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Adenovirus-Mediated Antisense Telomerase with Cisplatin Increased the Susceptibility of Cisplatin Resistant Ovarian Cancer Cell Line

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Abstract Telomerase adds telomeric repeats to chromosomal ends and is known to play an important role in carcinogenesis through cellular immortalization. Since telomerase is an essential pathogenomic factor in malignant tumors, inhibiting telomerase activity is thought to be possible to make telomerase positive tumors more sensitive to cisplatin treatment, which is effective in ovarian cancers, but clinical success is limited by chemo-resistance. In the present study, cisplatin-sensitive ovarian cancer cell line A2780 and cisplatin-resistant A2780/cp70 cell line were infected with antisense telomerase adenovirus Ad-OA. It was found that the Ad-OA suppressed ovarian cancer cell growth and this effect was mainly due to the induction of caspase-dependent apoptosis. Next, we infected the cisplatin resistant ovarian cancer cell line A2780/cp70 with Ad-OA and cisplatin concurrently. Interestingly, cisplatin treatment with Ad-OA was more effective to cisplatin-induced cell death in A2780/cp70 cells compared to cisplatin or the vector group only. These data suggest that cisplatin treatment with Ad-OA may be a new chemo-sensitizer for cisplatin resistant ovarian cancer.

Key words: Telomerase, ovarian cancer, adenovirus, cisplatin, targeted cancer gene therapy

Ovarian cancer accounts for only 4% of cancers in women, but it is the leading cause of death from gynecological malignancies in Western countries [2]. Since ovarian cancer tends to be asymptomatic until it is well advanced, it is difficult to diagnose in early stages. Furthermore, from the therapeutic point of view, surgical intervention has limited effect on long-term survival. The platinum-based drugs, such as cisplatin and carboplatin, play a major role in the chemotherapy of ovarian cancer [1]. The cytotoxicity of cisplatin is thought to be due to the formation of intra-strand and inter-strand cross-links in the DNA, which may induce cell cycle arrest and apoptosis [7]. The recent progress of chemotherapeutic agents in the field of chemotherapy leads to the improvement in survival rates, but perfect treatment of ovarian cancer remains limited because of chemo-resistance. Therefore, it is necessary to contrive a new approach to render tumors more sensitive to chemotherapeutic agents.

Telomerase is a ribonucleoprotein that functions to add telomeric repeats to the chromosomal ends, and to protect DNA from some kinds of genomic instability. Telomerase is highly active in about 70–95% of malignant tumors, such as lung cancers [11], colorectal and gastric cancers [6], hepatocellular carcinomas [20], hematologic malignancies [9], giant cell tumors of bone [22], prostate cancers [23], breast cancers [10], and ovarian carcinomas [8]. Theoretically, inhibition of telomerase activity may induce apoptosis in malignant tumors and make them more sensitive to DNA-damaging chemotherapeutic agents. Recently, an antisense sequence expressing adenovirus Ad-OA against the open part of hTR was constructed, and it showed cytotoxic effect on lung cancer [24]. In this study, the relationship between inhibition of telomerase and sensitivity to cisplatin in an ovarian cancer cell line was investigated.

Materials and Methods

Recombinant Adenovirus Vector

The recombinant adenovirus (Ad) vector Ad-OA is a E1a/E1b-deficient vector [24]. It contains residues 94 and 76 of the telomerase template sequence driven by the

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Table 1. Sequences of oligonucleotides complementary to human telomerase RNA.

Names	Sequences
OA	5'-**gatcc**cgcgcggggagcaaaagcacg**a**-3' 3'-**ggcgcgcccctcgttttcgtgc**ttcga-5'
M6	5'-**gatcc**cgc**C**cg**C**gg**T**gc**T**aa**T**gc**T**cg**a**-3' 3'-**ggcg**G**gc**G**cc**A**cg**A**tt**A**cg**A**g**cttcga**-5'

*Each oligomer, which has a *Bam*HI and *Hin*dIII site at the 5'-end and 3'-end, respectively, is in boldface; underlined capitals indicate mismatched nucleotides.

cytomegalovirus (CMV) immediate promoter, inserted into the E1 region. The vector was amplified and titrated in a 293 cell line. The negative control vector Ad-M6 contains six mismatched nucleotides in the antisense cassette, which would reduce or prevent binding with the telomerase RNA (Table 1). The other negative control vector (Ad5.CMV-Null; Ad-Null) was purchased from Quantum-Appligene (Quantum-Appligene, U.S.A.). Ad-Null is an empty vector, which contains no coding sequences between the CMV promoter and PA (polyadenylation site). The infection efficiency was determined by the method described previously [21].

Cell Culture

A2780 (cisplatin-sensitive ovarian carcinoma cell line) and A2780/cp70 (cisplatin resistant ovarian carcinoma cell line) were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, U.S.A.) supplemented with 10% FBS (HyClone, Logan, U.S.A.), penicillin (50 units/ml), and streptomycin (50 μg/ml) in the presence of 5% CO_2. All cell lines were kindly provided from Dr. Robert Brown, Glasgow University, U.K.

Telomerase Activity Assay (TRAP assay)

Telomerase activity was examined by a telomeric repeat amplification protocol (TRAP) assay with 2,000 cells using a Telosay Kit in accordance with the manufacturer's instructions (Intron, Korea). This method was based on an original method described by Kim *et al.* [14]. The PCR products were electrophoresed in a 10% nondenaturing PAGE in TBE buffer and stained with the Silver Staining System (Promega, U.S.A.). For RNase treatment, the extract was incubated with RNase A for 30 min at 25°C.

RT-PCR

The total RNA was isolated from the A2780 and A2780/cp70 cells using an RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol and treated with DNase I to avoid any genomic DNA contamination. The cDNA was then synthesized from 1 μg of RNA using a SuperScript II reverse-transcriptase (GIBCO BRL, Germany) with the Oligo$(dT)_{15}$ primer (Promega, U.S.A.). For amplification, 2 μl aliquots of the cDNA were subjected to 30 cycles of a PCR (at 94°C for 1 min, 55°C for 1 min, and 72°C for 90 sec) with an hTR specific primer set (5'-TTTGTCTAACCCTAACTGAGAAGG-3' as forward and 5'-TGTGAGCCGAGTCCTGGGTGCACG-3' as backward), which gave rise to 400 bp. The efficiency of the cDNA synthesis from each sample was estimated using a PCR with GAPDH-specific primers (5'-TTCGTCATGGGTGT-GAACCA-3' as forward and 5'-TGGCAGGTTTTTCTA-GACGG-3' as backward) producing a 350 bp DNA segment.

Caspase Inhibition Assay

Cells were seeded at 5×10^3 cells/well (0.1 ml) in 96-well flat-bottomed micro titer plates and incubated overnight at 37°C. The cells were pre-incubated for 1 h with either cell-permeable YVAD-CHO [caspase-1 (ICE) inhibitor] or cell-permeable DEVD-CHO, [Caspase-3 (CPP32) inhibitor (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, U.S.A.)] solublized in DMSO at the concentration of 50 μM of each inhibitor. After washing the cells with PBS, the cells were treated with 100 multiplicity of infection (MOI) of Ad-M6 or Ad-OA. After 6 days, the cell viability was determined using a trypan blue dye exclusion assay.

Drug Sensitivity Assay

Cisplatin (cis-$[PtCl_2(NH_3)_2]$) was purchased from Sigma (St. Louis, U.S.A.) and 1 mM solution prepared in 0.9% saline. Cytotoxicity was assessed using the MTT colorimetric assay. Briefly, 10^4 cells were seeded onto each well of 96-well plates (Falcon, Becton-Dickinson, Cowley, U.K.) and allowed to attach overnight. Serial dilutions of platinum drugs were added for 24 h, and IC_{50} values were determined as the drug concentration that reduced the cell density to 50% of that in untreated control wells.

RESULTS

Telomerase Activity and hTR Expression in Experimental Cell Lines

To ascertain whether A2780 and A2780/cp70 cell lines had telomerase activity, a TRAP assay was performed. As shown in Fig. 1, telomerase activity was clearly shown in both A2780 and A2780/cp70 cell lines, but not in RNase A treated A2780 cells. To confirm the presence of the target molecule hTR, the RT-PCR method was performed in the experimental cell lines. As shown in Fig. 1, both cell lines had hTR mRNA.

Inhibition of Ovarian Cancer Cell Growth by Ad-OA *In Vitro*

To determine whether overexpression of antisense telomerase affected ovarian cancer cell growth, A2780 cells were infected with Ad-OA, Ad-M6, or Ad-Null at different MOI ranging from 0 to 100 (Fig. 2). Growth of Ad-OA-infected

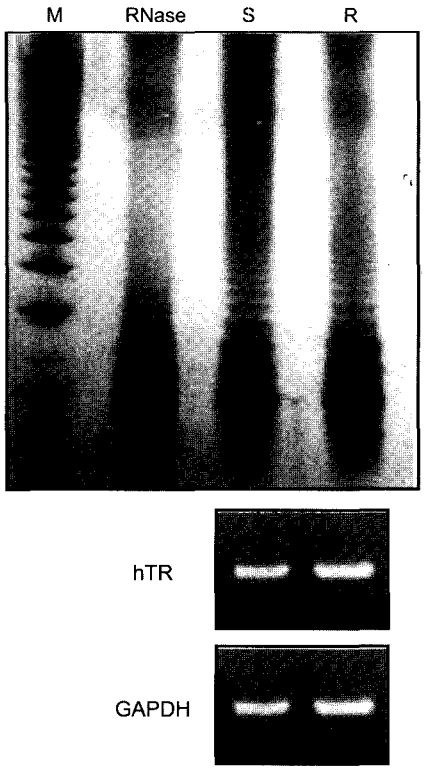

Fig. 1. Telomerase activity of A2780 and A2780/cp70.
Upper panel: The telomerase activity was assayed by a telomeric repeat amplification protocol (TRAP) assay using 2,000 cells. For RNase treatment, an extract was incubated with RNase A for 30 min at 25°C. M: 100 bp marker. RNase: A2780 extract treated with RNase A. S: A2780. R: A2780/cp70. Lower panel: The total RNA was isolated and used for a RT-PCR in the presence of hTR and GAPDH-specific primers. The PCR products were visualized after 1.5% agarose gel electrophoresis and ethidium bromide staining.

A2780 cells were significantly suppressed during 6 days and morphological changes were observed with cells rounding up at day 4 (data not shown). Cell growth was inhibited up to 70% at a MOI of 100, whereas inhibition by Ad-M6 was similar to that caused by Ad-Null (only 5–10% at a MOI of 100) (Fig. 2). These findings indicate that overexpression of antisense telomerase sequences inhibits ovarian cancer cell proliferation.

Caspase Inhibition Assay

After treatment with caspase inhibitors (ICE or CPP32 inhibitors), A2780 cells were infected with Ad-OA or Ad-M6 at a MOI of 100. Since DMSO was used for the solvent of caspase inhibitors, the same amount of DMSO was added without caspase inhibitors for the control. After 6 days of treatment, the viability of Ad-OA-infected A2780 cells reduced to about 35% compared to Ad-M6-infected cells (Fig. 3). Although Ad-OA was slightly inhibited by DMSO, incubation with ICE and CPP32 inhibitors resulted in the recovery of cell viability to about 85% and 75%, respectively. This experiment showed that A2780 cells

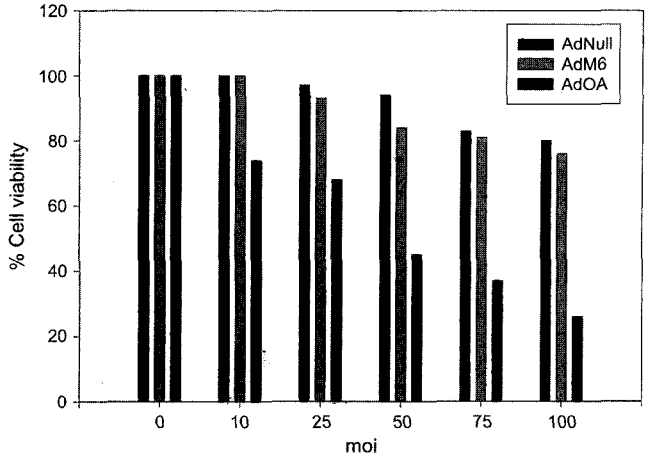

Fig. 2. Effect of MOI on cell growth by cell count assay.
A2780 cells were infected with Ad-Null, Ad-M6, or Ad-OA at the indicated MOI. Triplicate dishes of each treatment were counted relative to the mock-infected cells, 6 days after infection. The cell viability was determined by trypan blue exclusion.

infected with Ad-OA might have undergone cell death by caspase-dependent apoptosis.

Effect of Ad-OA on Cisplatin-Induced Cell Death

Using an MTT assay, the IC_{50} of cisplatin for A2780 and A2780/cp70 was calculated to be 8 and 32 μM, respectively, indicating the A2780/cp70 cells were 4 times more resistant to cisplatin than A2780 cells. To determine the effect of Ad-OA on cisplatin-induced cell death, we infected Ad-OA with cisplatin to A2780/cp70 (Fig. 4). First of all, to determine the best effective order of medication of Ad-OA and cisplatin, A2780/cp70 cells were infected with Ad-OA, followed by treatment with 32 μM cisplatin 2 days later (Lane 4). Second, A2780/cp70 cells were treated first with cisplatin, then infected with Ad-OA

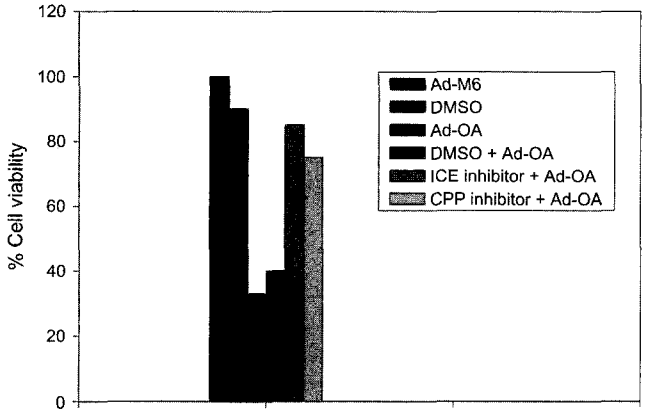

Fig. 3. Caspase activation by Ad-OA.
Cytotoxic effect of Ad-OA with or without caspase inhibitors (ICE or CPP32 inhibitor) was determined by trypan blue exclusion. A2780 cells were treated for 4 days. Results are the mean SD of three independent experiments in triplicate cultures.

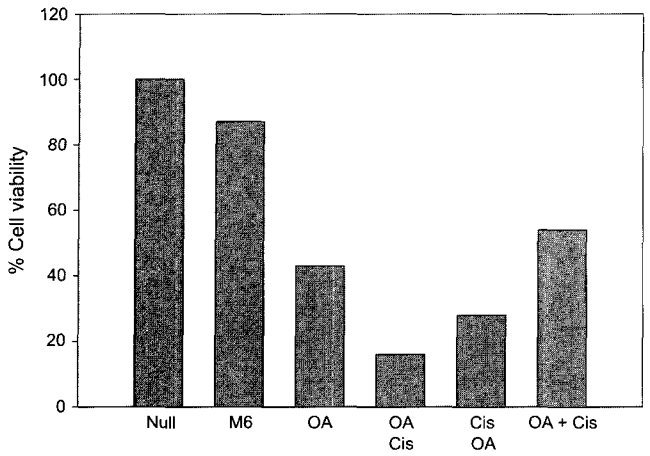

Fig. 4. Effect of Ad-OA with cisplatin on cisplatin-induced cell death of cisplatin resistant A2780/cp70 cells.
A2780/cp70 cells were infected with Ad-OA at a MOI of 100, and then treated with 32 μM cisplatin. Results are the mean±SD of three independent experiments in triplicate cultures. Lane 1: Ad-Null infection; Lane 2: Ad-M6 infection; Lane 3: Ad-OA infection; Lane 4: Ad-OA infection, then cisplatin treatment; Lane 5: cisplatin treatment, then Ad-OA infection; Lane 6: concurrent treatment of Ad-OA infection and cisplatin.

2 days later (Lane 5). Lastly, the cells were treated with Ad-OA together with cisplatin (Lane 6). The result showed that A2780/cp70 cells were most effectively killed if the cells were infected first with Ad-OA followed by cisplatin 2 days after the infection, about 80% of tumor cells being inhibited by Ad-OA and cisplatin, compared to Ad-Null-infected control (Fig. 4).

DISCUSSION

Although telomerase activity is undetectable in most human somatic tissues, it is present in about 70–95% of the malignant tumors, including ovarian cancer. As telomeres play a role in protecting DNA, it is possible that if telomerase activity in cancer cells is inhibited, they might be more susceptible to DNA-damaging agents, like cisplatin. Therefore, inhibition of telomerase activity is thought to be a very strong candidate for targeted cancer gene therapy. Up to the present, there have been several attempts to inhibit telomerase activity; for example, inhibitors of retroviral reverse transcriptase [25], peptide nucleic acid [19], cisplatin [5], hammerhead ribozyme [13], hTR antisense RNA [3], and hTR gene deletion [4]. Ever since the first attempt to inhibit gene expression by an antisense sequence [27], there have been numerous studies on the relantionship between the structure of an antisense sequence [3, 12, 13] and modulation of gene expression.

In order to investigate the relationship between telomerase inhibition and susceptibility to cisplatin, the apoptotic mechanisms of Ad-OA must be elucidated in advance. Kondo *et al.* reported that it took about 1 month to induce apoptosis of 50% U251-MG cells by telomere shortening following telomerase inhibition [17], but only 4 days after treatment with 2-5A-anti-hTR activating function of caspase and inducing apoptosis in 70% of U251-MG cells [16]. Based on this evidence, they hypothesized that the effect of 2-5A-anti-hTR (antisense OA) might be due to an active induction of caspase-dependent apoptosis, which is independent of telomere length [15]. It is possible that the cytotoxic effects of Ad-OA are mainly due to caspase-dependent apoptosis. To prove this hypothesis, caspase inhibition assay was performed in the present study. Although Ad-OA was slightly inhibited by DMSO, ICE and CPP32 inhibitors clearly recovered the cell viability (Fig. 3), indicating that Ad-OA induced caspase-activated apoptosis of A2780 cells.

Telomerase has the function to protect cellular DNA by elongation of the telomere and prevents the cell death crisis by some kind of genomic instability. Hence, it is possible that inhibition of telomerase activity may increase the susceptibility of tumor cells to chemotherapeutic agents. There have been several approaches of combined use of telomerase antisense plasmid and DNA damaging drugs to inhibit the growth of malignant cancer cells [18], but not with antisense telomerase adenovirus.

In this study, the relationship between cisplatin sensitivity and telomerase activity was revealed. The chemotherapeutic agent cisplatin is particularly effective against ovarian carcinoma, although its clinical success is limited by recurrent drug resistant disease. This study is the first trial that uses antisense telomerase adenovirus with cisplatin. It was found that the antisense telomerase adenovirus suppressed ovarian cancer cell growth (Fig. 2), and this effect was mainly due to the induction of caspase-dependent apoptosis (Fig. 3). Cisplatin treatment with Ad-OA was the most effective to induce susceptibility of cisplatin resistant A2780/cp70 cells to cisplatin-induced apoptic cell death, compared with cisplatin or vector only groups. A2780/cp70 cells were the most effectively killed, if the cells were infected first with Ad-OA, and then treated with cisplatin after 2 days of infection (Fig. 4). It seems that Ad-OA may be inhibited by cisplatin slightly, because cisplatin has antiviral activity [26]. The ability of cisplatin to react with DNA to form intra- and inter-strand cross-linkers has been considered to be the basis of its therapeutic effect [7], therefore, cisplatin might have hindered the action of adenovirus [26].

In summary, the present results suggest that cisplatin treatment together with antisense telomerase adenovirus vector Ad-OA might be a new chemo-sensitization tool for tumors resistant to cisplatin, and this approach would be a good strategy for anticancer gene therapy.

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