Abstract. Cancer cells developing multidrug resistance (MDR) is one of the most serious clinical problems responsible for the failure of cancer chemotherapy. P-glycoprotein (P-gp) overexpression and inhibitor of apoptosis proteins (IAPs) overexpression in cancer cells are the two common mechanisms of MDR. However, the relationship between IAPs and P-gp in MDR cancer cells is unknown. We investigated the expression levels of two IAPs, Survivin and XIAP, and their interaction with P-gp in MDR cancer cells. We have found that the human epidermoid carcinoma cells KBv200 and breast cancer cells MCF-7/Adr overexpress not only P-gp but also XIAP and Survivin, and showed high resistance to chemotherapeutic drugs doxorubicin, docetaxel and vincristine, in contrast to their parental cells KB and MCF-7. Furthermore, upregulation of Survivin or XIAP through transfection with the plasmid pECFPN1-Survivin or pcDNA3-6myc-XIAP in these four cell sublines did not affect the P-gp expression. Downregulation of Survivin or XIAP through transfection with the Survivin or XIAP siRNA did not have an effect on the P-gp expression in these resistant cells. Additionally, our immunoprecipitation results showed that Survivin or XIAP did not directly bind to P-gp. In summary, our study suggested that the overexpression of Survivin and XIAP in MDR cancer cells does not directly interact with P-gp.

Introduction

Cancer cells developing resistance to chemotherapeutic drugs is a frequent clinical problem encountered in the treatment of human cancers. After obtaining resistance to a single drug or a class of drugs, cancer cells show cross-resistance to other functionally and structurally unrelated drugs which include the anthracyclines, vinca alkaloids, taxanes, topoisomerase I inhibitors, and other natural products. This phenomenon is known as multidrug resistance (MDR) which is one of the most serious problems responsible for the failure of cancer chemotherapy (1). Various cellular mechanisms result in MDR, including increased drug efflux, alteration in cell cycle checkpoints, failure of apoptotic mechanisms, changes in targeted enzymes, altered DNA repair and scavenging enzymes (2,3). Among these mechanisms of MDR, the most common and extensively studied mechanism is cancer cells overexpressing P-glycoprotein (P-gp), an ATP-binding cassette (ABC) transporter, which is a 170-kD plasma membrane glycoprotein encoded by the MDR1 gene acting as a drug efflux pump to remove chemotherapeutic drugs from cancer cells, and its expression level decides the magnitude of resistance. Additionally, it has been suggested that, aside from the drug efflux pump, P-gp might also confer resistance to apoptosis through specific mechanisms by inhibiting the activation of the cell death effector enzymes caspases from different stimuli, such as anticancer drugs, serum starvation, UV or γ irradiation, and ligation of the cell surface death receptors Fas and tumor necrosis factor receptor (4-6). Nevertheless, this inhibitory effect of P-gp might be reversed by using specific P-gp antagonists, such as anti-P-gp antibodies or the pharmacological inhibitor verapamil (4,5).

The inhibitor of apoptosis proteins (IAPs), which are able to inhibit apoptosis induced by a variety of stimuli, are always overexpressed in cancer cells that give rise to resistance to apoptosis. IAPs are a class of structurally related proteins that were initially identified in baculoviruses. At present, eight human IAPs have been identified: c-IAP-1, c-IAP-2, ILP-2,
neuronal apoptosis inhibitory protein (NAIP), X-linked IAP (XIAP), Survivin, Livin, and Apollon/Bruce (7). IAPs are defined by one or more repeats of a highly conserved ~80 amino-acid zinc-binding domain located at the amino-terminus, termed the baculovirus IAP repeat domain. It has been shown that IAPs block apoptosis either by binding and inhibiting specific caspases, or through caspase-independent mechanisms (7). In addition, IAPs exhibit other biological functions that include involvement in protein degradation (8), and c-IAP1 and c-IAP2 are integral parts of the type-2 TNF-receptor complex (9), while Survivin plays a role in cell mitosis, mainly in microtubule organization (10). Recently, several studies showed that the MDR leukemia cells HL-60R significantly overexpressed not only P-gp but also both the mRNAs and all the IAPs studied, i.e. c-IAP-1, c-IAP-2, XIAP, NAIP and survivin except for c-IAP-1 mRNA, and their expression levels of IAPs were much less affected by the treatments with chemotherapeutic drugs in comparison with their parent sensitive cells HL-60 (11,12). The overexpression of either P-gp or IAPs in cancer cells can lead to MDR, and MDR cancer cells may overexpress P-gp and/or IAPs. Here, we speculated if there are interactions between P-gp and IAPs during the process of MDR cancer cells developing resistance to apoptosis induced by various stimuli.

In this study, we examined the protein expression of Survivin and XIAP, the two most important and studied IAP family members, in the human epidermoid carcinoma cells KB, breast cancer cells MCF-7, and their resistant cells KBV_{200} and MCF-7/Adr, and explored the interactions between XIAP, Survivin and P-gp in these two resistant cells.

Materials and methods

Materials. RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM medium, fetal bovine serum and Lipofectamine 2000 were purchased from Invitrogen Life Technologies Inc. (Carlsbad, CA, USA). Doxorubicin was purchased from Hisun Pharmaceutical Co., and vincristine was generously provided by Professor X.Y. Liu (National Cancer Institute, USA). All four cell lines were grown as adherent monolayers in flasks in RPMI-1640 or DMEM culture medium with 10% fetal bovine serum, benzylpenicillin (50 U/ml), and streptomycin (50 mg/ml) at 37˚C in a humidified atmosphere of 5% CO_2.

**MTT cytotoxicity assay.** Cells were harvested with trypsin and resuspended in a final concentration of 2x10^6 cells/ml respectively. Aliquot (190 μl) for each cell suspension was distributed evenly into 96-well multiplates. After cells were incubated for 24 h, designated wells were treated with different concentrations of doxorubicin, docetaxel or vincristine (10 μl/well). After 68 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution 10 μl (5 mg/ml) was added to each well, and the plate was further incubated for 4 h at 37˚C in 5% CO_2, allowing viable cells to reduce the yellow MTT into dark-blue formazan crystals which were dissolved in 100 μl dimethylsulfoxide. The absorbance in individual wells was determined at 540 and 655 nm by a microplate Reader (Bio-Rad). The inhibition of cell growth was evaluated by the MTT method using triplicate assay. The concentrations required to inhibit growth by 50% (IC_{50}) values were calculated from cytotoxicity curves by Bliss method.

**siRNA.** The chemic-modified double-strand siRNAs were purchased from GenePharma Co. (Shanghai, P.R. China) after being chemically synthesized, annealed, desalted and purified. The sense strand sequences of siRNAs were as follows: si-Survivin, 5'-AAGGCUGGGAGCCAGAUGACGTT-3' (target site: 315-335) (13); si-XIAP, 5'-GGAGAUACCUGUGCGGUGCUTT-3' (target site: 300-318) (14), and si-mock, 5'-UUCCUGGAACGUUGACGUUTT-3'. The mock siRNA was used as the nonsilencing negative control.

**Transfection plasmid and siRNA.** According to the manufacturer's instructions, 2.0 μg plasmid or 20 pmol siRNA and 4 μl Lipofectamine 2000 were respectively diluted with 100 μl OPTI-MEM medium for 5 min. The two dilutions were mixed gently and incubated for 30 min at room temperature. Cells were plated in a 12-well plate with a concentration of 4x10^3 cells/well in 800 μl of growth medium without antibiotics and immediately transfected with the above 200 μl plasmid/siRNA-Lipofectamine 2000 mixture (15). Cells were harvested after transfection for 48 h, and the effect of gene silencing was examined with Western blotting.
Western blot analysis. Cells were harvested and rinsed twice with PBS. Cell extracts were prepared with RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin) for 30 min with occasional rocking and clarified by centrifugation at 12,000 g for 15 min at 4°C. Identical amounts (100 µg of protein) of cell lysates were boiled for 10 min and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. After being incubated in blocking solution containing 5% powered milk in TBST buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 1 h at 4°C, the membranes were immunoblotted for 2 h with primary antibodies including anti-P-gp, anti-Survivin, anti-XIAP and anti-Hsp70 at 1:500 dilution. The expression of Hsp70 was used as loading control. The membranes were then incubated for 1 h with HRP-conjugated secondary antibody at 1:1000 dilution. Protein-antibody complex was detected by chemiluminescence. Protein expression was quantified by Scion Image software (Scion Co, USA) (16,17).

Immunoprecipitation assay. Protein preparations were similar as for Western blotting. 1.0 mg of total protein was incubated with 10 µl of control IgG or anti-P-gp, anti-Survivin or anti-XIAP antibody overnight at 4°C with gentle rocking after adjusting the volume to 1.0 ml with ice-cold RIPA buffer. Then 20 µl protein A/G plus-agarose was added to the complex with incubation at 4°C for 2 h on the rocker platform. The immune complex was collected by centrifugation at 1,000 x g for 30 sec at 4°C and washed three times with PBS, and then resuspended with sample loading buffer and boiled for 10 min and resolved by SDS-PAGE and electrotransferred onto PVDF membrane as previously mentioned. The membrane was incubated and immunoblotted with anti-P-gp, anti-Survivin or anti-XIAP antibody. Protein-antibody complex was detected by chemiluminescence.

Statistical analysis. All experiments were repeated at least three times and differences were determined by using the Student’s t-test. Significance was determined at P<0.05.

Results

The MDR characteristics of KBv200 and MCF-7/Adr cancer cells. (A) P-gp expression was detected with Western blotting in the KB, KBv200, MCF-7 and MCF-7/Adr cells. Cell lysates were prepared as described in Materials and methods. Equal amounts (100 µg proteins) of cell lysates were separated by SDS-PAGE, and then transferred onto PVDF membrane. The membranes were immunoblotted with primary antibodies including anti-P-gp and anti-Hsp70 at 1:500 dilution. The expression of Hsp70 was used as loading control. The membranes were then incubated for 1 h with HRP-conjugated secondary antibody at 1:1000 dilution. Protein-antibody complex was detected by chemiluminescence. Measurements were performed at least three times and a representative experiment is shown (A). (B) The growth curve of KB, KBv200, MCF-7 and MCF-7/Adr cells treated with different concentrations of doxorubicin, docetaxel and vincristine. The inhibition of cell growth was evaluated with the MTT assay as described in Materials and methods. The concentrations required to inhibit growth by 50% (IC50 values) were calculated from cytotoxicity curves by Bliss method. Data points are the means ± SD of triplicate determinations. Representative experiments are shown.

Immunoprecipitation assay. Protein preparations were similar as for Western blotting, 1.0 mg of total protein was incubated with 10 µl of control IgG or anti-P-gp, anti-Survivin or anti-XIAP antibody overnight at 4°C with gentle rocking after adjusting the volume to 1.0 ml with ice-cold RIPA buffer. Then 20 µl protein A/G plus-agarose was added to the complex with incubation at 4°C for 2 h on the rocker platform. The immune complex was collected by centrifugation at 1,000 x g for 30 sec at 4°C and washed three times with PBS, and then resuspended with sample loading buffer and boiled for 10 min and resolved by SDS-PAGE and electrotransferred onto PVDF membrane as previously mentioned. The membrane was incubated and immunoblotted with anti-P-gp, anti-Survivin or anti-XIAP antibody. Protein-antibody complex was detected by chemiluminescence.

Statistical analysis. All experiments were repeated at least three times and differences were determined by using the Student’s t-test. Significance was determined at P<0.05.

Results

The MDR characteristics of KBv200 and MCF-7/Adr cancer cell lines. To explore the MDR characteristics of KBv200 and MCF-7/Adr cancer cells, we detected the P-gp expression levels with Western blotting in MDR cancer cells KBv200, MCF-7/Adr and their parental sensitive cells KB and MCF-7, and the cytotoxic effect of several chemotherapeutic agents to these cells with MTT assays. As determined by immunoblot analysis, the membranes of the two MDR cancer cells KBv200 and MCF-7/Adr were a rich source of P-gp, however, the membranes of their parental sensitive cells KB and MCF-7 did not detect P-gp (Fig. 1A). Meanwhile, KBv200 cells showed 53.0-, 52.8- and 70.0-fold resistance to doxorubicin, docetaxel and vincristine respectively compared with KB cells, and MCF-7/Adr cells exhibited 95.3-, 29.8- and 51.4-fold resistance to doxorubicin, docetaxel and vincristine respectively compared with MCF-7 cells (Fig. 1B and Table I). These data suggested that KBv200 and MCF-7/Adr cancer cells possessed the classic MDR phenotypes: overexpression of P-gp and cross-resistance to functionally and structurally unrelated drugs. KBv200 and MCF-7/Adr overexpressed Survivin and XIAP in comparison with their parental sensitive cells. In an attempt to investigate whether the expression levels of IAP family members in MDR cancer cells were different from their parental sensitive cells, we chose the two most important and
well-studied IAP members, Survivin and XIAP, and detected their protein expression levels in the KBv 200 and MCF-7/Adr cells and their parental sensitive cells KB and MCF-7 by using Western blot analysis. As shown in Fig. 2, the protein level of Survivin in KBv200 cells was a little higher than that in KB cells, whereas the protein level of XIAP in KBv 200 cells was much higher than that in KB cells. Similarly, MCF-7/Adr cells showed a slightly high protein level of Survivin and significantly high protein level of XIAP in comparison with MCF-7 cells. These results suggested that in contrast to their parental sensitive cells, MDR cancer cells overexpressed the protein levels of Survivin and XIAP, which were least expressed in KBv200 and MCF-7/Adr cells, and that cancer cells giving rise to MDR were at least in part associated with the overexpression of Survivin and XIAP.

Neither upregulation nor downregulation of Survivin or XIAP affects the expression of P-gp. As the expression levels of P-gp, Survivin and XIAP were high in MDR cancer cells, we explored whether the expression change of Survivin or XIAP could have an effect on the P-gp expression. To address this possibility, we used two strategies. Firstly, we upregulated the expression of Survivin or XIAP by transfection with the plasmids which coded the Survivin or XIAP gene. After being transfected with the plasmid pECFPN1-Survivin which expressed the ECFP-Survivin fusion protein, KB and KBv200 cells showed increased expression of Survivin protein and ECFP-Survivin fusion protein. The Survivin protein expression of KB and KBv200 cells was not altered in the empty plasmid pECFPN1 transfected group. However, after transfection with pECFPN1-Survivin, KB cells did not show expression of P-gp, and KBv200 cells did not show increased or decreased expression of P-gp (Fig. 3A). MCF-7 and MCF-7/Adr cells also showed increased expression of Survivin protein and ECFP-Survivin fusion protein following transfection with pECFPN1-Survivin, but MCF-7 cells did not express P-gp.

Table I. Cytotoxicity of doxorubicin, docetaxel and vincristine in KB, KBv200, MCF-7 and MCF-7/Adr cells.

<table>
<thead>
<tr>
<th>Agents</th>
<th>KB</th>
<th>KBv200</th>
<th>FR</th>
<th>MCF-7</th>
<th>MCF-7/Adr</th>
<th>FR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (μM)</td>
<td>0.023±0.002b</td>
<td>1.22±0.05</td>
<td>53.0</td>
<td>0.334±0.022</td>
<td>31.82±3.80</td>
<td>95.3</td>
</tr>
<tr>
<td>Docetaxel (nM)</td>
<td>1.2±0.2</td>
<td>63.0±5.0</td>
<td>52.5</td>
<td>8.4±0.05</td>
<td>250.0±36.0</td>
<td>29.8</td>
</tr>
<tr>
<td>Vincristine (μM)</td>
<td>0.045±0.006</td>
<td>3.15±0.18</td>
<td>70.0</td>
<td>0.106±0.014</td>
<td>5.45±0.35</td>
<td>51.4</td>
</tr>
</tbody>
</table>

*Cell survival was determined by MTT assay as described in Materials and methods. bData are means ± SD of at least three independent experiments performed in triplicate. cFR (fold-resistance) was the IC50 value of the resistant cell line divided by the IC50 of the parental sensitive cell line.

Figure 3. Upregulation of Survivin did not affect the expression of P-gp. Cell lysates were prepared as described in Materials and methods. The protein expression of P-gp and Survivin was detected with Western blotting after transfection for 48 h. The following experiments were the same as described for Fig. 1A. The membranes were immunoblotted with primary antibodies including anti-P-gp, anti-Survivin and anti-Hsp70 at 1:500 dilution. (A) KB and KBv200 cells were transfected with the plasmids pECFPN1-Survivin and pECFPN1. (B) MCF-7 and MCF-7/Adr cells were transfected with the plasmids pECFPN1-Survivin and pECFPN1. The protein relative expression level of Survivin in the control group was defined with 1.00, and other values expressed the protein relative expression level in other groups. Measurements were performed at least three times and a representative experiment is shown. *P<0.05 compared with the corresponding control group.
and MCF-7/Adr cells did not show altered expression of P-gp (Fig. 3B). Similar results were obtained after transfection with the plasmid pcDNA3-6myc-XIAP which expressed the XIAP protein. In the pcDNA3-6myc-XIAP transfected group, KB, MCF-7, KBv200 and MCF-7/Adr cells exhibited increased protein expression of XIAP, but the expression level of P-gp still was not detectable in KB and MCF-7 cell lines and the expression level of P-gp was not altered in KBv200 and MCF-7/Adr cells. In the empty pcDNA3 transfected group, the expression levels of XIAP in all four cells were not changed (Fig. 4A and B).

Secondly, we knocked down the expression of Survivin or XIAP by using transfection with the Survivin or XIAP siRNA to investigate the effect on the expression of P-gp. As shown in Fig. 5A, both KBv200 and MCF-7/Adr cells exhibited increased protein expression of XIAP, but the expression level of P-gp still was not detectable in KB and MCF-7 cell lines and the expression level of P-gp was not altered in KBv200 and MCF-7/Adr cells. In the empty pcDNA3 transfected group, the expression levels of XIAP in all four cells were not changed (Fig. 4A and B).
decreased protein levels of XIAP and unchanged expression of P-gp (Fig. 5B). Moreover in the mock siRNA transfected group, neither the protein expression of Survivin nor XIAP in KBv200 and MCF-7/Adr cells was changed. Taken together, these experiments revealed that the increase or decrease of Survivin or XIAP expression did not affect the expression of P-gp, and that the overexpression of P-gp leading to MDR in cancer cells was independent of the overexpression of Survivin or XIAP.

Survivin or XIAP did not directly bind to P-gp. Although we have shown that changing Survivin or XIAP expression did not affect the expression of P-gp, it was necessary to explore the possibility of a physical interaction between P-gp and Survivin or XIAP. The immunoprecipitation assay was used to investigate this issue. We firstly immunoprecipitated P-gp from the cell lysates of KBv200 and MCF-7/Adr cells with the anti-P-gp antibody, and immunoblotted the immunoprecipitation complex with anti-P-gp, anti-Survivin and anti-XIAP antibodies. The cell lysates of KBv200 and MCF-7/Adr cells were used as the control to confirm the validity of immunoblotting. As shown in Fig. 6A, there was only P-gp in the immunoprecipitation complex formed with anti-P-gp antibody, not Survivin or XIAP, and neither P-gp, Survivin nor XIAP was contained in the control IgG immunoprecipitation complex. On the other hand, we immunoprecipitated Survivin or XIAP protein from the cell lysates of KBv200 and MCF-7/Adr cells with the anti-Survivin or anti-XIAP antibodies. As a result, there was only Survivin and no P-gp in the immunoprecipitation complex formed with the anti-Survivin antibody (Fig. 6B). Similarly, there was XIAP but no P-gp contained in the anti-XIAP antibody immunoprecipitation complex (Fig. 6C). Therefore, these data indicated that neither Survivin nor XIAP directly interacted with or bound to P-gp.

Discussion

In the present study, our results showed that the two classic MDR cancer cells KBv200 and MCF-7/Adr overexpressed high protein levels of P-gp, XIAP and Survivin, and conferred high levels of resistance to chemotherapeutic drugs doxorubicin, docetaxel and vincristine in contrast to their parental sensitive KB and MCF-7 cells. This result was consistent with another docetaxel and vincristine in contrast to their parental sensitive levels of resistance to chemotherapeutic drugs doxorubicin, protein levels of P-gp, XIAP and Survivin, and conferred high MDR cancer cells KBv200 and MCF-7/Adr overexpressed high in the present study, our results showed that the two classic MDR cancer cells KBv200 and MCF-7/Adr overexpressed high protein levels of P-gp, XIAP and Survivin, and conferred high levels of resistance to chemotherapeutic drugs doxorubicin, docetaxel and vincristine in contrast to their parental sensitive KB and MCF-7 cells. This result was consistent with another docetaxel and vincristine in contrast to their parental sensitive levels of resistance to chemotherapeutic drugs doxorubicin, protein levels of P-gp, XIAP and Survivin, and conferred high MDR cancer cells KBv200 and MCF-7/Adr overexpressed high in the present study, our results showed that the two classic MDR cancer cells KBv200 and MCF-7/Adr overexpressed high protein levels of P-gp, XIAP and Survivin, and conferred high levels of resistance to chemotherapeutic drugs doxorubicin, docetaxel and vincristine in contrast to their parental sensitive KB and MCF-7 cells. This result was consistent with another docetaxel and vincristine in contrast to their parental sensitive levels of resistance to chemotherapeutic drugs doxorubicin, protein levels of P-gp, XIAP and Survivin, and conferred high MDR cancer cells KBv200 and MCF-7/Adr overexpressed high in the present study, our results showed that the two classic MDR cancer cells KBv200 and MCF-7/Adr overexpressed high protein levels of P-gp, XIAP and Survivin, and conferred high levels of resistance to chemotherapeutic drugs doxorubicin, docetaxel and vincristine in contrast to their parental sensitive KB and MCF-7 cells. This result was consistent with another docetaxel and vincristine in contrast to their parental sensitive levels of resistance to chemotherapeutic drugs doxorubicin, protein levels of P-gp, XIAP and Survivin, and conferred high MDR cancer cells KBv200 and MCF-7/Adr overexpressed high
to verify our speculation. At first, we transfected the two sensitive cell lines KB and MCF-7 which do not express P-gp with the plasmids pECFPN1-Survivin or pcDNA3-6myc-XIAP to upregulate the expression of Survivin or XIAP and to observe whether P-gp expression follows transfection. However, our results suggest that neither Survivin nor XIAP upregulation can regulate the P-gp expression of both KB and MCF-7 cell lines. Secondly, we transfected the two MDR cells KBv200 and MCF-7/Adr which overexpress P-gp with the same plasmids of pECFPN1-Survivin or pcDNA3-6mcy-XIAP to observe whether after transfection, P-gp expression could be altered. Our experimental results were coincident that P-gp expression in both cell lines was unchanged. From these two experiments, we conclude that upregulating the expression of either Survivin or XIAP does not affect the expression of P-gp. Thirdly, we used RNAi strategies to downregulate Survivin or XIAP expression and to observe whether P-gp expression follows transfection. However, our results suggest that neither Survivin nor XIAP downregulation of Survivin or XIAP affected the expression of P-gp in these four cell sublines. We concluded that either Survivin or XIAP does not directly interact with P-gp. The mechanisms of the overexpression of IAPs in MDR cancer cells and P-gp affecting caspase activation need to be studied further.

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