ine exposure may render certain prostate tumor cells resistant to spermine growth inhibition. The failure to up-regulate antizyme does not result from a primary defect in polyamine transport because there is no significant difference in spermine uptake between the spermine-sensitive and spermine-resistant cell lines at the time of antizyme induction. We recognize, however, that defects in spermine transport may occur in some prostate cancer cells and could represent an additional mechanism for spermine resistance in prostate cancer.

Antizyme may also have a role in regulating tumor cell growth and survival in other tumors. In A375 melanoma cells, Interleukin-1 inhibition of cell growth was shown to correlate with antizyme up-regulation, indicating that antizyme regulation of cell growth can occur in response to stimuli other than polyamines (21). The recent finding that antizyme levels are reduced in carcinogen-induced oral cancers provides additional evidence that antizyme levels may regulate tumor development in vivo (22). Our findings extend these results to prostastic carcinoma cells that usually grow slowly in the polyamine-rich prostatic microenvironment. We speculate that failure to up-regulate antizyme in prostate carcinoma cells can lead to a subpopulation of cells that are no longer growth inhibited by spermine and that consequently grow preferentially in the later stages of prostate cancer progression.

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