FaDu cell characteristics induced by multidrug resistance

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Abstract. The major obstacle to tumor chemotherapy is drug resistance. In the present study, we investigated the characteristics of FaDu cells (a hypopharyngeal carcinoma cell line) with multidrug resistance (MDR) induced by Taxol. The resistant cell line, FaDu/T, was grown by exposing normal FaDu cells to escalating concentrations of Taxol stepwise for over 12 months. The multidrug resistant sensitivities of the FaDu/T cells to cisplatin (DDP), 5-fluorouracil (5-FU), doxorubicin (Dox) and vincristine (VCR) were investigated by methyl-thia-zolyl-tetrazolium (MTT) assay. Cell apoptosis was measured by acridine orange and Hoechst 33342/propidium iodide double staining. Cell cycle distribution and the cell apoptosis index were quantified using flow cytometry. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted to determine the mRNA levels of the MDR-related genes MDR1/ABCB1 and BCRP/ABCG2. Western blotting was used to assay the expression of MDR1/ABCB1 and BCRP/ABCG2 and the apoptosis-related proteins caspase-3, Bcl-2 and Bax. Compared with the FaDu cells, the drug resistance of FaDu/T cells to DDP, 5-FU, Dox and VCR was increased 8.99-, 21.96-, 31.42- and 10.00-fold, respectively. The percentages of FaDu/T cells in the G0/G1 and G2/M phases were increased while the cell percentage in the S phase decreased as compared with the percentages of FaDu cells. The anti-apoptotic ability increased prominently, as the index of apoptosis decreased. Furthermore, caspase-3, Bcl-2 and Bax expression was altered accordingly to resist apoptosis in the FaDu/T cells. MDR1/ABCB1 expression increased significantly at both the mRNA and protein levels, while BCRP/ABCG2 expression appeared to inversely affected, i.e. decreased, in a concentration-dependent manner. These findings may provide theoretical support for the prevention of MDR in clinical cancer chemotherapy.

Introduction

Hypopharyngeal carcinoma, one of the most common malignancies in the head and neck region, results in substantial morbidity and mortality annually. Despite the fact that multimodal approaches have been applied to treat this tumor, the survival rate has not considerably improved during the last two decades (1,2). Traditionally, the main treatment option for primary hypopharyngeal carcinoma is surgical resection (3). This strategy, however, has evolved from the point of view of pure treatment to maximum preservation of hypopharyngeal function, laying emphasis upon the quality of life of these patients. Thus, the standard approach for treatment for these patients is still chemotherapy.

A large number of chemotherapeutic agents are currently in use. Paclitaxel (Taxol®) was first approved by the Food and Drug Administration (FDA) for the treatment of refractory ovarian cancer in 1992 but demonstrated activity against head and neck squamous cell carcinoma (HNSCC) (4). Yet, the effect of Taxol for HNSCC is still far from satisfactory. An important limitation is the occurrence of multidrug resistance (MDR), by which cancer cells escape the toxic effect of most commonly used cancer drugs in spite of their different chemical structure and different mechanism of intracellular activity (5). Drug concentrations targeted to cells decrease and the sensitivity of cells decline, both of which lead to chemotherapeutic failure and limited clinical effect of chemotherapy. Thus, elucidation of the underlying molecular mechanisms leading to MDR and therapy failure is crucial (6).

With the aim to elucidate the mechanism of MDR, we carried out the present study using the human hypopharyngeal carcinoma cell line FaDu. A multidrug resistant cell line to Taxol (FaDu/T) was established by exposing normal FaDu cells to escalating concentrations of Taxol stepwise for over 12 months. The main characteristics between the FaDu and FaDu/T cells were investigated. Our results showed that, compared with FaDu cells, the percentage of FaDu/T cells in the G0/G1 phase and their anti-apoptotic ability were both increased. Expression of caspase-3, Bcl-2 and Bax was altered accordingly to resist apoptosis in the FaDu/T cells. Multidrug resistance-related genes MDR1/ABCB1 increased and BCRP/ABCG2 decreased to counteract the Taxol toxicity in FaDu/T cells. These findings may provide theoretical support for the prevention of MDR in clinical cancer chemotherapy.
Materials and methods

Cell culture and the establishment of resistant cell line FaDu/T. The human hypopharyngeal carcinoma cell line FaDu was obtained from the American Type Culture Collection (ATCC). Media and serum were purchased from Gibco (USA). Chemotherapeutic drugs cisplatin (DDP), 5-fluorouracil (5-FU), doxorubicin (Dox) and vincristine (VCR) were obtained from Sigma. Anti–ABCBl, -ABCG2, -caspase-3, -Bcl-2, -Bax and -β-actin antibodies were from Santa Cruz Biotechnology. All other agents were from Sigma (St. Louis, MO, USA). FaDu cells were cultured as a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg streptomycin at 37°C in a humidified atmosphere composed of 95% air and 5% CO2.

The resistant cell line, FaDu/T, was developed by continuous exposure of FaDu cells to stepwise escalating concentrations of Taxol® (Bristol-Myers Squib Co.) for over 12 months. The initial concentration was determined using the IC25 of Taxol in FaDu cells by MTT. In order to ensure the optimum establishment of the resistant cell line (FaDu/T), stepwise escalating concentrations of Taxol (5-200 nM for over 12 months) were used continuously as medium content in the progression of the culture, and the dead cells were discounted every 48-72 h by replacement with new medium containing Taxol. Cell passage was carried out just until achieving cell confluence, and FaDu/T cell lines resistant to different concentrations of Taxol were preserved at various times. FaDu/T cells were labeled according to their resistance to Taxol at various concentrations, such as ‘FaDu/T-200 nM’ which stands for the resistant cell line FaDu/T whose tolerance to Taxol was 200 nM.

Assessment of cell viability. Methyl-thiazolyl-tetrazolium (MTT) assay was employed to assess the drug resistant sensitivities of FaDu/T-200 nM cells. The cells (15x10^4/ml) were subcultured in a 96-well cluster (Corning, USA) were treated with different concentrations of Dox, VCR, 5-FU and DDP for 72 h. MTT (5 mg/ml, 20 µl) was added to each well 4 h prior to the indicated time points. After 4-h of incubation at 37°C, the medium was removed, and the precipitate was dissolved in dimethyl sulfoxide (DMSO). The optical density (OD) values were measured at 570 nm using an ELISA reader (Multiskan MA). The relative OD ratio was calculated with NIH software Image J by comparison with actin in three experiments.

Analysis of the cell growth curve. FaDu and FaDu/T-200 nM cells (10x10^4/ml) were subcultured in a 96-well cluster, and the OD570 nm values were measured as described above every 24 h for 6 days. Experiments were repeated three times.

Morphological observation. FaDu, FaDu/T-200 nM and FaDu cells treated with Taxol (200 nM) for 24 h were seeded (15x10^4/well) in 24-well dishes containing 1 ml culture medium to observe the morphological changes. Cells were washed twice with phosphate-buffered solution (PBS), fixed using 95% alcohol for 10 min, and stained with 0.01% acridine orange for 5 min, or with Hoechst 33342 (10 µg/ml) and propidium iodide (PI) (50 µg/ml) at 37°C for 30 min. Morphological changes were examined by fluorescence microscopy.

Flow cytometric analysis of cell cycle distribution and apoptosis. The cell cycle distribution and apoptosis in the FaDu, FaDu/T-200 nM and FaDu cells treated with Taxol (200 nM) for 24 h were analyzed by flow cytometry. In brief, cells (15x10^6/ml) were trypsinized and washed with PBS, respectively. Cells were then pelleted by centrifugation at 1000 rpm for 5 min and resuspended at a concentration of 1x10^6 cells/ml in staining solution (50 µg/ml PI and 100 µg/ml RNase A). After incubation at 4°C in the dark for 30 min, cells were analyzed using an Epics XL flow cytometer (Beckman Coulter Co., Miami, FL, USA). The proportions of cells at G0/G1, S and G2/M phases were analyzed by the Multicycle software program (Beckman Coulter).

For assessment of apoptosis, Annexin V-FITC and PI were used. After trypsinization, cells were resuspended in binding buffer (20 µl) containing Annexin V-FITC (5 µl, 20 µg/ml) and PI (10 µl, 20 µg/ml) for at least 10 min at room temperature, and then binding buffer (300 µl) was added before analysis with the System II, version 3.0 (Beckman Coulter).

Reverse transcription-PCR. Total RNA was extracted using TRIzol (Invitrogen). The reverse transcription reaction was performed using the ExScript RT reagent kit (Takara, Dalian, China) in a final volume of 20 µl containing 1 µg total RNA, 4 µl 5X ExScript buffer, 1 µl dNTP mix, 1 µl Oligo(dT) primer, 0.5 µl ExScript RTase, 0.5 µl RNase inhibitor, and RNase-free water to a volume of 20 µl. The reverse transcription reaction was performed at 42°C for 15 min, and the reaction was terminated by heating at 95°C for 2 min. PCR was performed following the instructions of Takara Taq™ under the following conditions: pre-degeneration at 95°C for 3 min, degeneration at 95°C for 60 sec, renaturation at 58°C for 45 sec, and elongation at 72°C for 60 sec, for a total of 25 cycles. All experiments were conducted 3 times. The primers were as follows: MDR1/ABCB1: forward 5’-CTGCTCAAGTTAAAGGGGCTAT-3’ and reverse 5’-AACGGTTCGGAAGTTTTCTATT-3’; BCRP/ABCG2: forward 5’-AAGCGTTCAGGCCCCATTTAC-3’ and reverse 5’-GAGTCTGCCACTTTATCCA-3’.

Western blot analysis. Total protein was extracted using radioimmunoprecipitation buffer (RIPA), a protein lysis buffer, according to standard protocols. The Bradford method was used to determine the protein concentration of the supernatant. Samples (40 µg of total protein each) were analyzed with Western blotting using the primary antibodies (MDR1/ABCB1 1:400, mouse anti-human; BCRP/ABCG2, 1:200, mouse anti-human; caspase-3 1:200, rabbit anti-human; Bcl-2 1:200, rabbit anti-human; Bax 1:200, rabbit anti-human; actin, 1:2000, mouse anti-human). The bands of MDR1/ABCB1, BCRP/ABCG2, caspase-3, Bcl-2, Bax and actin were visualized at apparent molecular weights of 170, 70, 32, 26, 23 and 43 kDa, respectively. The relative OD ratio was calculated with NIH software Image J by comparison with actin in three experiments.

Statistics. Data are presented as mean ± standard error of the mean (SEM). Statistical calculations were performed using
SPSS16.0 software package. One-way analysis of variance (ANOVA) was applied for the analyses; P-values <0.05 were considered significant.

**Results**

*Establishment of a Taxol-resistant cell line FaDu/T and growth curve analysis.* We established FaDu/T by exposing normal FaDu cells to escalating concentrations of Taxol step-wise for over 12 months. FaDu/T showed a marked change in cellular morphology with many elongated cell dendrites, and slow growth was observed (Fig. 1A). The growth curves for FaDu and FaDu/T cells are shown in Fig. 1B. The resistant cells grew more slowly than the parental cells (P<0.05).

*Cross-resistance profiles of the FaDu/T cells.* Chemotherapeutic drugs of different categories including Taxol, Dox, VCR, 5-FU and DDP were used to evaluate the different resistances of the FaDu/T cells. As shown in Table I, the IC<sub>50</sub> values for Taxol in the FaDu and FaDu/T cells were 0.13±0.02 and 15.32±3.22 µM, respectively. FaDu/T cells were 117.85-fold more resistant to Taxol than the parental cells. Our results further indicated that the FaDu/T cells also had cross-resistance to DDP, 5-FU, DOX and VCR, with 8.99-, 21.96-, 31.42- and 10.00-fold resistance, respectively.

*Morphological changes between FaDu and FaDu/T cells.* Morphological changes were examined in normal FaDu cells, FaDu cells treated with Taxol (200 nM) for 24 h and FaDu/T-200 nM cells. As illustrated in Fig. 2, using acridine orange (AO) staining, normal FaDu cells exhibited a polygonal shape, while cells treated with Taxol (200 nM) for 24 h became rounded and exhibited cytoplasmic contraction and chromatin condensation. Apoptotic bodies, the main morphological characteristic of apoptosis, were also present. However, FaDu/T cells showed a similar morphology to that of the normal FaDu cells, with intact polygonal nuclei.

For the Hoechst 33342 and PI double staining, blue intact nuclei indicated normal cells, red staining was interpreted as necrosis, while blue nuclear fragmentation was an indication of apoptosis. As shown in Fig. 2, compared with normal FaDu cells, red nuclei and blue nuclear fragmentation were detected in the cells treated with Taxol (200 nM) for 24 h. However, the FaDu/T cells (with tolerance to Taxol at 200 nM) showed a similar morphology to that of the normal FaDu cells, with blue intact nuclei. Our data indicate that FaDu/T cells exhibit anti-apoptosis when stimulated by Taxol.

*Cell cycle distribution of the FaDu and FaDu/T cells.* As shown in Fig. 3, compared with the normal FaDu cells, the percentages of FaDu/T-200 nM cells in the G0/G1 and G2/M phases were higher - G0/G1: FaDu 38.0±3.12%, FaDu/T 61.70±2.82%; G2/M: FaDu 8.33±2.65%, FaDu/T 19.54±3.49%, while the percentage of FaDu/T-200 nM cells in the S phase was inversely reduced: FaDu 53.67±5.66%, FaDu/T 18.76±2.47%. These results explain the reason for the slow cell growth of FaDu/T cells as mentioned above.

<table>
<thead>
<tr>
<th>Drug</th>
<th>FaDu (µM)</th>
<th>FaDu/T (µM)</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol</td>
<td>0.13±0.02</td>
<td>15.32±3.22</td>
<td>117.85</td>
</tr>
<tr>
<td>DDP</td>
<td>2.81±0.57</td>
<td>25.25±3.78</td>
<td>8.99</td>
</tr>
<tr>
<td>5-FU</td>
<td>15.85±3.61</td>
<td>347.99±46.95</td>
<td>21.96</td>
</tr>
<tr>
<td>Dox</td>
<td>1.88±0.59</td>
<td>59.07±9.03</td>
<td>31.42</td>
</tr>
<tr>
<td>VCR</td>
<td>6.70±2.31</td>
<td>66.97±8.43</td>
<td>10.00</td>
</tr>
</tbody>
</table>

FaDu and FaDu/T cells were exposed to different concentrations of Taxol, DDP, 5-FU, Dox and VCR, respectively for 72 h, and then the relative survival rates were detected by MTT. IC<sub>50</sub> values were used to evaluate the cross-resistance sensitivities of FaDu/T cells. RI, resistance index. *P<0.05.
Apoptosis of the FaDu and FaDu/T cells induced by Taxol. Compared with the apoptotic rate of the untreated cells (4.45±1.60%), the rates in the cells exposed to Taxol (200 nM, 24 h) and FaDu/T cells whose tolerance to Taxol was 200 nM were 32.9±2.39 and 4.53±1.1%, respectively (Fig. 4). These results further indicate that the anti-apoptosis function of FaDu/T cells increased significantly.

mRNA levels of MDR1/ABCB1 and BCRP/ABCG2 in the FaDu and FaDu/T cells. Compared with the untreated cells,
the mRNA level of MDR1/ABCB1 was up-regulated while BCRP/ABCG2 was down-regulated in the FaDu/T cells (Fig. 5A).

By densitometry, the mRNA expression of MDR1/ABCB1 increased but BCRP/ABCG2 reversely decreased in FaDu/T cells compared with that in FaDu cells, particularly in the FaDu/T-200 nM cells (*P<0.05). (A) RT-PCR analysis of MDR1/ABCB1 and BCRP/ABCG2 in FaDu and FaDu/T cells. (B) Graphs show the quantification of DNA bands by densitometric scanning. The relative mRNA level was presented as the ratio of densities of MDR1/ABCB1 and BCRP/ABCG2 to actin bands. Results are expressed as the mean ± SD (n=3).

Statistical analysis showed that a significant difference existed between the FaDu and FaDu/T cells (P<0.05).

Changes in MDR1/ABCB1 and BCRP/ABCG2 protein levels. In comparison with the untreated FaDu cells, the expression of MDR1/ABCB1 was up-regulated while that of BCRP/ABCG2 was down-regulated when β-actin was taken as the standard in both the FaDu/T-50 nM and FaDu/T-200 nM cells (Fig. 6A). Image J software was used to analyze the relative photodensity. Considering the control as 100%, the ratio of the density of ABCB1/actin was 115.47±12.74% in the FaDu/T-50 nM and 176.86±37.88% in the FaDu/T-200 nM cells. The ABCG2/actin level in the FaDu/T-50 nM cells was 87.20±11.81% and 39.26±9.08% in the FaDu/T-200 nM cells (Fig. 6B). Statistical analysis showed that a significant difference existed between the FaDu and FaDu/T cells (P<0.05).

Caspase-3, Bcl-2 and Bax expression as assessed by Western blotting. In comparison with the untreated FaDu cells, the expression of caspase-3 and Bax was up-regulated while that of Bcl-2 was down-regulated in both the FaDu/T-50 nM and FaDu/T-200 nM cells when β-actin was used as the standard (Fig. 7A). Image J software was used to analyze the relative photodensity. Considering the control as 100%, the
density ratio of caspase-3, Bcl-2 and Bax was 57.92±6.55, 199.69±29.96 and 89.03±13.56% in the FaDu/T-50 nM and 40.56±7.93, 223.85±45.84 and 53.82±11.17% in the FaDu/T-200 nM cells, respectively (Fig. 7B). The relative Bcl-2/Bax level was analyzed between the different cells and a significant increase was detected in the FaDu/T cells compared with the control. Considering the control as 1.00, the ratio of Bcl-2/Bax was 1.87±0.16 in FaDu/T-50 nM and 3.83±0.47 in the FaDu/T-200 nM cells (Fig. 7C). Statistical analysis showed that a significant difference was achieved between the FaDu and FaDu/T cells (P<0.01). These results further explain the mechanism of anti-apoptosis in FaDu/T cells.

Discussion

The major obstacle to tumor chemotherapy is drug resistance, including intrinsic and acquired resistance to multiple chemotherapeutic drugs, yet the mechanisms responsible for MDR remain unclear (7). To elucidate the mechanism responsible for MDR is critical. Here, we established a multidrug resistant cell line to Taxol (FaDu/T) by exposing normal FaDu cells to escalating concentrations of Taxol stepwise for over 12 months and investigated the basic characteristics of FaDu/T cells in an effort to provide theoretical support for the study of the reversal of MDR in HNSCC.

Firstly, the multidrug resistance of FaDu/T to DDP, 5-FU, Dox and VCR was assayed by MTT. Results showed varying degrees of resistance, respectively. Morphological changes were then analyzed by microscopic observation using phase contrast microscopy. The MDR cell line FaDu/T grew significantly slower when compared with the parental cells as determined by morphological observation and growth curve analysis. The biological activity of Taxol is based on its ability to promote microtubule assembly and stabilize tubulin polymers against depolymerization. This arrests cells in the G2/M phase, inducing cell apoptosis (consequently, leaving the cells in the G0/G1 phase to survive). This may be the reason for the slower growth rate of FaDu/T cells (8). Analysis of the cell cycle distribution by flow cytometry revealed an increased proportion of cells in the G0/G1 and G2/M phases in this study, which verified the growth curve analysis.

Upon acridine orange staining and Hoechst 33342/PI double staining, FaDu/T cells showed powerful apoptosis to Taxol compared with the control cells. Chromosome condensation and nuclear fragmentation were detected under fluorescence microscopy. Quantitative analysis of apoptosis by flow cytometry revealed a similar result, which further confirmed the anti-apoptotic ability of FaDu/T cells. In general, the mechanism of apoptosis induced by Taxol involves Bcl-2, caspase-3, and the Fas/Fas ligand (9). Expression of caspase-3, Bcl-2 and Bax in FaDu and FaDu/T cells was detected by Western blot analysis in this study, and significant alterations to resist apoptosis were detected. This further proves the anti-apoptosis of the MDR cells.

Many genes have been reported to be related with drug resistance or sensitivity, such as ATP-binding cassette (ABC) genes (10). The human has 49 ABC genes, arranged in eight subfamilies and named through divergent evolution (11). The variable expression of ABC transporters results in ATP-driven efflux of antitumor drugs from cancer cells, thereby leading to decreased intracellular drug accumulation and consequent MDR (12). The main elements related to MDR include P-glycoprotein (P-gp) translated by MDR1/ABCB1, the multidrug-resistance protein (MRP1) and the breast cancer multi-drug resistance protein 1 (BCRP/ABCG2) (13).

In the present study we focused on MDR1/ABCB1 and BCRP/ABCG2. The overexpression of MDR1/ABCB1 has been reported to be associated with resistance to a wide range of anticancer drugs, including Taxol (14-17). Here, we assessed the mRNA and protein levels of MDR1/ABCB1 in the FaDu/T cells, and up-regulation was obviously detected in a dose-dependent manner. Yet, there is a disagreement concerning the role of BCRP/ABCG2 during MDR progression and whether or not Taxol is a substrate of ABCG2. Studies have found that Taxol is not the substrate of BCRP/ABCG2 during MDR (18,19). They reported, although a mutation at position 482 can alter the substrate specificity of BCRP/ABCG2, Taxol was not the substrate of either wild-type BCRP/ABCG2 or its mutants (20). Meanwhile, various studies found that BCRP/ABCG2 was involved during MDR induced by Taxol (21,22). At the gene and protein levels, we observed a decreased expression of BCRP/ABCG2 in the FaDu/T cells compared with the parental cells. For the first time we report the role of BCRP/ABCG2 in MDR progression induced by Taxol in FaDu/T cells. Controversy concerning the relationship between MDR1/ABCB1 and BCRP/ABCG2 during MDR also exists. A recent study reported the inverse trend of expression between ABCB1 and ABCG2 mRNA at the blood-brain barrier in ABCB1 (-/-) and ABCG2 (−/−) knockout mice (23). Other studies have also demonstrated an inverse relationship between them (24,25). Meanwhile, a positive relationship between MDR1/ABCB1 and BCRP/ABCG2 expression has also been reported (26,27). According to RT-PCR and Western blot analysis, we reported that the levels of two transporters in FaDu and FaDu/T cells were MDR1/ABCB1: FaDu<FaDu/T whereas BCRP/ABCG2: FaDu>FaDu/T, respectively. When the cancer cells were exposed to the chemotherapy drugs, due to coexpression, inverse or positive relationships among the ABC transporters have not been particularly investigated. It could be hypothesized that to protect the cells from cytokine-induced oxidative damage, the lack of a specific gene may be offset by expression and protein synthesis of another compensatory gene. The variable expression of ABC transporters may be regulated in an abnormal manner in the presence of an anticancer drug. The mechanism involved in the decreased expression level of BCRP/ABCG2 in FaDu/T cells warrants further studied.

In conclusion, the MDR cell line FaDu/T provides us with a base for the further study of the resistant mechanisms of clinical HNSCC drug resistance. Further study must be carried out on the mechanism involved in the reversed trend of molecular expression between ABCB1 and ABCG2 in FaDu/T cells, which would be valuable for the prevention of MDR in clinical cancer chemotherapy.

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References


