Adenovirus type 5 E1A gene therapy for ovarian clear cell carcinoma: a potential treatment strategy

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Abstract
Resistance of ovarian clear cell carcinoma (CCC) to platinum-based chemotherapy is associated with poor prognosis, and an effective treatment for advanced disease is urgently needed. HER2/neu is up-regulated more often in CCC than in other histologic types of epithelial ovarian cancer. The purpose of this study was to assess possible treatment for ovarian CCC with the anti-HER2 antibody trastuzumab or human adenovirus type 5 E1A. We treated 10 CCC cell lines with trastuzumab or E1A and assessed cell viability, proliferation, and colony formation and the expression of HER2 and wild-type p53 and assessed cell viability, proliferation, and colony formation and the expression of HER2 and wild-type p53 and poor prognosis than does serous adenocarcinoma of the ovary (2, 3), and lack of responsiveness of CCC to conventional platinum-based chemotherapy is also associated with poor prognosis (4, 5). Currently, the preferred treatment for CCC is complete resection of the tumor, but this is difficult to accomplish when the disease is advanced. Hence, effective novel treatment strategies for advanced CCC are urgently needed (6).

Molecular analyses of various types of ovarian tumors recently showed HER2/neu to be overexpressed in CCC relative to other major histologic types of epithelial ovarian cancer (7). The HER2 proto-oncogene encodes a 185-kDa transmembrane growth factor receptor tyrosine kinase (8). Several types of tumors that overexpress HER2 have shown poor sensitivity to conventional anticancer agents and poor prognosis (9, 10). In ovarian cancer, HER2 protein is overexpressed as a consequence of HER2 gene amplification in 20% to 25% of cases and is a marker of poor prognosis (11, 12).

In this study, we explored two molecules thought to target HER2: trastuzumab (Herceptin; Genentech, South San Francisco, CA) and adenovirus type 5 E1A. Trastuzumab was developed initially from a murine monoclonal antibody (4D5) directed against an extracellular epitope of HER2 protein that reacts with murine and human cells overexpressing HER2 (13). Trastuzumab, the humanized form of 4D5, is a recombinant anti-HER2 monoclonal antibody that has antiproliferative activity against HER2-overexpressing cells (14). Human adenovirus type 5 E1A is an early viral gene that codes for two major proteins by alternative splicing of two exons. The two early viral proteins (243 and 289 amino acids in length) can activate significant (P < 0.05) suppression of proliferation and enhancement of cell death; this effect required stabilization of p53 (but not p73) protein and was associated with the up-regulation of Bax and the cleavage of caspase-9. Other mechanisms, such as p53-independent apoptosis, may also be involved in E1A-mediated cell death in CCC. Finally, treatment with E1A prolonged survival in a CCC xenograft model (P < 0.001). E1A gene therapy, because of its ability to stabilize wild-type p53, is worth exploring as a treatment modality for women with ovarian CCC, which typically expresses wild-type p53. [Mol Cancer Ther 2007;6(1):227–35]
or repress transcription of several viral or cellular genes and thereby regulate the cell cycle (15). The ability of E1A to inhibit HER2 expression was first noted in rodent and human cancer cells (16).

The human HER2 oncogene is overexpressed in many types of cancer in humans and E1A acts as a tumor suppressor by down-regulating HER2 transcription (16). However, E1A has also been shown to reduce tumor growth in cancer cells that do not overexpress HER2 (17–19). The expression of E1A can reduce the anchorage-independent growth and tumorigenicity of several malignant cell lines, including ovarian cancer, sarcoma, and lung cancer (17–19). E1A has shown promising therapeutic effects in phase I trials of breast cancer (20) and head and neck cancer (21). Here, we assessed HER2 expression in 10 CCC cell lines and tested the potential cytotoxicity of trastuzumab and E1A in those cell lines, with the ultimate goal of finding a novel treatment strategy for CCC.

Materials and Methods

Cell Lines and Cell Cultures

The 10 human ovarian CCC cell lines used in this study (RMG-I, RMG-II, KK, KOC-7c, HCH-1, SMOV-2, OVAS, OVISE, OVTOKO, and OVSAYO) were obtained as follows: RMG-I and RMG-II from Professor Shiro Nozawa (Keio University, Tokyo, Japan); KK from Dr. Yoshhiro Kikuchi (National Defense Medical College, Tokorozawa, Japan); KOC-7c from Dr. Toru Sugiyama (Kurume University, Kurume, Japan); HCH-1 from Dr. Takashi Yamada (Osaka Medical College, Takatsuki, Japan); SMOV-2 from Dr. Tomohiro Iida (St. Marianna University, Kawasaki, Japan); and OVAS, OVISE, OVTOKO, and OVSAYO from Dr. Hiroshi Minaguchi (Yokohama City University, Yokohama, Japan). The three human ovarian serous adenocarcinoma cell lines used (2774-C10, SKOV-3, and OVCAR-3) were obtained from the American Type Culture Collection (Manassas, VA). The KK, KOC-7c, HCH-1, SMOV-2, OVAS, OVISE, OVTOKO, OVSAYO, SKOV-3, and OVCAR-3 cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum instead of the primary antibody and used as a negative control.

Western Blot Analysis

Cells were washed thrice with PBS and then lysed in lysis buffer [20 mmol/L Na₂PO₄ (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% aprotinin, 1 mmol/L phenylmethylsulfonylfluoride, 100 mmol/L NaF, and 2 mmol/L Na₂VO₃]. Protein content was determined against a standardized control by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 50 μg of protein were separated by SDS-PAGE on a 10% SDS gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Nonspecific binding on the membrane filter paper was minimized with blocking buffer consisting of 5% nonfat dry milk and 0.1% (v/v) Tween 20 in PBS. The treated filter paper was then incubated, first with the primary antibody and then with the secondary antibody (horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit antibody; 1:5,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). The specific antibodies used were mouse anti–adenovirus type 5 E1A antibody (1:500 dilution; PharMingen, San Diego, CA), mouse anti-human HER2 antibody (1:500 dilution; Oncogene Research Products, San Diego, CA), rabbit anti–phospho-HER2 antibody (1:500 dilution; Upstate Biotechnology, Lake Placid, NY), mouse anti-human p53 antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human Bax antibody (1:200 dilution; Santa Cruz Biotechnology), rabbit anti-human Bcl-2 antibody (1:100 dilution; Santa Cruz Biotechnology), rabbit anti-human caspase-9 antibody (1:500 dilution; Cell Signaling Technology, Beverly, MA), and rabbit antiactin antibody (1:5,000 dilution; Sigma, St. Louis, MO).

Immunohistochemical Staining

For the immunohistochemical studies, 4-μm-thick sections were cut from paraffin blocks and deparaffinized. Sections were then subjected to heat-induced epitope retrieval by immersion in 0.01 mol/L citrate buffer (pH 6.0) preheated to >90°C and heated in an electric vegetable steamer (Black and Decker, Shelton, CT) for 15 min. Endogenous peroxidase activity was blocked by 5 min of incubation with 3% hydrogen peroxide in absolute methanol. The slides were then incubated with the anti-HER2 monoclonal antibody e2-4001 (1:100 dilution) in an autostainer (DAKO, Carpinteria, CA) for 60 min at room temperature. Immunoperoxidase staining was done with the LSAB2 peroxidase kit (DAKO). The antigen-antibody immunoreaction was visualized with 3,3-diaminobenzidine as the chromogen. The slides were counterstained with Mayer’s hematoxylin. For each run, a composite slide consisting of three formalin-fixed human breast carcinoma cell lines representing different levels of HER2 protein expression [MDA-MB-231 (0), MDA-MB-175 (1+), and SK-BR-3 (3+)] was used as the control. In addition, for each case, one slide was incubated with normal rabbit serum instead of the primary antibody and used as a negative control.

Fluorescence In situ Hybridization

Gene amplification was assessed by fluorescence in situ hybridization with the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, IL) according to the manufacturer’s instructions and with reagents, probes, and positive controls provided by the manufacturer. HER2 gene copy number was evaluated by epifluorescence microscopy (Zeiss, Thornwood, NY). The PathVysion kit uses two directly labeled fluorescent DNA probes: LSI HER-2/neu, which is specific for the HER2 gene locus, and CEP17, which is specific for the α satellite DNA sequence at the centromeric region of chromosome 17. The expected ratio

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of LSI HER2 to CEP 17 is 2.0 for normal (unamplified) breast tissue specimens; a ratio >2.0 was considered amplified. Signals were counted in 60 tumor cell nuclei for each cell type.

**Sensitivity to Trastuzumab**

The sensitivity of the cell lines to trastuzumab (Roche, Basel, Switzerland) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were diluted with culture medium supplemented with 1% or 10% FBS to a seeding density of 4 × 10^4/mL, plated on 96-well tissue culture plates at 100 μL/well, and incubated at 37°C overnight. The next day, the cells were incubated with various concentrations (0–100 μg/mL) of trastuzumab. After incubation for 120 h, 10 μL of MTT solution (5 mg/mL) were added to each well, and the plates were incubated for another 4 h. At the end of that incubation, 100 μL of DMSO (Sigma) were added to each well to solubilize the MTT formazan product, and the plates were incubated for an additional 4 h. Absorbance at 570 nm was measured with a Benchmark Microplate Reader (Bio-Rad).

**Plasmids and Colony Formation Assay**

Plasmids used for these experiments were pSV2-neo (vector alone), which encodes the neomycin resistance gene as a selection marker, and pAd5E1A-neo, which encodes both the neomycin resistance gene and cDNA of adenovirus type 5 E1A. All 10 CCC cell lines were transfected in 100-mm plates with 10 μg of pAd5E1A-neo or pSV2-neo. Three weeks after selection with G418 (Sigma), colonies were stained with 0.5% crystal violet (Sigma) in 70% ethanol.

**Adenoviral Vectors**

Recombinant, replication-deficient adenoviral vectors were propagated in HEK293 cells. Viruses were purified by using the Adeno-X Virus Purification Kit (BD Biosciences, Palo Alto, CA), aliquoted, and stored at −80°C. Viral titers were measured with the Adeno-X Rapid Titer Kit (BD Biosciences). Three types of adenoviral vectors were used: the control vector Ad.E1A(−) dl312, which lacks E1A, E1B, and E3; Ad.E1A(+) (ref. 22), an adenovirus type 5 containing E1A but lacking EIB and E3; and Ad.RSVlacZ (23), an adenovirus type 5–based vector lacking E1A, E1B, and E3 but containing the Rous sarcoma virus long terminal repeat as a promoter driving the *Escherichia coli* LacZ gene.

**In vitro Cytotoxicity Assays**

The cytotoxicity of E1A was assessed by trypan blue exclusion in RMG-I, SMOV-2, OVTOKO, and OVSAYO cells. Cells (2 × 10^5) were plated on 35-mm tissue culture dishes, and Ad.E1A(−) or Ad.E1A(+) virus was added in replicates of three. After 0, 24, 48, 72, 96, or 120 h of incubation, the cells were stained with trypan blue (Sigma), and numbers of viable and dead cells were counted under a microscope. The numbers of viral infectious units (80 multiplicity of infection for RMG-I, SMOV-2, and OVTOKO cells and 160 multiplicity of infection for OVSAYO cells) were chosen based on the results of an infection efficiency assay. Briefly, cells were plated in six-well culture plates at 2 × 10^5 to 4 × 10^5 per well; infected with Ad.RSVMlacZ at 5, 10, 20, 40, 80, or 160 multiplicity of infection for 48 h; and then rinsed with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS, washed again with PBS, and incubated with 5-bromo-4-chloro-3-indoly1-β-D-galactopyranoside solution (5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L MgCl2 in PBS) at 37°C overnight. Infection efficiency was calculated as (number of positive cells / number of total cells) × 100.

**DNA Laddering**

The DNA Laddering Kit (Cayman Chemical Company, Ann Arbor, MI) was used to detect apoptosis according to the specifications of the manufacturer. DNA was isolated from both adherent and suspended cells and subjected to electrophoresis on a 1.8% agarose gel in 89 mmol/L Tris-HCl, 89 mmol/L boric acid, 2 mmol/L EDTA, pH 8.0 buffer at 50 V for 2 h. A 100-bp DNA ladder was used as the standard. DNA was visualized by ethidium bromide staining and photographed under UV illumination.

**Annexin V Staining**

The Annexin V-FITC Fluorescence Microscopy Kit I (BD Biosciences PharMingen) was used to assess apoptosis in terms of the externalization of phosphatidylserine residues according to the specifications of the manufacturer. Briefly, cells were washed twice with cold PBS and once with 1× Annexin V binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl2]. Then, the cells were stained with Annexin V-FITC diluted 1:10 in 1× Annexin V binding buffer for 15 min at room temperature. Finally, the cells were washed with 1× Annexin V binding buffer; 500 μL of 1× Annexin V binding buffer were added to each well; and the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Small Interfering RNA**

Cells were seeded in six-well culture plates at 2.5 × 10^5 per well (30–50% confluence) in DMEM/F12 medium supplemented with 10% FBS. The next day, cells were transfected with small interfering RNA (siRNA) against p53 (Cell Signaling Technology) or a control siRNA (Santa Cruz Biotechnology) at a final siRNA concentration of 100 nmol/L by using Lipofectamine 2000 (Invitrogen, Palo Alto, CA).

**E1A Gene Therapy in an Ovarian Cancer Xenograft Model**

For these experiments, RMG-I cells in log-phase growth were trypsinized, washed twice with PBS, and centrifuged at 250 × g. Viable cells were counted and then 1 × 10^7 viable cells (in 0.5 mL of PBS) were injected under aseptic conditions into the peritoneal cavities of female severe combined immunodeficient mice. Mice were then randomly divided into three groups (10 mice per group) and treatment was started 4 days later as follows. Group 1 was given i.p. PBS weekly; group 2, i.p. Ad.E1A(−) weekly (500 μL per injection); and group 3, i.p. Ad.E1A(+) weekly (500 μL per injection) for 5 weeks. Tumors were collected...
on day 28, fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries, Osaka, Japan), and embedded in paraffin for immunohistochemical analysis. Paraffin blocks were sliced in 4-μm sections and deparaffinized. The expression of E1A protein and p53 in the tumor tissue sections was detected with the Histofine Simple Stain PO kit (Nichirei, Tokyo, Japan). Slides were counterstained with hematoxylin. The primary antibodies used were anti-E1A (dilution 1:40; Oncogene Research Products) and anti-p53 (dilution 1:50; DAKO).

**Statistical Analysis**

Statistical analyses were done with the JMP Version 5J program (SAS Institute, Inc., Cary, NC). Data are presented as mean ± SD. Means for all data were compared by one-way ANOVA with post hoc testing, and the significance of apparent differences in survival distribution between groups was tested with log-rank tests. P < 0.05 was considered statistically significant.

**Results**

**HER2 Expression in CCC Cell Lines**

Levels of HER2 and phosphorylated HER2 protein expression were examined in all cell lines by Western blotting and immunohistochemical analyses (Fig. 1). HER2 protein was detected, at various levels, in all 10 CCC cell lines by Western blotting and in eight of those cell lines by immunohistochemical staining. All 10 CCC cell lines expressed substantial amounts of phosphorylated HER2 protein as compared with the three serous adenocarcinoma cell lines (2774-C10, SKOV-3, and OVCAR-3). In the immunohistochemical staining, only RMG-I cells showed 3+ staining for HER2 protein and four other CCC cell lines showed 2+ (weakly positive) staining (Fig. 1). Gene amplification was detected by fluorescence in situ hybridization in only 1 of 10 cell lines, RMG-I, which also had shown 3+ staining on immunohistochemical analysis.

**Effects of Trastuzumab on CCC Proliferation**

In breast cancer, HER2 gene amplification is the best predictor of response to trastuzumab (14, 24). However, whether this is also true for CCC has not been addressed. Thus, we examined the effects of trastuzumab on the proliferation of four CCC cell lines (RMG-I, SMOV-2, OVTOKO, and OVSAYO) that expressed a range of HER2 protein levels. Trastuzumab did not inhibit proliferation of any of these cell lines, regardless of whether the cultures had been supplemented with 10% FBS or 1% FBS (data not shown). Moreover, expression of phosphorylated Akt protein (pAkt), which is regulated by HER2 signaling (25), by those cell lines was not affected by treatment with 100 μg/mL trastuzumab (data not shown).

**Effects of E1A on CCC Colony Formation**

We next examined the cytotoxicity of E1A in CCC cell lines by measuring the ability of 10 CCC cell lines that had been transfected with pAd5E1A-neo or pSV2-neo (control), after which cells were selected with medium containing the neomycin analogue G418, stained with crystal violet, and the numbers of colonies counted. Transfection with the E1A construct suppressed colony formation in all 10 cell types tested.

**Effects of E1A on CCC Proliferation and Cell Death**

Next, to test whether infection with Ad.E1A(+) would suppress cell growth in vitro, we infected four CCC cell lines expressing different amounts of HER2 with a control construct, with Ad.E1A(−), or with Ad.E1A(+) (>90% of cells can be infected at 80 multiplicity of infection for RMG-I, SMOV-2, and OVTOKO cells and at 160 multiplicity of infection for OVSAYO cells) and counted the numbers of...
live and dead cells daily for 5 consecutive days. Cell proliferation was significantly suppressed in the Ad.E1A(+) infected cells compared with either the control or the Ad.E1A(−) infected cells, and cell death rate was correspondingly higher in the Ad.E1A(+) treated group (Fig. 3A). DNA fragmentation was observed only in the Ad.E1A(+) infected cells (Fig. 3B). Expression of HER2 and pAkt, determined by Western blotting, showed that HER2 expression had been down-regulated 48 h after Ad.E1A(+) infection in those four cell lines but pAkt expression did not change (data not shown), suggesting that E1A operates independently of HER2.

Stabilization of p53 by E1A Activates an Apoptotic Pathway

Evidence that CCC rarely expresses mutated p53 (26, 27) and that E1A can stabilize the p53 protein (28) led us to hypothesize that E1A-induced apoptosis depends on p53 expression. To test this hypothesis, we used siRNA to p53 to block the expression of wild-type p53 protein and examined E1A-induced cell death in RMG-I and SMOV-2 cells. We first confirmed that these two CCC cell lines express endogenous wild-type p53 (data not shown). Next, after confirming that Ad.E1A(+) was more cytotoxic than Ad.E1A(−) in RMG-I cells (P < 0.05), we treated the cells with both Ad.E1A(+) and siRNA to p53 and found that this combination partially reduced the cytotoxic effect of E1A in RMG-I cells compared with the other treatment conditions. Points, mean from four dishes; bars, SD. B, SMOV-2 cells were collected 36 h after treatment with the indicated combinations of constructs and tested for the expression of p53, Bax, Bcl-2, and caspase-9 by Western blotting. Cells in which p53 had been knocked down did not show up-regulation of Bax or cleavage of caspase-9.

Figure 3. Cytotoxicity of Ad.E1A(+) in CCC cells. A, SMOV-2 cells were treated with PBS (control), Ad.E1A(−), or Ad.E1A(+) stained with trypan blue at the indicated times; and numbers of live and dead cells were counted. Cell proliferation was significantly suppressed by Ad.E1A(+) compared with either control or Ad.E1A(−). Inset, results from Western blot analysis confirming the expression of the E1A protein. B, DNA fragmentation was observed 48 h after Ad.E1A(+) infection in SMOV-2 and RMG-I cells. M, 100-bp DNA ladder as standard; lane 1, no treatment; lane 2, Ad.E1A(−); lane 3, Ad.E1A(+).

Figure 4. E1A-induced apoptosis and stabilization of p53 protein in CCC cells. A, cells were untreated (control) or treated with siRNA to p53 (si-p53) or a control (si-c), followed by Ad.E1A(−) or Ad.E1A(+). Cells were then stained with trypan blue and counted, and apoptosis was determined by Annexin V-FITC staining. The combination of E1A and siRNA to p53 reduced the cytotoxic effect of E1A in RMG-I cells compared with the other treatment conditions. Points, mean from four dishes; bars, SD. B, SMOV-2 cells were collected 36 h after treatment with the indicated combinations of constructs and tested for the expression of p53, Bax, Bcl-2, and caspase-9 by Western blotting. Cells in which p53 had been knocked down did not show up-regulation of Bax or cleavage of caspase-9.
cytotoxicity affected by knocking down p73 with siRNA to p73 (data not shown) in RMG-I and SMOV-2 cells, suggesting that p73 is not involved in the E1A-induced apoptosis of CCC.

Finally, to determine whether the cytotoxic effect of E1A was attributable to an increase in p53 protein, we examined the expression of p53, Bax, Bcl-2, and caspase-9 proteins after Ad.E1A(+) treatment in SMOV-2 cells. At 36 h after infection with Ad.E1A(+), the expression of p53 and Bax proteins was up-regulated and cleaved caspase-9 was seen in the Ad.E1A(+) -treated cells. However, cells in which p53 had been knocked down showed reduced p53 expression and no up-regulation of Bax or cleavage of caspase-9 (Fig. 4B). Similar results were obtained with RMG-I cells (data not shown). These findings suggest that E1A-induced apoptosis in CCC cells may require stabilization of p53 protein.

E1A Gene Therapy Prolongs Survival in Mice with CCC Cells

After confirming that E1A reduced cell viability and enhanced apoptosis in vitro, we examined the effect of E1A gene therapy on survival in a xenograft model of CCC. Female severe combined immunodeficient mice were given i.p. injections of RMG-I cells and then treated with PBS, Ad.E1A(-), or Ad.E1A(+). Immunohistochemical analysis of tumor tissues was used to verify that the tumor cells were being transfected by the Ad.E1A(+) and to confirm the stability of p53 in those cells. As expected, E1A and p53 proteins were expressed only in tumors from mice treated with Ad.E1A(+) (Fig. 5A, b and d). The mice treated with Ad.E1A(+) survived significantly longer than those treated with either PBS or Ad.E1A(-); median survival times were 76 days for the PBS group, 84 days for the Ad.E1A(-) group, and 218 days for the Ad.E1A(+) group (P < 0.001). These findings indicate that the E1A gene can, through the stabilization of p53 protein, induce apoptosis of RMG-I cells, resulting in prolonged survival of severe combined immunodeficient mice bearing RMG-I cells.

Discussion

In this exploration of the cytotoxicity of trastuzumab or E1A in CCC cells with known levels of HER2 protein expression, we found that trastuzumab was not cytotoxic and that E1A induced apoptosis independent of HER2 expression levels. We previously found that E1A gene therapy is not effective in two of four low-HER2-expressing ovarian serous adenocarcinoma cell lines (30). These findings suggest that E1A inhibits cell growth in these cells by a mechanism independent of HER2 suppression.

The gene expression profile of CCC cells, particularly the up-regulation of HER2, can distinguish CCC from other types of epithelial ovarian cancer (7). In one study (31), rates of HER2 overexpression were higher among patients with CCC (43%) than among patients with serous adenocarcinoma (29%). Our findings from Western blot analysis and immunohistochemical staining support other evidence that up-regulation of HER2 protein is more common than HER2 DNA amplification in CCC cell lines and, by extension, clinical evidence that trastuzumab is not effective in CCC. Lack of DNA amplification of HER2 in patients with breast cancer has been shown to correlate with reduced tumor response rate (14). In our study, only one cell line showed HER2 DNA amplification (RMG-I), and trastuzumab had no cytotoxic effect in that cell line (or, indeed, in any other cell line). Trastuzumab inhibits cell growth by inhibiting Akt kinase activity through the down-regulation of the HER2 receptor, leading to apoptosis (32); however, expression of pAkt protein was not affected by trastuzumab in CCC cells in this study, suggesting that HER2 may not be necessarily an important therapeutic target in CCC.
E1A is known to be cytotoxic in HER2-overexpressing cells, and that cytotoxic effect is thought to result, in part, from down-regulation of HER2 (16). Interestingly, we found here that E1A suppressed colony formation in all 10 CCC cell lines tested. Moreover, E1A inhibited proliferation and induced cell death in these CCC cells without down-regulating pAkt expression. These results also support the concept that E1A-induced cytotoxicity does not depend on down-regulation of the HER2 receptor.

E1A has been reported to suppress tumor growth by inducing apoptosis through a variety of mechanisms, including down-regulation of HER2 (16) and induction of p19ARF, which leads to accumulation and stabilization of p53 and subsequent apoptosis (28). E1A can also induce apoptosis through p53-independent mechanisms (29). Studies of murine melanoma cells have shown that the CR2 (pRb-binding) domain is required for E1A-mediated apoptosis (33); E1A-mediated apoptosis also involves polyubiquitination and degradation of topoisomerase II (34) by E2 enzyme activity (35). E1A sensitizes cells to many different stimuli, including serum starvation (36), ionizing radiation (37), DNA-damaging agents (38), tumor necrosis factor (39), and tumor necrosis factor–related apoptosis-inducing ligand (40), any of which can lead to apoptosis. E1A also induces the expression of p21, a cyclin-dependent kinase inhibitor (41), and induces apoptosis by binding to p21 and restoring cyclin-dependent kinase-2 activity in DNA-damaged cells (42). Finally, in head and neck squamous cell lines, E1A has been shown to down-regulate the expression of the epidermal growth factor receptor (43).

Several analyses have been reported of genetic and molecular events in the development of ovarian cancer (7, 44). Changes in the p53 gene are seen in ~50% to 70% of cases of advanced serous adenocarcinoma (45, 46). In contrast, p53 mutation is rare in CCC (0–10%; refs. 26, 27), and CCC tends to express little or no p53 protein on immunohistochemical staining (27). However, these results could reflect the short half-life of wild-type p53; indeed, overexpression of p53 protein usually occurs in tandem with a mutation of the p53 gene that leads to expression of an abnormal and stabilized protein (47). We found by Western blotting that all 10 CCC cell lines showed weak expression of p53 protein, which we determined to be of wild-type sequence (data not shown). Similarly, sequencing of p53 in RMG-I and SMOV-2 cells also showed the wild-type sequence (data not shown). Thus, we chose to focus our study on the p53-dependent apoptotic pathway induced by E1A.

One of the most important links between p53-mediated transactivation and apoptosis comes from the ability of p53 to control transcription of proapoptotic members of the Bcl-2 family such as Bax (48). An increase in Bax protein results in the release of cytochrome c from mitochondria, which leads to caspase-9 cleavage and activation of executioner caspases (49). In this study, E1A seemed to induce apoptosis by up-regulating p53 and Bax proteins. Moreover, silencing p53 through transfection with siRNA to p53 partially restored E1A-induced apoptosis by down-regulating p53, which had been up-regulated after Ad.E1A(+) treatment, thereby inhibiting the up-regulation of Bax and the cleavage of caspase-9.

E1A is known to override the cellular control of the Rb-E2F interaction by binding hypophosphorylated Rb and freeing E2F. E2F signaling indirectly to p53 facilitates caspase activation and simultaneously produces increases in caspase expression (50). This p53-independent pathway may partially contribute to E1A-induced apoptosis in CCC cells. These findings suggest that the p53-Bax pathway probably has a key role in the induction of apoptosis by E1A in these cells.

In 1997, Kaghad et al. (51) reported identifying p73, which maps to the short arm of chromosome 1; they further found that a p73 protein encoded by this gene shares 63% homology with the DNA-binding region of p53 as well as 38% identity with the tetramerization domain and 29% identity with the transactivation domains. p73 has been reported to be required for p53-dependent apoptosis in response to DNA damage through the induction of PERP, bax, and NOXA (52); p73 has also been shown to be involved in p53-independent apoptosis induced by E2F1, c-Myc, and E1A (29). In this study, however, p73 protein expression was not up-regulated by infection with Ad.E1A(+), nor was E1A-induced cytotoxicity affected by knocking down p73 with siRNA. These findings suggest that p73 is not necessary for E1A-induced apoptosis in CCC cells. Whether other mechanisms, such as p53-independent apoptosis, are also involved in E1A-mediated cell death in CCC is not fully understood, but this question is being addressed in the authors’ laboratories.

Currently, approaches to gene therapy tested in clinic for ovarian cancer include adenovirus vectors carrying wild-type p53 (Adp53), ONIX-015, herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV), and E1A (53). Adp53 and ONIX-015 are given to patients with ovarian cancers harboring p53 mutations; therefore, these approaches may not be suitable strategies for the treatment of CCC, which usually has wild-type p53 (26, 27). At present study, we chose E1A because CCC has wild-type p53 and HER2 overexpression, although suicide gene therapy, such as HSV-tk/GCV, might be effective against CCC.

Ovarian cancer is regarded as a particularly good candidate for gene transfer using viral vectors because the disease generally remains confined to the abdominal cavity throughout its course. The doses of adenovirus vectors range from $1 \times 10^9$ to $3 \times 10^{12}$ viral particles, and no dose-limiting toxicity has been observed (54). Adeno-virus-mediated gene transfer depends on adequate virus uptake and thus on the presence of coxsackie-adenovirus receptor (CAR) on tumors. Adenoviral infection is mediated by binding to CAR on cell surface. Zeimer et al. (55) reported that expression of CAR was observed in 36 of 37 ovarian carcinomas by immunohistochemistry. Therefore, adenovirus gene therapy may be useful for ovarian cancer.
The presence of adenovirus-neutralizing antibody, whether the rapid development of antibody against vectors or preimmunized, in the ascitic fluid of patients with ovarian cancer may reduce gene transfer to targeting tumor cells with adenoviral vectors. One of the promising strategy to modulate immune response to the vector has been developed using the adenovirus vector that is covalently coated with a multivalent hydrophilic polymer poly[N-(2-hydroxypropyl)methacrylamide] (ref. 56). By coating all the structural viral antigens, the initial neutralizing responses should be delayed. However, the generation of more efficient vectors to improve gene transfer into the cancer cells by reconsidering alternative types of viral and nonviral transfection systems, such as liposomeseems to be mandatory.

In summary, we found that E1A had marked cytotoxic effects on CCC cells, even on cell types known to be resistant to conventional platinum-based chemotherapy. We also found that p53 status probably affected the efficient induction of apoptosis by E1A, although CCCs rarely have mutations in p53 (26, 27). Therefore, we conclude that E1A gene therapy, through its ability to stabilize p53 protein, is worth exploring as a treatment modality for CCC. Currently, a phase II clinical trial of E1A gene therapy in combination with paclitaxel is under way at M.D. Anderson Cancer Center. In this trial, E1A is given by i.p. injection to patients with ovarian cancer; the patient group includes both those with HER2-overexpressing tumors and those with low-HER2-expressing tumors.

References


Molecular Cancer Therapeutics

Adenovirus type 5 E1A gene therapy for ovarian clear cell carcinoma: a potential treatment strategy

Hiroaki Itamochi, Junzo Kigawa, Yasunobu Kanamori, et al.


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