Restoration of Wild-Type p53 by Adenovirus-Mediated Gene Transfer May Enhance the Therapeutic Efficacy of Chemotherapy in Human Ovarian Cancer Cells

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Objective: In an effort to develop a more effective therapeutic strategy for ovarian cancer, we examined whether the restoration of the wild-type p53 gene can enhance the therapeutic effect of chemotherapy.

Methods: In this study, Ov-ca-2774 cells, which are known to have p53 point mutation and cisplatin-resistance, were selected and currently used chemotherapeutic agents including cisplatin, carboplatin, paclitaxel, etoposide, topotecan, and doxorubicin were added concurrently or sequentially with adenovirus-mediated p53 gene transfer (Ad5CMV-p53).

Results: Transfer of the wild-type p53 cDNA gene into Ov-ca-2774 cells showed 55% cell killing in vitro at a multiplicity of infection (MOI) of 40. Although the combination of carboplatin or paclitaxel followed by p53 gene transfer with an interval of 48 h manifested no enhanced cell killing compared with cells infected with Ad5CMV-p53 alone, the other combinations of chemotherapeutic agents and p53 gene transfer resulted in 15% to 37% further cell killing (P<0.05). Furthermore, p53 gene transfer followed by doxorubicin with an interval of 24 h and concurrent combination of etoposide with p53 gene transfer showed significant difference in cell killing in contrast to the other combination strategies in the respective chemotherapeutic agent exposure groups (P<0.05).
Conclusion: Our data demonstrated that combination of p53 gene transfer and chemotherapeutic agents had higher cell killing than either of these two modality alone.

Key Words: Wild-type p53, Adenoviral vector, Chemotherapy, Ovarian cancer.

Introduction

Ovarian cancer is the leading cause of gynecologic cancer death in the United State, with approximately 22,000 new cases and 13,000 deaths estimated in 1994. Unfortunately, the inaccessibility of the ovary to examination and the lack of early symptoms make the diagnosis of ovarian cancer difficult. No screening test has yet been shown to be effective for prevention or early diagnosis. Two-third of patients present with advanced stage disease, wherein the survival is only 15%-20%. Based on the prospective trials by the Gynecologic Oncology Group, the combination of paclitaxel and cisplatin has become the new standard regimen in patients with suboptimal stage III and stage IV ovarian cancer in the United States. Responses were reported in 73% of those patients. But, median progression-free survival was 17.9 months. Recent studies by genetic analysis have shown that multiple alteration of genes such as K-ras, HER-2/neu, c-myc, and p53 occur in ovarian cancers. Mutations of the p53 gene has been identified in 27% to 79% of epithelial ovarian cancers, which represent the most common genetic alteration in ovarian cancers and correlate with poor prognosis. Some authors observed that a strong correlation had emerged between p53 alteration and resistance to chemotherapy in ovarian carcinomas and bladder cancer. Furthermore, from relapsed clinical cases, increased resistance to chemotherapy was found to be correlated with acquired mutations of the p53 gene, which were not found in primary tumor. So, p53 gene status and the ability of p53 to induce apoptosis may be determinant of sensitivity to chemotherapy.

Materials and Methods

Cell culture. The well-characterized platinum-resistant human epithelial ovarian cancer cell line Ov-ca-2774 was selected for this experiment. These cells are known to contain p53 mutation and are found to be resistant to gene therapy by other investigator. Cells were maintained in HGD MEM (Dulbecco's Modified Eagle Medium with high glucose, Gibco) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamine at 37°C and 5% CO2 in a humidified incubator.

Construction of recombinant adenoviral vector. The construction and isolation of recombinant adenoviral vector have been described previously. The recombinant p53 adenoviral vector (Ad5CMV-p53) contains the cytomegalovirus promotor, wild-type p53 cDNA and SV40 polyadenylation signal in a minigene cassette which is inserted into the E1-deleted region of modified adenovirus.

Transduction efficiency and virus-related cyto-
Transduction efficiency was investigated by infecting Ov-ca-2774 cells with the adenoviral vector containing the β-galactosidase gene (Ad5CMV-LacZ). Cells were seeded at 1.0 x 10^5 cells in 6-well tissue culture plates. 24 h later, cell were infected with Ad5CMV-LacZ at an multiplicity of infection (MOI) ranging from 0-500. Infection of cells was carried out by adding the desired amount of virus to the cell monolayer in HGDMEM medium and 10% FBS. Cells were incubated 37°C for 60 minutes with agitation every 15 minutes. Additional medium was added and cells were incubated at 37°C. 24 h after infection, cells were fixed in 0.5% glutaraldehyde at room temperature for 5 minutes, washed twice with phosphate-buffered saline and then incubated for 4 h with X-gal solution (1.3 mM MgCl₂, 15 mM NaCl, 44 mM Tri, pH 7.4, 3 mM potassium ferricyanide, 3 mM potassium ferricyanide, and 2% X-gal in n,n'-dimethylformamide) to measure β-galactosidase activity. 500 cells in each dish were counted three times and percentages of cells stained positive were determined. To assess the virus-related toxicity, cell were infected with Ad5CMV-LacZ at a multiplicity of infection (MOI) ranging from 0-1000 and incubated for 6 days. Following incubation, a percentage of cell survival was determined using MTT assay that measures viable cell dehydrogenase activity as described previously. 26,27 1mg/ml of MTT (Sigma Chemical Co., St. Louis, MO) was added to each well and plates were incubated for 4 h. After that, 100 μl of 100% DMSO (Sigma) was added to each well and the absorbance at 595 nm of each well was measured using a microplate reader (Biorad Laboratories Model 550). After correction for background media-only absorbance, data for each well were expressed as a absorbance or a percentage (% cell survival) to the absorbance in well containing uninfected control cells.

Cytotoxic Effect of chemotherapeutic agents. Cells were plated at a density of 1 x 10^3 in 96-well flat-bottomed plates and allowed to attach overnight. Various concentrations of each chemotherapeutic agents were added to media to test concentrations required to kill 50-70% of control cells.

Combination of Chemotherapeutic Agents and Adenovirus-mediated p53 Gene Transfer. Cells were plated at a density of 1 x 10^3 in 96-well flat-bottomed plates and allowed to attach overnight. Considering the observations by others that systemic cisplatin administration before p53 gene transfer or concurrent p53 gene transfer with cisplatin administration produced a synergistic effect in inhibition of tumor growth, 18,19 cells were treated with one of the three different combination strategies in this study: p53 gene transfer concurrently with chemotherapeutic agents exposure, p53 gene transfer followed by chemotherapeutic agents exposure with an interval of 24 hours, or chemotherapeutic agents exposure followed by p53 gene transfer with an interval of 48 hours. We chose platinum agents, topoisomerase inhibitors, antimicrotubule agent, antitumor antibiotics. Selected concentrations, required to kill about 50-70% of control cells, of chemotherapeutic agents including cisplatin (Bristol-Myers Squibb Co., Princeton, NJ), carboplatin (Bristol-Myers Squibb Co.), topotecan (SmithKline Beecham Pharmaceuticals, Philadelphia, PA), etoposide (Bristol-Myers Squibb Co.), paclitaxel (Bristol-Myers Squibb Co.), and doxorubicin (Pharmacia Inc., Columbus, Ohio) were added to the culture media. Following incubation for 6 days after initial treatment, a percentage of cell survival was determined using MTT assay.

Statistics. All results was expressed as the mean±standard error of 8 identically treated wells in triplicates. The means were compared utilizing analysis of variance (ANOVA). When significant difference was found, Student t-test was used to test among groups. (P<0.05 was considered significant)

Results

Transduction efficiency and virus-related cytotoxicity. 50% of transduction efficiency was noted using
Ad5CMV-LacZ with an MOI of 40 and there was virus-related cytotoxicity with Ad5CMV-LacZ at an MOI of 500 or more. (data not shown)

Cytotoxic Effect of chemotherapeutic agents. Concentrations of each chemotherapeutic agents required to kill 50-70% of control cells were summarized. (Table 1)

Cytotoxic effect of adenovirus-mediated p53 gene transfer on Ov-ca-2774 cells. The cells were infected with Ad5CMV-LacZ or Ad5CMV-p53 at a multiplicity of infection (MOI) of 40 and the absorbance for virus-infected or mock-infected wells was assessed by MTT assay on day 1, day 3, and day 6. By day 3, the absorbance of Ad5CMV-p53 infected wells was lower than that of Ad5CMV-LacZ or mock-infected wells. 6 days after infection, the absorbance of Ad5CMV-p53 infected wells significantly decreased to about 45% in comparison to that of mock-infected wells, while the absorbance of Ad5CMV-LacZ infected wells was 99% of that of mock-infected wells. (Fig. 1)

Combined cytotoxic effect of chemotherapeutic agents and adenovirus-mediated p53 gene transfer on Ov-ca-2774 cells. The combined cytotoxic effect was assessed in vitro using various concentrations of several chemotherapeutic agents, required to kill 50%-70% of uninfected control cells, and Ad5CMV-p53 with an MOI of 40. Although the combination of carboplatin or paclitaxel followed by p53 gene transfer with an interval of 48 h manifested no enhanced cell killing compared to cells infected with Ad5CMV-p53 alone, the other combinations of chemotherapeutic agents and p53 gene transfer resulted in 15% to 37% further cell killing than p53 transfer alone (P<0.05, Fig. 2). Accordingly, p53 gene transfer followed by doxorubicin with an interval of 24 h and concurrent combination of etoposide with p53 gene transfer showed significant difference in contrast to the other combination strategies in the respective groups (P<0.05). However, there were no difference in cell killing among three combinations of cisplatin or topotecan with p53 gene transfer in the respective groups. In conclusion, based on the data we have, combination therapy will enhance therapeutic efficacy in most circumstances.

![Graph: Growth curves of mock-infected, Ad5CMV-LacZ infected, and Ad5CMV-p53 infected Ov-ca-2774 cells with a multiply of infection (MOI) of 40. The average values of samples and standard deviations are shown.](image)

**Table 1.** Concentrations of each chemotherapeutic agents required to kill 50-70% of control cells.

<table>
<thead>
<tr>
<th>chemotherapeutic agent</th>
<th>concentrations</th>
<th>% of control cells</th>
</tr>
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<tbody>
<tr>
<td>cisplatin</td>
<td>2.5 ng/ml</td>
<td>69%</td>
</tr>
<tr>
<td>carboplatin</td>
<td>10 ng/ml</td>
<td>72%</td>
</tr>
<tr>
<td>topotecan</td>
<td>10 ng/ml</td>
<td>63%</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>2 ng/ml</td>
<td>68%</td>
</tr>
<tr>
<td>etoposide</td>
<td>0.2 ng/ml</td>
<td>46%</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>1.2 ng/ml</td>
<td>77%</td>
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It is well known that the p53 gene operates by stopping the cell cycle in G1 to allow necessary repairs to DNA damage which can be induced either by a malfunctioning DNA replication machinery or by DNA damaging drugs and radiation. If the damage can not be repaired, cell death by apoptosis is triggered by the p53 gene. Therefore, alterations in the p53 gene lead to loss in this critical guardian function and might be associated with resistance to chemotherapy. It was already known that p53 gene transfer combined with chemotherapy has greater efficacy than chemotherapy alone in ovarian cancer whose p53 gene is deleted. \(^{21,22}\) Novel strategies for anticancer therapy focus on the replacement of mutant p53 protein to avoid resistance to chemotherapy. In this study, we demonstrated that the restoration of the wild-type p53 gene in ovarian cancer containing p53 mutation could enhance the therapeutic efficacy of chemotherapy.

In order to determine the efficacy of p53 gene transfer combined with chemotherapy, we chose the dosage of viral vector or concentrations of chemotherapeutic agents which can kill about 50% of cancer cell alone. As described above, most combinations achieved better cell killing efficacy. However, some combinations, such as combination of carboplatin or paclitaxel followed by p53 gene transfer with an interval of 48 h, failed to manifest enhanced cell killing compared to cells infected with Ad5CMV-p53 alone. This might be partially explained by the different expression patterns of the exogenous p53 gene in individual cells after exposure to chemotherapeutic agents.

The highest level of p53 expression can be affected by many factors such as interval between gene transfer and exposure of chemotherapeutic agents, \(^{18}\) concentration of chemotherapeutic agent, \(^{19}\) cancer cell type. Our results also showed that the timing of chemotherapeutic agents exposure and p53 gene transfer was shown to be important: p53 gene transfer followed by doxorubicin with an interval of 24 h, and concurrent combination of etoposide and p53 gene transfer showed significant difference in cell killing of

**Discussion**

Although contemporary mainstay of treatment for patients with advanced stage of ovarian cancer remains aggressive surgery followed by chemotherapy, the major problem of chemotherapy in ovarian cancer is development of resistance. Mutation of the p53 gene, which is the most common mutation in ovarian cancer, has been found to be associated with chemotherapy resistance.
Ov-ca-2774 cells in contrast to the other combination strategies in the respective groups. In ovarian cancer cells, doxorubicin was reported to be less cytotoxic in cells expressing wild-type p53 protein than in cells expressing no p53 or mutant p53,28,29 and sensitivity of paclitaxel was shown to be not affected by the status of the p53 gene.30 However, our experimental study revealed that all combination strategies of doxorubicin with p53 gene transfer and two combination strategies of paclitaxel with p53 gene transfer can enhance cell killing of Ov-ca-2774 cells. Each chemotherapeutic agent has different mechanism of action. Some work fast or others do slow. So we should try to find out the timing when high level of p53 expression can be achieved and chemotherapeutic agents has best activity.

There was a report about clinical trial of p53 gene therapy in lung cancer patients31 and head and neck cancer patients.32 In these studies, it was proven to be safe even through long term toxicity or mutagenesis by p53 gene transfer is uncertain. More clinical studies are needed to get informations about the toxicity and efficacy of p53 gene transfer combined with chemotherapy.

- 참고문헌 -


목적: 진행된 난소암의 치료는 수술 및 수술 후 항암 화학요법제의 사용이 근간을 이루고 있으나, 완치율이 낮고, 치료 실패의 대부분 원인은 수술 후 항암 화학요법제에 대한 내성이 발생한다. 난소암에 있어서 가장 많은 유전자 변이는 p53 유전자 변이이며, 이는 항암 화학요법제에 대한 내성과 관련이 있는 것으로 알려져 있다. 진행된 난소암에 대한 진보된 치료법을 찾기 위하여, 난소암 세포주에 이입된 p53 유전자가 항암 화학요법제의 작용을 중가시키는 지를 알아보고자 한다.

제료 및 방법: p53 유전자의 변이와 cisplatin에 대한 내성을 갖고 있는 것으로 알려진 Ov-ca-2774 세포주에 난소암 치료에 널리 사용되는 항암 화학요법제의 투여와 정상 p53 유전자 (Ad5CMV-p53)를 동시에 혹은 순차적으로 이입하였다. 항암화학제를 단독으로 투여한 경우에 비하여 정상 p53 유전자를 40 MOI(multiplicity of infection)의 농도로 항암화학제와 병행하여 Ov-ca-2774 세포주에 이입한 경우 대부분에서 15% 내지 37%의 증가된 암세포 성장억제가 관찰하였다(P<0.05). 그러나 carboplatin이나 paclitaxel의 경우, 투여 48시간 후 정상 p53 유전자를 이입한 경우에는 정상 p53 유전자만 이입한 경우에 비하여 증가된 암세포 성장억제가 없었다. doxorubicin의 경우, 정상 p53 유전자와 동시에 투여하거나, doxorubicin 투여 48시간 후 정상 p53 유전자를 이입한 경우보다 정상 p53 유전자 이입 24시간 후 doxorubicin을 투여한 경우에 유의한 암세포 성장억제가 관찰되었다 (P<0.05). etoposide의 경우, etoposide와 정상 p53 유전자를 동시에 투여한 경우가 이들은 순차적으로 투여한 경우보다 의의 있는 암세포 성장억제를 보였다(P<0.05).

결론: 이상의 결과로 보아 정상 p53유전자와 항암 화학요법제를 병합한 경우 각각의 방법을 단독으로 처리하는 것에 비하여 증가된 난소암 세포주의 성장억제를 시험관내에서 관찰할 수 있었다.