Establishment and biological characteristics of oxaliplatin-resistant human colon cancer cell lines

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Abstract

Background and Objective: Chemotherapy is the main treatment for colon cancer, while multidrug-resistance is the main reason for chemotherapy failure and tumor relapse. This study was to establish two oxaliplatin-resistant colon cancer cell lines and evaluate their biological characteristics. Methods: Oxaliplatin-resistant colon cancer cell lines SW620/L-OHP and lovo/L-OHP were established in vitro by continuous exposure to oxaliplatin (L-OHP) of low and gradually increased concentration. Growth curve, cross-resistance and resistance index of the oxaliplatin-resistant cell lines to various anti-cancer agents were determined by CCK8 assay. The expressions of P-glycoprotein (P-gp), multidrug-resistance protein 1 (MRP1) and MRP2 were detected by Western blot. Cell cycle distribution as well as the expression of CD133 and CD44 were measured by flow cytometry. Results: It took 10 months to establish the SW620/L-OHP and LoVo/L-OHP cell lines with stable resistance to oxaliplatin. Cross-resistance to 5-fluorouracil, etoposide, cisplatin, vincristine and epirubicin but not to paclitaxel was observed. Longer doubling time, higher proportion of cells in G0/G1 phase and lower proportion in G2/M phase were observed in the two oxaliplatin-resistant cell lines compared with their parental cell lines. The expression of MRP2 in the oxaliplatin-resistant cells was up-regulated, while those of P-gp and MRP1 had no significant change. CD133 was overexpressed while CD44 level remained unchanged in SW620/L-OHP and LoVo/L-OHP cells. Conclusions: SW620/L-OHP and LoVo/L-OHP cell lines show a typical and stably resistant phenotype and may be used as research models.

Key words: Drug resistance, oxaliplatin, colon neoplasms, P-gp, MRP1, MRP2, CD133

Colon cancer is one of the common types of cancer in China. Chemotherapy is an important treatment for colon cancer[6]. Oxaliplatin (L-OHP), a third-generation platinum-based drug, is one of the first-line drugs in colon cancer treatment. It has been reported in some clinical trials that as the standard first-line treatment of advanced colon cancer, the FOLFOX regimen [L-OHP, 5-fluorouracil (5-FU) plus leucovorin (LV)] can significantly increase the response rate up to 54% and the median survival to nearly two years. However, when switching to second-line treatment as the first-line chemotherapy fails, the response rate drops to only 4%[6]. Failure of chemotherapy greatly results from the acquired or congenital multidrug resistance (MDR) against various chemotherapeutic agents in a few cancer cells[6]. Certain cancer cells survive the chemotherapy and proliferate infinitely, which finally causes the death of the patient. Some researches showed that cancer stem cells may play an important role in this drug-resistant course[6]. Hence, it is necessary to investigate the mechanism of L-OHP-resistance in colon cancer cells and provide new methods of overcoming this resistance in clinical practice. In this study, we used L-OHP as an in vitro inducer to establish two L-OHP-resistant human colon cancer cell lines and explore the mechanism of this resistance.
Materials and Methods

Materials

RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from Gibco BRL company; CCK8 from Dojindo company; L-OHP from Sanofi-Aventis company; 5-FU from Jinyao Amino Acid company (Tianjin); cisplatin (DDP) from Gejiu Bio-Pharmaceutical (Yunnan); paclitaxel (PTX) from Taiji company; epirubicin (EPI) from Pfizer company; etoposide (VP-16) from Sunnyhope Pharmaceutical company (Sichuan); and vincristine (VCR) from Minsheng Pharmaceutical company (Hangzhou).

Establishment of drug-resistant cell lines SW620/L-OHP and LoVo/L-OHP

The human colon cancer cell lines SW620 and lovo were preserved by our own lab. Oxaliplatin-resistant cell lines SW620/L-OHP and LoVo/L-OHP were induced by continuous exposure to L-OHP of low and gradually increased concentrations. When the cells were in logarithmic growth phase, L-OHP was added to the medium to a final concentration of 0.01 mg/L. After a 24-hour incubation, the old medium was discarded and fresh medium was added. Cells were passed when they were 80% confluent and L-OHP of 0.01 mg/L was then added. The concentration of L-OHP was gradually increased after the cells had grown stably. Finally, a cell line resistant to L-OHP of 0.2 mg/L was derived from SW620 and kept in complete medium containing L-OHP of 0.2 mg/L; a cell line resistant to L-OHP of 2 mg/L was derived from lovo and kept in complete medium containing L-OHP of 2 mg/L.

Cytotoxicity assay of various anti-cancer drugs using CCK8

Single cell suspension (8 × 10^3/mL, 100 μL) was dispensed in a 96-well plate. After a 24-hour pre-incubation, the old medium was replaced by medium containing L-OHP of 10 different concentrations, which was replaced in turn with fresh medium after another 24-hour incubation. After 72 h, CCK8 was added for another 4-hour incubation. Then the absorbance (A value) at 490/630 nm was measured using a UV spectrophotometer. An effect-dose curve was drawn to calculate 50% inhibition concentration (IC_{50}) and resistance index (RI). RI = IC_{50} of drug-resistant cell line / IC_{50} of parent cell line.

Population doubling time and growth curves

Drug-resistant cells were dispensed in a 96-well plate (800 cells/well). Three wells were taken each day and CCK8 was added in for a 4-hour incubation. A value at 490/630 nm was measured using a UV spectrophotometer. This procedure was repeated for 6 continuous days. Growth curves were drawn to calculate population doubling time according to the Patterson formula.

Flow cytometry of cell cycle distribution

Single cell suspension was collected and washed with cold PBS for 3 times, then fixed with 70% ethanol at 4°C overnight. After centrifugation, the supernatant was discarded and the cells were washed with cold PBS twice. Then the cells were stained with propidium iodide (PI). Flow cytometry (FCM) was performed to analyze cell cycle.

Detection of P-gp, MRP1, and MRP2 expressions in SW620, SW620/OHP, LoVo and LoVo/L-OHP cells by Western blot

After 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto a PVDF membrane. The membrane was blocked at room temperature for 2 h and then washed. Then the membrane was incubated with 1:500 diluted P glycoprotein (P-gp) antibody, 1:500 diluted multidrug-resistant protein 1 (MRP1) antibody and 1:500 diluted MRP2 antibody at 4°C overnight, washed with TBST and then incubated with secondary antibody at room temperature for 2 h. After washed with TBST, the membrane was analyzed using an Odyssey Infrared Fluorescence Detector.

Flow cytometry for CD133 and CD44 expression detection in SW620, SW620/OHP, LoVo and LoVo/L-OHP cells

After dissociation and centrifugation, the cells were washed with PBS once and incubated with FITC-labeled anti-CD133 and PE-labeled anti-CD44 in an ice bath in dark for 45 min. Then the cells were washed with FACS buffer for 3 times. FCM was performed, with anti-mouse IgG being the isotype control.

Statistical analysis

The significance of difference between two paired groups was determined by the Student’s t-test using the
Successful establishment of drug-resistant cell lines from SW620 and LoVo

Two L-OHP-resistant cell lines SW620/L-OHP (resistant to L-OHP of 0.2 mg/L) and LoVo/L-OHP (resistant to L-OHP of 2 mg/L) were successfully induced by continuous exposure to L-OHP of low and gradually increased concentrations after 10 months for more than 100 cell passages. The cell lines were used for experiments after another 2 months of culture in drug-free medium. Figure 1 shows the morphologic differences between the resistant cells and their parent cells under light microscope.

Stability of drug resistance

According to the results of CCK8 assay, the IC_{50} of L-OHP for SW620/L-OHP cells was 0.337 mg/L, and RI was 21.06. After 2 months of drug-free culture, the IC_{50} was 0.316 mg/L and RI was 19.750. Thus 93.78% drug-resistance was preserved. The IC_{50} for lovo/L-OHP was 3.513 mg/L, and RI was 13.780. After 2 months of drug-free culture, the IC_{50} was 3.162 mg/L and RI was 12.4. Thus 90.00% resistance was preserved. In addition, the SW620/L-OHP and LoVo/L-OHP cells grew stably after they had been frozen for 3 months and then thawed, and the IC_{50} then was 0.337 mg/L and 3.513 mg/L, respectively. Thus freezing and thawing did not influence the drug-resistance of these cell lines.

Multidrug-resistance

The SW620/L-OHP and LoVo/L-OHP cells also showed cross-resistance to 5-FU, VP-16, DDP, VCR and EPI to various degrees, but were still sensitive to PTX (Table 1).

Population doubling time and growth curves

The growth curves of the parent cells and the drug-resistant cells (Figure 2) show that the drug-resistant cells grew more slowly. The population doubling time of SW620/L-OHP cells was significantly longer than that of SW620 cells by 5.41 h (P = 0.006), and the population doubling time of LoVo/L-OHP cells was significantly longer than that of lovo cells by 3.34 h (P = 0.005).

Cell cycle distribution

After gaining drug-resistance by L-OHP induction, the cells proliferated more slowly in the logarithmic phase. The results of FCM showed an increase in the proportion of cells in G_{0}/G_{1} phase (38.9% vs. 29.0%, P = 0.003) and a decrease in the proportion in G_{2}/M phase (15.6% vs. 31.5%, P < 0.001) in SW620/L-OHP cells compared with SW620 cells, and an increase in the proportion of cells in G_{0}/G_{1} phase (61.2% vs. 50.1%, P = 0.001) and a decrease in the proportion in G_{2}/M phase (10.6% vs. 18.1%, P = 0.001) in LoVo/L-OHP cells compared with LoVo cells. All these changes were of statistical significance (Figure 3).

P-gp, MRP1 and MRP2 expressions in SW620, SW620/OHP, LoVo and LoVo/L-OHP cells

Western blot analysis showed that comparing with their parental cells, the expression of MRP2 protein in the resistant cells was up-regulated, while those of P-gp and MRP1 had no significant change (Figure 4).

CD133 and CD44 expressions in SW620, SW620/OHP, LoVo and LoVo/L-OHP cells

The results of FCM showed that comparing with their parental cells, CD133 was overexpressed (9.6% vs. 4.6%)
significant change (0.2% vs. 0.2%) in LoVo/L-OHP cells, and similarly CD133 was overexpressed (0.9% vs. 0.3%) while CD44 level did not significantly change (0.2% vs. 0.2%) in SW620/L-OHP cells, and similarly CD133 was overexpressed (0.9% vs. 0.3%) while CD44 level did not significantly change (0.2% vs. 0.2%) in LoVo/L-OHP cells (Figure 5).

**Discussion**

Oxaliplatin, a third-generation platinum-based drug, is the only platinum-based drug that has anti-tumor activity in colon cancer. Its cytotoxicity is resulted from inhibition of DNA synthesis by cross-linking adjacent guanine bases or adjacent guanine and adenine [6]. Resistance occurs when this DNA damage is repaired by nucleotide excision repair (NER) mechanism [6]. However, recent studies on the mechanism of platinum drug-resistance mainly focus on cisplatin, but seldom on L-OHP. Studies on the establishment of in vitro L-OHP-resistant model are also rarely seen. Therefore, in

### Table 1  Sensitivity and cross-resistance of SW620, SW620/L-OHP, LoVo and LoVo/L-OHP cells to seven chemotherapeutic drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} (mg/L) SW620</th>
<th>IC_{50} (mg/L) SW620/L-OHP</th>
<th>RI</th>
<th>P</th>
<th>IC_{50} (mg/L) LoVo</th>
<th>IC_{50} (mg/L) LoVo/L-OHP</th>
<th>RI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-OHP</td>
<td>0.016 ± 0.004</td>
<td>0.337 ± 0.084</td>
<td>21.06</td>
<td>0.022</td>
<td>0.255 ± 0.025</td>
<td>3.513 ± 0.329</td>
<td>13.78</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.018 ± 0.005</td>
<td>0.105 ± 0.017</td>
<td>5.83</td>
<td>0.001</td>
<td>0.085 ± 0.007</td>
<td>0.249 ± 0.018</td>
<td>2.93</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>VP-16</td>
<td>0.175 ± 0.084</td>
<td>0.400 ± 0.027</td>
<td>2.29</td>
<td>0.011</td>
<td>1.241 ± 0.251</td>
<td>2.354 ± 0.484</td>
<td>1.90</td>
<td>0.024</td>
</tr>
<tr>
<td>PTX</td>
<td>0.015 ± 0.001</td>
<td>0.015 ± 0.002</td>
<td>1.00</td>
<td>0.815</td>
<td>0.738 ± 0.142</td>
<td>0.847 ± 0.114</td>
<td>1.15</td>
<td>0.359</td>
</tr>
<tr>
<td>VCR</td>
<td>0.014 ± 0.002</td>
<td>0.027 ± 0.007</td>
<td>1.93</td>
<td>0.038</td>
<td>0.289 ± 0.027</td>
<td>0.478 ± 0.058</td>
<td>1.65</td>
<td>0.007</td>
</tr>
<tr>
<td>DDP</td>
<td>0.122 ± 0.065</td>
<td>0.634 ± 0.168</td>
<td>5.20</td>
<td>0.008</td>
<td>0.525 ± 0.113</td>
<td>3.188 ± 0.725</td>
<td>6.07</td>
<td>0.003</td>
</tr>
<tr>
<td>EPI</td>
<td>0.004 ± 0.001</td>
<td>0.034 ± 0.003</td>
<td>8.50</td>
<td>&lt; 0.001</td>
<td>0.252 ± 0.020</td>
<td>1.271 ± 0.041</td>
<td>5.04</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

L-OHP, oxaliplatin; 5-FU, 5-fluorouracil; VP-16, etoposide; PTX, paclitaxel; VCR, vincristine; DDP, cisplatin; EPI, epirubicin. The sensitivity of colon cancer cell lines to seven antitumor drugs was evaluated using the CCK8 assay as described in methods. The 50% inhibition concentration (IC_{50}) of antitumor drugs was calculated. The IC_{50} values are presented as mean ± standard deviation. SW620/L-OHP and LoVo/L-OHP cells exhibit moderate cross-resistance to cisplatin and epirubicin, but are sensitive to paclitaxel.

Figure 2  Growth curves of SW620, SW620/L-OHP, LoVo and LoVo/L-OHP cells

The curves of L-OHP-resistant cells rise up slowly. The doubling time of SW620 cells (●) is 29.53 h and that of SW620/L-OHP cells (●) is 34.94 h, on the other hand, the doubling time of LoVo cells (●) is 24.77 h and that of LoVo/L-OHP cells (●) is 28.11 h.

Figure 3  Cell cycle distribution of parental cells and L-OHP-resistant cells

Cell cycle determined by FCM shows a decrease of the proportion of cells in G2/M phase and an increase of the proportion in G0/G1 phase in SW620/L-OHP cells (D) and LoVo/L-OHP cells (B) compared with SW620 cells (C) and LoVo cells (A).

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In the current study, we established two in vitro L-OHP-resistant cell models to provide the basis for further studies on the mechanism of L-OHP-resistance in colon cancer.

One of the major approaches of studying the mechanism of MDR in tumor cells is to establish drug-resistant cell lines in vitro. In this study, it took 10 months for us to successfully establish two L-OHP-resistant human colon cancer cell lines SW620/L-OHP and LoVo/L-OHP through continuous exposure to L-OHP of low and gradually increased concentrations. These two cell lines have shown stable drug-resistance and consistent biological characteristics.

Cell cycle is arrested in G2/M phase by platinum-based drugs through their inhibitory effect on cyclin-dependent kinase (CDK) activity. Carole Voland et al. reported that cells were arrested in G2 phase by L-OHP platinum as a result of inhibition of G2/M phase transition and thus inhibition of cell division [7], an increase in cells in G2/M phase finally led to apoptosis, which was showed as
cytotoxicity of the drug[8]. In the current study, an increase in the proportion of cells in G2/M phase and S phase as well as a decrease in the proportion in G0/M phase were observed in the two L-OHP-resistant cell lines. Activation of DNA repair mechanisms in the resistant cells might be the possible reason of these changes, which helped the cells pass through G0 phase to M phase and subsequently divide and proliferate, which in turn resulted in a shift of cell cycle distribution. These findings may provide the basis for the selection of cell cycle-specific chemotherapeutic drugs for L-OHP-resistant patients in clinical treatment.

The L-OHP-resistant cell lines also showed cross-resistance to platinum-based drugs such as DDP, as well as to some other anti-cancer drugs that act via different mechanisms. Some researchers have suggested a grading system depending on RI: low (< 5), medium (5–15) and high (> 15)[9]. According to this system, SW620/L-OHP and lovo/L-OHP cells both showed medium resistance to DDP and EPI and low resistance to VP-16 and VCR, but no resistance to PTX. In addition, SW620/L-OHP and LoVo/L-OHP cells showed medium and low resistance, respectively, to 5-FU.

One common mechanism of drug-resistance is a decrease in the accumulation of platinum compounds in the cells, which is thought to be closely related to the exporter ATP7B but not associated with P-gp and MRP1[10]. Liedert et al. [7] demonstrated an increase in both MRP2 mRNA and protein levels in cells resistant to platinum-based drugs, but no significant change in mRNA and protein levels of other ATP-binding cassette transporters, which is consistent with the L-OHP-resistant cell lines in our present study. It has also been reported that it is the ATP-dependent GS-X pump instead of P-gp that accounts for the enhanced exportation of platinum drugs in the platinum-resistant cells.

MRP (ABCC) proteins are membrane transporters that can use the energy of ATP hydrolysis to carry out their “drug pump” function [11]. These proteins are 15% homologous to P-gp in amino sequence but do not act synergistically with P-gp in term of their “drug pump” function. P-gp acts on a variety of drugs, including doxorubicin (ADM), VCR, VP-16, PTX, and anthrancene derivatives [12], whereas MRP transfers cytotoxic drugs that are able to bind reduced glutathione (GSH), such as ADM, VP-16, and VCR[13], and alters their intracellular distribution[14]. Resistance to EPI, VP-16, and VCR [15–17] is P-gp/MPR-mediated; however, neither P-gp nor MRP1 was up-regulated in LoVo/L-OHP and SW620/L-OHP cells, which suggests that the cross-resistance to EPI, VP-16, and VCR in these two cell lines was possibly MRP2-mediated. Some researches showed that overexpression of MRP2 resulted in cross-resistance to VP-16, DDP, ADM, and EPI[18]. MRP2-mediated resistance to certain drugs is associated with GSH level, and the MRP2-mediated resistance to VP-16, ADM, and DDP can be reversed by the GSH inhibitor BSA. Moreover, in our present study, the L-OHP-resistant cells also demonstrated low to medium resistance to 5-FU. 5-FU-resistance is believed to result from several mechanisms [19], including changes in the activities of thymidylate synthetase (TS), dihydropyrimidine dehydrogenase (DPD) and folic acid metabolism-related enzymes, DNA mismatch repair (MMR)[20], and so on, but not associated with the expressions of P-gp and MRP. These imply that aberration of DNA repair mechanisms may also play an important role in 5-FU-resistance. In addition, the two L-OHP-resistant cell lines in our study showed no cross-resistance to PTX, which may be because PTX-resistance is mediated by P-gp rather than by MRP2[21,22]. This provides an important basis for selecting sensitive second-line drugs when patients fail to respond to L-OHP as the first-line drug in clinical treatment.

Cancer stem cells are stem cell-like cells that exist in the hematopoietic system or some solid tumors. The cancer stem cell hypothesis suggests that during chemotherapy tumor size reduces but a small number of stem cells survive, which cause relapse and metastasis [23]. The self-protective mechanism of stem cells against cytotoxic drugs remains to be elucidated. But it is currently believed that the major mechanisms involve the drug pump activity of ABC transporters [24], overexpression of anti-apoptotic genes, DNA repair, increase in cells in G0/G1 phase, and slowdown of the proliferation of stem cells to evade the action of chemotherapeutic drugs [25]. Stem cells enriched from U87MG cells overexpress CD133 and are cross-resistant against ADM, VP-16, carboplatin and BCNU [26]. In our present study, MRP2 up-regulation, slowdown of cell proliferation, and an increase in G0/G1 phase cells were observed in the L-OHP-resistant cell lines, which implied a stem cell phenotype. Furthermore, the proportion of CD133+ cells also increased as the cells became resistant to L-OHP. These all may suggest an important role of cancer stem cells in the development of MDR in the L-OHP-resistant cell lines. Thus it is possible to sensitize the tumor tissue to L-OHP and reverse its resistance by using stem cell-targeting therapy.

In the current study, we successfully established two L-OHP-resistant human colon cancer cell lines SW620/L-OHP and lovo/L-OHP. They are stable in cell passage and drug-resistance, and their biological characteristics are highly consistent. These cell lines may serve as ideal models for the study of the mechanism and the way of reversal of L-OHP-resistance in colon cancer.

References


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