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Original Article

Generation of Cisplatin-Resistant Ovarian Cancer Cell Lines

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Abstract

Ovarian cancer is the most lethal gynecological cancer in which cisplatin-based treatment plays a fundamental role as the first line chemotherapy option. However, development of platinum-resistance is a critical and poorly understood problem in ovarian cancer treatment. Although *in vitro* generation of platinum-resistant ovarian cancer cell lines is a long established approach to uncover the molecular mechanisms underlying resistance development, the methodology of this resistance induction is poorly explained in publications. The aim of this study was to propose a method for induction of resistance in ovarian cancer cell lines. To reach this aim, A2780 human ovarian cancer cell line was continuously exposed to stepwise increasing concentrations of cisplatin (0.5–2.6μM) over a period of 6 months and three resistant sublines were collected. Cisplatin resistance was examined by clonogenic survival assay and growth curve analysis was carried out in order to evaluate the proliferation characteristics of the established sublines. The A2780 resistant sublines exhibited 5.1 to 11.7 fold resistance to cisplatin, compared to their parental cells, and although growth rate and plateau saturation density significantly decreased by cisplatin resistance enhancement, all three resistant sublines presented a typical growth curve even though they were cultured in the cisplatin containing medium. These results suggest that reliable drug resistant human ovarian cancer cell lines can be successfully established by this method.

Keywords: A2780, A2780-CP, cell line model, cisplatin, ovarian cancer, resistance induction.

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1. Introduction

Ovarian cancer is considered to be one of the most sensitive solid tumors with high range of objective responses (60-80%) even in advanced stage patients [1]. However, despite the considerable initial response to chemotherapy [2], the majority of patients experience an early relapse [3] and the long-term

survival rate of the patients with relapsed ovarian cancer is disappointingly low [4]. This turns the ovarian cancer to be the most deadly gynaecological neoplasia worldwide [5]. One of the major factors contributing to loss of chemotherapy effectiveness which results in high relapse rate is chemoresistance development following several rounds of chemotherapy [6]. As a result, the curative potential of cisplatin, one of the first line treatment options in ovarian cancer, has been significantly limited [7].

Resistance to chemotherapy agents may be inherent or acquired [8]. In the process of acquiring resistance, cancer cells may develop cross resistance to a wide range of chemotherapeutic drugs with different mechanisms of action. This makes the acquired resistance a particular problem in cancer treatment and ultimately leads to treatment failure in more than 90% of patients with metastatic neoplasia [9, 10]. Numerous studies over the past decades have revealed that chemoresistance involves multiple complex mechanisms [11-13]. Despite this progress, our knowledge of biological pathways in chemoresistance still remains limited [14].

Several methods have been developed to evaluate the mechanisms underlying drug resistance and the biological factors involved in chemoresistance pathways in recent years [15]. Among all of the common methods, those based on human cancer-derived cell lines have played an important role in our current knowledge of anticancer drug resistance [16]. The establishment of chemoresistant cancer cell lines might be one of the useful model systems to study molecular mechanisms leading to cancer drug resistance [17]. However, most of the published scientific research papers poorly explained resistance induction methods in details [18, 19]. There are many differences in protocols for *in vitro* induction of resistance and although exposing cancer cells to stepwise increasing concentrations of anticancer drugs is the cornerstone in the development of resistant sublines, there are many differences in initial dose of anticancer agent and the intervals of drug exposure. Besides numerous studies in which the initial drug exposure concentration is extremely below the IC_{50} [11, 20], there are multiple studies that initially treated the cells with IC_{50} concentration [21, 22]. Moreover, the exposure time widely differs in various studies [20, 21, 23] and in some investigations exposure period was followed by a recovery period [24] while others used a continual exposure model [23].

The aim of the present study is to develop a simple and effective method to establish cisplatin resistant human ovarian cancer cell lines as a model to study chemoresistance in ovarian cancer.

2. Materials and Methods

2.1. Materials

Cisplatin solution was obtained from Kokak Farma®, Turkey. All other materials used in this study were purchased from Gibco®, Life Technologies®, unless otherwise specified.

2.2. Cell Lines

A2780S (human ovarian carcinoma-sensitive to cisplatin) and A2780CP (human ovarian carcinoma-resistant to cisplatin) were obtained from the Pasteur Institute of Iran, Tehran, Iran.

2.3. Methods

2.3.1. Cell Culture

Cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin in a 37ºC incubator with a humidified atmosphere containing 5% CO2. Exponentially growing cells were used in experiments.

2.3.2. Initial Dose and Dosing Interval Determination

Optimal initial dose and dosing intervals were determined using clonogenic assay [25]. Briefly, cells were seeded into 6-well plates (SPL Lifesciences®, Korea) at a density of 250 cells per well and allowed to adhere overnight at 37ºC. After 24 hours the cells were exposed to different concentrations of cisplatin and incubated for 2, 24, 48, 72 and 168 hours. Then, the medium was removed by aspiration and 2 ml of fresh medium was added to each well.The incubation continued until visible colonies could be identified (typically 8 days after cell seeding). At this point the medium was removed and formed colonies were fixed in ethanol (96%) for 10 minutes and stained with Methylen blue (0.4%) for 30 minutes. Finally the plates were gently washed with water and air-dried. The results were quantified by comparison with the control cells exposed to cisplatin-free solvent (0.9% sodium chloride).

2.3.3. Induction of Cisplatin-Resistance in A2780 Cell Line

Cisplatin-resistant A2780 cell lines were derived from original parental cell line by continuous exposure to stepwise increasing concentrations of cisplatin. Initially, the exponentially growing cells were exposed to IC_{50} concentration obtained from clonogenic assay. These cells were maintained in cisplatin containing RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin and subcultured upon reaching 70 - 80% confluency for 4 weeks. At this point the concentration was increased and the above process was repeated. Aliquots of cell sublines were cryopreserved at each incremental concentration. Drug concentration was increased approximately 1.5 fold in the initial steps and 1.25 fold in the final steps. This development period was carried out for about 6 months and 3 resistant sublines were collected named A2780-R1, A2780-R2 and

A2780-R3. These sublines were exposed to 400, 600 and 800ng/ml cisplatin respectively. Additionally, vehicle treated parental cell line was kept in culture during this period as control cell line.

2.3.4. Measurement of Drug Resistance

Cisplatin sensitivity was determined in the parental sensitive cell line (A2780), its cisplatin resistance variant (A2780-CP) and three established sublines (A2780–R1, R2, R3) using clonogenic assay as described above. The cells were treated with different concentrations of cisplatin for 7 days and the IC_{50} values and its ratio between the resistant and parental cell lines were defined.

2.3.5. Growth Curve Analysis

Cells were plated at a density of 10^5 cells in $25cm^2$ flasks. Parental A2780 cell line was plated in 5 ml of cisplatin free culture medium; while A2780-R1, A2780-R2 and A2780-R3 were plated in medium containing 400, 600 and 800ng/ml cisplatin respectively. Viable cells were counted using trypan blue exclusion test every 24 hours for 10 consecutive days. Finally, cell growth data were plotted on a semi-log scale and doubling time was calculated for each cell line. According to the Patterson equation, cell doubling time was calculated as follow: Td = t lg2/lg(Nt/N0) where Td: doubling time (h); t: required time when cell numbers increased from N0 to Nt; N0: cell numbers in the inoculation; Nt: cell numbers after culture for t hours [26].

2.3.6. Microscopic Images

Microscopic images of the cells were taken using a camera focused on the optic lenses of a Nikon® inverted microscope with 10× objective.

2.4. Statistical Analysis

Data are presented as mean ± SEM (Standard Error of the Mean). The Graph Pad Prism® softwere (Graph Pad Prism Software, Inc.) was used to construct graphs and statistical analysis. Statistical significance was determined using one-way ANOVA followed by Tukey's test. p value ≤ 0.05 was considered significant.

3. Results and Discussion

3.1. Initial Dose and Dosing Interval Determination

The appropriate initial dose and dosing intervals were determined by evaluating the colony-forming-ability of each single cell in the presence of cisplatin. Figure 1 shows the dose response clonogenic survival of A2780 cells after exposure to increasing concentrations of cisplatin for 2,24,48,72 and 168 hours. The IC_{50} values are shown in table 1. Although the IC_{50} value is significantly higher upon 2 hours exposure to cisplatin, there is no significant difference in IC_{50} values of the A2780 cells exposed to cisplatin for 24, 48, 72 and 168 hours. These data suggest that the IC_{50} reduction is not linearly related to the prolongation of cisplatin exposure time and while exposure time

Table 1. Cytotoxicity of cisplatin in A2780 human ovarian cancer cell line following different drug exposure time.

	Drug Exposure Time (h)				
	2	**24**	**48**	**72**	**168**
IC50 ± SEM (ng/ml)	2128 ± 1.083	199.3 ± 1.059	198.7 ± 1.054	161.1 ± 1.07	151.1 ± 1.07

prolongation initially increases cisplatin cytotoxicity in the first 24 hours, the inhibitory effect does not significantly increase after 24 hours cisplatin treatment. These results were also supported by previous studies [27, 28]. Considering these results together with the fact that resistant models that are selected by pulse selection are often less stable than their continuously selected counterparts [19], we decided to expose A2780 cells to stepwise increasing concentrations of cisplatin in a continuous manner. Since continuous exposure model was selected for resistance induction, the cells were initially exposed to IC_{50} concentration obtained from exposing A2780 cells to different concentration of cisplatin for seven days (168 hours).

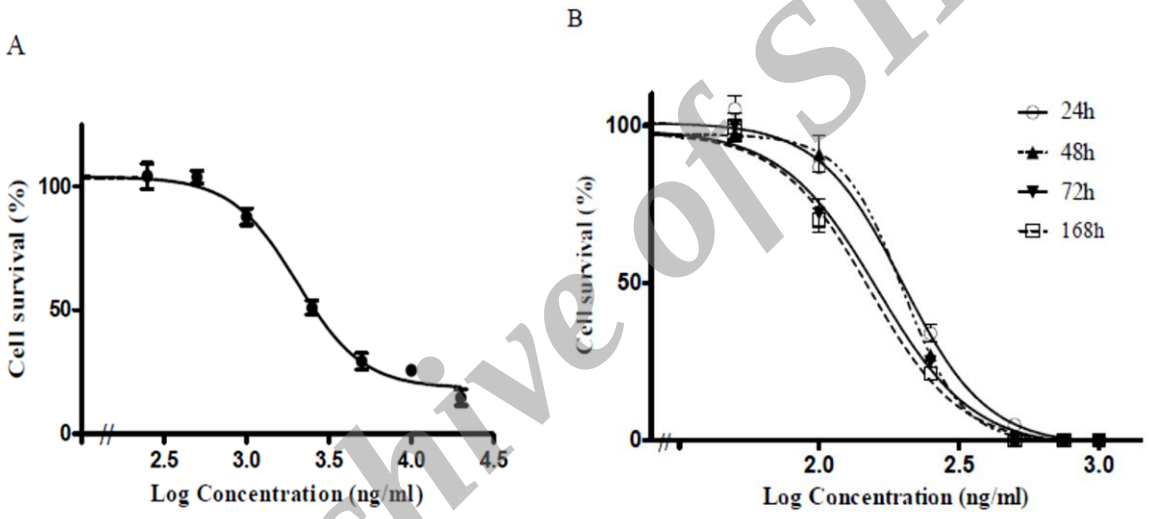

Figure 1. Dose- response clonogenic survival of A2780 cells after exposure to increasing concentrations of cisplatin for 2 hours (A) and 24, 48, 72 and 168 hours (B).

3.2. Measurement of Drug Resistance

The sensitivity of parental cell line, its resistant variant and three established sublines to cisplatin was evaluated by clonogenic assay and the data are summarized in table 2. The resistance index (RI) was determined as the ratio

Table 2. Cisplatin resistance ratios induced in three established resistant sublines (A2780 – R1, R2, R3) comparing to parental cell line (A2780) and its resistance variant (A2780-CP).

Cell line	IC_{50} ± SEM (ng/ml)	Resistance Ratio
A2780	151.1 ± 1.07	1
A2780 – R1	782 ± 1.106	5.17
A2780 – R2	1036 ± 1.066	6.83
A2780 – R3	1778 ± 1.54	11.77
A3780-CP	2199 ± 2.065	14.56

of the IC_{50} of the cisplatin-resistant cell line to the IC_{50} of the sensitive parental A2780 cell line.

3.3. Growth Curve Analysis

In order to evaluate the proliferation abilities

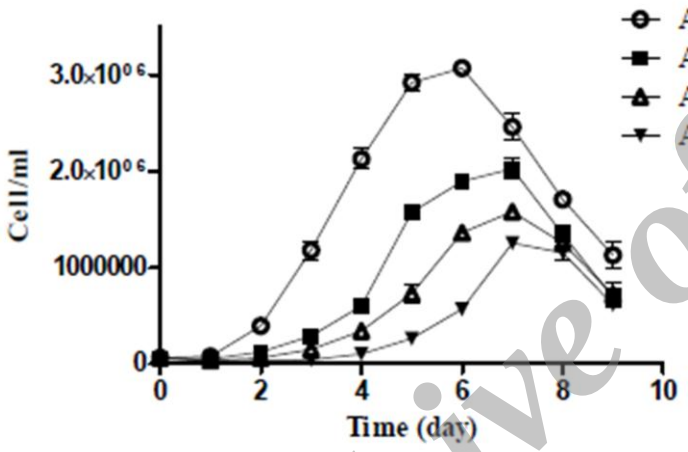

Figure 2. Growth curve of the established sublines (A2780 – R1, R2, R3) comparing to A2780 parental cell line. Parental A2780 cell line was plated in cisplatin free culture medium; while A2780-R1, A2780-R2 and A2780-R3 were plated in medium containing 400, 600 and 800ng/ml cisplatin respectively.

as shown in table 2, the established sublines exhibited 5.1 to 11.7 fold resistance to cisplatin, compared to their parental cells and the A2780-CP cell line exhibited 14.56 fold resistance to cisplatin in comparison with its sensitive cell line. These results indicate that the described method can be used for the development of sublines with different degrees of resistance in ovarian cancer cell line.

of the established sublines in the presence of cisplatin, growth curve analysis was performed. As shown in figure 2 all of the three resistant sublines present a typical growth curve (consisting of lag, log, stationary and death phase) even though they were cultured in the cisplatin containing medium. The results of table 3 reveal that cell growth rate and plateau saturation density were significantly decreased by cisplatin resistance enhancement. These data

Table 3. Growth characteristics of A2780 cell line and the established cisplatin resistant sublines (A2780 – R1, R2, R3).

Cell line	Population Doubling Time (h)	Plateau Saturation Density (cell number/cm^2)
A2780	24.66 ± 0.191	1.2×10^5
A2780 – R1	30.19 ± 0.188	8.1×10^4
A2780 – R2	42.55 ± 2.508	6.3×10^4
A2780 – R3	75.66 ± 2.454	5.0×10^4

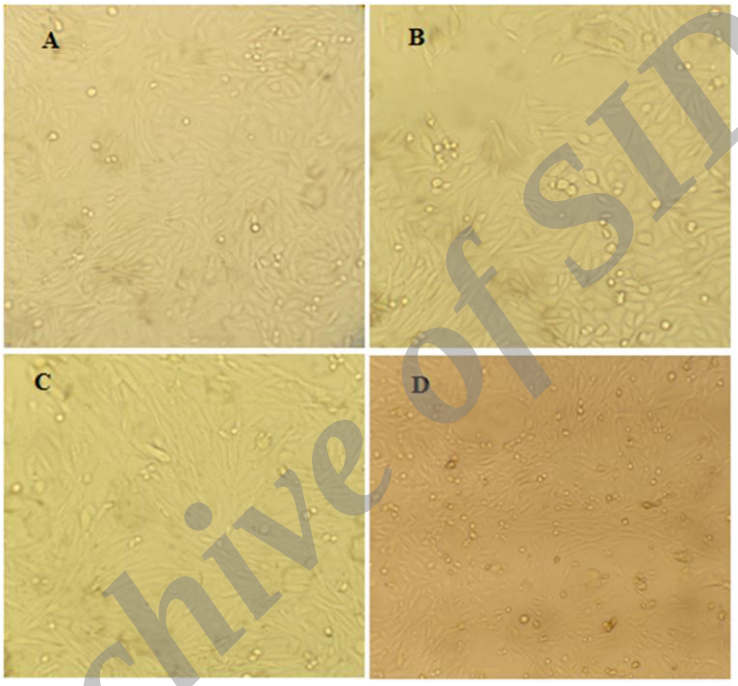

Figure 3. Cell morphology of A2780 cell line (A) and three established cisplatin-resistant sublines A2780 – R1 (B), A2780 – R2 (C) and A2780 – R3 (×10).

are consistent with previous observation in resistant sublines of human breast cancer MCF-7 and human hepatoma cell line SKHep1 [29, 30].

3.4. Microscopic Images

As shown in figure 3, resistant sublines' morphologies were approximately similar to those of the parental cells with small alterations.

4. Conclusion

Cell line models have been shown to be effective tools in ovarian cancer research and the resistant cell lines which have been selected following exposure to anticancer drugs played an important role in our understanding of mechanisms underlying chemoresistance development in ovarian cancer [31]. Although the cell lines that have been derived from cancer

patients before and after chemotherapy are ideal models for chemoresistance development, there are limitations for the availability of tumor biopsy specimens in large quantity [32]. Consequently, the models based on the *in vitro* establishment of chemoresistant sublines have been used in many investigations as a valuable tool for the illumination of the factors underlying drug resistance. There are two possible models for *in vitro* development of resistant cell lines: pulse treatment and continuous treatment [33]. Several studies have been performed to evaluate and compare the effectiveness of these two methods in the last decades [34-36]. The investigators found that the schedule of drug exposure can markedly influence the resistance index and according to most of the studies, continuous drug exposure is considered to be more effective in producing resistance than intermittent treatment [32, 34, 35]. These results were supported by Kuppen *et al*., who observed that a six time repeated pulse exposure of ovarian cancer cells to cisplatin for l hour did not result in a cell line with a higher survival in cisplatin-containing medium, while exposing the parental cell line to increasing concentration of cisplatin in a continuous manner induced resistance [37]. In addition, as shown in some studies, partial loss of resistance occurs in absence of drug and the resistant sublines should be cultured in drug-containing medium in order to maintain resistant phenotype [32, 35, 36]. Taking these together, we continuously exposed the A2780 cells to stepwise increasing concentrations of cisplatin in order to induce resistance.

As shown in results, cell growth rate and plateau saturation density significantly decreased by cisplatin resistance enhancement. These results, together with those previously described by other studies [29, 30], suggest the slower growth as a component of drug resistance. Wosikowski *et al*., performed serum starvation experiments to determine whether there is a relationship between reduced growth rate and drug resistance. Interestingly, their results showed that decreased proliferative activity due to serum starvation resulted in a marked increase in resistance to doxorubicin and paclitaxel in the MCF-7 parental cells. By contrast, serum starvation had lower effect on the sensitivity to cytotoxic agents in the resistant sublines, possibly because the underlying reduction in growth rate already contributed maximally to their resistant phenotype [29]. It is well established that actively proliferating cells are more vulnerable to effects of the most anticancer drugs than the quiescent cells [38]. One hypothesis is that reduced growth rate could constitute a significant event in the survival of cancer cells following a major stress like cisplatin treatment.

In conclusion, the results of this study can provide a suitable method to establish cisplatin resistant subpopulations of ovarian cancer cells. These cisplatin resistant cell lines might be useful to study molecular mechanisms leading to

cisplatin resistance development in ovarian cancer.

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