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 (PI3K) 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 development of adenovirus vector-based 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 PI3KCA 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 pathway involved in cell growth. A growth suppressor gene (PTEN) on chromosome 3q26, which encodes a phosphatidylinositol phosphatase. The PTEN product opposes activation of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, second messengers downstream of PI3K (3–7). Although mutations of the PTEN gene were reported to be rare in ovarian cancers (8, 9), gene transfer may be an effective therapy for ovarian cancers (8, 9), gene transfer indeed can 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 exogeous PTEN on transcription of PTEN, PIK3CA, and receptors for the adenovirus vector. We found that the level of transcription of Integrin α 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 [γ-32P]ATP-labeled internal primers. Signal intensities were measured with a bioimaging 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′-TTT CCG CAG AGG CCT CCC A-3′; reverse (R), 5′-TCC AGT TTT ATT TTC CCA AAG GAT-3′

PIK3CA: F, 5′-GTA GTA TTA TCC GCC ACA TGA TGT-3′; R, 5′-CAC GTA TGG TGG ATT TTA AGA GAA-3′

HCAR: F, 5′-ACA ACT TCA ACT AGC GGA-3′; R, 5′-GAT CAG GAA-3′

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2 The abbreviations used are: PI3K, phosphatidylinositol 3′-kinase; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; MOI, multiplicity of infection.

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TGA TTA CTG CCG ATG-3′; and I, 5′-GAA GTT CAT CAC GAT ATC AG-3′

Integrin αv: F, 5′-GAC TCC TGC TAC CTC TGT G-3′; R, 5′-GCT CTC GCT CCT GTT TCA TC-3′; and I, 5′-TTC AAC CTA GAC GTG GAC AG-3′

Integrin β3: F, 5′-AGG ATT ACC TGG ACA TC-3′; R, 5′-TCA CTA CCA ACA TGA CAC TG-3′; and I, 5′-GTG AAG AAG CAG AGT GTG TC-3′

Integrin β5: F, 5′-GGA GTG CAT TCA GAT GAC AC-3′; R, 5′-TGG TTG GTC GCA GTG TAC-3′; and I, 5′-GGA GTG CAT TCA GAT GAC AC-3′

**β2-microglobulin:** F, 5′-CAC CCC CAG TAA AAG AGA TGA-3′; R, 5′-TAC CTG TGG AGC AAC CTG C-3′; and I, 5′-ATC TTC AAC CTC

**Construction of Recombinant Adenovirus and Conditions of Infection.** To construct AdCAPTEN viruses, the cDNA of PTEN was cloned by RT-PCR using placental mRNA as a template and the following primers: 5′-CTT CAG GCC CCAG GCT CCA-3′ and 5′-TGG TGT TTAT TCC CTT GTG A-3′. A 1.2-kb blunt-ended fragment of PTEN cDNA was inserted into the SmaI site of the cosmid pAxCAwt (TaKaRa; Ref. 13) that contained the CAG promoter (composed of the cytomegalovirus enhancer and chicken β-actin promoter; Ref. 14) and an entire genome of type 5 adenovirus except for the E1 and E3 regions. This procedure generated pAxCAPTEN. Recombinant adenoviruses were constructed by in vitro homologous recombination in the human embryonic kidney cell line 293 using pAxCAPTEN and the adenovirus DNA terminal-protein complex (TaKaRa; Ref. 13). As a control, AdCA viruses were generated from the cosmid pAxCAwt without a transgene. AdCA LacZ viruses encoding the β-galactosidase gene under the control of the CAG promoter were constructed from the control cosmid pAxCAiLacZ (TaKaRa). Viruses were propagated in the 293 cell line and purified by two rounds of CsCl density centrifugation. Viral titers were measured in a limiting-dilution bioassay using AG-3999. Viral titers were measured in a limiting-dilution bioassay using AG-3999. Viral titers were measured in a limiting-dilution bioassay using AG-3999.

**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 × 10^6 cells of each ovarian cancer cell 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...
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 $\beta$-galactosidase transduction assays using AdCA$\beta$-LacZ, which carries the $\beta$-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 $\beta$-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-

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Table 1  PTEN gene transfer to ovarian cancer cells and quantified transcription levels of PTEN, PIK3CA, and adenovirus receptors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Growth inhibition$^a,b$ (%)</th>
<th>Transduction efficiency$^c,d$ (%)</th>
<th>PTEN$^d$</th>
<th>PIK3CA$^d$</th>
<th>Integrin subunit$^d$</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\alpha^b$</td>
</tr>
<tr>
<td>MDAH 2774</td>
<td>87</td>
<td>100</td>
<td>1.8</td>
<td>5.1</td>
<td>2.4</td>
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<td>TYK-nu</td>
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<td>93</td>
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<tr>
<td>SW 626</td>
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<td>100</td>
<td>0.1</td>
<td>1.2</td>
<td>0.7</td>
</tr>
<tr>
<td>NIH:OVCAR-3</td>
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<td>83</td>
<td>1.0</td>
<td>1.9</td>
<td>1.0</td>
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<tr>
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<td>SK-OV-3</td>
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<tr>
<td>Caov-3</td>
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<td>0.3</td>
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<tr>
<td>ES-2</td>
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<td>1.2</td>
<td>2.3</td>
<td>0.4</td>
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<tr>
<td>MCAS</td>
<td>15</td>
<td>9</td>
<td>0.2</td>
<td>0.3</td>
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</table>

$^a$ The growth-inhibitory effects of AdCAPTEN infection were calculated as $\frac{(x - y)}{x}$ ($x$, mean cell number 5 days after AdCA infection at 100 MOI; $y$, mean cell number 5 days after AdCAPTEN infection at 100 MOI).

$^b$ The correlations between transcription levels of Integrin $\alpha v$ and adenoviral transduction efficiencies or growth-inhibitory effects were both statistically significant (Integrin $\alpha v$ versus growth inhibition, $P = 0.009$, $r = 0.924$; Integrin $\alpha v$ versus transduction efficiency, $P = 0.014$, $r = 0.869$; Spearman’s rank correlation).

$^c$ Mean percentages of 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside-positive cells were measured microscopically 24 h after AdCA$\beta$-LacZ infection at 100 MOI.

$^d$ The transcription levels of PTEN, PIK3CA, Integrin $\alpha v$, Integrin $\beta 3$, and Integrin $\beta 5$ were examined using semiquantitative RT-PCR. The intensity of each band was calculated as a ratio against the intensity of the corresponding $\beta 2$-microglobulin band by densitometric analysis.

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Fig. 3. Inhibition of human ovarian cancer cells by AdCAPTEN. Nine cell lines were infected with a PTEN-expressing viral construct (AdCAPTEN) or control virus (AdCA). Mean cell numbers on the 5th day after infection are plotted; bars, SD (three replicates).
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 and β3 and β5 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 these cells correlates with the efficiency of gene transfer and with the degree of growth inhibition by exogenous PTEN (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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