Differences in Effects of Oncogenes on Sensitivity to Anticancer Drugs

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Oncogene/SHOK cells/Chemosensitivity/IC50.

Methods to predict the responsiveness of a particular tumor to a particular anticancer drug are desirable not only for chemotherapy but also for chemoradiotherapy. Here, we examined the effects of viral or activated oncogenes on sensitivity to anticancer drugs by using SHOK (Syrian hamster Osaka-Kanazawa) cells and their transfectants. The IC50 of each transfectant was compared with that of the pSV2Neo transfected control. Cells transfected with the c-myc, v-mos, or v-fgr gene increased their sensitivity to bleomycin, while those transfected with the H-ras gene developed resistance. Resistance to cisplatin was conferred by the introduction of the H-ras or c-cot gene. In the case of adriamycin, the c-myc or c-cot transfected increased sensitivity and the H-ras transfected decreased it. Mitomycin C resistance was observed by the introduction of the K-ras gene. Thus, the H-ras gene was found to be involved in the development of resistance to three of the four anticancer drugs. In addition, we have for the first time shown that mos and cot have an effect on sensitivity to three and all of the four anticancer drugs, respectively. These results suggest that the expression of each oncogene would differentially affect sensitivity to the four anticancer drugs used in this study, and this property could be a possible marker to predict chemosensitivity.

INTRODUCTION

Radiotherapy, along with surgery and chemotherapy, is one of the three most important treatments for cancer. Some cancers at their early stages are completely cured by radiotherapy alone, whereas in many advanced cases local control rates are low with the therapy.1 To improve local tumor control and/or eradication of distant metastases, the combination of radiotherapy and chemotherapy (chemoradiotherapy) is currently used. With this combined modality therapy, improved local control and/or increased survival has been shown in malignant lymphoma and many types of carcinoma, such as head and neck, nasopharynx, lung, esophagus, stomach, rectum, prostate, cervix, breast, anus, and bladder.2,3 On the other hand, because anticancer drugs have a narrow therapeutic index and a great potential for causing harmful side effects,4 the introduction of chemotherapy potentially increases both the spectrum and magnitude of normal-tissue toxicities compared with radiotherapy alone.3 Therefore, if patients who are resistant to chemotherapy could be identified before treatment, this would prevent unnecessary toxicity.3 Thus, methods to enable accurate prediction of the responsiveness of a particular tumor to a particular anticancer drug are desirable for not only chemotherapy but also chemoradiotherapy.

We conducted an investigation of the intracellular factors that might be useful to predict chemosensitivity, beginning with oncogenes. Oncogenes have been related to malignant progression and poor prognosis in several types of human tumors.5-8 The effect of expression of an oncogene on drug sensitivity has been demonstrated by previous reports, using the following: mouse fibroblast cell line NIH3T3,9-12 mink lung epithelial cell line,13 Friend murine erythroleukemia cell line,14-15 human cell line of teratocarcinoma16 and small cell lung carcinoma.17 In studies using NIH3T3 cells, Sklar18 showed that the activated H-ras, v-K-ras, or N-ras decreased sensitivity to cisplatin (CDDP). The same results were obtained by Isonishi et al.19 with activated H-ras. Peters et al.20 also observed decreased sensitivity to CDDP and adriamycin (ADR) in H-ras transformed lines. However, in activated H-ras transfected cells, Tofti et al.21 reported no change in sensitivity to CDDP or ADR; further, Niimi et al.22 and Gao et al.23 presented findings of no change or, rather, increased sensitivity to ADR and increased sensitivi-
ties to CDDP. Thus, even in the case that the same parent cells are used, the results are not always the same, and the contribution of oncogene activation to chemosensitivity is currently not well established.

For purposes of our study, we selected Syrian hamster Osaka-Kanazawa (SHOK) cells, a clonal cell line derived from Syrian/golden hamster embryo cells, because this is a stable cell line, suitable for the introduction of foreign genes. In a previous study using SHOK cells, we reported the effects of mutated or viral oncogenes on sensitivity to γ-rays, ultraviolet light (UV), and heat shock. In this study, we examined the effects of various oncogenes on sensitivity to several anticancer drugs, which are often used in chemoradiotherapy.

**MATERIALS AND METHODS**

**Anticancer drugs**

Bleomycin (BLM) and CDDP were kindly supplied by Nippon Kayaku Co., Ltd. (Tokyo, Japan). ADR and mitomycin C (MMC) were provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan).

**Cell line and culture**

Characteristics of cells used in this study are shown in Table 1.

The establishment of the SHOK cell line and the transfectants of these cells containing various oncogenes has been reported elsewhere. These transfectants, except for SHOK (neo), SHOK (myc), and SHOK (erbB), were originated from foci formed after the introduction of mutated cellular and viral oncogenes. In the case of SHOK (neo), SHOK (myc), and SHOK (erbB), transfectants were established in a selection medium containing geneticin. Because SHOK (myc) and SHOK (erbB) cells did not give rise to foci, their expression of an introduced gene was confirmed by RNA blot analysis.

Cells were cultured in Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (Biosciences PTY Ltd., Australia) and 20 mM HEPES at 37°C in a humidified atmosphere with 5% CO₂.

**Drug sensitivity assay**

Inhibitory effects of anticancer drugs on growth of SHOK cells and their transfectants were determined by applying the WST-1 assay using a "Cell Counting Kit" (Dojindo Laboratories, Kumamoto, Japan). Cells were inoculated onto 96-well microtiter plates at a concentration of 1 x 10⁵ cells/well in 100 μl of medium. Following a 24-h incubation, BLM, CDDP, ADR, or MMC was added in 100 μl of medium with nine concentrations obtained by two-fold serial dilution of the drug. All drug concentrations were tested in triplicate wells. Before dilution with medium, ADR and MMC were dissolved in water and CDDP was dissolved in dimethylsulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA). BLM was dissolved directly in medium. The final concentration of DMSO in CDDP-treated cells did not exceed 0.5%, a concentration which has no inhibitory effect on cell growth. After drug exposure for 48 h, a mixture (20 μl) of 4-(3-(4-indophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1) and 1-methoxy-5-methyl-phe

<table>
<thead>
<tr>
<th>Table 1. Characteristics of cells used in this study.</th>
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<tbody>
<tr>
<td>cells</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>SHOK</td>
</tr>
<tr>
<td>SHOK(neo)</td>
</tr>
<tr>
<td>SHOK(myc)</td>
</tr>
<tr>
<td>SHOK(H-ras)</td>
</tr>
<tr>
<td>SHOK(K-ras)</td>
</tr>
<tr>
<td>SHOK(N-ras)</td>
</tr>
<tr>
<td>SHOK(cot)</td>
</tr>
<tr>
<td>SHOK(mos)</td>
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</table>

of drug concentration versus the percent of treated cells that survived.

Statistical analysis
Data were first analyzed by F-test for equality of variance. If the test revealed equality of variance, a Student’s t-test was used to evaluate the statistical significance of differences between cell lines. In the case of inequality of variance, a Welch’s t-test was used. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION
The survival curves of SHOK cells and their transfectants for BLM, CDDP, ADR and MMC are shown in Fig. 1 and Fig. 2. Each drug dose-dependently inhibited proliferations of cells used in this study in the range of each concentration under our exposing condition. But there was a slight difference in patterns of inhibition of SHOK cells, for example, among the drugs. BLM showed biphasic growth inhibitory effect, which inhibited the cell growth moderately at lower concentrations, but more strongly at concentrations higher than about 200 μg/ml. In contrast, CDDP, ADR and MMC inhibited the cell growth strongly at lower concentrations.

To express the differences in chemosensitivity of each cell line observed in Fig. 1 and Fig. 2 numerically, we determined IC50. Table 2 summarizes the IC50s of the anticancer drugs for all the cell lines. SHOK (neo), transfected with pSV2Neo plasmid only, showed no significant differences in IC50s compared with those of SHOK cells to BLM, CDDP, or MMC, though the cells were more resistant to ADR. Therefore, to consider the effect of the introduction of a foreign gene, the IC50s of each transfectant were compared with that of SHOK (neo). Differences in the effect of pSV2Neo plasmid on sensitivity to anticancer drugs were reported by Gao et al., who found no change in sensitivity to BLM and ADR, but more did find higher resistance to CDDP and higher sensitivity to MMC, based on a comparison with parental NIH3T3 cells. Discrepancies between their results and ours may be due to differences in the characteristics of each parental cell.

In the case of BLM, SHOK (myc) cells were most sensitive, and SHOK (fgr) cells and SHOK (mos) cells were significantly more sensitive than SHOK (neo) cells, the IC50 value of which reduced to 4.1%, 42.9%, and 64.4% of SHOK (neo) cells, respectively. The IC50 of SHOK cells

![Fig. 1](image_url) Survival curves for SHOK cells and their neo transfectant and oncogene transfectants following exposure to BLM and CDDP. Comparison of survival curves for SHOK cells and neo transfectant with those for myc, H-ras, K-ras, and N-ras transfectants (BLM-1 and CDDP-1), or those for cot, mos, erbB, and fgr transfectants (BLM-2 and CDDP-2) was made. Each point represents the mean of the % survivals obtained at each concentration. Error bars have been omitted for clarity.

containing the N-ras and erbB genes also reduced, but not significantly. In contrast, SHOK (H-ras) cells were 2.69 times more resistant than SHOK (neo) cells. The opposite results were reported by Gao et al.,18 who demonstrated, using NIH3T3 cell lines, that the v-H-ras or c-K-ras (Val-12) transfectant increased, and that the v-erbB transfectant decreased, sensitivity to BLM. Le-Ruppert et al.16 reported that N-ras activation had no effect on sensitivity to BLM, based on an investigation using human ovarian teratocarcinoma sublines. BLM uptake is known to be limited by the plasma membrane, and cell electroporation study has demonstrated that its cytotoxicity is closely related to cell permeabilization and to direct internalization of BLM into the cytosol.23 Therefore, an elevation in BLM influx may be responsible for the sensitization of our five cell lines. In addition to membrane alterations, mechanisms reported to influence BLM cytotoxicity include metabolic inactivation of BLM by a cytosolic hydrolase and elevated DNA repair activity.24,25 Further investigation is needed to elucidate which of these mechanisms makes the most significant contribution to BLM resistance of SHOK (H-ras) cells.

BLM, like ionizing radiation, efficiently generates double-strand DNA breaks and is therefore regarded as radiomimetic. We previously reported that SHOK (mos) cells, SHOK (cot) cells, and SHOK (N-ras) cells developed resistance to γ-rays,20 which dose not coincide with the data of BLM in this study. Because BLM molecules, unlike radiation, need to bind to DNA in order to exhibit their cytotoxic activity,26 one or more of the above-mentioned mechanisms that could influence BLM cytotoxicity may contribute to this difference.

In the case of CDDP, no cells were more sensitive than SHOK (neo) cells. SHOK (cot) cells and SHOK (H-ras) cells were 1.81 and 1.75 times significantly more resistant than SHOK (neo) cells, respectively. The IC50 value of SHOK cells containing the K-ras and mos genes was elevated to 1.69 and 1.92 times that of SHOK (neo) cells, respectively, though not significantly. This is the first report showing CDDP resistance as a result of cot transformation. The effect of transformation by the oncogenes used in this study on sensitivity to CDDP has previously been reported by several groups. Sklar27 reported that NIH3T3 cells transformed by mutated H-ras, K-ras or N-ras were 4.5- to 8.5-fold more resistant to CDDP, and that transformation of these cells by v-mos also conferred 2-fold CDDP resistance. With regard to H-ras, K-ras, and mos, our results resemble those of

Fig. 2. Survival curves for SHOK cells and their neo gene transfectant and oncogene transfectants following exposure to ADR and MMC. Comparison of survival curves for SHOK cells and neo transfectant with those for myc, H-ras, K-ras, and N-ras transfectants (ADR-1 and MMC-1), or those for cot, mos, erbB, and fgr transfectants (ADR-2 and MMC-2) was made. Each point represents the mean of the % survivals obtained at each concentration. Error bars have been omitted for clarity.

Table 2. Sensitivity of SHOK cells and their transfectants to anticancer drugs

<table>
<thead>
<tr>
<th>Cells</th>
<th>BLM (µg/ml)</th>
<th>CDDP (µg/ml)</th>
<th>ADR (µg/ml)</th>
<th>MMC (µg/ml)</th>
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<tr>
<td>SHOK</td>
<td>277.8 ±56.1</td>
<td>1.60±0.26</td>
<td>0.248±0.029*</td>
<td>0.0360±0.0032</td>
</tr>
<tr>
<td>SHOK(neo)</td>
<td>219.4 ±14.0</td>
<td>1.59±0.18</td>
<td>0.469±0.091</td>
<td>0.0323±0.0032</td>
</tr>
<tr>
<td>SHOK(myc)</td>
<td>9.08± 1.60**</td>
<td>1.44±0.24</td>
<td>0.199±0.029*</td>
<td>0.0302±0.0048</td>
</tr>
<tr>
<td>SHOK(H-ras)</td>
<td>591.0 ±64.9**</td>
<td>2.78±0.37*</td>
<td>0.939±0.082*</td>
<td>0.1398±0.0415*</td>
</tr>
<tr>
<td>SHOK(K-ras)</td>
<td>214.4 ±58.1</td>
<td>2.68±0.60</td>
<td>0.588±0.066</td>
<td>0.0600±0.0042**</td>
</tr>
<tr>
<td>SHOK(N-ras)</td>
<td>146.8 ±31.8</td>
<td>1.86±0.36</td>
<td>0.315±0.065</td>
<td>0.0371±0.0050</td>
</tr>
<tr>
<td>SHOK(cot)</td>
<td>180.4 ±29.3</td>
<td>2.87±0.16**</td>
<td>0.248±0.040*</td>
<td>0.0414±0.0033</td>
</tr>
<tr>
<td>SHOK(mos)</td>
<td>141.2 ±3.0*</td>
<td>3.05±0.72</td>
<td>0.464±0.049</td>
<td>0.0407±0.0028</td>
</tr>
<tr>
<td>SHOK(erbB)</td>
<td>150.9 ±21.1</td>
<td>2.01±0.11</td>
<td>0.340±0.036</td>
<td>0.0405±0.0076</td>
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<tr>
<td>SHOK(fgr)</td>
<td>94.1 ±21.8**</td>
<td>1.66±0.45</td>
<td>0.469±0.071</td>
<td>0.0324±0.0022</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of 3-5 separate experiments. Significantly different from SHOK(neo), *p < 0.05, **p < 0.01 by Student’s or Welch’s t-test. # Welch’s t-test was used.

Sklar. Resistance to CDDP in H-ras transfectants has also been reported by other groups. However, no change or rather increase in sensitivity to CDDP was observed in H-ras transformed NIH3T3 cells. In a study by Gao et al., c-K-ras(Val-12)-transfected NIH3T3 cells were shown to have increased sensitivity to CDDP. Le-Ruppert et al., by using cells from human ovarian teratocarcinoma sublines, reported that N-ras activation increased sensitivity to CDDP. With regard to myc, CDDP resistance was observed in c-myc transfected NIH3T3 cells. Sklar et al., using Friend murine erythroleukemia cells, demonstrated that increased expression of c-myc induces increased CDDP resistance. Further, Van Waardenburg et al., reported that down-regulation of endogenously expressed c-myc in a CDDP-resistant human small cell lung carcinoma subline resulted in increased sensitivity to CDDP. In contrast, Gao et al., found no change in sensitivity to CDDP in v-myc-transfected NIH3T3 cells, which is in agreement with our data. In v-erbB-transfected NIH3T3 cells, Gao et al. found increased sensitivity to CDDP, which does not change with our data. It has been reported that the major mechanisms appearing to contribute to CDDP resistance include impairment of CDDP accumulation, elevated levels of methallothioneins (MTs) or glutathione (GST), and enhanced DNA repair. Ionishita et al., demonstrated that CDDP resistance in mutant c-H-ras overexpressed NIH3T3 cells was associated with an impairment of cellular CDDP accumulation, and with an increase in MT content but without change in GST content. Their results may be a clue to identifying which mechanism contributes to CDDP resistance of SHOK (H-ras) cells.

In the case of ADR, SHOK (myc) cells and SHOK (cot) cells were significantly more sensitive than SHOK (neo) cells; their IC50 value reduced to 42.4% and 52.9% of SHOK (neo) cells, respectively. In contrast, SHOK (H-ras) cells were 2.0 times more resistant than SHOK (neo) cells. This is the first report showing increased ADR sensitivity as a result of cot transformation. In myc-transformed NIH3T3 cells, increased sensitivity to ADR, which is consistent with our results, has been previously observed, though resistance to ADR has also been observed. In agreement with our observations, resistance to ADR has been reported in H-ras transformed NIH3T3 cells, as well as Mink lung epithelial cells. However, no change or increase in sensitivity to ADR has been observed in H-ras transformed NIH3T3 cells. In N-ras-transformed NIH3T3 cells, Gao et al. showed increased sensitivity to ADR. A correlation has been reported between intracellular ADR accumulation and sensitivity to ADR in NIH3T3 cells transfected with several oncogenes. Because ADR efflux did not differ among the group including the ras-family transfecants and the vector-transfected control, Gao et al. postulated that changes in ADR influx might be responsible for the changes in ADR sensitivity in those transfecants. Decreased ADR accumulation is known to be related to an overexpression of P-glycoprotein. Peters et al. observed that expression of P-glycoprotein was not different between NIH3T3 cells and their H-ras transfectant, although the ADR accumulation in the H-ras transfectant was significantly lower. The same non-P-glycoprotein-related resistance to ADR, which is associated with a lower accumulation of ADR, was also observed in other H-ras transformed lines. Although we did not examine ADR accumulation and the P-glycoprotein expression of our cell lines, a similar mechanism may contribute to the ADR resistance of SHOK (H-ras) cells.

In the case of MMC, as well as that of CDDP, no cells were more sensitive than SHOK (neo) cells. SHOK (K-ras) cells were 1.86 times significantly more resistant than SHOK (neo) cells. The IC₅₀ value of SHOK (H-ras) cells was elevated to as much as 4.33 times that of SHOK (neo) cells, though elevation itself was not significant. In H-ras-transformed NIH3T3 cells, no change or increase in sensitivity to MMC was reported. MMC is bioreductively activated by a number of oxidoreductases, and produces lethal adducts with DNA. In those enzyme systems, decrease in DT-diaphorase (DTD) activity has been known to be associated mainly with MMC resistance. Further investigation is needed to ascertain whether decrease in DTD activity contributes to MMC resistance of SHOK (K-ras) and SHOK (H-ras) cells.

Some of our data in the present study did not agree with the previously published data, especially with that of Gao et al. using NIH3T3 cells, although their results are not always the same as other studies using the same parent cells. Studies using NIH3T3 cells have raised some problems requiring clarification, such as the heterogeneity of their chromosome constitution and their difference in cell cycle distribution. The former has been relevant to clonal heterogeneity of radiation response, along with the malignancy and growth properties of cells, while the latter has been associated with the sensitivity of cells. Because we previously confirmed that there is no alteration in either modal karyotype or cell cycle distribution among SHOK cells and their transfectants whereas Gao et al. did not refer to these points, we speculate that some differences in chemosensitivity of transfectants between NIH3T3 cells and SHOK cells may come from these differences in characteristics of the parent cells.

Our results suggest that the expression of each oncogene would differently affect sensitivity to the four anticancer drugs used in this study. Our previous report, using SHOK cells, showed that each of mos, cot, and N-ras transfectants developed resistance to γ-rays. In the present study, mos transfectant and cot transfectant increased sensitivity to BLM and ADR, respectively. Therefore, if a clinical tumor tissue specimen is revealed to express mos or cot oncogene, we might have to choose BLM or ADR, respectively, instead of radiotherapy. Further, SHOK (H-ras) presented resistance significantly for BLM, CDDP, and ADR, and without significance but with about four times higher IC₅₀ value necessary for MMC. We also reported findings of SHOK (H-ras) resistance to UV and heat shock, and an absence of resistance to γ-rays. Therefore, if a specimen shows H-ras expression, radiotherapy might have to be chosen rather than these drugs and hyperthermia. Investigation of sensitivity to additional anticancer drugs having different mechanisms of action than those investigated herein would further the understanding of the drug resistance profile of SHOK (H-ras) cells.

The expression of mos or cot in human neoplasia has not been reported, until recently that of mos in lung carcinoma and astrocytic tumors, as well as overexpression and mutation of cot in Hodgkin’s disease, nasopharyngeal carcinoma, gastric and colonic adenocarcinomas, and breast cancer has been shown. In this study we for the first time shown that effect of mos on sensitivity to three (except CDDP) of the four anticancer drugs, and effect of cot on that to all of the four anticancer drugs. Therefore, our results, along with our previous results of γ-rays and heat shock, would be useful information on the selection of the most suitable modality to treat tumors that express mos or cot.

In breast cancer, both in vitro studies and clinical studies have shown that amplification of the HER-2/neu oncogene may play a role in predicting sensitivity to anticancer drugs. Accumulation of clinical studies could confirm that each oncogene that affects sensitivity to one or several anticancer drugs investigated in this study could be a marker to predict chemosensitivity in a particular human tumor.

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4567–4579.


Received on January 5, 2005
1st Revision received on February 3, 2005
Accepted on February 4, 2005


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