Increased Sensitivity to Long-term 5-Fluorouracil Exposure of Human Colon Cancer HT-29 Cells Resistant to Short-term Exposure

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A 5-fluorouracil (5-FU)-resistant subline of human colon cancer HT-29 cells was developed by repeated 1-h exposure in vitro to 5-FU. This subline (HT-29/5-FU/S) had 8-fold resistance to 5-FU in a 1-h exposure assay. However, it had rather increased sensitivity to 5-FU when assayed after a continuous 96-h exposure to it. Significantly less 5-fluorouridine-5′-triphosphate was produced in the resistant cells, leading to a lower level of 5-FU incorporation into the cellular RNA. The reduced activity of orotate phosphoribosyltransferase might explain these results. In contrast, the HT-29/5-FU/S cells were more sensitive to the inhibition of in situ thymidylate synthase (TS) by 5-FU than were the parent cells. The lower in situ TS activity may have made HT-29/5-FU/S cells more sensitive to TS inhibition by 5-FU as compared with the parent cells. The fact that HT-29/5-FU/S was more resistant to short-term 5-FU exposure but more sensitive to long-term exposure than the parent line confirmed the existence of different modes of action of 5-FU, depending on the exposure time.

Key words: HT-29 human colon cancer — 5-Fluorouracil-resistance — Dual actions of 5-FU — Exposure time

5-FU is a unique anticancer agent with two major pharmaco-biochemical actions: of its active metabolites, FUTP is fraudulently incorporated into RNA and impairs the multiple functions of RNA, while FdUMP blocks the catalytic activity of TS by forming a ternary covalent complex with its co-substrate, 5,10-methylene-5,10-methylenetetrahydrofolate. Thus 5-FU potentially exerts both RNA- and DNA-directed actions against target cells.

One of the factors determining which action 5-FU preferentially exerts on the target cells seems to be their innate biochemical characteristics of 5-FU metabolism. If the resultant intracellular concentration of FUTP or FdUMP is lower than the effective level, 5-FU may exert only one of its dual actions. However, it seems to exert dual actions against the majority of cancer cells, although the efficacy of such actions varies. Another determinant is the length of the exposure time to 5-FU. We have previously developed a method for the kinetic analysis of the cell-killing action of anticancer agents which can classify them into cell cycle phase-nonspecific (type I) drugs and cell cycle phase-specific (type II) drugs.1 According to this analysis, FUrd and FdUrd showed typical kinetic profiles for type I and II drugs, respectively. 5-FU had a FUrd-like kinetic profile for cell killing action when cells were exposed for a short time and a FdUrd-like profile when they were continuously exposed for a long time.3,4 These results suggested that 5-FU exerts RNA-directed action under short exposure conditions and DNA-directed action under long, continuous exposure conditions. The former requires a high 5-FU concentration, while for the latter, a low concentration is sufficient.

In view of the dual actions of 5-FU, which depend on the exposure time, it might be possible to develop a subline which acquires resistance to short/long exposure to 5-FU, but does not show cross-resistance to long/short exposure. Based on this idea, we have established some 5-FU-resistant sublines of human gastrointestinal cancer cell lines by repeated long-term exposure to 5-FU. However, we failed to obtain a subline of the type we had expected. In the present study, we tried repeated 1-h exposure of human colon cancer HT-29 cells to 5-FU, and established a subline named HT-29/5-FU/S. This subline is resistant to short-term exposure to 5-FU, exhibited no cross-resistance to long-term exposure to 5-FU, and had rather higher sensitivity than its parent cells.

MATERIALS AND METHODS

Chemicals [3H]5-FU (655 GBq/mmol) was purchased from NEN Life Science Products (Wilmington, DE); [3H]FdUMP (481 GBq/mmol), [3H]FUrd (740 GBq/mmol) and [3H]FdUrd (740 GBq/mmol) were from Moravek Biochemicals (Brea, CA); Sulforhodamine B was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.
Cell lines  HT-29, a human colon cancer cell line, was obtained from American Type Culture Collection (Rockville, MD) through Dainippon Pharmaceutical Co., Tokyo. A 5-FU-resistant subline, HT-29/5-FU/S, was established by repeated 1-h exposure of HT-29 cells to increasing concentrations of 5-FU (0.5 to 10 mM).

Drug sensitivity assays  The sensitivity of cells in vitro was determined by means of the sulforhodamine B assay\(^1\) with a minor modification. Briefly, cells were cultured with the drug for 96 h (continuous exposure), or for 1 h with the drug followed by 96 h without the drug (1-h exposure). After culture, the adherent cells were fixed in 10% trichloroacetic acid, washed and dried. Sulfurhodamine B (0.4% wt/vol. in 1% acetic acid) was added to stain the cells, and unbound sulfurhodamine B was removed by washing with 1% acetic acid. The cells were air-dried, and the bound sulforhodamine B was solubilized in 10 mM unbuffered Tris base (pH 10.5). The optical density was read on an automated spectrophotometric plate reader at a wavelength of 525 nm.

5-FU incorporation into RNA  Cells at the logarithmic growth phase were incubated with 300 µM \([^{3}H]\)5-FU for 1 h at 37°C. Four million cells harvested with trypsin were washed twice with phosphate-buffered saline, then treated twice with 2.5 ml of 10% (wt/vol.) perchloric acid, and RNA was extracted by shaking the precipitated residue in 5 ml of 1 N perchloric acid for 18 h at 4°C. After centrifugation, the supernatant was transferred to a tube, to which another 5 ml of 1 N perchloric acid washing the residue was added. The radioactivity levels in aliquots of the RNA extract were measured using a Beckman model LS 7500 scintillation counter (Fullerton, CA) through Dainippon Pharmaceutical Co., Tokyo. The eluate was analyzed using a Shimadzu model LC-10A HPLC (Tokyo) equipped with an Inertsil ODS column (250×6.0 mm, 4-mm particle size; GL Sciences, Tokyo). The elution proceeded as described previously.\(^2\) The radioactivity of fractions containing FUTP or FdUMP was measured.

Assay for 5-FU-anabolizing enzymes  The activities of uridine/thymidine phosphorylase and kinase and OPRT were measured as described by Peters et al.\(^3\) Briefly, phosphorylase was assayed using 0.5 mM \([^{3}H]\)FdUrd or \([^{3}H]\)FdUrd, 2.5 mM β-mercaptoethanol and 100 mM sodium phosphate buffer (pH 6.4); kinase was assayed using 0.5 mM \([^{3}H]\)FdUrd or \([^{3}H]\)FdUrd, 5 mM ATP, 5 mM MgCl\(_2\), 15 mM 2-glycerophosphate and 50 mM Tris-HCl (pH 8.0); OPRT was assayed using 0.5 mM \([^{3}H]\)5-FU, 2 mM PRPP, 5 mM MgCl\(_2\), 15 mM 2-glycerophosphate, 0.6 mM α,β-methyladenosine diphosphate and 50 mM Tris-HCl (pH 8.0). These mixtures were incubated at 37°C with 40–50 µl of the cytosol in a total volume of 100 µl for 20 min, and the reaction was stopped by heating at 90–100°C. After centrifugation at 14,000 rpm for 2 min in a Tomy model MR-150 centrifuge (Tokyo), 20 µl of the supernatant was charged with the cold carrier on a PEI-cellulose thin-layer chromatography sheet and developed with water. The spots of substrate and product were distinguished under UV and excised, and the level of radioactivity in each was measured.

In situ TS assay  The in situ TS activity was measured as described previously.\(^3\) Cells (8×10^5 cells/ml) were incubated with or without 5-FU for 5 h at 37°C. \([^{3}H]\)dTdUrd (final 37 kBq/ml) was added 2 h after the start of the incubation. The reaction was terminated by transferring 100-µl aliquots into centrifuge tubes containing 200 µl of a 10% activated charcoal suspension in 4% aqueous trichloroacetic acid. The tubes were vigorously stirred by vortexing, then centrifuged at 14,000 rpm for 2 min in a Kubota model KM-15200 microfuge (Tokyo). The radioactivity in 150 µl of the supernatant was determined in a scintillation counter.

RESULTS

Sensitivity to 5-FU and FdUrd  When cells were exposed to various concentrations of 5-FU for 1 h and cultured without the drug for 96 h, the IC_(50) values of 5-FU for HT-29 and HT-29/5-FU/S were 0.1 and 0.77 mM, respectively (Fig. 1A), which indicated 8-fold resistance. However, when cells were continuously exposed to 5-FU for 96 h, the IC_(50) values of 5-FU for HT-29 and HT-29/5-FU/S were 0.14 and 0.033 µM, respectively. These results demonstrate that HT-29/5-FU/S was resistant to short-term exposure, but became more sensitive to long-term exposure to 5-FU (Fig. 1B). HT-29/5-FU/S also showed significant collateral sensitivity to FdUrd (Fig. 1C).

5-FU incorporation into RNA  The amount of 5-FU incorporated into the cellular RNA was measured after incubation of the cells with 300 µM \([^{3}H]\)5-FU for 1 h and
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After post-incubation for 24 h in drug-free medium. Under both conditions, the amount of 5-FU incorporated into RNA in HT-29/5-FU/S was 60% less than in HT-29 cells (Fig. 2). The P values (Student’s t test) were 0.0003 for A and 0.0002 for B.

**Levels of active metabolites of 5-FU** Intracellular FUTP level was assessed after 1-h incubation with 300 µM [3H]5-FU (Table I). The level in HT-29/5-FU/S cells was one-fourth of that in HT-29 cells. FdUMP level was measured after 5-h incubation with 10 µM [3H]5-FU. It was also lower in the resistant cells, but the difference was not as marked as in the case of FUTP.

**Activities of 5-FU-anabolizing enzymes** The activities of five enzymes involved in 5-FU anabolism were measured (Table II). Only OPRT showed lower activity in HT-29/5-FU/S cells. Uridine phosphorylase/kinase and thymidine phosphorylase exhibited rather higher activity in the resistant cells. There was no significant difference in thymidine kinase activity between the two cell lines.

**In situ TS activity and its sensitivity to 5-FU** Cells were incubated with or without 5-FU for 5 h and the in situ TS activity was determined for the last 3 h (Fig. 3). The basal levels of TS activity measured in the absence of 5-FU were 70.4±7.1 and 35.3±2.7 (pmol/mg protein/3 h) for HT-29 and HT-29/5-FU/S cells, respectively; the TS activity in the 5-FU resistant cells was about 50% lower than that in the parent cells (P=0.0004). The values of percentage inhibition of in situ TS in HT-29 and HT-29/5-FU/S cells were 0 and 21% at 1 µM 5-FU, and 40 and 85% at 10 µM 5-FU, respectively. This demonstrates that HT-29/5-FU/S cells were more sensitive to TS inhibition by 5-FU than HT-29 cells.

Fig. 1. Sensitivity of HT-29 and HT-29/5-FU/S cells to 5-FU and FdUrd. Cells were exposed to various concentrations of 5-FU for 1 h then cultured without the drug for 96 h (A) or cultured with 5-FU (B) or FdUrd (C) continuously for 96 h. The relative cell number was measured by a sulforhodamine B assay. Each point is the mean of 4 determinations with a coefficient of variation of less than 10%. ○ HT-29; ● HT-29/5-FU/S.

Fig. 2. The incorporation of 5-FU into RNA in HT-29 and HT-29/5-FU/S cells. Cells were exposed to 300 µM 5-FU for 1 h (A) or incubated without the drug for 24 h after 1 h exposure to 300 µM 5-FU (B) and then RNA was extracted as described in “Materials and Methods.” Each column is the mean of 3 determinations with the bar indicating the standard deviation. The open and shaded columns denote HT-29 and HT-29/5-FU/S, respectively.
DISCUSSION

We have established two sublines of human stomach and colon cancer cell lines with acquired resistance to long-term exposure to 5-FU. Both of them also showed resistance to short-term exposure to 5-FU. The major mechanism of resistance in these sublines was the decreased production of two active metabolites, FUTP and FdUMP. In the present study, we established a subline of human colon cancer HT-29 cells by repeated 1-h exposure to stepwise-increasing concentrations of 5-FU. This subline manifested 8-fold resistance to 5-FU in a 1-h exposure assay. In contrast, it showed rather higher sensitivity to 5-FU and FdUrd under a 96-h exposure condition (Fig. 1).

Aschele et al. also established 5-FU-resistant sublines of human colon cancer (HCT-8) using two different exposure schedules. One subline developed by repeated 4-h exposure to 5-FU showed 3-fold resistance to short-term exposure to 5-FU but retained full sensitivity to long-term exposure. Decreased incorporation of 5-FU into cellular RNA was found in this cell line. Another subline developed after repeated 7-day exposure to 5-FU exhibited 10-fold increased resistance to long-term exposure and marked cross-resistance to short-term exposure to 5-FU. Decreased expression of folypolyglutamate synthetase was reported as a novel mechanism of resistance to 5-FU in this cell line.

We studied the mechanism of resistance to short-term exposure and the increased sensitivity to long-term exposure to 5-FU in HT-29/5-FU/S cells. A significant reduction of 5-FU incorporation into cellular RNA was found in this cell line. Another subline developed after repeated 7-day exposure to 5-FU exhibited 10-fold increased resistance to long-term exposure and marked cross-resistance to short-term exposure to 5-FU. Decreased expression of folypolyglutamate synthetase was reported as a novel mechanism of resistance to 5-FU in this cell line.

Table I. FdUMP/FUTP Levels in HT-29 and HT-29/5-FU/S Cells

| Substrate | Incubation time (h) | Products (pmol/mg protein) | P value |
|-----------|---------------------|----------------------------|---------|
|           | Metabolite          | HT-29          | HT-29/5-FU/S |         |
| 300 µM 5-FU | 1                 | FUTP           | 679±48     | 1602±14.6 | 0.0036 |
| 10 µM 5-FU  | 5                 | FdUMP          | 4.14±0.13  | 2.28±0.57 | 0.0209 |

Table II. Enzyme Activity of 5-FU Anabolism in HT-29 and HT-29/5-FU/S Cells

| Enzyme                   | Activity (nmol/mg protein/20 min) | P value |
|--------------------------|-----------------------------------|---------|
|                          | HT-29                              | HT-29/5-FU/S |         |
| Uridine phosphorylase    | 2.60±0.72                         | 7.15±1.05 | 0.0285  |
| Uridine kinase           | 4.62±0.48                         | 8.16±0.36 | 0.0105  |
| Orotate phosphoribosyltransferase | 7.47±0.34                    | 4.64±0.24 | 0.0041  |
| Thymidine phosphorylase  | 11.5±1.7                          | 21.7±4.7  | 0.0774  |
| Thymidine kinase         | 17.2±1.8                          | 14.5±1.2  | 0.261   |

Fig. 3. In situ thymidylate synthase inhibition by 5-FU in HT-29 and HT-29/5-FU/S cells. Cells were incubated with or without 5-FU for 5 h, and [3H]dUrd was added 2 h later after the start of incubation. In situ TS activity was measured as described in “Materials and Methods.” Each column is the mean of 3 determinations with the bar indicating the standard deviation. The open column denotes HT-29 and the shaded one denotes HT-29/5-FU/S.
ribose-1-phosphate pool, the decreased activity of OPRT may contribute to the decreased production of FUTP. An increased level of TS as a target enzyme of 5-FU can be a mechanism of resistance to 5-FU. Copur et al. reported elevated levels of TS protein and mRNA and amplification of the TS gene in human colon H630 cells which acquired resistance to continuous exposure to 5-FU. Therefore, we determined the basal level of the in situ TS activity and the inhibitory effect of 5-FU. The basal activity of the in situ TS in HT-29/5-FU/S cells was significantly lower than that in HT-29 cells. Probably as a result of the lower TS activity, the inhibitory effect of 5-FU on in situ TS was more potent in HT-29/5-FU/S than in HT-29 cells (Fig. 3). This could explain the increased sensitivity of HT-29/5-FU/S cells to long-term exposure to 5-FU, in which DNA-directed action through TS inhibition seems to be predominant. The difference in the effective concentrations of 5-FU (see Figs. 1 and 3) might be caused by the difference in exposure time between these two experiments.

In conclusion, an HT-29 subline that had acquired 8-fold increased resistance to short-term exposure to 5-FU showed collateral sensitivity to the same drug under a long-term exposure condition. This result provides additional evidence for the hypothesis that 5-FU exerts different modes of cytotoxic action depending on the exposure time and treatment schedule.

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