Mechanism for Resistance to 5-Fluorouracil in P388 Leukemia Cells

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In order to assess mechanisms for acquired resistance to 5-fluorouracil (5-FU) of P388 cells on a cellular basis, we compared sensitivities of P388 and its 5-FU-resistant subline (P388/5-FU) cells to 5-FU, 5-fluorouridine (FUr d) and 5-fluoro-2'-deoxyuridine (FdUrd). P388/5-FU cells exhibited an approximately 10-fold resistance to 5-FU and 170-fold cross-resistance to FUr d but not to FdUrd when they were exposed to each agent for 5 h in vitro. 5-FU-induced growth inhibition was hardly reversed with thymidine, suggesting its ribonucleic acid (RNA)-directed effect. This was supported by the fact that similar amounts of 5-FU were incorporated into cellular RNA in P388 and P388/5-FU when these cells were incubated with equitoxic concentrations of 5-FU. Furthermore, incorporation of 5-FU and FUr d into cellular RNA in P388/5-FU cells were significantly lower than in P388 cells when cells were exposed to them at the same concentration. These results suggest a major action of 5-FU is directed toward RNA in these cells at least under the present experimental condition, and 5-FU resistance of this cell line is closely associated with reduced uridine kinase activity among various enzymatic changes previously observed.

Keywords — 5-fluorouracil; resistance mechanism; P388 leukemia; uridine kinase

Introduction

5-Fluorouracil (5-FU) is one of the most useful antitumor agents effective against various solid tumors. However, the mechanism for resistance to 5-FU as well as its mode(s) of action are still controversial because of its intricate metabolism and dual actions against ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). In several studies on the mechanism for 5-FU resistance, altered activities of enzymes involved in 5-FU anabolism were reported.1-5

We established a 5-FU-resistant subline of P388 leukemia (P388/5-FU) by a therapeutic means,6 and Tezuka et al.7 investigated enzymatic characteristics of this resistant cell line. As a result, they found that activities of uridine kinase, deoxyuridine phosphorylase, and orotate phosphoribosyltransferase were significantly lower in the resistant cells than in the sensitive parental cells. In contrast, thymidine kinase activity was much higher in the resistant cells than in the sensitive cells. No difference in uridine phosphorylase activity was observed between two cell lines. However, it is not entirely clear which difference(s) among them actually contributes to the cellular resistance.

To assess the actual mechanism for resistance to 5-FU, therefore, it seems important to compare the characteristics observed in not only the cell-free but the intact cell system between the sensitive and resistant cells. In the present study using P388 leukemia model, we investigated the reversibility of resistance with thymidine (dThd), the pattern of cross-resistance to 5-fluorouridine (FUr d) and 5-fluoro-2'-deoxyuridine (FdUrd), and incorporation of 5-FU, FUr d or FdUrd into the RNA of the cells treated with each of them.

Materials and Methods

Chemicals — 5-FU was kindly supplied by Kyowa Hakko Kogyo Co., LTD., Tokyo. FUr d was a gift from Dr. Ohashi, Research Institute for Polymers and Textiles, Tsukuba, who synthesized it. FdUrd was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [6-3H]5-FU, [6-3H]FUr d and [6-3H]FdUrd were purchased from New England Nuclear, Boston, MA, U.S.A. Their radiochemical purities were 99% and specific activities were 12.9, 16.4 and 14.3 Ci/mmol, respectively.

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Cell Lines — P388 leukemia was supplied by the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD, U.S.A. P388/5-FU cells were developed by an in vivo procedure. 6

Cell Growth Inhibition Assay — P388 and P388/5-FU cells were grown in glass tubes containing RPMI1640 (M. A. Bioproducts, Walkersville, MD, U.S.A.) supplemented with 10% fetal calf serum (M. A. Bioproducts) and 100 µg/ml Kanamycin (Meiji Seika Kaisha, Ltd., Tokyo) at an initial cell density of 5 × 10⁴ cells/ml as a suspension culture.

Cells were exposed to varying concentrations of 5-FU, FUr 후 DfUr at 37 °C for 5 h. After adequate washing with phosphate-balanced saline, they were cultured for 48 h at 37 °C in a CO₂ incubator. At the end of the culture period, the number of cells was determined in a model ZBI Coulter counter after cells had been incubated with 0.25% trypsin for 5 min for cell dissociation.

Incorporation of Drug into RNA — One million cells in 1 ml of complete culture medium were incubated with 1 µM [³H]5-FU, [³H]FUr or [³H]DfUr for 3 h at 37 °C, and then RNA was extracted by a modification of the Ogor-Rosen method. 8 In detail, 2.5 ml of ice-cold 10% (w/v) perchloric acid was added to 1 ml of the cell suspension, followed by centrifugation. After washing by the same procedure, the precipitate obtained was suspended in 5 ml of ice-cold 1 N perchloric acid and shaken at 4 °C for 18 h to extract RNA. The residue was washed again with 1 N perchloric acid, and the washings were combined with the RNA extract. By measuring radio-activities of 1 ml aliquots, we estimated the molar content of these drugs incorporated into total RNA from 10⁶ cells.

Results

Cross-Resistance Pattern

We first compared sensitivities to 5-FU of P388 and P388/5-FU cells under the assay condition of 5 h drug exposure. P388/5-FU was approximately 10-fold resistant to 5-FU in vitro (Fig. 1A). We next studied their sensitivities to 5-FU nucleosides, FUr and FdUr, under the same condition. As a result, P388/5-FU exhibited up to approximately 170-fold cross-resistance to FUr (Fig. 1B), while it showed no cross-resistance to FdUr (Fig. 1C).

Effects of Thymidine on 5-FU-Induced Growth Inhibition

Cellular sensitivities to 5-FU shown in Fig. 1A were observed by exposing cells to it for 5 h to mimic as closely as possible in vivo pharmacokinetics that result from its bolus injection. To know how 5-FU acts on each of these cell lines under this condition, we observed the reversing
TABLE I. Reversing Effects of Thymidine on FdUrd- and 5-FU-Induced Growth Inhibition

<table>
<thead>
<tr>
<th>Cell</th>
<th>Drug concentration (μM)</th>
<th>Growth (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>dThd (-)</td>
</tr>
<tr>
<td>P388</td>
<td>FdUrd 10</td>
<td>5.6 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>5-FU 100</td>
<td>1.3 ± 2.0</td>
</tr>
<tr>
<td>P388/5-FU</td>
<td>FdUrd 10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-FU 300</td>
<td>0</td>
</tr>
</tbody>
</table>

Cells were exposed to either FdUrd or 5-FU for 5 h and then cultured in a fresh medium with or without 8—10 μM dThd for 48 h. Each value represents the mean ± S.D. of 3 determinations.

effects of dThd on 5-FU-induced growth inhibition. As a positive control, its effects on FdUrd-induced growth inhibition were also observed. As shown in Table I, the growth-inhibition by 5-FU on P388 and P388/5-FU cells was hardly reversed by dThd, while their FdUrd-induced inhibitions were significantly reversed by dThd. 

Incorporation of 5-FU, FUrld and FdUrd into Cellular RNA

First, 5-FU amounts incorporated into cellular RNA of 10^6 cells treated with equitoxic concentrations, 50% growth-inhibitory concentration (IC50's), of [%H]5-FU were compared between these cell lines. As shown in Table II, P388 and P388/5-FU cells exhibited a similar level of incorporation into cellular RNA despite significantly different extracellular drug concentrations.

Second, the amount of 5-FU incorporated into cellular RNA was compared when cells were incubated with the same concentration (1 μM) of [%H]5-FU. As demonstrated in Table III, cellular RNA of P388/5-FU contained only one third of the amount incorporated by P388. The incorporation of [%H]FUrld was also compared between these cell lines. It should be noted that its incorporation into the RNA of P388/5-FU cells was much less than that in P388 cells. In addition, the similarly small amount of FdUrd was incorporated into RNA in both cell lines.

TABLE II. Incorporation of 5-FU into Cellular RNA in P388 and P388/5-FU Cells at Its Equitoxic Concentrations

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (μM)</th>
<th>Incorporation of 5-FU into RNA (pmol/RNA of 10^6 cells/5 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388</td>
<td>7</td>
<td>18.1 ± 1.27</td>
</tr>
<tr>
<td>P388/5-FU</td>
<td>60</td>
<td>19.0 ± 0.25</td>
</tr>
</tbody>
</table>

Cells were exposed to [%H]5-FU at each IC50 for 3 h at 37 °C, and radioactivity associated with RNA isolated from 10^6 cells was counted. Each value represents the mean ± S.D. of 3 determinations.

Discussion

As enzymatic comparative study on P388 and P388/5-FU has already been reported.7 Significant changes in the activities of some anabolic enzymes for 5-FU, such as: decreased uridine kinase, deoxyuridine phosphorylase and orotate

TABLE III. Incorporation of 5-FU, FUrld and FdUrd into Cellular RNA

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incorporation of 5-FU into RNA (pmol/RNA of 10^6 cells/3 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-FU</td>
</tr>
<tr>
<td>P388</td>
<td>1.98 ± 0.013</td>
</tr>
<tr>
<td>P388/5-FU</td>
<td>0.66 ± 0.022</td>
</tr>
</tbody>
</table>

Cells were exposed to 1 μM [%H]5-FU, [%H]FUrld or [%H]FdUrd for 3 h at 37 °C, and radioactivity associated with RNA isolated from 10^6 cells was counted. Each value represents the mean ± S.D. of 3 determinations.
phosphoribosyl-transferase activities and increased thymidine kinase activity, were observed.
with P388/5-FU. However, it is not necessarily clear which change(s) among them make the
major contribution to resistance to 5-FU. In the present study, in order to assess mechanism for
this acquired resistance in P388/5-FU on a cellular basis, we investigated them in a non-
enzymatic manner.

P388/5-FU was developed by bolus i.p. injections of 5-FU once daily for 5 d after each succes-
sive transplantation.  As a result, this cell line became almost completely resistant in vivo
to 5-FU by the fifth transplant generation. Accordingly, in vitro sensitivity was determined by
relatively short exposure (5 h) to the drug instead of continuous long exposure because the phar-
macokinetic condition in vitro was not so different from the in vivo situation. Under this assay
condition, P388/5-FU exhibited an approximately 10-fold resistance to 5-FU (Fig. 1).

To determine how 5-FU acts on P388 and P388/5-FU cells under this drug exposure con-
dition, we examined the reversibility of 5-FU-induced growth inhibition by dThd, because the
mechanism for resistance to a certain agent is generally closely related to the mode of action
of the agent. As a result, reversal of 5-FU cytotoxicity with dThd was found to be much less
than that of FdUrd (Table I), suggesting that the major action of 5-FU against these cells, at least
under this condition, is directed toward its incorporation into RNA rather than the inhibition
of thymidylate synthase by 5-fluoro-2'-deoxy-yridylate.

To further confirm the RNA-directed action of 5-FU on these cell lines, we compared
amounts of 5-FU incorporated into cellular RNA of 10^6 cells of each line treated with its equitox-
ic concentration. In spite of very different extracellular concentrations, similar amounts of
5-FU were incorporated into cellular RNA in P388 and P388/5-FU (Table II). This result also
supports the view that the incorporation of 5-FU into RNA is an important determinant of its
cytotoxicity against at least P388 cells. The present finding was in good agreement with the
result of our recent kinetic analysis of cell killing action of 5-FU on P388 cells.  

Based on the RNA-directed effect of 5-FU on P388 leukemia cells, evident cross-resistance of
P388/5-FU to FdUrd but not to FdUrd strongly suggests decreased metabolism of FdUrd to
5-fluorouridylate as a possible mechanism for 5-FU resistance of this cell line. Furthermore, sig-
nificantly reduced incorporation of not only 5-FU but FdUrd into RNA in P388/5-FU cells
(Table III) supported this hypothesis. In conclusion, these results clearly indicate that decreased
activity of uridine kinase, which would probably result in decreased production of 5-fluo-
rouridine-5'-triphosphate, plays a major role in the resistance to both 5-FU and FdUrd in this
P388 model.

It should be noted that the degree of cross-resistance to FdUrd was much higher than that of
resistance to 5-FU. This implies that anabolism of 5-FU at some pathway other than phosphory-
lation of FdUrd is more efficient in P388/5-FU rather than P388 cells. Remarkably high
resistance observed with FdUrd seems to be weakened by that pathway in case of the treat-
ment with 5-FU. As a pathway, for example, the process of 5-FU to FdUrd might be possible.
Although uridine phosphorylase activity alone has been reported to be similar in both cell
lines, it is possible that the actual metabolism of 5-FU to FdUrd is more efficient in P388/5-FU
cells due to a higher intracellular concentration of ribose 1-phosphate. In any case, it is certain
that uridine kinase is a key enzyme for this re-
sistance. Decreased activity of uridine kinase has been reported with 5-FU-resistant sublines of
L1210 leukemia and Ehrlich carcinoma. However, it is uncertain how much such change
actually contributes to their cellular resistance, because activities of other anabolizing enzymes
such as uridine/deoxyuridine phosphorylase are also reduced, as were P388/5-FU cells.

In the present study, we found it very impor-
tant to study the mechanisms for 5-FU resistance
in view of not only the enzymatic but also the
cellular level of difference. We are currently in-
vestigating mechanisms for 5-FU resistance in
human colon and gastric cancer cell lines in this
respect.

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References


