Targeting stathmin in prostate cancer

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Abstract

Stathmin is the founding member of a family of microtubule-destabilizing proteins that regulate the dynamics of microtubule polymerization and depolymerization. Stathmin is expressed at high levels in a variety of human cancers and provides an attractive molecule to target in cancer therapies that disrupt the mitotic apparatus. We developed replication-deficient bicistronic adenoviral vectors that coexpress green fluorescent protein and ribozymes that target stathmin mRNA. The therapeutic potential of these recombinant adenoviruses was tested in an experimental androgen-independent LNCaP prostate cancer model. Adenovirus-mediated transfer of anti-stathmin ribozymes resulted in efficient transduction and marked inhibition of stathmin expression in these cells. Cells that were transduced with the anti-stathmin adenoviruses showed a dramatic dose-dependent growth inhibition. This was associated with accumulation of LNCaP cells in the G2-M phases of the cell cycle. A similar dose-dependent inhibition of clonogenic potential was also observed in cells infected with anti-stathmin adenoviruses. Morphologic and biochemical analysis of infected cells showed a marked increase in apoptosis characterized by detachment of the cells, increased chromatin condensation, activation of caspase-3, and fragmentation of internucleosomal DNA. If these findings are confirmed in vivo, it may provide an effective approach for the treatment of prostate cancer. [Mol Cancer Ther 2005; 4(12):1821–9]

Introduction

Microtubules are dynamic protein polymers that are essential for a myriad of cellular functions, including mitosis, intracellular transport, polarity, and motility (1). During cell division, the mitotic spindle, which is composed of microtubule polymers of α/β-tubulin heterodimers, plays the primary role in the segregation of chromosomes to the two daughter cells (1). The movement of chromosomes toward the spindle poles is made possible by the dynamic instability of microtubules that switch between phases of elongation and shortening (1, 2). The transition from a phase of growth to a phase of shrinkage is called catastrophe, and the transition from a phase of shrinkage to a phase of growth is called rescue (1). The dynamics of microtubule polymerization/depolymerization during the different phases of the cell cycle are regulated by a balance between the activities of two major classes of proteins, microtubule-stabilizing and microtubule-destabilizing factors (2). Changes in the phosphorylation of these proteins are responsible for cell cycle-specific alterations of the microtubule network.

Stathmin is a founding member of a family of microtubule-destabilizing proteins that play a critical role in the regulation of mitosis (3–5). The initial clue that stathmin may have a direct role in the regulation of mitosis came from genetic studies that showed that manipulation of stathmin expression interferes with the progression of cells through mitosis (6, 7). High levels of stathmin expression were described in a variety of human malignancies, including acute leukemia (8), malignant lymphoma (9), neuroblastoma (10), ovarian carcinoma (11), prostate carcinoma (12), and breast carcinoma (13, 14). Interestingly, in many of these cancers, a high level of stathmin expression was shown to correlate with bad prognosis (12, 13, 15). This suggested that a high level of stathmin expression may play an important role in the malignant phenotype. We have shown previously that plasmid-mediated antisense inhibition of stathmin expression in K562 leukemic cells results in abrogation of the malignant phenotype (16). These observations led us to propose that stathmin may be an attractive new target for cancer therapeutics whose aim is to disrupt microtubule dynamics and the mitotic spindle.

Although stathmin is expressed at high levels in a variety of human cancers, we believe that prostate cancer provides one of the best models for the development of therapeutics that target stathmin. When biopsy specimens from human prostate cancers were immunostained with an anti-stathmin antibody, immunoreactivity was seen in poorly differentiated tumors but not in hyperplastic or highly differentiated prostate cancer (12). More importantly, the level of expression of stathmin was shown to correlate with the malignant behavior of prostate cancer cells (12). In fact, it was proposed that the level of expression of stathmin may serve as an important prognostic marker in prostate cancer (12). The fact that the prostate is relatively accessible for local injections adds to the attractiveness of this model for the local delivery of a therapeutic gene that targets stathmin expression.

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In a previous study, we designed and tested several ribozymes that target and degrade stathmin mRNA (17). A major advantage of ribozymes over other RNA-interfering strategies is that ribozymes cleave target mRNA catalytically, resulting in highly efficient mRNA degradation, thus providing a more efficient approach for down-regulating genes like stathmin that are expressed at high levels in cancer cells (18, 19). We tested three different hammerhead ribozymes that targeted two GUC motifs at nucleotides 184 (Rz184) and 305 (Rz305) and a GUU motif at nucleotide 197 (Rz197) of stathmin mRNA (17). Rz184 and Rz305 showed efficient catalytic cleavage of stathmin mRNA in vitro, whereas Rz197 was significantly less efficient (17). In this report, we describe the generation and characterization of adenoviral vectors that carry gene encoding Rz184 and Rz305. Our studies show that these recombinant adenoviruses can degrade stathmin mRNA efficiently in prostate cancer cells and result in marked inhibition of their proliferation and clonogenicity and increase in apoptosis.

Materials and Methods

Cell Lines

The human androgen-independent LNCaP prostate cancer cells used in this study were described previously (20). These cells were grown in RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum, 5 μg/mL human insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin. The 293 packaging cell line was maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C in a humidified 5% CO₂ environment.

Production of Recombinant Adenoviruses

The first step in the generation of the recombinant adenoviruses involved cloning of the anti-stathmin ribozyme genes, Rz184 and Rz305 (17), in adenoviral transfer vectors. The oligonucleotides encoding Rz184 and Rz305 (17) were flanked by BglII restriction sites to facilitate cloning at the unique BglII site of the adenoviral transfer vector, pAd-CMV5-IRES-GFP (Quantum Technology, Montreal, Canada). All recombinant transfer plasmids were verified for the correct insertion of anti-stathmin ribozyme sequences by DNA sequencing. To generate a control virus, we used a similar adenoviral backbone in which the GFP coding sequences were placed under the control of the CMV5 promoter without the ribozyme sequences.

The second step involved the production of infectious viral particles by standard homologous recombination (21). This was achieved by cotransfection of each of the recombinant transfer plasmids with part of the adenovirus type 5 genome selected to promote in vivo homologous recombination between the two DNA molecules to yield infectious virus. Cotransfection of the recombinant transfer plasmid and the E1a-deleted adenoviral DNA was facilitated by calcium phosphate coprecipitation on 293 packaging cells, which complement the E1a and E1B adenovirus type 5 viral genes that are deleted from the recombinant adenoviruses. Recombinant adenoviruses were identified by screening of packaging cells for bright green plaques under a fluorescent microscope. Several individual plaques were picked for each virus and their viral lysates were analyzed by PCR for the presence of anti-stathmin ribozyme sequences in the adenovirus type 5 genome. Several adenoviral clones were positive for both anti-stathmin ribozyme and GFP sequences. For large-scale production of the virus, a single clone from each recombinant adenovirus was amplified in 293 cells and purified by ultracentrifugation on a cesium chloride gradient, dialyzed, and stored at −80°C. The infectious viral titers were determined by plaque assays in 293 cells (21).

Adenoviral Infections In vitro

Cells were seeded in six-well culture plates 24 hours before virus infection. For all the experiments described below, cells were infected with the recombinant adenoviruses at different multiplicity of infection (MOI) in a 2% reduced serum medium for 3 hours. After infection, the virus was removed and the cells were further incubated in complete growth medium. The efficiency of the adenoviral infections was assessed by fluorescence microscopy or flow cytometry at either 48 or 72 hours after infection.

Northern Analysis

Uninfected cells and cells infected with either control Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP adenoviruses at MOI of 25 were harvested at 24, 48, or 72 hours. Total RNA was isolated from these cells using the guanidinium thiocyanate-phenol-chloroform extraction method (22). Each RNA sample (20 μg) was denatured in glyoxal and separated by agarose gel electrophoresis as described (22). A 1.5-kb XbaI fragment from the stathmin cDNA (23) was labeled by random priming and used as a probe in hybridization experiments. The Northern filter was stripped and rehybridized to ribozyme, GFP, and 18S rRNA probes.

Western Analysis

Uninfected cells and cells infected with either control Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP adenoviruses at MOI of 25 were harvested 72 hours after infection. The cell pellets were lysed for 30 minutes on ice in a buffer consisting of 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, and 1% Triton X-100. The cell extracts were clarified by centrifugation and the protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Each protein extract (25 μg) was electrophoresed on a 15% SDS-polyacrylamide gel, transferred to membrane, and blocked in PBS containing 5% nonfat milk powder and 0.2% Tween 20 for 1 hour at room temperature. The filter was incubated overnight at 4°C with anti-stathmin antibody (BD PharMingen, San Diego, CA), each protein extract (25 μg) was electrophoresed on a 15% SDS-polyacrylamide gel, transferred to membrane, and blocked in PBS containing 5% nonfat milk powder and 0.2% Tween 20 for 1 hour at room temperature. The filter was incubated overnight at 4°C with anti-stathmin antibody (BD PharMingen, San Diego, CA) in PBS containing 0.1% Tween 20 followed by incubation with horseradish peroxidase–conjugated anti-mouse IgG (Sigma, St. Louis, MO) for 1.5 hours. The filter was then washed several times with PBS containing 0.2% Tween 20. As an internal control, the same filter was hybridized to anti-actin antibody (Oncogene Research Products, Hercules, CA).
San Diego, CA) followed by incubation with horseradish peroxidase–conjugated anti-mouse IgM (Calbiochem, San Diego, CA) as above. The bands were visualized by chemiluminescence using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

**Cell Proliferation Assay**

To assess the rate of proliferation, equal numbers of cells (2 × 10⁷) were infected with either control or anti-stathmin adenoviruses in triplicates at different MOIs. The cells (floating and detached) were harvested and stained with trypan blue to determine cell viability. Cells were counted on a hemocytometer on alternate days and the data were plotted in graphical form to generate growth curves.

**Cell Cycle Analysis**

We used propidium iodide staining of fixed whole cells to analyze the distribution of cells in the different phases of the cell cycle (4). Uninfected cells and cells infected with either control or anti-stathmin adenoviruses at different MOIs were harvested and divided into two fractions, one for cell cycle analysis and the other for GFP fluorescence analysis. For cell cycle analysis, the cells were fixed in 70% ethanol, washed in PBS, and resuspended in 1 mL propidium iodide solution (PBS containing 0.05 mg/mL, 0.1% sodium citrate and 1 μg/mL RNase). The cells were incubated for 30 minutes at 37°C. DNA content was analyzed within 2 hours in a Becton Dickinson (Bedford, MA) FACStar Plus flow cytometer at 488 nm single laser excitation. For GFP fluorescence analysis, the cells were fixed in 1% paraformaldehyde. Transduction efficiencies of infected cells were assessed by measuring the fraction of cells that expressed GFP by flow cytometry. The cell cycle distribution and GFP positivity were analyzed using Win List software.

**Clonogenic Assay**

Anchorage-independent growth was assessed by colony formation in a methylcellulose semisolid medium. Equal numbers of cells were infected with either control or anti-stathmin adenoviruses in triplicates at MOI of 5, 10, and 25 for 3 hours. The cells were washed in PBS and resuspended in 5 mL methylcellulose-based semisolid medium (0.9% methylcellulose, 1% bovine serum albumin, and 0.1 mmol/L β-mercaptoethanol prepared in RPMI 1640 containing 30% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin). Cells (1 × 10⁵) were then plated in six-well tissue culture plates in triplicates and incubated at 37°C in 5% CO₂ atmosphere. The colonies that formed were counted after 2 weeks.

**Apoptosis Assays**

Cells were infected with either control or anti-stathmin adenoviruses as above. Both floating and attached cells were harvested 5 days after infection and the effect of ribozyme-mediated stathmin inhibition on apoptosis was quantified in several different assays.

**Analysis of Nuclear Morphology.** For the morphologic evaluation of the proportion of apoptotic cells, ~10,000 uninfected and infected cells were spun onto a microscope slide and stained with 4',6-diamidino-2-phenylindole at 1 μg/mL for 15 minutes. The cells were then scored under a fluorescence microscope as either normal (nuclei with smooth and homogeneous staining) or apoptotic (condensed nuclei with intense chromatin staining).

**Analysis of Caspase-3 Activation.** To detect caspase-3 activation, uninfected cells and cells infected with either Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP were harvested, fixed, and permeabilized using a caspase-3 apoptosis kit according to the manufacturer’s protocol (BD PharMingen). Cells were then stained with a monoclonal anti-caspase-3 antibody conjugated to phycoerythrin and analyzed by flow cytometry.

**Analysis of DNA Fragmentation.** DNA fragmentation was assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay using a Cell Death Detection kit (Roche) according to the manufacturer’s instructions. Briefly, uninfected cells and cells infected with either Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP were fixed, permeabilized, and incubated with the TUNEL reaction mixture. As a positive control, uninfected cells were treated with DNase. The free 3'-OH groups of fragmented DNA labeled with tetramethylrhodamine-labeled nucleotides were quantified by flow cytometry.

**Results**

**Generation of Recombinant Adenoviruses That Carry Anti-Stathmin Ribozymes**

The production of the recombinant adenoviruses was done in two steps. The first step involved the cloning of the anti-stathmin ribozymes in the adenoviral transfer vector. We used a bicistronic expression vector (pAd-CMV5-IRES-GFP) that contains an adenovirus origin of replication and 2.5 kb of human adenovirus type 5 DNA for efficient homologous recombination. This vector also contains an internal ribosome entry site element derived from the encephalomyocarditis virus that permits the translation of two open reading frames from the same mRNA (24). The internal ribosome entry site cassette of this vector is downstream from a unique cloning site to allow coexpression of a GFP selectable marker and the therapeutic gene of interest. Both the selectable and the therapeutic genes are placed under the control of CMV5, a CMV promoter-enhancer modified for maximal expression. This arrangement allows high-level expression of the transgene and easy visualization and/or selection of transduced cells.

We cloned the genes that encode Rz184 and Rz305 in the adenoviral gene transfer vectors as shown in Fig. 1. We tested the activity of these recombinant adenoviruses in an *in vitro* model of prostate cancer. The human LNCaP is the most widely used cell line for the study of prostate cancer (25). For our studies, we used an androgen-independent human LNCaP cell line that was derived by maintaining androgen-dependent LNCaP cells in an androgen-poor medium (20). This selective pressure led to the growth of androgen-independent cells with properties that mimic advanced prostate cancer (20).
The efficiency of adenovirus infection was assessed by infecting the LNCaP cells with anti-stathmin adenoviruses at increasing MOI. As controls, the cells were either mock infected or infected with the control Ad.GFP adenovirus at a similar MOI to determine if adenoviral infection results in cytotoxicity. The efficiency of gene transfer was determined by quantifying the fraction of cells that expressed GFP by flow cytometry. Infection of cells with control or anti-stathmin adenoviruses at increasing MOIs from 5 to 50 resulted in a progressive increase in the percentage of transduced cells. The optimal MOI for LNCaP cells was determined to be between 5 and 25. Infection at these MOIs resulted in efficient transduction without any significant cytotoxicity. Figure 1B represents fluorescent microscopy images showing >90% of the cells in the monolayer culture positive for GFP fluorescence after a single infection with the recombinant adenoviruses at MOI of 25 and visualized by fluorescence microscopy after 48 h.

Effect of Anti-Stathmin Ribozymes on Stathmin Expression

We first asked whether adenovirus-mediated gene transfer of anti-stathmin ribozyme would decrease the level of stathmin mRNA in LNCaP cells. We did a time course analysis in which cells were harvested at different intervals after infection with either the control Ad.GFP adenovirus at a similar MOI to determine if adenoviral infection results in cytotoxicity. The efficiency of gene transfer was determined by quantifying the fraction of cells that expressed GFP by flow cytometry. Infection of cells with control or anti-stathmin adenoviruses at increasing MOIs from 5 to 50 resulted in a progressive increase in the percentage of transduced cells. The optimal MOI for LNCaP cells was determined to be between 5 and 25. Infection at these MOIs resulted in efficient transduction without any significant cytotoxicity. Figure 1B represents fluorescent microscopy images showing >90% of the cells in the monolayer culture positive for GFP fluorescence after a single infection with the recombinant adenoviruses at MOI of 25.

Effect of Anti-Stathmin Ribozymes on Proliferation

We examined the effects of the anti-stathmin ribozymes on the proliferation of LNCaP cells. Figure 3 illustrates the growth rates of uninfected cells, cells infected with the control Ad.GFP, and cells infected with either Ad.Rz184.GFP or Ad.Rz305.GFP adenoviruses. When cells were infected with Ad.GFP virus at different MOIs, no significant growth inhibition was observed compared with uninfected cells (Fig. 3A). In contrast, cells transduced with Ad.Rz184.GFP showed a dose-dependent growth inhibition, with essentially complete cessation of growth at a MOI of 25. We also observed striking differences in the morphology of cells transduced with anti-stathmin adenoviruses. A vast majority of the cells infected with anti-stathmin adenoviruses rounded up and detached from the culture dish after 3 to 4 days (data not shown). The rounding up and the
detachment of cells in culture are typical of mitotic cells that undergo profound cytoskeletal changes. In contrast, cells infected with the control adenovirus remained attached as a monolayer.

**Effect of Anti-Stathmin Ribozymes on Cell Cycle Progression**

We also examined the effects of adenovirus-mediated transduction of LNCaP cells with anti-stathmin ribozymes on cell cycle progression. Cells were infected with either control Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP adenoviruses at different MOIs. After 48 hours, the cells were harvested and stained with propidium iodide and their DNA content was assessed in a flow cytometer (Fig. 4). When LNCaP cells were transduced with the control Ad.GFP at different MOIs, the DNA histograms were similar to that of uninfected cells (Fig. 4A). In contrast, cells transduced with Ad.Rz184.GFP or Ad.Rz305.GFP showed a marked accumulation of cells in the G2-M phases, with a corresponding decrease in the fraction of cells in the G0-G1 phases of the cell cycle (Fig. 4A). Moreover, the accumulation of cells in the G2-M phases in the infected cells increased with increase in MOI.

To make sure that the observed differences in the DNA histograms are not a result of differences in the efficiency of viral transduction, we quantified the transduction efficiency directly by measuring the fraction of cells that express GFP. Small aliquots of cells from the experiment described above were fixed in 1% paraformaldehyde and analyzed for GFP fluorescence by flow cytometry. The fractions of GFP-positive (i.e., transduced) cells were comparable in cells infected with Ad.GFP, Ad.Rz184.GFP, and Ad.Rz305.GFP (Fig. 4B). Thus, the observed differences in the cell cycle distribution of transduced cells are clearly not a result of differences in the efficiency of transduction.

**Effect of Anti-Stathmin Ribozymes on Clonogenicity**

We also studied the effects of anti-stathmin adenoviruses on the ability of LNCaP cells to form anchorage-independent colonies in semisolid medium. Figure 5 illustrates the relative clonogenicity of uninfected cells and cells infected with either Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP adenoviruses at different MOIs. No significant differences in the clonogenicity were seen when uninfected cells were compared with cells infected with the control Ad.GFP adenovirus (Fig. 5). In contrast, LNCaP cells infected with anti-stathmin adenoviruses at different MOIs showed a dose-dependent inhibition of colony formation, with a near-complete suppression of anchorage-independent growth at a MOI of 25. Thus, adenovirus-mediated anti-stathmin ribozyme expression can inhibit tumorigenicity of prostate cancer cells in vitro.

**Effects of Anti-Stathmin Ribozyme on Apoptosis**

In the cell proliferation assays described above, we observed massive cell death by trypan blue staining 5 days after infection with anti-stathmin adenoviruses. To investigate these observations further, we assessed the effects of anti-stathmin ribozymes on apoptosis of LNCaP cells (Fig. 6). We first evaluated the effects of anti-stathmin ribozymes on the morphology of the nuclei by 4',6-diamidino-2-phenylindole staining. Figure 6A illustrates nuclear chromatin condensation in cells transduced with ribozyme carrying adenoviruses. Nuclei of normal cells are stained homogeneously by 4',6-diamidino-2-phenylindole, whereas nuclei of apoptotic cells are highly irregular as a result of chromatin condensation. Infection of cells with Ad.Rz184.GFP or Ad.Rz305.GFP resulted in apoptosis in 29% and 25% of cells, respectively, compared with 0.6% in uninfected cells and 1% in cells infected with the control Ad.GFP virus (Fig. 6A). We also investigated the effects of anti-stathmin ribozymes on the activation of caspase-3. Uninfected cells and cells infected with either Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP were stained with anti-caspase-3 antibody and analyzed by flow cytometry. Cells infected with control Ad.GFP showed no significant caspase-3 activation (1.6%). In contrast, caspase-3 activation was seen in 43% and 37% of cells infected with Ad.Rz184.GFP and Ad.Rz305.GFP, respectively (Fig. 6B). We also assessed the effects of anti-stathmin ribozymes on DNA fragmentation in a flow cytometric TUNEL assay. Cells infected with control Ad.GFP showed virtually no TUNEL-positive cells (1.3%), whereas cells infected with

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**Figure 2.** Kinetics of anti-stathmin ribozyme expression and activity in LNCaP cells. Total RNA was isolated from uninfected cells or cells infected with either Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP at a MOI of 25. **A,** effects of Ad.Rz305.GFP on the level of stathmin mRNA. Lane 1, RNA (20 μg) isolated from uninfected cells; lanes 2 to 7, RNA (20 μg) isolated from cells transduced with Ad.GFP and Ad.Rz305.GFP at 24 h (lanes 2 and 3), 48 h (lanes 4 and 5), and 72 h (lanes 6 and 7). **B,** effects of Ad.Rz184.GFP on the level of stathmin mRNA. Lane 1, RNA (20 μg) isolated from uninfected cells; lanes 2 to 7, RNA (20 μg) isolated from cells transduced with Ad.GFP and Ad.Rz184.GFP at 24 h (lanes 2 and 3), 48 h (lanes 4 and 5), and 72 h (lanes 6 and 7). The filters were hybridized with stathmin, ribozyme (Rz), GFP, and 18S probes as indicated. **C,** effects of anti-stathmin adenoviruses on stathmin protein levels. Lanes 1 to 4, protein extracts (25 μg) isolated from uninfected, Ad.GFP-infected, Ad.Rz305.GFP-infected, and Ad.Rz184.GFP-infected cells, respectively.
Ad.Rz184.GFP and Ad.Rz305.GFP showed 59% and 56% TUNEL-positive cells, respectively (Fig. 6C). Thus, in all three assays, infection with adenoviral vectors that carry anti-stathmin ribozyme genes results in profound increase in apoptosis.

**Discussion**

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths in men in the United States. In the early stage of the disease, the treatments of choice are extensive surgery...
and/or radiation therapy. Although both treatment modalities are highly effective, they are associated with significant morbidity and mortality. The localized aspect of the disease in its early stages and its responsiveness to local therapy makes prostate cancer an attractive model for the development of a stathmin-based local therapy that might avoid the morbidity and mortality of extensive surgery and radiation therapy. When local therapies for prostate cancer fail and the disease progresses, systemic androgen ablation therapy, with or without chemotherapy, can frequently lead to tumor regression. However, the disease inevitably progresses to an androgen-independent state that is resistant to hormonal therapy and chemotherapy. Thus, the development of alternative therapeutic strategies for prostate cancer in the early and late stages remains a high priority. The aim of the studies described in this report is to develop a novel therapeutic strategy for the treatment of prostate cancer that targets the microtubules that make up the mitotic spindle.

Adenoviruses are efficient gene delivery vectors that are widely used in cancer gene therapy (26). They can be produced at very high titers and can infect a variety of cell types both in vitro and in vivo (26). Whereas retrovirus-mediated gene transfer results in stable integration of the transgene in transduced cells, adenoviruses do not integrate in the host genome (27). Moreover, adenoviruses induce an immune response in the host that can result in the clearance of transduced cells within 1 or 2 weeks after gene transfer (27). Although such an immune response is a major limitation in the gene therapy of genetic disease, it may be advantageous in cancer gene therapy because elimination of the transduced cells is an end point of therapy. Thus, we developed adenoviral gene transfer vectors to achieve efficient delivery of anti-stathmin ribozymes into prostate cancer cells. The experiments described above show that the two anti-stathmin ribozymes that we described previously can be used in adenoviral gene transfer vectors to achieve very effective down-regulation of stathmin expression in prostate cancer cells. The progressive increase in ribozyme expression correlated with a corresponding decrease in the levels of stathmin mRNA and protein.

We investigated the therapeutic potential of adenovirus-mediated anti-stathmin therapy in several biological assays. Cell cycle analysis of anti-stathmin ribozyme-infected cells

![Figure 5. Effects of anti-stathmin ribozymes on the clonogenic potential of LNCaP cells.](image)

Figure 5. Effects of anti-stathmin ribozymes on the clonogenic potential of LNCaP cells. Clonogenic potential of uninfected cells or cells infected with either Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP at different MOIs. The relative clonogenicity of infected cells was calculated relative to a clonogenic potential of uninfected cells of 100%. Open column, uninfected cells; filled column, Ad.GFP-infected cells; hatched columns, Ad.Rz184.GFP-infected cells; gray columns, Ad.Rz305.GFP-infected cells. Representative of three different experiments. Bars, SD.

![Figure 6. Effects of anti-stathmin ribozymes on apoptosis in LNCaP cells.](image)

Figure 6. Effects of anti-stathmin ribozymes on apoptosis in LNCaP cells. A, nuclear morphology of uninfected and infected cells as indicated. Apoptotic nuclei (arrowheads) show characteristic chromatin condensation and nuclear fragmentation in anti-stathmin adenovirus infected cells after 4',6-diamidino-2-phenylindole staining. B, flow cytometric histograms showing the expression of caspase-3 in uninfected and infected cells as indicated. The uninfected cells were essentially negative for the presence of active caspase-3. The histogram of uninfected cells (black) is overlaid on histograms of Ad.GFP, Ad.Rz184.GFP, or Ad.Rz305.GFP (red) as indicated. C, DNA histograms showing the percentage of TUNEL-positive cells in uninfected, Ad.GFP-infected, Ad.Rz184.GFP-infected, and Ad.Rz305.GFP-infected cells as indicated.
showed a marked accumulation of cells in the G₂-M phases of the cell cycle. These observations in prostate cancer cells are similar to previous observations that we and others made in leukemic cells (6, 7). Because stathmin is a catastrophe factor and/or sequester of tubulin that leads to depolymerization of microtubules (3, 4), inhibition of stathmin is expected to impede progression through mitosis. The mitotic block that we observed correlated with a dose-dependent inhibition of growth of LNCaP prostate cancer cells infected with the anti-stathmin adenoviruses. Similar antimitotic and antiproliferative effects were also observed in DU145 and PC3 prostate cancer cell lines (data not shown). The inclusion of the GFP gene in our adenoviral vectors allowed us to exclude the possibility that differences in the efficiency of transduction or cytotoxicity of the adenovirus itself may be the cause of the observed differences in proliferation and cell cycle progression.

Of all the in vitro assays of transformation, clonogenic anchorage-independent growth has the best correlation with in vivo assays of tumorigenicity (28, 29). Whereas nontransformed cells may divide once or twice in semisolid medium, transformed cells continue to divide until they form visible colonies. Using this assay, we showed that adenovirus-mediated anti-stathmin therapy results in a profound dose-dependent inhibition of the clonogenic growth of LNCaP cells. Interestingly, the reduced number of colonies that formed at lower MOIs in cells transduced with the anti-stathmin ribozymes were also much smaller compared with colonies that formed in uninfected or Ad.GFP-infected cells. Moreover, no further increase in the number or size of colonies was observed after 4 weeks in semisolid medium. We also assessed the effect of anti-stathmin ribozyme genes on apoptosis in LNCaP cells by analyzing cell morphology, caspase activation, and DNA fragmentation. Stathmin inhibition was associated with a marked increase in rounding and detachment of cells, increase in chromatin condensation, activation of caspase-3, and fragmentation of internucleosomal DNA. Although the increase in caspase-3 activation suggests that the apoptosis that we observed is induced by a caspase-dependent pathway, it is not clear what the exact mechanism of apoptosis is in those cells. We speculate that the mitotic spindle abnormalities that result from down-regulation of stathmin expression might activate mitotic checkpoints that lead to apoptosis.

Prostate cancer is generally considered a chemotherapy-resistant disease. Taxol is one of few chemotherapeutic agents that have activity against prostate cancer (30). The taxanes are a family of mitotic inhibitors that exert their antitumor effects by stabilizing the microtubules that make up the mitotic spindle (30). Although taxol has marked activity against prostate cancer cells in vitro, the results of clinical studies in which taxol was used as a single agent in prostate cancer have been disappointing (31, 32). Our previous studies have shown synergy between stathmin inhibition and taxol exposure in leukemic cells (33). If similar synergy is shown in prostate cancer cells that are inherently more sensitive to taxol than leukemic cells, combination therapy consisting of taxol and anti-stathmin ribozymes may provide a potentially effective therapeutic approach for prostate cancer.

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References

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