The Activated MEK/ERK Pathway may Potentiate Breast Cancer Resistance Protein /ABCG2 Function in SN38-selected MCF-7 Cells

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INTRODUCTION

Irinotecan is a semisynthetic analog of camptothecin, originally isolated from the Chinese/Tibetan ornamental tree Camptotheca acuminata. Irinotecan is a prodrug converted by carboxylesterase into its active form 7-ethyl-10-hydroxy-camptothecin (SN38) [1]. SN38 interferes with Topoisomerase I (Topo I) function by forming stable ternary complexes at the DNA single-strand breakage points. Collisions between SN38/Topo I single-strand cleavage complexes and DNA replication forks produce irreversible double-strand breaks, ultimately leading to irreversible DNA damage and to a series of events that
result in apoptosis. Irinotecan is used in the treatment of certain neoplasms such as inoperable and/or recurrent colorectal cancers, esophageal cancers, gastric cancers, non-small cell and small cell lung cancers, non-Hodgkin lymphoma, and breast cancers [1-3]. In spite of an initial response, most patients treated with irinotecan become resistant to this chemotherapy. Therefore, we aimed to determine the mechanism involved in resistance to SN38 in human cancer cells.

To this end, we exposed human breast cancer MCF-7 cells to SN38 continuously and progressively and obtained cells 160-fold more resistant than the parental cells (MCF-7/SNR). Breast cancer resistance protein (BCRP)/ABCG2 transports SN38 across the cell membrane out of cells [4]. Although MCF-7/SNR cells endogenously expressed as much BCRP as MCF-7/BCRP cells that overexpressed exogenous BCRP, MCF-7/SNR cells were far more resistant than MCF-7/BCRP cells. The higher levels of SN38 resistance of MCF-7/SNR cells were attributed to endogenous BCRP, since Ko-143, a specific BCRP inhibitor, completely reversed the resistance [5]. Further analyses revealed that the Ras/Raf/mitogen activated-protein kinase/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway was activated in MCF-7/SNR cells and that MEK inhibitors partially circumvented the SN38 resistance. Treatment of MCF-7/SNR cells with PD98059 resulted in a rapid increase of intracellular accumulation of topotecan, which is another substrate of BCRP, without affecting BCRP levels [6]. In addition, alteration of cellular BCRP distribution was observed after PD98059 treatment. These data suggest that activation of the MEK/ERK pathway may regulate BCRP trafficking and turnover, and potentiate BCRP function on the plasma membrane.

MATERIALS AND METHODS

Cell lines

MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂. MCF-7 cells were cloned by limiting dilution method. MCF-7 clone 3 was used for further analyses [7]. MCF-7 represents MCF-7 clone 3 hereinafter in this report, unless otherwise stated. MCF-7/SNR cells were established by continuous exposure to SN38 (stepwise increasing concentrations from 1.22 nM to 121.5 nM) over a period of six months. MCF-7/SNR cells were maintained in the presence of 121.5 nM SN38 until 24 h prior to any experiment.

Cell growth assay

Cells (3.0 x 10⁴/well) were seeded in 12-well plates each well of which contained 2 ml of DMEM supplemented with 7% FBS, and cultured in the medium with or without increasing doses of specific anticancer agents in the absence or presence of MEK inhibitors for 4 days. MEK inhibitors were replaced with fresh one 2 days after the start of the treatment. Cell numbers were then determined using a Coulter counter (Beckman Coulter), and presented as percentages relative to those of control cells. IC₅₀ values (drug dosages that cause 50% inhibition of cell growth) were calculated from the growth inhibition curves. Then, degrees of resistance were determined by dividing IC₅₀ values of resistant cells by those of control cells.

Western blot analysis of BCRP

Exponentially growing cells were harvested, washed and lysed in the lysis buffer (10 mM Tris-HCL pH 8.0, 0.1% Triton-X100, 10 mM MgSO₄, 2 mM CaCl₂, 1 mM AEBSF, 1 mM dithiothreitol). After centrifugation, the cell lysates were solubilized with the lysis buffer (2 SDS, 50 mM Tris-HCL pH 7.5, 5% 2-mercaptoethanol) and resolved by 5-20% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes, and blots were then incubated with 5 μg/ml of the anti-BCRP mouse monoclonal antibody BX-21 (Millipore). After washing, the blots were incubated with the anti-mouse peroxidase-conjugated secondary antibody (GE Healthcare). Membrane-bound peroxidase was visualized on XAR film (Kodak) after enhancement using a chemiluminescence detection kit (GE Healthcare). GAPDH expression was analyzed as a loading control.

Western blot analysis of ERK1/2

Cells were serum-starved for 24 h and then cultured in DMEM supplemented with 7% FBS for 1.5 h or 6 h. After harvesting the cells, phosphorylated ERK1/2 was detected by using the rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology) at a 1:200 dilution according to the manufacturer’s instruction. Then, the blots were stripped and reprobed with the rabbit polyclonal anti-p44/42 MAP kinase antibody (Cell Signaling Technology) at a 1:1000 dilution to detect ERK1/2. GAPDH expression was analyzed as a loading control.

cDNA microarray analysis

Extraction of total RNA was performed using an RNeasy Mini kit (QIAGEN) according to the manufacturer’s instruction. Integrity of extracted total RNA was checked by electrophoresis. 3D-Gene Human Oligo chip 25k (Toray) was used (25,392 distinct genes). For efficient hybridization, this microarray has 3-dimensions that is constructed with a well as the space between the probes and cylinder-stems with 70-mer oligonucleotide probes on the top. Total RNA was labeled with Cy3- or Cy5- using the Amino Alkyl MessageAMP II aRNA Amplification kit (Applied Biosystems). The Cy3- or Cy5- labeled aRNA pools were hybridized for 16 h according to the supplier’s protocols (www.3d-gene.com). Hybridization signals were scanned using ScanArray Express Scanner (PerkinElmer), and processed by GenePixPro version 5.0 (Molecular Devices). Detected signals for each gene were normalized by local normalization method (Cy3/Cy5 ratio median=1). Genes with Cy3/Cy5 normalized ratios greater than 2.0 or less than 0.5 were defined, respectively, as commonly up- or down-regulated genes.

Reverse transcription (RT)-PCR analysis

Up-regulation of the RASGRP1 and Jak-2 genes were validated by RT-PCR. Exponentially growing cells in DMEM with 7% FBS were harvested, and total RNA extraction, first-strand cDNA synthesis were performed as described previously [7]. PCR was carried out using 50 ng of total RNA as a template. The primers were 5’- TGC GGA ATG AGT GTG TGT CT - 3’ (forward) and 5’- ACT CCT CCA TAG TGT CTG TC - 3’ (reverse) for the RASGRP1 gene (319-bp fragment), and 5’- CAG TTT TTC AGG TGT ATC TT - 3’ (forward) and 5’- TCC TTT CTG TT TTT CAC TCA TT - 3’ (reverse)
for the *Jak-2* gene (171-bp fragment). As an internal control, amplification of *GAPDH* mRNA (551-bp fragment) was carried out with the primers 5’- ATC ACC ATC TTC CAG GAG CGA - 3’ (forward) and 5’- GCT TCA CCA CCT TCT TGA TGT - 3’ (reverse). The PCR conditions were as follows: a first denaturing at 94°C for 2 min, then amplifications with increasing cycle numbers of 94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 7 min. Data are representative of three independent experiments.

**Intracellular topotecan uptake**

Cells were cultured in the absence or presence of 10 μM of PD98059 for 48 h. After trypsinization, cells (5 x 10^5) were incubated with 40 μM of topotecan for 30 min at 37°C, washed in ice-cold PBS, and subjected to fluorescence analysis using FACSCalibur (Becton-Dickinson).

**Indirect immunofluorescence analysis of BCRP expression**

Cells were treated with 20 μM of PD98059 for 48 h and collected with a cell scraper. PD98059 was replaced with fresh ones 24 h after the start of the treatment. The cells were routinely formalin-fixed, paraffin-embedded as a cell block, and processed. Antigen-retrieval was performed by autoclaving (121°C) for 15 min. Then, the slides were incubated with 12.5 μg/ml of BXP-21 at 4°C overnight followed by incubation with a polyclonal anti-mouse immunoglobulin/FITC secondary antibody (1:20 dilution) (Dako) at 4°C overnight. BCRP was detected by fluorescent microscopy as green fluorescence (λex 470-490 nm) and cell nuclei counterstained with 0.8 μg/ml of 4', 6-diamidino-2-phenylindole (λex 330-385 nm) were indicated by purple fluorescence.

**Statistical analysis**

The two-sided unpaired Student’s t test was used to evaluate the statistical significance of differences between the two sets of data. The difference was considered significant when the p value was less than 0.05.

**RESULTS AND DISCUSSION**

**Establishment of MCF-7/SNR cells**

In order to overcome naturally-occurring irinotecan resistance, we established MCF-7/SNR cells and analyzed the mechanism of their SN38 resistance. To exclude contamination of intrinsic SN38 resistant cells, we first cloned MCF-7 cells and selected MCF-7 done 3 as reference one. This sensitive cell line was selected by SN38 over a period of six months, and MCF-7/SNR cells were obtained. Cell growth assay demonstrated that MCF-7/SNR cells were more resistant to SN38, even more than MCF-7/BCRP cells that were transfected with BCRP cDNA. However, MCF-7, MCF-7/BCRP, and MCF-7/SNR cells revealed similar resistances to vincristine and cisplatin, suggesting absence of cross-resistance to these anti-cancer drugs (Figure IA, Table 1).

Only two models using SN38 as a selecting agent have been described until now. One was obtained by developing SN38 resistance on the PC-6 human lung cancer carcinoma cell line [4]. The SN38 resistant PC-6 cells, 18- and 34-fold resistant compared with the parental cells, overexpressed BCRP. Another was obtained by developing SN38 resistance on the HCT-116 human colon cancer carcinoma cell line [8]. The SN38 resistant HCT-116 cells, 6- and 54-fold resistant compared with the parental cells, overexpressed BCRP, of which expression levels correlated with the resistance level. Thus, up-regulated BCRP was the common mechanism of the resistance in the two models. In the present study, MCF-7/SNR cells were also found to overexpress BCRP by western blot analysis (Figure 1B). In spite of the higher SN38 resistance, BCRP expression levels in MCF-7/SNR cells were similar to or somewhat lower than those in MCF-7/BCRP cells. The data suggested that the high degree of SN38 resistance may be caused not only by BCRP overexpression but by other factors.

Reportedly, the SN38 resistant HCT116-SN6 cells, 6-fold more resistant than the parental cells, were found to proliferate more slowly than the parental cells, and the authors speculated that a slower cell proliferation rate was a major cause of acquired resistance to SN38 [9]. Consistently, comparison of mRNA expression profiling revealed that 27% of differentially expressed genes in the resistant cells were related to negative regulation of cell proliferation. In the present study, MCF-7/SNR cells grew significantly more slowly than MCF-7 cells. The doubling time of MCF-7/SNR cells was 22.7 ± 1.7 h and that of MCF-7 cells 16.6 ± 0.6 h, respectively (p < 0.05). However, a specific BCRP inhibitor, Ko-143 (1 μM), completely abolished SN38 resistance of not only MCF-7/BCRP cells but also MCF-7/SNR cells (Figure 1C). From these results, we expected further unknown factors contributing to SN38 resistance by potentiating BCRP function.

**Gene expression analysis**

The following mechanisms of irinotecan-resistance have been reported to date: (i) variable levels of the enzymes involved in the conversion of irinotecan into SN38 or SN38 into inactivated form SN38G by glucuronidation [10,11]; (ii) reduced cellular accumulation from active drug efflux [12-14]; (iii) reduced levels of Topo I [15]; (iv) alterations in the structure of Topo I from different mutations [16]; (v) alterations in the cellular resistance to SN38-Topo I-DNA complex formation, which involves proteasome degradation of Topo I and/or enhanced DNA repair [17]; (vi) activation of NF-κB by DNA damage and subsequent suppression of apoptosis [18,19]. We then performed cDNA microarray analysis between MCF-7/SNR cells and MCF-7 cells. cDNA expression levels were similar between these cells in 90.8% of the total examined genes. After global normalization, 1,515 out of the 25,392 genes were found to be up-regulated by more than two-fold, and 822 genes were down-regulated by 90.8% of the total examined genes. After global normalization, 1,515 out of the 25,392 genes were found to be up-regulated by more than two-fold, and 822 genes were down-regulated by more than two-fold. The overall microarray data are available in GEO web page (accession no. GSE27534). *BCRP* was found to be significantly up-regulated (Cy3/Cy5 60.3), consistent with the result of western blot analysis. Expression levels of Topo I and UDP-glucuronosyltransferase (UGT) 1A1 which is a major determinants of SN38 inactivation and excretion in the living body [1] were not found to be significantly altered. Another ABC transporter *ABCC2/cmOAT2*, encoding a known transporter of SN38 [1], was not also up-regulated. In summary, expression levels of genes involved in activity and metabolism of irinotecan and SN38 were not significantly altered in MCF-7/SNR cells, except for *BCRP*. 


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Figure 1 Establishment of MCF-7/SNR cells. A, drug sensitivity studies. Cells (3 \times 10^4 / well) were treated with increasing doses of anti-cancer agents for 4 days, and cell numbers were determined and presented as percentages relative to those of control cells cultured in the absence of anti-cancer drugs. The data shown are means ± SD of triplicate determinations, and are representative of three independent experiments. Square, MCF-7 cells; lozenge, MCF-7/BCRP cells; circle, MCF-7/SNR cells. Where a vertical bar is not shown, the SD is within the symbol. B, western blot analysis of BCRP. Cells were cultured free of SN38 for 24 h before harvest. Protein sample (20 µg) was loaded in each lane, and BCRP was detected by using BXP-21. The data are representative of three independent experiments. C, effects of Ko-143 on SN38 resistance of MCF-7/BCRP (C-1) and MCF-7/SNR (C-2) cells. Cells (3 \times 10^4 / well) were treated with increasing doses of SN38 for 4 days in the absence or presence of Ko-143 (1 µM), and cell numbers were determined and presented as percentages relative to those of control cells cultured in the absence of anti-cancer drugs. The data shown are means ± SD of triplicate determinations, and are representative of two independent experiments.

Table 1: IC_{50} values of anticancer drugs in MCF-7-derived cells.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>MCF-7 (nM)</th>
<th>MCF-7/BCRP (DR)</th>
<th>MCF-7/SNR (DR)</th>
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<tr>
<td>SN38</td>
<td>0.85 ± 0.02</td>
<td>15.6 ± 2.04**</td>
<td>132 ± 13.9**</td>
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<td></td>
<td></td>
<td>(18.4 ± 2.16)</td>
<td>(157 ± 13.9)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>1.43 ± 0.08</td>
<td>1.03 ± 0.02**</td>
<td>1.59 ± 0.09**</td>
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<td></td>
<td></td>
<td>(0.72 ± 0.04)</td>
<td>(1.41 ± 0.09)</td>
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<tr>
<td>Cisplatin</td>
<td>1350 ± 90</td>
<td>1200 50^*</td>
<td>1960 ± 60^*</td>
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<tr>
<td></td>
<td></td>
<td>(0.83 ± 0.02)</td>
<td>(1.17 ± 0.03)</td>
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IC_{50} values that cause 50% inhibition of cell growth; DR: Degree of resistance.

On the other hand, RAS guanyl releasing protein 1 (RASGRP1) was found to be markedly up-regulated (Cy3/Cy5 34.0). RASGRP1 has been reported to activate the Ras oncoprotein by releasing GDP from Ras and in turn combining GTP with Ras, leading to Ras activation [20]. We therefore searched for other genes associated with activation of Ras and its downstream pathway with special interest. The Jak-2 gene, whose translation product activates the MEK/ERK pathway [21], was also found to be up-regulated (Cy3/Cy5 7.3). Up-regulations of these genes were confirmed by RT-PCR (Figure 2A). These data suggested that the Ras/Raf/MEK/ERK pathway may be activated in MCF-7/SNR cells.

Western blot analysis of ERK1/2

We next investigated the phosphorylation status of ERK1/2 in MCF-7/SNR cells. After serum starvation for 24 h, addition of FBS to the medium resulted in increased phosphorylation of ERK1/2 in the both cells, but the serum-responsiveness was found to be enhanced in the resistant cells (Figure 2B).

Effects of MEK inhibitors on SN38 resistance

Because the MEK/ERK pathway would likely be activated in MCF-7/SNR cells, growth inhibition studies by SN38 were performed in the absence or presence of MEK inhibitors. Growth inhibitory effects were less than 30% when cells were cultured...
Figure 2 Activation of the Ras/Raf/MEK/ERK pathway in MCF-7/SNR cells. A, semi-quantitative RT-PCR of RASGRP1 and Jak-2 mRNAs. MCF-7/SNR cells were cultured free of SN38 for 24 h before harvest. Exponentially growing cells were then harvested and total RNA was extracted. First-strand cDNA was synthesized with 50 ng of total RNA, and RASGRP1 and Jak-2 cDNA fragments were amplified by PCR using the indicated cycle numbers. Amplification of GADPH mRNA was carried out as an internal control. The data are representative of two independent experiments. B, serum-induced ERK1/2 phosphorylation. Cells were serum-starved for 24 h, followed by medium exchange to that supplemented with 7% FBS. ERK1/2 phosphorylation was examined 1.5 h and 6 h after the medium change by Western blotting. The data are representative of three independent experiments.

Table 2: IC_{50} values of SN38 in MCF-7 and MCF-7/SNR cells in the presence of MEK inhibitors.

<table>
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<tr>
<th>MEK inhibitors</th>
<th>(µM)</th>
<th>IC_{50} values (nM)</th>
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<tr>
<td></td>
<td></td>
<td>MCF-7</td>
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<tr>
<td>PD98059</td>
<td>0</td>
<td>1.22</td>
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<td>0.97</td>
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<td>10</td>
<td>0.97</td>
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<td>U0126</td>
<td>0</td>
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Table 2: IC_{50} values of SN38 in MCF-7 and MCF-7/SNR cells in the presence of MEK inhibitors.

IC_{50} drug dosages that cause 50% inhibition of cell growth; DR: Degree of resistance. 
"p < 0.05 compared with untreated cells; *p < 0.01 compared with untreated cells.
Effects of PD98059 on BCRP expression in MCF-7/SNR and cetuximab, anti-epidermal growth factor receptor (EGFR) antibody, was more effective than cetuximab monotherapy in the treatment of irinotecan-refractory metastatic colorectal cancers [23]. The results suggested that EGFR inhibition by cetuximab might circumvent irinotecan resistance. Since EGFR lies in the upstream of the Ras/Raf/MEK/ERK pathway, the study also suggested the importance of the pathway for circumventing irinotecan resistance.

CONCLUSION

We established MCF-7 cells that are highly and specifically resistant to SN38. The SN38 resistance was caused by overexpression of endogenous BCRP, whose drug efflux function was likely to be potentiated by the activated MEK/ERK pathway. These data may give preclinical evidences for usefulness of co-treatment with irinotecan and MEK inhibitors for BCRP-overexpressing refractory cancers.

REFERENCES


