[CANCER RESEARCH 59, 6063–6067, December 15, 1999]

Advances in Brief

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 G_1 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 (*PTEN*) on chromosome 10q23, 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 (5–7). Although mutations of the *PTEN* gene were reported to be rare in ovarian cancers (8, 9), gene transfer may be an effective therapy for 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 G_1 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 G_1 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-^{32}P]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′-CTT CAG CCA CAG GCT CCC A- 3′; reverse (R), 5′-TGG TGT TTT ATC CCT CTT GAT A-3′; and internal (I), 5′-GAC CAA TGG CTA AGT GAA GAT-3′

PIK3CA: F, 5′-GTA TGT CTA TCC GCC ACA TGT-3′; R, 5′-CAG TCA TGG TTG 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

Received 7/28/99; accepted 10/29/99.

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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 CTG 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 CGT CAT TCA GAT GAC AC-3′; R, 5′-TGG TTG GAT GCA GTG TAC TC-3′; and I, 5′-GGA CGT CAT TCA GAT GAC AC-3′

β2-microglobulin: F, 5′-CAC CCC CAC TGA AAA AGA TGA-3′; R, 5′-TAC CTG TGG AGC AAC CTG C-3′; and I, 5′-ATC TTC AAC CTC CAT GAT G-3′

Construction of Recombinant Adenovirus and Conditions of Infection. To construct AdCA*PTEN* viruses, the cDNA of *PTEN* was cloned by RT-PCR using placental mRNA as a template and the following primers: 5′-CTT CAGCCACAGGCTCCCA-3′ and 5′-TGGTGTTTTATCCCTCTTGATA-3′. A 1.2-kb blunt-ended fragment of *PTEN* cDNA was inserted into the *Swa*I 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 pAxCA*PTEN*. Recombinant adenoviruses were constructed by *in vitro* homologous recombination in the human embryonic kidney cell line 293 using pAxCA*PTEN* 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 pAxCAi*LacZ* (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 the 293 cells. Cancer cells were infected by viral solutions to cell monolayers and incubated at 37°C for 1 h with brief agitation every 15 min. Culture medium was added, and the infected cells were returned to the 37°C incubator.

Immunodetection of PTEN Protein. SK-OV-3 cells were infected with AdCA*PTEN* at a MOI of 100 plaque-forming units/cell. After 0, 6, 12, 18, and 24 h, the cells were washed twice with PBS and harvested in lysis buffer [150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1 mM DTT, and 1× Complete Protease Inhibitor Cocktail (Boehringer Mannheim)]. After cells were homogenized and centrifuged at 10,000 × *g* for 30 min, the supernatants were standardized for protein concentration by the Bradford assay (Bio-Rad). Proteins were separated by 8% SDS-PAGE and immunoblotted with goat anti-PTEN and goat anti-actin polyclonal antibodies (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) served as the secondary reagent for the ECL Detection System (Amersham).

Cellular Proliferation Assay. Each line of ovarian cancer cells was seeded at 1×10^4 cells/well in six-well culture plates for 20 h before viral infection. The cells were infected in triplicate with AdCA*PTEN* or AdCA at a MOI of 5, 20, or 100. Triplicate dishes of each treatment were counted by the trypan blue exclusion method using a hemocytometer on the 5th day after infection.

β-Galactosidase Transduction Assay. To assess efficiency of the adenovirus-mediated gene transfer, 1×10^5 cells were plated in triplicate in six-well plates and infected 20 h later with AdCA*LacZ* at 100 MOI for 1 h. Virus-containing solutions were removed at 1 h; cells were washed with PBS, refed with culture medium, and returned to the 37°C incubator. After 24 h, the cells were fixed with 2% paraformaldehyde/PBS, washed, and stained in a 1 mg/ml

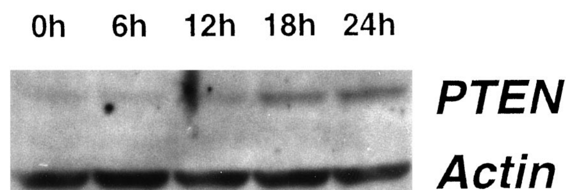

Fig. 1. Chronicity of expression of PTEN protein in transfected ovarian cancer cell line SK-OV-3. Proteins were extracted from the cells at the time points indicated. The levels of PTEN expression were compared with expression of actin.

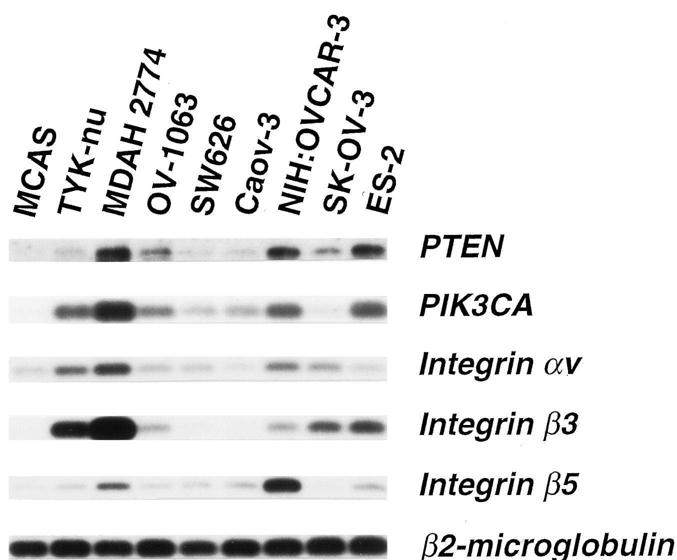

Fig. 2. Semiquantitative RT-PCR analysis of the transcription levels of PTEN, PIK3CA, and Integrin subunits αv, β3, and β5 in all nine cell lines. The integrity of each RNA template was controlled through amplification of β2-microglobulin.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution consisting of 0.1 M sodium phosphate (pH 7.3), 1.3 mM $MgCl_2$, 3 mM $K_3[Fe(CN)_6]$, and 3 mM $K_4[Fe(CN)_6]$ in PBS. β-galactosidase-positive cells in triplicate wells were counted microscopically.

Flow Cytometry. Cells were plated at a density of 5×10^5 cells/100-mm dish and infected 20 h later with 100 MOI of AdCA*PTEN* 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 G_0-G_1, S, and G_2-M phases of the cell cycle and sub-G_1 population were determined from at least 20,000 ungated cells.

Results and Discussion

Expression of recombinant *PTEN*-containing adenovirus (AdCA*PTEN*) 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, 1×10^4 cells of each ovarian cancer lines were infected in triplicate with AdCA*PTEN* 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 AdCA*PTEN* 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

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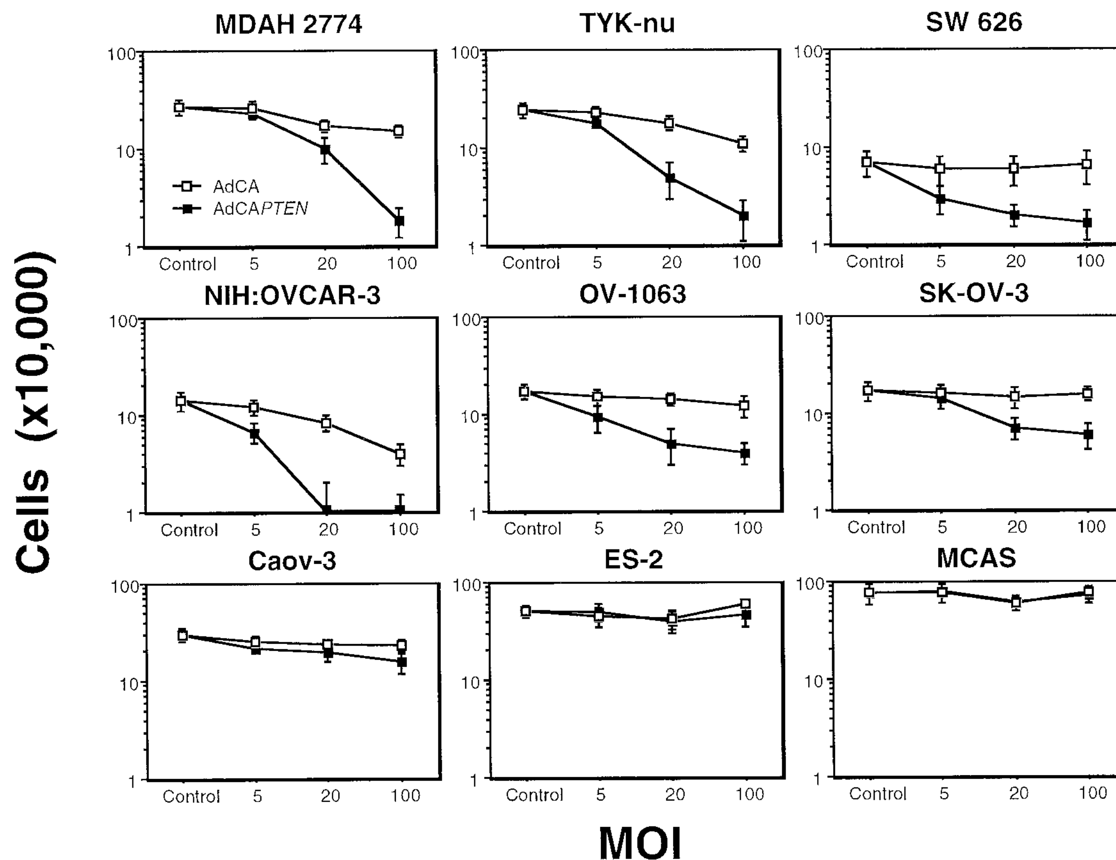

Fig. 3. Inhibition of human ovarian cancer cells by AdCA*PTEN*. Nine cell lines were infected with a PTEN-expressing viral construct (AdCA*PTEN*) or control virus (AdCA). Mean cell numbers on the 5th day after infection are plotted; *bars,* SD (three replicates).

MCAS, had little or no effect by transfection of AdCA*PTEN*. 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 AdCA*LacZ*, 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 AdCA*PTEN* 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-G_1 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-G_1 population and a significant increase in the proportion of G_0-G_1 phase population. OV-1063 showed a moderate increase in the sub-G_1 population. The cell line Caov-3, which showed a little growth suppression and moderate (44%) gene trans-

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

Cell line	Growth inhibition[a,b] (%)	Transduction efficiency[b,c] (%)	*PTEN*[d]	*PIK3CA*[d]	*Integrin subunit*[d] αv[b]	$\beta 3$	$\beta 5$
MDAH 2774	87	100	1.8	5.1	2.4	23.4	1.0
TYK-nu	82	93	0.2	1.5	1.0	3.5	0.1
SW 626	75	100	0.1	1.2	0.7	0.4	0.4
NIH:OVCAR-3	75	83	1.0	1.9	1.0	1.0	1.4
OV-1063	67	70	0.5	1.2	0.5	0.8	0.3
SK-OV-3	62	71	0.3	0.6	0.7	1.9	0.0
Caov-3	32	44	0.1	1.0	0.3	0.2	0.1
ES-2	23	12	1.2	2.3	0.4	2.5	0.4
MCAS	15	9	0.2	0.3	0.3	0.0	0.0

[a] The growth-inhibitory effects of AdCA*PTEN* infection were calculated as $(x - y)/x$ (x, mean cell number 5 days after AdCA infection at 100 MOI; y, mean cell number 5 days after AdCA*PTEN* infection at 100 MOI).

[b] The correlations between transcription levels of *Integrin* αv and adenoviral transduction efficiencies or growth-inhibitory effects were both statistically significant (*Integrin* αv *versus* growth inhibition, $P = 0.009$, $\rho = 0.924$; *Integrin* αv *versus* transduction efficiency, $P = 0.014$, $\rho = 0.869$; Spearman's rank correlation).

[c] Mean percentages of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-positive cells were measured microscopically 24 h after AdCA*LacZ* infection at 100 MOI.

[d] The transcription levels of *PTEN, PIK3CA, Integrin* αv, *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 β_2-*microglobulin* band by densitometric analysis.

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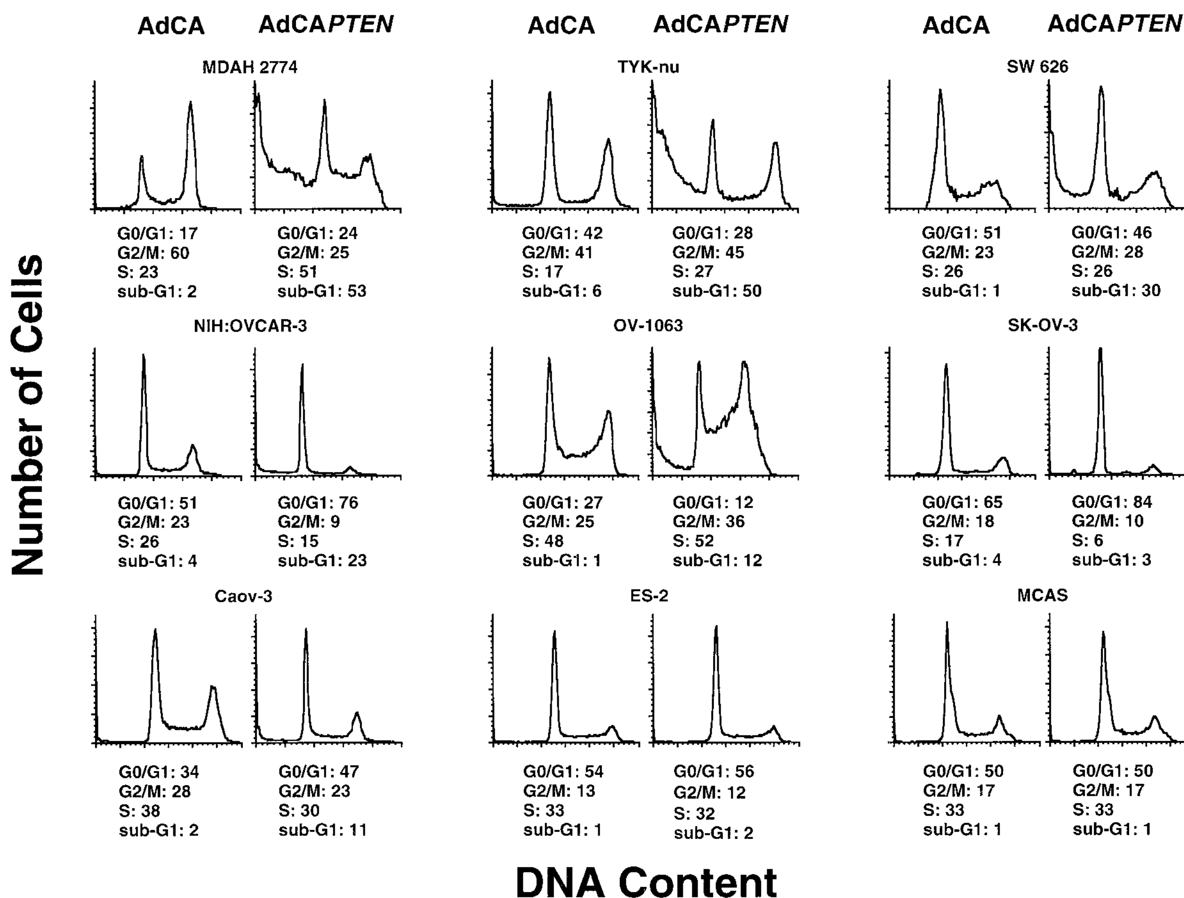

Fig. 4. Flow cytometric analysis of AdCA or AdCA*PTEN*-infected ovarian cancer cells. A representative profile from a single experiment is shown for each cell line analyzed, along with the percentages of the cell cycle phase distribution and sub-G_1 population.

duction efficiency, revealed a moderate increase of sub-G_1 population and a moderate increase in the proportion of G_0-G_1 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 G_1 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 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 ovarian cancer cells correlated significantly with the efficiency of adenoviral gene transfer (P = 0.014; P = 0.869) and also 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 G_1 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.

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

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Cancer Res 1999;59:6063-6067.

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