Growth Suppression of Human Ovarian Cancer Cells by Adenovirus-mediated Transfer of the PTEN Gene

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Abstract

A tumor suppressor gene on chromosome 10q23, PTEN, encodes a phosphatidylinositol phosphatase that antagonizes activation of the phosphatidylinositol 3'-kinase-mediated pathway involved in cell growth. A gene encoding the catalytic subunit of phosphatidylinositol 3'-kinase (PIK3CA) is frequently activated in ovarian cancers; therefore, overexpression of the PTEN product through gene transfer might be an effective strategy for treating ovarian cancers. To test the potential for this type of gene therapy, we constructed a recombinant adenovirus encoding wild-type PTEN and examined its effects on nine cell lines derived from human ovarian carcinomas. Transduction of the PTEN gene significantly inhibited growth of six of these cell lines compared with infection with virus alone, and the degree of inhibition correlated with the efficiency of gene transfer as determined by β-galactosidase assay. Results of flow cytometry suggested that the observed effects were mediated by two mechanisms, apoptosis and/or arrest in the G1 phase of the cell cycle, and that high adenoviral transduction efficiency of cells was associated with induction of apoptosis. We also found that the level of transcription of Integrin αv in ovarian cancer cells correlated with the efficiency of transduction (P = 0.014) and with the degree of growth inhibition after PTEN gene transfer (P = 0.009). These findings carry significant implications for adenovirus vector-based PTEN gene therapies for ovarian cancers.

Introduction

Among gynecological malignancies, ovarian cancer is the leading cause of death. Despite introduction of new chemotherapeutic agents into treatment protocols, to date no overall improvement has been achieved in long-term survival. Hence, developing alternative strategies is a matter of urgency. The PIK3CA gene on chromosome 3q26, which encodes the catalytic subunit of PI3K,2 is frequently increased in copy number in ovarian cancers (1); PI3K mediates a major growth-control pathway, acting both to stimulate cell growth and to block apoptosis (2–4). This pathway is antagonized by a tumor suppressor gene on chromosome 3q26, which encodes a phosphatidylinositol phosphatase that antagonizes activation of the phosphatidylinositol 3,4,5-trisphosphate, second messengers downstream of PI3K (5–7). Amplification of the PTEN gene was reported to be rare in ovarian cancers (8, 9), gene transfer may be an effective therapy for this type of tumors, if a high dose of the PTEN product is able to block the activated PI3K-mediated cell growth pathway. Transfection of a PTEN expression plasmid into glioma cells indeed can suppress growth by arresting cells in the G0 phase (10). Moreover, adenovirus-mediated PTEN gene transfer into glioma cells is able to suppress tumorigenicity (11) and induces apoptosis initiated by disruption of the interactions of the cells with the extracellular matrix (12).

To test the potential for PTEN gene therapy of ovarian cancers, we introduced wild-type PTEN genes into each of nine cell lines derived from human ovarian carcinomas and correlated the effect of exogenous PTEN on transcription of PTEN, PIK3CA, and receptors for the adenovirus vector. We found that the level of transcription of Integrin αv in these cell lines correlated with the efficiency of transduction and also with the extent of growth inhibition by overexpressed PTEN. Flow cytometry suggested that the growth-inhibitory activity of PTEN was mediated by two mechanisms, apoptosis and/or cell cycle arrest in the G1 phase, and that high adenoviral transduction efficiency of cells associated with induction of apoptosis. Our results carry significant implications for development of adenovirus vector-based gene therapies for this disease.

Materials and Methods

Ovarian Cancer Cell Lines. Cell lines Caov-3, ES-2, MDAH 2774, NIH:OVCAR-3, OV-1063, SK-OV-3, and SW 626 were obtained from the American Type Culture Collection (Manassas, VA). MCAS and TYK-nu cell lines were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). NIH:OVCAR-3 and OV-1063 cells were maintained in RPMI 1640 with 10% FBS. ES-2 and SK-OV-3 cells were grown in McCoy’s 5A medium with 10% FBS. MDAH 2774 and SW 626 cells were maintained in Leibovitz’s L-15 medium with 10% FBS. MCAS and TYK-nu cells were grown in Eagle’s MEM with 10% FBS, and Caov-3 cells were maintained in DMEM with 10% FBS.

RT-PCR Analysis. Using a semiquantitative RT-PCR method, we assessed transcription levels of PTEN, PIK3CA, HCAR, Integrin αv, Integrin β3, and Integrin β5 relative to β2-microglobulin in all nine ovarian cancer cell lines. Total RNAs were isolated using Trizol reagent (Life Technologies, Inc.), treated with DNase I, and reverse-transcribed using Superscript II reverse transcriptase (Life Technologies, Inc.). Each RT-PCR reaction consisted of 25 or 30 cycles of 30 s at 94°C, 30 s at 55°C (for PTEN, PIK3CA, and β2-microglobulin) or 60°C (for Integrin αv, β3, and β5) and 1 min at 72°C. Amplification of β2-microglobulin revealed similar signal strengths in all samples, as a control for the integrity of each RNA template. PCR products were electrophoresed in 2% agarose gels, blotted onto nylon membranes, and hybridized with [32P]ATP-labeled internal primers. Signal intensities were measured with a bioimage analyzer (BAS 1000; Fujifilm) and autoradiography. The RNA preparations were checked for contamination by genomic DNA by comparing PCR reactions for all sets of primers with or without reverse transcriptase. Primers used for amplification and internal probes for hybridization were as follows:

PTEN: forward (F), 5'-CTT CAG CCA CAG GCT CCC A-3'; reverse (R), 5'-TGG TGT TTT TTC CTT CTT GAT A-3'; and internal (I), 5'-GAC CAA TGG CTA AGT GAA GAT-3'

PIK3CA: F, 5'-GTA TGG CTA TCC GCC ACA TGG-3'; R, 5'-CAG TCA TGG TGG ATT TTC AGA G-3'; and I, 5'-TCA CCA GAA TTG CCA AAG CAC-3'

HCAR: F, 5'-ACA ACT GTC AGA TAT TGG CAC-3'; R, 5'-GAT GAA...
Fig. 1. Chronicity of expression of PTEN protein in transfected ovarian cancer cell line SK-OV-3. Proteins were extracted from the cells at the time points indicated. The levels of PTEN expression were compared with expression of actin.

Fig. 2. Semiquantitative RT-PCR analysis of the transcription levels of PTEN, PIK3CA, and Integrin subunits αv, β3, and β5 in all nine cell lines. The integrity of each RNA template was controlled through amplification of β2-microglobulin.

5-bromo-4-chloro-3-indoly-β-D-galactopyranoside solution consisting of 0.1 M sodium phosphate (pH 7.3), 1.3 mM MgCl₂, 3 mM K₃[Fe(CN)₆], and 3 mM K₄[Fe(CN)₆] in PBS. β-galactosidase-positive cells in triplicate wells were counted microscopically.

Flow Cytometry. Cells were plated at a density of 5 × 10⁵ cells/100-mm dish and infected 20 h later with 100 MOI of AdCAPTEN or AdCA. Forty-eight h after infection, cells were trypsinized, collected in PBS, and fixed in 70% cold ethanol. After RNase treatment, cells were stained with 50 μg/ml propidium iodide in PBS. Flow cytometry was performed on a Becton Dickinson FACScan and analyzed by ModFit software (Verity Software House, Inc., Topsham, ME). The percentages of nuclei in G₀-G₁, S, and G₂-M phases of the cell cycle and sub-G₁ population were determined from at least 20,000 ungated cells.

Results and Discussion

Expression of recombinant PTEN-containing adenovirus (AdCAPTEN) was examined in the ovarian cancer cell line SK-OV-3 by Western blotting of cell lysates (Fig. 1). Overexpression of PTEN protein was detected as early as 18 h after infection. To evaluate the effect of the transgene on proliferation of ovarian cancer cells, we selected an additional eight cell lines derived from human ovarian carcinomas. We examined genetic alterations of the PTEN gene in each line by direct sequencing of the coding region and found that none contained mutations in this gene. We also examined the transcription level of PTEN and PIK3CA in each line, using semiquantitative RT-PCR (Fig. 2). Interestingly, cell lines showing a high level of endogenous PTEN transcription showed a high level of PIK3CA, and those showing a very low level of PTEN expression revealed a relatively low level of PIK3CA expression except one cell line, TYK-nu.

To examine effects of PTEN transfection using the recombinant adenovirus on the cellular proliferation, 1 × 10⁴ cells of each ovarian cancer line were infected in triplicate with AdCAPTEN or AdCA at a MOI of 5, 20, or 100, and the cell numbers were counted on the 5th day after infection. As shown in Fig. 3, six of the nine cell lines, MDAH 2774, TYK-nu, SW 626, NIH-OVCAR-3, OV-1063, and SK-OV-3, showed significant decreases in cell number by infection with AdCAPTEN compared with cells infected with control virus alone; the numbers of these cells were decreased to 13–38% of the control cells. The remaining three cell lines, Caov-3, ES-2, and

Genetic Alterations of PTEN in Ovarian Cancer Cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Genetic Alterations</th>
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<tbody>
<tr>
<td>SK-OV-3</td>
<td>None</td>
</tr>
<tr>
<td>Caov-3</td>
<td>None</td>
</tr>
<tr>
<td>ES-2</td>
<td>None</td>
</tr>
<tr>
<td>MDAH 2774</td>
<td>None</td>
</tr>
<tr>
<td>TYK-nu</td>
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</tr>
<tr>
<td>SW 626</td>
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</tr>
<tr>
<td>NIH-OVCAR-3</td>
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</tr>
<tr>
<td>OV-1063</td>
<td>None</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>None</td>
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</tbody>
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Fig. 2. Semiquantitative RT-PCR analysis of the transcription levels of PTEN, PIK3CA, and Integrin subunits αv, β3, and β5 in all nine cell lines. The integrity of each RNA template was controlled through amplification of β2-microglobulin.
MCAS, had little or no effect by transfection of AdCAPTEN. These findings indicate that genotypically wild-type PTEN cells can be growth inhibited by a high dose of exogenous PTEN. Similar results were reported by Li et al. (15), where adenoviral PTEN gene transfer suppressed the growth of breast cancer cells, regardless of the mutational status of endogenous PTEN.

To investigate a relationship between the efficiency of the gene transfers by the virus vector and the growth-suppressive effects, we performed β-galactosidase transduction assays using AdCALacZ, which carries the β-galactosidase gene of Escherichia coli. Six of the nine ovarian cancer cell lines that revealed significant growth suppression in the cellular proliferation assay by transfection of AdCAPTEN showed transduction efficiencies as high as 70–100% when transfected at a MOI of 100 (Table 1). In contrast, the two cell lines, ES-2 and MCAS, that had no growth-suppressive effect showed 12 and 9% efficiencies of β-galactosidase transduction, respectively.

To further examine the molecular mechanisms of growth suppression, we performed flow cytometric analysis and found that a marked increase in the sub-G1 population in three of the six cell lines that showed significant growth suppression, MDAH 2774, TYK-nu, and SW 626 (Fig. 4). Among the other three growth-inhibited cell lines, the two lines NIH:OVCAR-3 and SK-OV-3 showed a moderate increase of sub-G1 population and a significant increase in the proportion of G0-G1 phase population. OV-1063 showed a moderate increase in the sub-G1 population. The cell line Caov-3, which showed a little growth suppression and moderate (44%) gene trans-
duction efficiency, revealed a moderate increase of sub-G₁ population and a moderate increase in the proportion of G₀-G₁ phase population. The two cell lines that had no growth-suppressive effect showed no alterations by flow cytometric analysis (Fig. 4). These findings suggested that the growth-inhibitory activity of exogenous PTEN is mediated by two mechanisms, apoptosis and/or arrest at the G₁ phase of the cell cycle, and that high adenoviral transduction efficiency of cells was associated with induction of apoptosis by PTEN gene transfer.

Expression of fiber receptors (HCAR in particular) and of αv-integrins is thought to be required for efficient entry of adenovirus into cells and subsequent gene transfer (16–21). Therefore, we examined transcription of HCAR and of integrin subunits αv, β3, and β5 by RT-PCR analysis (Fig. 2). No transcript of HCAR was detected, even after 30 cycles of PCR amplification, a result consistent with Northern blot analyses reported by Tomko et al. (21). According to those investigators, no signal was detected in the ovary, whereas the brain revealed the highest level of HCAR transcription among the tissues tested. Susceptibility of glioma cells to adenovirus-mediated gene transfer has been correlated with expression of HCAR but not αv-integrins (22). Although we detected no HCAR expression in the ovarian cancer cell lines examined, we obtained transduction efficiencies comparable with those reported in glioma cells (23). Hence, we suspect that ovarian cells express molecules having functions similar to HCAR. Furthermore, our RT-PCR analysis found that transcription of Integrin αv in ovarian cancer cells correlated significantly with the efficiency of adenoviral gene transfer (P = 0.009 and P = 0.924; Spearman’s rank correlation; Table 1). These findings suggest that different limiting factors affect adenoviral gene transfer, and that they may be tissue specific. Vanderkwaak et al. (24) reported that incorporation of an integrin-binding RGD motif to the adenovirus fiber knob enhanced the gene transfer efficiency by modified adenovirus vector in the context of ovarian cancer.

We have demonstrated here that recombinant adenovirus expressing wild-type PTEN can significantly inhibit the growth of ovarian cancer cells through two mechanisms, apoptosis and/or cell cycle arrest at the G₁ phase. Moreover, we have shown that transcription of Integrin αv in these cells correlates with the efficiency of gene transfer and with the growth-inhibitory effect of transduced PTEN. Our data suggest that adenovirus-mediated transfer of the PTEN gene may be a potential approach for treatment of patients with ovarian cancer.

References

Fig. 4. Flow cytometric analysis of AdCA or AdCAPTEN-infected ovarian cancer cells. A representative profile from a single experiment is shown for each cell line analyzed, along with the percentages of the cell cycle phase distribution and sub-G₁ population.


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