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RESISTANT MECHANISMS OF CISPLATIN IN HUMAN LUNG ADENOCARCINOMA CELL LINE A_{549}DDP

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To study the resistant mechanisms of cisplatin in human lung adenocarcinoma cell line A_{549}DDP. A_{549}DDP cells was established by stepwise increasing concentration of cisplatin (CDDP) in medium. Interstrand cross-linked DNA (ICL) was measured by ethidium bromide fluorescence assay. The intracellular and intranuclear accumulation of cisplatin was measured by atomic absorption spectrometry. The removal of GS-X was determined by FCM and fluorescence microscopy. Results: The A_{549}DDP cell line was 8.9-fold resistance relative to the parental A_{549} cell line. The formation of ICL in A_{549} was 6.28 times higher than that in A_{549}DDP cells. The intracellular and intranuclear accumulation of cisplatin in A_{549} cells was 5.9 times and 4.1 times higher than that in A_{549}DDP cells, respectively. The ability of GS-X pump pumped GS-X complex (GS-Pt) in A_{549}DDP cells was higher than that in A_{549}. The repair rate in A_{549}DDP cells was 2 times higher than that in A_{549}. Conclusions: Decreased accumulation and increased export of cisplatin might be the main mechanism of cisplatin resistant A_{549}DDP cells while the enhanced repair capacity of DNA may play a role in CDDP resistance.

Key words: Resistance mechanism, Human A_{549}, Cisplatin, Interstrand cross-link, Accumulation, DNA repair capacity.

Cisplatin (CDDP) is one of the most effective antitumor agents and has been used broadly in the treatment of lung, testicular, ovarian, head and neck cancers.[1] However, its usefulness is limited by the rapid development of acquired resistance, and which is often regarded as one the causes of chemotherapeutic failure. Therefor, an understanding of the mechanisms underlying the process of acquired (CDDP) resistance is the main problem to be solved preclinically. The resistant mechanisms of cisplatin are considered to be as following: (a) decreased accumulation of cisplatin; (b) enhanced repair capacity of cisplatin-DNA adducts; (c) increased inactivation of cisplatin by detoxification system, etc.[2,3] In an attempt to understand the resistant mechanism of cisplatin further, a cisplatin-resistant human lung adenocarcinoma cell line A_{549}DDP cells was established by stepwise increasing concentration of CDDP in cell culture medium. The formation of interstrand cross-link, the intracellular and intranuclear accumulation of cisplatin, the excretion of GS-X, the repair capacity in A_{549}DDP cell lines as well as in its parental cell line A_{549} was studied.

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MATERIALS AND METHODS

Materials

RPMI-1640 was purchased from Gibco; Fetal bovine serum was obtained from Institute of Hematology, Tianjin; Cisplatin was purchased from Shandong Qilu Pharmaceutical Factory; Thiazollblue, ethidium bromide, sarkosyl, nonidet P-40 were purchased from Sigma; Monochlorobimane (MCB)

was purchased from Calbiochem.

Cell Culture

A_{549} cell line was incubated in RPMI-1640 medium containing 15% fetal bovine serum and with a stepwise increasing concentration of CDDP for 12 months. The selected A_{549}DDP cell line was incubated in the medium containing 12 μMol.L^{-1} CDDP for more than 4 months, and the resistant phenotype of the A_{549}DDP cell was stable for more than 5 months in the absence of CDDP.

Cytotoxicity

The cytotoxicity of CDDP on A_{549} and A_{549}DDP was determined by MTT assay. The ratio of IC_{50} of the resistant cell line A_{549}DDP/that of the sensitive cell line A_{549} was designated as resistant fold.

Determination of Interstrand Cross-link (ICL)

Renaturation of DNA after denaturation and rapid cooling is related to the formation of DNA cross-links. Double stranded renatured DNA were stained selectively by ethidium bromide and measured by fluorescence assay (EFA).[4] The A_{549} and A_{549}DDP cell lines were exposed to various concentrations of CDDP for 1 h., washed with hank's balanced solution, after lysed, ICL was determined by fluorescence assay. For repair study, the cells were incubated with 200 μMol.L^{-1} CDDP for 2 hr. After removed the drug, the cells were maintained in fresh medium free of CDDP, then the ICL was measured at 0 and 24 hr., the ratio between the ICL at 24 hr. and at 0 h. was designated as repair rate.

Accumulation of Intracellular and Intranuclear Concentration of Cisplatin

Accumulation: 2×10^7 A_{549} and A_{549}DDP cells were treated with various concentrations of CDDP for 1 h., washed twice and solubilized in 0.5 ml nitric acid at an electric dry plate (260 ℃). The final volume was adjusted to 200 μl and measured by atomic absorption spectrometry.[5] For determining the accumulation of CDDP in intranuclear, the treated cells were washed twice, the cells pellets were resuspended in NTE buffer (0.1 M NaCl, 0.01 M Tris-EDTA) and lysed with 5% Np40. Nuclei were obtained by centrifugation.

Retention: After the A_{549} and A_{549}DDP cells were treated with 60 μMol.L^{-1} CDDP for 1 h., the cells were washed with cold PBS three times at 4 ℃ and then maintained in fresh medium without drugs, the concentrations of cisplatin were measured at different intervals. We take the concentration at 0 h. as 100%, the relative content at other time was compared to it.

Removal of GS-X

Monochlorobimane (MCB), a nonfluorescence compound, is specially conjugated with glatathione (GSH) in the cell by the action of glutathione S-transferase, and the resulting glatathione-S conjugate (GS-X) exhibits intense fluorescence. GS-X was excreted by GS-X export pump.[6] GSH reacts with CDDP, forms GSH-cisplatin complex (GS-Pt) which was also excreted by the GS-X pump.[2] Therefor, we use MCB as a indicator to study the excretion of GS-Pt indirectly by FCM and fluorescence microscopy.[7] 1×10^6 cells/ml were incubated with 20 μM MCB at 37 ℃ for 20 min., washed and incubated in the MCB-free medium for 0, 45, 90 min. The fluorescence intensity of the cells was analysed by FCM and observed under fluorescence microscopy.

RESULTS

Cytotoxicity Results

After have exposed to CDDP. The IC_{50} of both cell lines was calculated. The IC_{50} for A_{549} and A_{549}DDP was 9, 80 μMol.L^{-1}, respectively. The resistant fold was 8.9 (Figure 1).

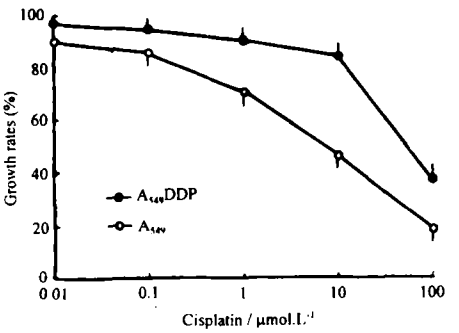

Fig. 1. Relationship between cisplatin concentration and survival rate of A_{549} and A_{549}DDP cell lines. n=3, $\bar{x}\pm s$

Formation of ICL

The formation of ICL in parental A_{549} cells was 6.28-fold higher than that of the resistant A_{549}DDP cells. The ICL was directly proportion to the concentration of CDDP (Figure 2).

Accumulation of Cisplatin

Intracellular and intranuclear concentrations of cisplatin in A_{549}DDP cells was 5.9-fold and 4.1-fold lesser than that in A_{549} cells, respectively (by the ratio of slope of regression line). Both of them were also directly proportional to the concentrations of CDDP (Figure 3). The CDDP content exported from A_{549}DDP cells more quickly than that from A_{549} cells, especially during the first 4 h. There is only a little difference between the A_{549}DDP cells and A_{549} cells about the excretion of cisplatin from nucleus (Table 1).

The nuclei-cytoplasm ratio (N/S) in A_{549}DDP cells was 50% that in A_{549} (Table 2).

Removal of GS-X

After incubated the A_{549} cells and the A_{549}DDP cells with MCB, the florescence intensity of the A_{549}DDP cells decreased dramatically during 0–90 min., although the initial fluorescence intensity in resistant A_{549}DDP cells was even higher than that in A_{549} cells (Figure 4). This was also showed by FCM as a shift of the fluorescence intensity peak (Figure 5).

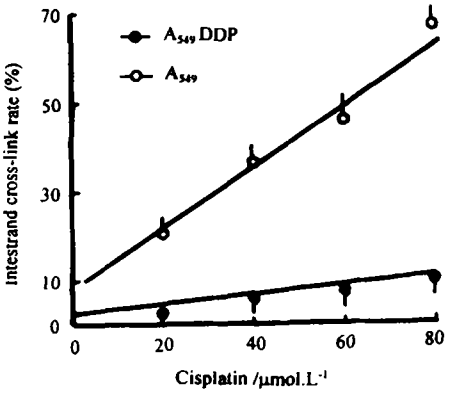

Fig. 2. Relationship between cisplatin concentration and intrastrand cross-link rate in A_{549} and A_{549}DDP cell lines. n=3, $\bar{x}\pm s$

The Repair of ICL

The repair rate in the A_{549}DDP cells is 2 times higher than that in A_{549} cells (Table 3).

Table 1. Relationship between the relative cellular (C) and nuclear (N) retention of cisplatin and the time of post-cisplatin in A_{549} and A_{549}DDP n=3, $\bar{x}\pm s$

	0 h	4 h	8 h	12 h
A_{549} (C)	519.8± 78 (100%)	445.7± 39.6 (85.7%)	396.2± 59 (76.2%)	336± 3.65 (64.7%)
A_{549}DDP (C)	174.2± 27 (100)%	99± 13 (56.8%)	75.5± 8.9 (43.31 %)	78.7± 9.8 (22.2%)
A_{549} (N)	98.45± 8.6 (100%)	86± 8 (87.7%)	84± 9.1 (85.3%)	72.3± 5.3 (73.8%)
A_{549}DDP (N)	16.8± 1.54 (100%)	14.33± 3.2 (85.3%)	13.29± 2.89 (79.1%)	10.3± 0.74 (61.2%)

Table 2. Intracellular (C) and intranuclear (N) accumulation of cisplatin after exposed of the A_{549} *and* A_{549}*DDP cell lines to 60 μMol.L^{-1} cisplatin for 1 h, n=3, $\bar{x}\pm s$, ng 2×10^7 cells^{-1}*

	A_{549}	A_{549}DDP
C	519.8± 78	174.2± 27*
N	98.5± 8.6	16.8± 1.54*
N/C	0.198	0.096

*$P<0.01$

DISCUSSION

The mechanism of resistance to cisplatin is still controversial, although several kinds of processes have been proposed. For elucidation of the mechanism of CDDP resistance, we examined the formation of DNA interstrand cross-links (ICL), and its repair capacity in CDDP-resistant human non-small cell lung cancer cells (A_{549}DDP/8.9-fold resistance).

We measured the intracellular and intranuclear concentrations of CDDP and the N/C ratio. We also examined the pumping of GS-X in both of the A_{549}DDP and the A_{549} cell lines. Our results showed a 6.28-fold reduction of formation of ICL in the resistant A_{549}DDP cells than that in the sensitive A_{549} cells. A 6.28-fold reduction is close to the 8.9-fold resistance of A_{549}DDP to A_{549}, therefore it suggests that ICL was the main cytotoxical form of CDDP. These results are very similar to those of Bungo's work.[8]

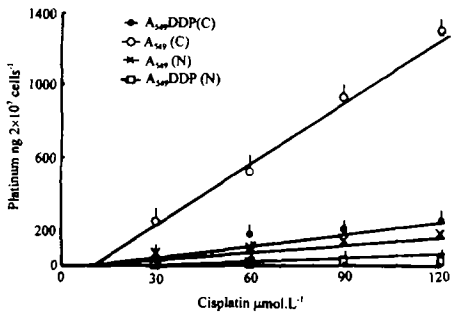

Fig. 3. Relationship between intracellular (C) and intranuclear (N) accumulation of cisplatin and cisplatin contation in A_{549} and A_{549}DDP cell lines. n=3, $\bar{x}\pm s$

Table 3. Percentage of ICL in the A_{549} and the A_{549}DDP cell lines after exposed to cisplatin 100 μMol.L⁻¹ for 2 h, n=3, $x\pm s$.

	0 h	21 h	Repair (%)
A_{549}	0.775± 0.093	0.517± 0.11	33.3
A_{549}DDP	0.468± 0.081	0.151± 0.042	67.8*

*$P<0.01$

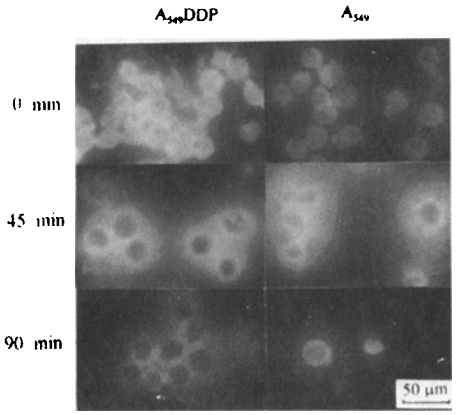

Fig. 4. Fluorescence microscopic analysis of the A_{549} and A_{549}DDP incubated with monochloribimane.

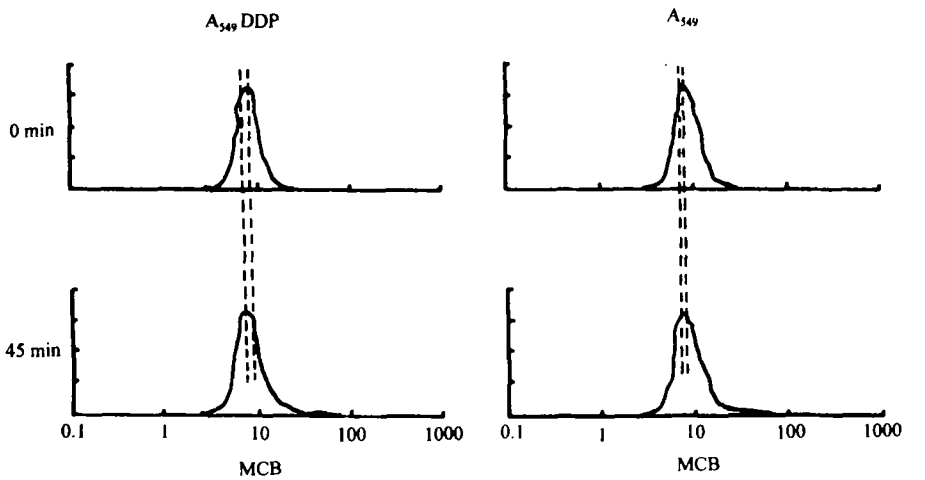

Fig. 5. Flow cytometric analysis of the fluorescence of A_{549} and A_{549}DDP incubated with monochloribimane.

In an attempt to elucidate the cause of reduction of ICL, a 5.9-fold decreased intracellular accumulation of cisplatin was found in the resistant A_{549}DDP cells than that of the A_{549} cells, which suggested that the decreased accumulation of CDDP caused the reduction of ICL.

The decreased accumulation of cisplatin may be the results of enhanced export and decreased uptake. Our results shew the former was the main mechanism. Apart from decreased accumulation of cisplatin, the lower nuclei-cytopalsm ratio suggested that the CDDP was obstructed from entering nuclei in the resistant A_{549}DDP cells.

The 2-fold increased repair activity in the resistant A_{549}DDP cells also suggested it might make some contribution to CDDP resistance.

We have showed that the A_{549}DDP resistance related to GSH levels, GST contents in our previous study.[9] In this investigation, our results suggested that decreased accumulation, enhanced export of DNA-Pt consisted one of the main resistant mechanisms while the increased repair activity also play some roles in it.

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