Establishment and characterization of lung adenocarcinoma cell lines with multidrug resistance☆

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Received October 15, 2006

Abstract

Objective. Many discoveries of multidrug resistance (MDR) have resulted from studies with drug-resistant tumor cell lines as their models. Till now, there has been no report on the detailed characterization of such a cell line from lung adenocarcinoma (LA). By long-term exposure of an established LA cell (A549 cell) to increase concentrations of paclitaxel, we established a series of subcultures that were considerably more resistant to this drug. *Methods*. Paclitaxel-resistant sublines (A549/TXL) were established *in vitro* by exposing to stepwise increased concentrations of the drug in a cell culture medium. Biological morphology was analyzed by morphometry and flow cytometry. The chemoresistance indexes of cells were measured by methyl tetrazolium assay. Evaluation of growth, *in vitro* drug sensitivity, and a pharmacokinetic study were performed. *Results*. Compared with parent cells, the resistant sublines were smaller and mixed with giant cells in different sizes and with different numbers of nuclei. The resistant cells, A549/TXL20 were 19.3 times more resistant to paclitaxel and 67.4 times more resistant to cisplatin than the parent cells. The resistant cells also demonstrated cross-resistance to mitomycin, vinblastine, hydroxycamptothecine, and 5-fluouracil (5-FU). Compared with the A549 cell line, an unreasonably higher level of drug-resistance and lower drug concentration was detected in A549/TXL20 cells after exposure to the drug in the culture medium. *Conclusion*. The paclitaxel-induced MDR sublines may be used as an experimental system for the search of a means to overcome drug resistance and elucidate possible mechanisms of acquired MDR involved in human lung adenocarcinoma. [Life Science Journal. 2007;4(1):13-16] (ISSN: 1097-8135).

Keywords: lung adenocarcinoma; multidrug resistance; paclitaxel

1 Introduction

Chemotherapy has proven effective in the therapy or palliation of many human tumors such as testicular cancer and leukemia; however, drug resistance remains a major obstacle in the treatment of other carcinomas. Human lung adenocarcinoma (LA) displays a characteristically high degree of chemoresistance toward a broad spectrum of natural cytotoxic compounds that do not possess obvious functional or structural similarities[1,2]. This phenomenon is termed multidrug resistance (MDR)[3]. Since reliable therapeutic alternatives to chemotherapy are still lack, this resistance contributes considerably to the poor prognosis of patients with disseminated LAs.

Paclitaxel, an anti-microtubule agent isolated from *Taxus brevifolia*, has been shown to demonstrate clinical efficacy in LA[4]. This agent binds to and stabilizes microtubules, and consequently, induces mitotic arrest and apoptotic cell death[5,6]. Paclitaxel-based chemotherapy produced high response rates and satisfied prognosis, and is considered to be the international standard regimen against LA[7]. However, acquired-resistance to paclitaxel has become a serious clinical issue with the increasing prescription[8]. With the recent demand to analyze the biological behavior of paclitaxel-resistant tumors and uncover the paclitaxel-resistance mechanism to improve the therapeutic efficacy against recurrent LA, it has become necessary to establish and analyze paclitaxel-resistant LA cell lines which properly and faithfully simulate the clinical situation.

The aim of this study is to establish MDR cell lines of human LA against the clinically important chemotherapeutic compound paclitaxel, to trace the underlying resistance mechanisms of acquired MDR.

☆Supported by the National Natural Science Foundation of China (No. 30471952).

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2 Materials and Methods

2.1 Establishment of LA MDR cell lines

Culture medium RPMI-1640 containing 300 mg/L glutamine, fetal calf serum (FCS), and antibiotics were purchased from Gibco (Gaithersburg). A human LA cell line, A549, was used in this study. It was maintained in a RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 mg/mL streptomycin at 37 ℃ in an atmosphere of 5% CO_2 and 100% humidity. A549 cells were first incubated in 0.5 nM paclitaxel for 4 weeks. These cells were then periodically treated with 1.0 nM paclitaxel for 2 hours followed by incubation in 0.5 nM paclitaxel until the cells were again almost 100% confluent. This cyclic treatment was repeated five times over a period of 18 weeks. The cells were then cultured in 5 nM paclitaxel, thus generating the paclitaxel-resistant subline A549/TXL5. On stable growth, the paclitaxel concentration was increased every 6 weeks. Stable sublines were acquired from 5 nM to 20 nM paclitaxel and named as A549/TXL5, A549/TXL10 and A549/TXL20. Before experimental use, A549/TXL cells were maintained in a paclitaxel-free culture medium and subcultured at least three times.

2.2 Growth morphometry

The growth rate, saturation density, size, and nucleus to cytoplasm (N/C) ratio were analyzed individually. HE stained, and measured for size and N/C ratio under image analyzer (L2 system, Yen-Hau, Taiwan).

2.3 MTT cytotoxicity assay

Cell number was counted on a hemocytometer and 2,000 cells were pipetted into each well of a 96-well microtiter plate in 100 μl RPMI-1640 medium. The cells were left to adhere overnight and paclitaxel was then added at increasing concentrations in a volume of 100 μl medium. After a 10-day incubation period, 20 μl of MTT stock solution (0.05 mg/100 mL) were added to each well. 4 hours later, the liquid was removed, the formazan crystals released and then solubilized by the addition of 150 μl DMSO (Merck). The extinction of the purple color, which is directly proportional to the number of viable cells, was measured at a wave-length of 540 nm in the ELISA photometer (Titertek Multiscan Plus MK Ⅲ). The percentage of viable cells was calculated by the following formula: living cells = (sample ext. − blank ext.)/(control ext. − blank ext.) × 100%. The IC_{50} value is defined as the dosage of drugs in which 50% of cellular death (50% reduction of absorbance at 540 nm) occurred after 48-hour treatment.

2.4 Evaluation of drug sensitivity by a colonyforming assay

Cells were suspended in 6-well culture plates at various cell concentrations (10^3, 5×10^2, 10^2, and 50 cells/well for untreated controls; 5×10^3, 10^3, 5×10^2, and 10^2 cells/well for the 5 nM paclitaxel-treated group; 10^4, 5×10^3, 10^3, and 5×10^2 cells/well for the 50 nM drug-treated group; and 5×10^4, 10^4, 5×10^3, and 10^3 cells/well for the 500 nM drug-treated group). After overnight incubation, cultures were exposed to various concentrations of paclitaxel (0 nM, 10 nM, 100 nM, and 1000 nM) for 24 hours at 37 ℃, and thereafter the drug was removed and each well was washed with cold PBS and re-incubated by a complete medium. 10 days later, the plates were fixed with Carnoy's fixative (ethanol: chloroform: glacial acetic acid = 6 : 3 : 1) and stained with crystal violet. The numbers of visible colonies, consisting approximately of more than 50 cells, were counted and the plating efficiency (P.E.) was calculated as (number of the colonies) / (number of the seeded cells). The surviving fraction was calculated as (P.E. in treated well)/ (P.E. in untreated well). The mean value ± SE was calculated in triplicate.

2.5 Intracellular drug concentration

The subconfluently cultured cells of A549 and A549/TXL20 were exposed to 100 nM paclitaxel for 2 hours. After rinsing twice with cold PBS, cells were harvested into tubes and cell pellets were collected after centrifugation (2,700 rpm, 5 minutes), and were analyzed by solid-phase extraction high performance liquid chromatography (SBS, Sagamihara, Japan). Intracellular paclitaxel concentration of cultured cells was indicated as the total amount of paclitaxel per 10^6 cultured cells. The detection wavelength was 227 nm, and the limit of detection for paclitaxel was 5.0 ng. The intra-day and inter-day coefficients of variation of paclitaxel were 3.5% and 4.1% over the concentration range from 70 ng to 5.0 ng.

2.6 Statistical analysis

The data were collected and analyzed by origin 7.0 software. The results were expressed as mean ± SE. Comparisons between groups of data were carried out using Student's paired or unpaired *t*- test. Comparisons in one group of data were carried out using One-way ANOVA. *P*-value less than 0.05 was considered statistically significant. IC_{50} values were determined by non-linear regression by GraphPad Prism software.

3 Results

3.1 Changes of cellular morphology and kinetics

A series of MDR sublines, from A549/TXL5 to A549/TXL20, were established after culturing for more than 1 year, with paclitaxel concentration in the medium increased from 5 nM to 20 nM. Cells adapted to clonal growth, smaller in size and with a lobulated giant nuclei after the drug challenge. They showed increased N/C ratio (Table 1). However, they didn't show any significant changes in growth, saturation density, cell

area and cell perimeter (Table 1 and Figure 1).

3.2 Drug-resistant strength of MDR sublines

The drug resistance of MDR subline A549/TXL20 to paclitaxel was 19.3-fold stronger than the native line at the IC_{50} level (Table 2). The resistance against cisplatin was increased 67.4 fold. The resistance index remained stable for several weeks even after paclitaxel was withdrawn from the culture medium. Cross-resistance to mitomycin, vinblastine, hydroxycamptothecine, and 5-fluouracil (5-FU) was also observed in LA MDR sublines (Table 3).

Table 1. The biological characteristics of the A549 cell line and paclitaxel-induced multidrug resistance sublines.

Cell line	A549	A549/TXL5	A549/TXL10	A549/TXL20
Doubling time (h)	72.4	74.7	75.5	77.3
Saturation density ($\times 10^5$ cell/cm^2)	4.36	4.28	4.15	4.03
Cell area(μm^2)	352	261	242	214
N/C ratio	0.48±0.1	0.76±0.12	0.75±0.16	0.69±017*
Cell perimeter(μm)	76	75	72	68

*$P < 0.05$, compared with native cell line.

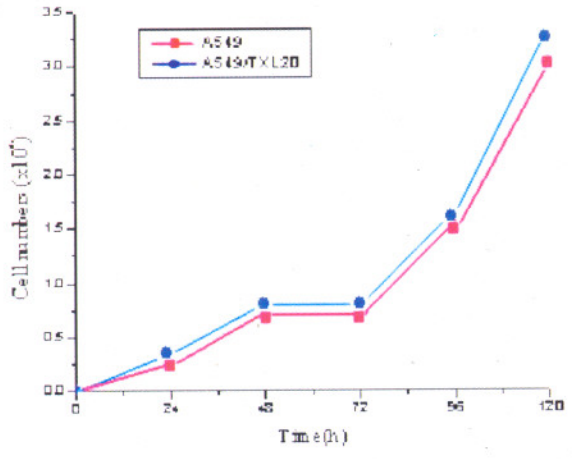

Figure 1. Growth curve of A549 cell line and A549/TXL20 cell line

3.3 Drug sensitivity evaluated by a colony-forming assay

Using the colony-forming assay, the *in vitro* drug sensitivity was evaluated by the surviving fraction of colony formation, after 24-hour exposure to paclitaxel (Figure 2). A549 cell line curves showed the colony forming ability was impaired after exposure to 0 − 1000 nM paclitaxel. On the other hand, the A549/TXL20 subline showed higher drug resistance, characterized by a much shallower slope in the curve of the surviving fraction versus drug dose, and its colony-forming ability was not impaired, even against 100 nM paclitaxel. Compared with the surviving fractions of A549, the resistance of A549/TXL20 was approximately 6,300 fold higher the resistance of against 1,000 nM paclitaxel.

Table 2. IC_{50} and resistance index of cell lines to paclitaxel and cisplatin.

Cell line	Paclitaxel		Cisplatin	
	IC_{50} (nM)	Resistance index	IC_{50} (μg/ml)	Resistance index
A549	81		0.065	
A549/TXL5	308	3.8	0.494	7.6
A549/TXL10	102	12.6	0.637	9.8
A549/TXL20	1560	19.3	4.381	67.4

Table 3. IC_{50} and resistance index of A549/TXL20 and A549 to nonrelated anticancer drugs

Anticancer drugs	IC_{50}(μg/ml)		Resistance index
	A549	A549/TXL20	
Mitomycin	0.039	0.298	7.64
Vinblastine	0.091	0.692	7.06
Hydroxy-camptothecine	0.072	0.096	1.33
5-FU	0.194	2.896	14.93

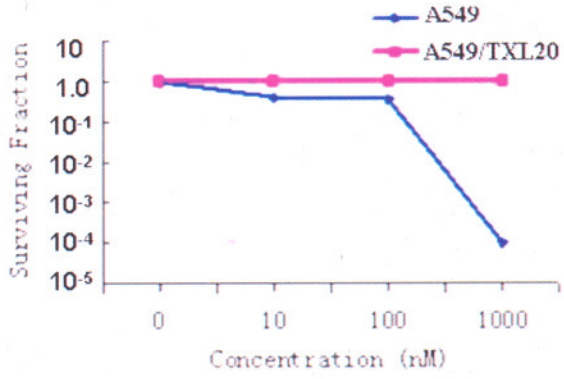

Figure 2. *In vitro* drug sensitivity of A549 and A549/TXL20 evaluated by a colonyforming assay. 10 days after 24-hour exposure to different concentrations of paclitaxel, the plating efficiency was determined by counting the colony number.

3.4 Intracellular drug concentration

Figure 3 showed intracellular paclitaxel concentrations in the cultured cells of A549 and A549/TXL20. At the 2nd hour and the 4th hour of paclitaxel exposure, A549/TXL20 cells contained about 5.6 ng, 0.91 ng paclitaxel respectively, which were much lower than that of A549 cells (64.1 ng and 63.8 ng respectively, $P < 0.05$).

4 Discussion

Clinical multidrug resistance to chemotherapeutic agents is a major obstacle to potentially curative treatment for LA[1]. Therefore, one approach to improve treatment is to study the biologic character of the multidrug resistance (MDR) to find ways to reverse it. MDR is characterized by cross-resistance to structurally and functionally unrelated drugs[9,10]. Although it has been reported that there are different kinds of mechanisms

which are responsible for MDR[11-13], the mechanism for MDR of LA is not fully elucidated[14,15]. In this study, we established the pacitexal - resistant human lung adenocarcinoma cell lines (A549/TXL) by exposing the stepwise increased concentration of pacitaxal in cell media to trace the underlying resistance mechanisms of acquired MDR.

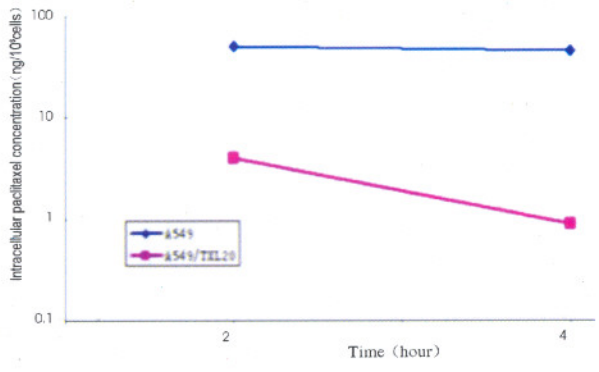

Figure 3. Intracellular paclitaxel concentration of cultured tumor cells. Paclitaxel concentration was measured by solid-phase extraction high performance liquid chromatography. Intracellular concentration of paclitaxel after 2-hour exposure of 100 nM paclitaxel.

As shown by tumor growth curves (Figure 1) and Table 1, the growth property of A549/TXL20 didn't change significantly compared with A549. In addition, compared with A549, decreased cellular size, and increased N/C ratio were shown in the A549/TXL20 (Table 1).

The paclitaxel-induced LA MDR sublines, A549/TXL20, demonstrated cross-resistance to various anticancer drugs, including mitomycin, vinblastine, hydroxycamptothecine and 5-FU, in addition to paclitaxel and cisplatin which are in the same family. The resistance of A549/TXL20 to cisplatin is much higher than to the original MDR-inducing drug, paclitaxel. The underlying mechanisms are not yet defined. These anticancer drugs have difference in structure and anticancer mechanisms. That means some common pathways may participate in the antidrug function of acquired MDR LA cells at the same time.

A549/TXL20 showed a higher level of paclitaxel-resistance which could be evaluated by drug sensitivity assays (Figure 2). Meanwhile, there was a significant difference in pharmacokinetics between A549 and A549/TXL20 after paclitaxel administration. The intracellular paclitaxel concentration in A549/TXL20 was significantly lower than in A549. These results suggested that the decreased influx and/or the increased efflux of the drug was into the A549/TXL20 cells.

In conclusion, morphological adaptation and intracellular changes could be evoked by drug challenge on LA cancer cells with acquired high drug resistance. The well-characterized MDR sublines may be used as an experimental system for the search of a means to overcome drug resistance and elucidate possible mechanisms of acquired MDR involved in human lung adenocarcinoma.

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