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Reduced Activity of Anabolizing Enzymes in 5-Fluorouracil-resistant Human Stomach Cancer Cells

Makoto Inaba,[1,3] Junko Mitsuhashi,[1] Hiroko Sawada,[1] Naoko Miike,[1] Yoshinori Naoe,[1] Aiko Daimon,[1] Kazuhisa Koizumi,[2] Hiroaki Tsujimoto[2] and Masakazu Fukushima[2]

[1]*Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-Ikebukuro 1-37-1, Toshima-ku, Tokyo 170 and* [2]*Cancer Research Laboratory, Hanno Research Center, Taiho Pharmaceutical Co., Ltd., Nakayashita 216-1, Yaoroshi, Hanno, Saitama 357*

The mechanism of resistance to 5-fluorouracil (5-FU) was studied with NUGC-3/5FU/L, a human stomach cancer cell line which had acquired resistance as a consequence of repeated 5-day exposures to stepwise-increasing concentrations of 5-FU *in vitro*. NUGC-3/5FU/L was 200-fold and over 16-fold resistant to 96-h and 1-h exposures to 5-FU, respectively. NUGC-3/5FU/L incorporated less 5-FU into RNA, indicating resistance to the RNA-directed action of 5-FU. On the other hand, NUGC-3/5FU/L also showed resistance to *in situ* thymidylate synthase (TS) inhibition by 5-FU. Polymerase chain reaction-single-strand conformation polymorphism analysis of TS cDNA and a FdUMP ligand binding assay showed that quantitative and qualitative alterations of TS are not responsible for this resistance. In contrast, the ability to metabolize 5-FU to its active metabolites, FUTP and FdUMP, was reduced in NUGC-3/5FU/L. We found that not only the activities of uridine phosphorylase/kinase and orotate phosphoribosyl-transferase (OPRT), but also the level of phosphoribosyl pyrophosphate, a cosubstrate for OPRT, were significantly lower in NUGC-3/5FU/L than in the parent NUGC-3. These results indicated that resistance to 5-FU in NUGC-3/5FU/L is due to reduced activities of 5-FU-anabolizing enzymes, but not to an alteration of TS. 2′-Deoxyinosine effectively enhanced TS inhibition by 5-FU in the resistant cells, thus markedly sensitizing them to 5-FU.

Key words: 5-Fluorouracil — Mechanism of resistance — Human stomach cancer line — Anabolizing enzyme — 2′-Deoxyinosine

5-FU[4] is clinically useful against solid cancers, particularly stomach and colon cancers, being unique among several antimetabolic anticancer drugs. As with other anticancer drugs, cancers initially responsive to 5-FU acquire resistance to it after several treatments. Thus, it is important to understand how human gastrointestinal cancers become resistant to 5-FU. Although 5-FU has been clinically used for very long periods, there are rather few reports on resistance to this agent in human cancers.

A number of investigators have studied FdUrd resistance,[1–11] but not 5-FU resistance of human cancer cell lines. Defective membrane transport,[3,4] low FdUMP levels,[2,10] reduced TS affinity to FdUMP[1,6,7,9,11] and elevated TS contents[5] have been proposed as mechanisms for resistance to FdUrd. However, it is important to distinguish strictly between 5-FU and FdUrd with respect to the mechanism of resistance, because the modes of cytotoxic action and the metabolic pathways are quite different between the two drugs.[12]

Only a few investigators have studied acquired resistance to 5-FU. Aschele *et al.*[13] developed two sublines of human colon cancer, HCT-8, by repeatedly exposing the cells to 5-FU for only 4 h or continuously for 7 days. The subline developed by 4-h exposure showed less incorporation of 5-FU into RNA, although the precise mechanism remains unknown, and the subline established by the 7-day exposure exhibited less inhibition of TS, possibly due to a defect in the polyglutamylation of the folate cofactor. Copur *et al.*[14] have reported that two sublines of H630 (a human colon cancer cell line), which were developed by long-term continuous exposure to various concentrations of 5-FU, had significantly higher basal levels of TS. Innate resistance to 5-FU in human gastrointestinal cancers has also been studied in some experi-

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[3] To whom requests for reprints should be addressed.

[4] The abbreviations used are: 5-FU, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FUrd, 5-fluorouridine; FdUMP, 5-fluoro-2′-deoxyuridylate; FUMP, 5-fluorouridylate; FUTP, 5-fluorouridine-5′-triphosphate; dUrd, 2′-deoxyuridine; dIno, 2′-deoxyinosine; R-1-P, ribose-1-phosphate; dR-1-P, 2′-deoxyribose-1-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; 5,10-CH_2-FH_4, 5,10-methylene-tetrahydrofolate; PBS(−), Ca^{++}, Mg^{++}-free phosphate-buffered saline; SRB, sulforhodamine B; OPRT, orotate phosphoribosyltransferase; TS, thymidylate synthase; HPLC, high-performance liquid chromatography; AGPC method, acid guanidinium thiocyanate-phenol-chloroform method; cDNA, complementary DNA; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; PAGE, polyacrylamide gel electrophoresis.

mental models[15, 16] as well as in clinical tumors,[17, 18] and multiple mechanisms have been found.[15–18]

In this study, we developed a 5-FU-resistant subline of NUGC-3, a cultured cell line of a human stomach cancer, by repeated 5-day exposures to stepwise-increasing concentrations of 5-FU *in vitro*. We found a different mechanism of resistance from those reported for colon cancer cells.[13, 14]

MATERIALS AND METHODS

Chemicals [^{3}H]5-FU (17.7 Ci/mmol) and [8-^{14}C]hypoxanthine (55 mCi/mmol) were purchased from Du Pont Co. (Wilmington, DE); [^{3}H]FUrd (20 Ci/mmol), [^{3}H]FdUrd (22 Ci/mmol), [^{3}H]FdUMP (13 Ci/mmol) and [^{3}H]dUrd (20 Ci/mmol) were from Moravek Biochemicals (Brea, CA); dIno, leucovorin and SRB from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Cell lines NUGC-3, a human stomach cancer cell line,[19] was supplied by Japanese Cancer Research Resources Bank (Tokyo). A 5-FU resistant subline of NUGC-3, NUGC-3/5FU/L, was developed as follows: NUGC-3 cells were seeded at approximately 4×10^5 cells in 60-mm dishes and cultured with RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (M.A. Bioproducts) in the presence of 0.02 m*M* 5-FU for 120 h. The dishes were cultured in drug-free medium until the surviving cells regrew. These cells were repeatedly exposed for 120 h to stepwise-increasing concentrations of 5-FU up to 0.4 m*M*.

Drug sensitivity assays The sensitivity of cells *in vitro* was assessed by means of the SRB assay[20] 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 the culture, the adherent cells were fixed in 10% TCA, washed and dried. SRB (0.4% wt/vol in 1% acetic acid) was added to stain the cells, and unbound SRB was removed by washing with 1% acetic acid. The cells were air-dried, and bound SRB was solubilized in 10 m*M* unbuffered Tris base (pH 10.5). The optimal densities were read on an automated spectrophotometric plate reader at a single wavelength of 525 nm.

Procedure for the colony assay was as described.[21]

5-FU incorporation into RNA Cells at the logarithmic growth phase in culture plates (15 cm in diameter) were incubated with 300 μM [^{3}H]5-FU for 1 h at 37°C. Some plates were further cultured in a drug-free medium for 24 h. Cells harvested from 5 plates with trypsin were transferred to a polypropylene tube and washed with phosphate-buffered saline. RNA was isolated using the AGPC method.[22] The radioactivity of an aliquot of RNA was measured using a Beckman scintillation counter (Model LS7500) and the RNA content was estimated from the UV absorbance at 260 nm.

***In situ* TS assay**[23] Cells (8×10^4 cells/ml) were incubated with or without 5-FU at 37°C for 5 h. [5-^{3}H]dUrd (final concentration 1 μCi/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 TCA. The tubes were vigorously stirred by vortexing then centrifuged at 14,000 rpm for 2 min in a Kubota microfuge (model KM-15200). The radioactivity in 150 μl of the supernatant was determined in a scintillation counter.

PCR-SSCP analysis of TS cDNA fragments As described by Orita *et al.*,[24] we examined whether or not cDNAs prepared from mRNAs of NUGC-3 and NUGC-3/5FU/L cells were identical with respect to the base sequence. Briefly, total RNA was prepared from both cell lines using the AGPC method,[22] and cDNAs of the TS gene were synthesized by RT-PCR[25] using a TS cDNA probe prepared as described by Kaneda *et al.*[26] Eight ^{32}P-labeled segments (A-H) of TS cDNA covering the entire coding region were synthesized by PCR using the cDNAs from both cell lines and 8 pairs of primers (Fig. 4). PCR consisted of 35 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 1 min. The formed segments were resolved by PAGE after dilution in formamide and heating to dissociate the DNA strands. Bands on the gel were detected by autoradiography and analyzed for TS cDNA mutation in the resistant cells.

FdUMP ligand TS binding assay Cytosol was prepared by harvesting over 10^7 cells at the logarithmic growth stage, sonicating them in homogenate buffer (50 m*M* Tris-HCl, 1 m*M* EDTA and 5 m*M* $MgCl_2$, pH 7.4) at maximum output (Sonifier cell disruptor 350; Smith-Kline), and centrifuging at 75,000*g* at 4°C for 20 min in a Beckman ultra-centrifuge (model TL-100). The supernatant was divided into several tubes and frozen at −80°C until use.

The cellular basal TS contents were measured by a [^{3}H]FdUMP binding assay combined with gel filtration. The cytosol was incubated with [^{3}H]FdUMP and 5,10-CH_2-FH_4 at 37°C for 30 min as described by Spears *et al.*,[27] then the mixture was gel-filtered using a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) to separate TS-bound from free [^{3}H]FdUMP. The sample was eluted with PBS(−) and the total radioactivity of the fractions containing protein was measured. Protein content of the cytosol was measured using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

Assay of intracellular FUTP and FdUMP About 10^6 cells were incubated at 37°C with 400 μM [^{3}H]5-FU for 10 min to measure the FUTP level or with 1 μM [^{3}H]5-FU for 3 h to measure FdUMP level. Cells were washed

twice with PBS(−), disrupted in 1 ml of 1% NP-40 in a lysis buffer (10 m*M* Tris-HCl, 5 m*M* EDTA, 50 m*M* NaF, 50 m*M* NaCl; pH 7.5) and centrifuged at 75,000*g* for 20 min at 4°C. Cold 6 *M* TCA was added to 500 μl of the supernatant to give a final concentration of 0.6 *M* and the mixture was centrifuged. The supernatant was added to 2 vol. of cold Freon containing 17.7% tri-*n*-octylamine, and the mixture was vortex-mixed and centrifuged. The last procedure was repeated until the pH of the aqueous phase became 7, when this phase was separated and filtered through ultrafree C3HV. The filtrate was analyzed by HPLC (Shimadzu, model LC-10A) equipped with an Inertsil ODS column (250×6.0 mm, 4-μm particle size; GL Sciences, Tokyo). The elution proceeded as described.[28] The radioactivity levels in the FUTP and FdUMP fractions were measured.

Assay of 5-FU-anabolizing enzymes Activities of pyrimidine nucleoside phosphorylase and kinase and OPRT were measured as reported by Peters *et al.*[29] Briefly, phosphorylase was assayed using 0.5 m*M* [^{3}H]FUrd or [^{3}H]FdUrd, 2.5 m*M* β-mercaptoethanol and 100 m*M* sodium phosphate buffer (pH 6.4). Kinase was assayed using 0.5 m*M* [^{3}H]FUrd or [^{3}H]FdUrd, 5 m*M* ATP, 5 m*M* $MgCl_2$, 15 m*M* 2-glycerophosphate and 50 m*M* Tris-HCl (pH 8.0). OPRT was assayed using 0.5 m*M* [^{3}H]5-FU, 2 m*M* PRPP, 5 m*M* $MgCl_2$, 15 m*M* 2-glycerophosphate, 0.6 m*M* α, β-methylene-adenosine diphosphate and 50 m*M* 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 them at 90–100°C. After centrifugation at 14,000 rpm for 2 min in a Tomy centrifuge (model MR-150), 20 μl of the supernatant was charged together with cold carrier on a PEI-cellulose thin-layer chromatography sheet and developed with water. The spots of the substrate and product were distinguished under UV and excised, and the level of radioactivity in each was measured.

Measurement of PRPP The intracellular PRPP level was measured as the formation of [^{14}C]IMP from [^{14}C]hypoxanthine in the presence of hypoxanthine-guanine phosphoribosyltransferase (Sigma) and boiled cytosol.[30] Briefly, the cytosol was prepared as described above except for heating at 90°C for 1 min after sonication. Cytosol (50 μl) was incubated with the enzyme preparation (10 units), [^{14}C]hypoxanthine (10 nmol) and 5 m*M* $MgCl_2$ at 37°C for 10 min, and the reaction mixture was ultrafiltered and subjected to HPLC to quantify the amount of [^{14}C]IMP formed.

RESULTS

Degree of resistance to 5-FU NUGC-3/5FU/L was established by repeated 5-day exposures to stepwise-increasing concentrations of 5-FU. As shown in Fig. 1, NUGC-3/5FU/L exhibited high resistance to 5-FU during exposure for 1 and 96 h. In the 96-h exposure experiment, the IC_{50}s for NUGC-3 and NUGC-3/5FU/L were 2.5 and 520 μM, respectively, indicating about 200-fold resistance. In 1-h exposure, those values were 640 and above 10,000 μM, respectively, implying over 16-fold resistance.

Less incorporation of 5-FU into RNA in the resistant cells Cells of both sensitive and resistant lines were

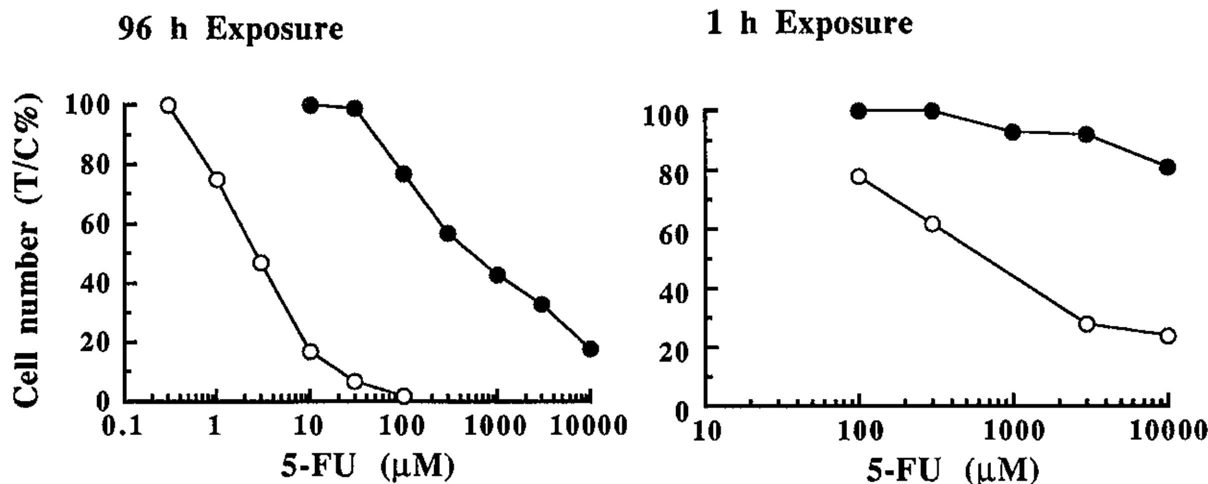

Fig. 1. Sensitivity of NUGC-3 and NUGC-3/5FU/L cells to 5-FU. Cells were cultured with various concentrations of 5-FU for 96 h, or exposed to 5-FU for 1 h then cultured without the drug for 96 h. The relative cell number was measured by a sulforhodamine B assay. ○, NUGC-3; ●, NUGC/5FU/L.

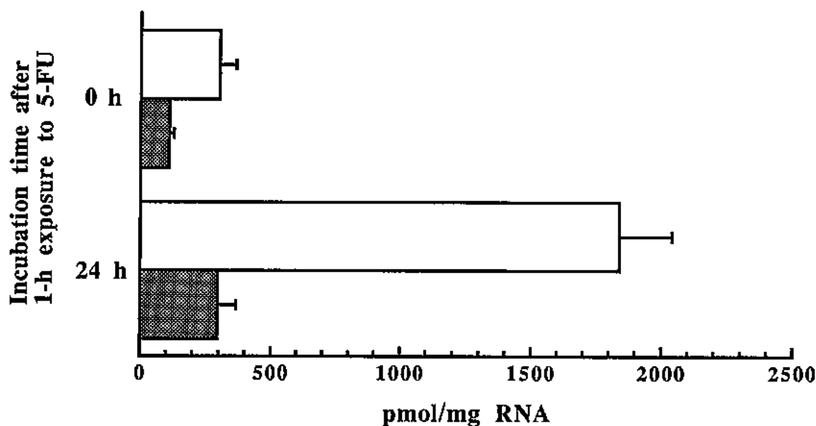

Fig. 2. The incorporation of 5-FU into RNA in NUGC-3 and NUGC-3/5FU/L cells. Cells were exposed to 300 μM 5-FU for 1 h, and RNA was isolated using the AGPC method immediately after the drug exposure or after 24 h post-incubation without the drug. Unfilled columns, NUGC-3; filled columns, NUGC-3/5FU/L.

incubated with 300 μM 5-FU for 1 h, and the amount of 5-FU incorporated into cellular RNA was measured. The results indicated that 5-FU content per mg RNA in NUGC-3/5FU/L cells was 36% of that in NUGC-3 cells (Fig. 2). Following a 1-h exposure to 5-FU, cells were further incubated for 24 h in the absence of 5-FU, and then the 5-FU contents in the RNA were assessed. As shown in Fig. 2, the 5-FU in the RNA from NUGC-3 cells was elevated 6-fold from that of the 1-h incubation, whereas in NUGC-3/5FU/L cells it increased only 2.8-fold, thereby indicating a marked difference in the 5-FU contents per mg of RNA between the two cell lines: the level of 5-FU in RNA of NUGC-3/5FU/L cells was only 16% of that of NUGC-3 cells.

Less *in situ* TS inhibition by 5-FU in the resistant cells Cells were incubated with 3 concentrations of 5-FU for 5 h, and the inhibition of *in situ* TS activity during the last 2 h was measured. As shown in Fig. 3, at 30 μM 5-FU, *in situ* TS activity of NUGC-3 cells was inhibited by 90% (T/C value), while that of NUGC-3/5FU/L cells was inhibited by only 17%, with half of the original activity remaining even at 300 μM. These results demonstrated that NUGC-3/5FU/L cells are significantly resistant to the inhibitory effect of 5-FU on intracellular TS.

Qualitative and quantitative analysis of TS To determine whether there is any alteration of amino acid sequence in TS of NUGC-3/5FU/L cells, we compared the TS cDNAs from the two cell lines by PCR-SSCP analysis. Eight DNA segments covering the entire coding region of TS cDNA were prepared (Fig. 4), and compared between the two cell lines by SSCP analysis (Fig. 5). No differences were evident, indicating that there was no qualitative difference between the TS molecules of these lines.

The basal level of intracellular TS was measured in terms of [^{3}H]FdUMP binding to TS in the cytosol. No significant difference in TS content was found between the cell lines (Table I). This finding indicated that there was also no quantitative difference in TS between the two lines.

Reduced production of 5-FU active metabolites Cells were incubated with 400 μM 5-FU for 10 min, and the amounts of FUTP produced by the two cell lines were

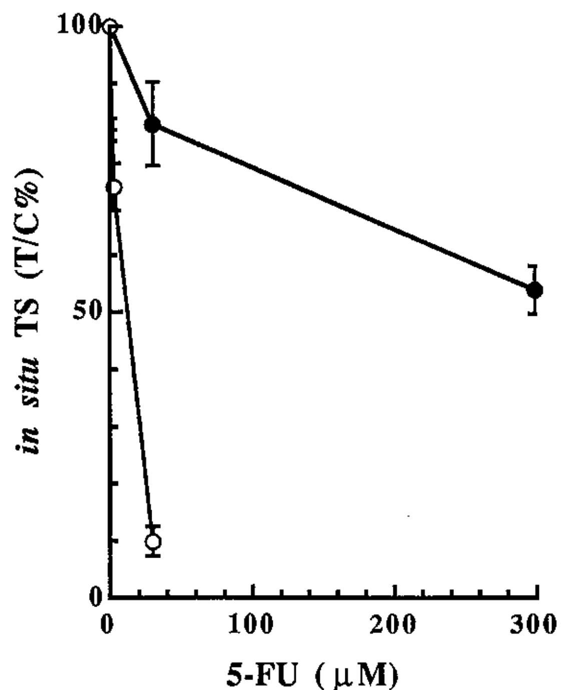

Fig. 3. Sensitivity of *in situ* TS to 5-FU in NUGC-3 and NUGC-3/5FU/L cells. Cells were incubated with 3 concentrations of 5-FU for 5 h, and [5-^{3}H]dUrd was added 2 h after the start of incubation. The *in situ* TS activities were measured (see "Materials and Methods"). ○, NUGC-3; ●, NUGC/5FU/L.

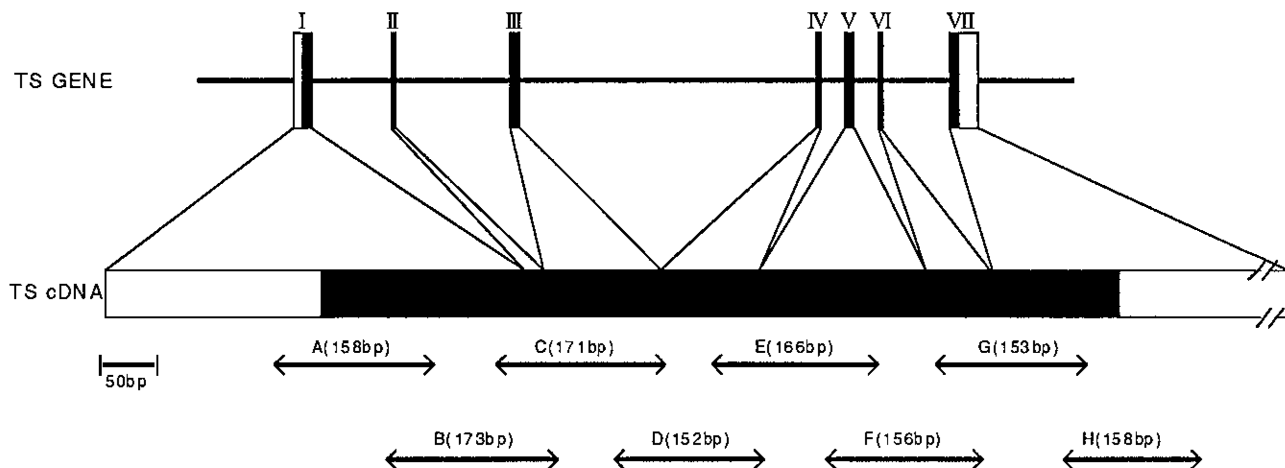

Fig. 4. Regions of the TS gene analyzed by PCR-SSCP. Eight DNA fragments (A–H) covering the entire coding region of TS cDNA were prepared by PCR using eight pairs of primers. Filled column, coding region; unfilled column, non-coding region; ▬, intron; ⟷, amplified fragments.

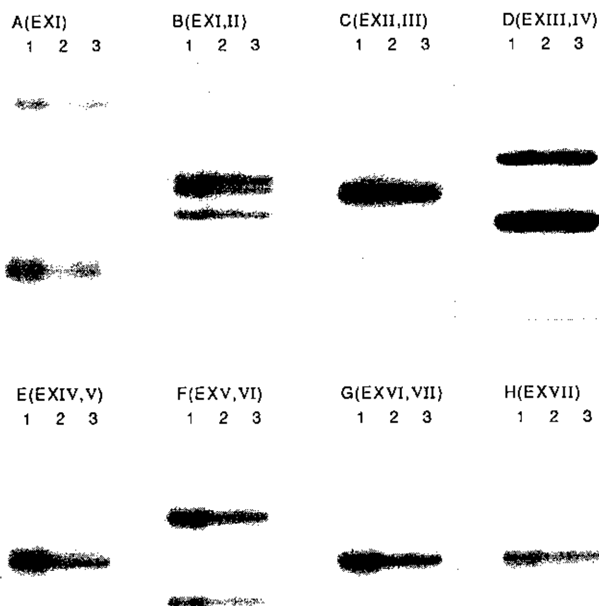

Fig. 5. PCR-SSCP analysis of TS cDNA fragments in NUGC-3 and NUGC-3/5FU/L cells. The eight DNA segments were resolved by PAGE after dilution in formamide and heated to dissociate the DNA strands. DNA bands on the gel were detected by autoradiography and mutations of the TS cDNA from the resistant cells were analyzed. 1, control (cloned TS cDNA); 2, NUGC-3; 3, NUGC-3/5FU/L. See "Materials and Methods."

Table I. Intracellular Baseline Levels of TS in NUGC-3 and NUGC-3/5FU/L Cells

Cell line	TS content (pmol/mg protein)
NUGC-3	1.24±0.24
NUGC-3/5FU/L	0.906±0.17

Cytosol prepared by sonication was incubated with [^{3}H]FdUMP and 5,10-CH_2-FH_4 at 37°C for 30 min, and the TS complex was isolated by gel-filtration. The data represent the mean±SD of 5 determinations.

Table II. Intracellular Metabolism of 5-FU into FUTP and FdUMP in NUGC-3 and NUGC-3/5FU/L Cells

5-FU (μM)	Incubation time	Metabolite (pmol/mg protein)		
		FUTP/FdUMP	NUGC-3	NUGC-3/5FU/L
400	10 min	Free FUTP	84.4±8.5	14.1±2.1
1	3 h	Free FdUMP	1.26±0.18	0.083±0.033
		Bound	3.34±0.99	0.941±0.45
		Total	4.60	1.02

Cells were incubated with [^{3}H]5-FU under the conditions shown above and its active metabolites were separated by HPLC. The data represent the mean±SD of 3–5 determinations.

compared (Table II). The FUTP level in the resistant cells was significantly lower, being only 17% of that of the parent cells. Then, cells were exposed to 1 μM 5-FU for 3 h, and the total (free and bound) FdUMP level was measured. We found that the total FdUMP levels were 4.6 for NUGC-3 and 1.0 (pmol/mg protein) for NUGC-

Table III. Activities of 5-FU-anabolizing Enzymes in NUGC-3 and NUGC-3/5FU/L Cells

Enzyme	Activity (nmol/h/mg protein)	
	NUGC-3	NUGC-3/5FU/L
Uridine phosphorylase	29.7±1.56	8.40±2.25
Uridine kinase	6.48±0.69	2.05±0.31
Orotate phosphoribosyltransferase	14.7±1.59	4.50±1.38
Thymidine phosphorylase	88.5±16.7	90.5±8.58
Thymidine kinase	14.6±1.48	28.7±3.54

Cytosol was incubated with 0.5 m*M* [^{3}H]FUrd (for uridine phosphorylase/kinase), [^{3}H]5-FU (for orotate phosphoribosyltransferase) or [^{3}H]dFUrd (for thymidine phosphorylase/kinase) at 37°C for 20 min and metabolites were separated by thin-layer chromatography. The data represent the mean±SD of 3 determinations.

Table IV. Intracellular PRPP Levels in NUGC-3 and NUGC-3/5FU/L cells

Cell line	PRPP levels (pmol/10^7 cells)
NUGC-3	127±12.7
NUGC-3/5FU/L	45.5±4.1

PRPP was measured enzymically by reacting cytosol with [^{3}H]hypoxanthine and hypoxanthine-guanine phosphoribosyltransferase. The data represent the mean±SD of 3 determinations.

3/5FU/L cells, indicating that less FdUMP was produced from 5-FU in the resistant cells.

5-FU-anabolizing enzyme activities and PRPP levels The activities of five major enzymes involved in 5-FU anabolism were measured and compared between the two cell lines (Table III). The activities of uridine phosphorylase, kinase and OPRT were significantly lower in NUGC-3/5FU/L cells, being 28, 32 and 31%, respectively, of those in NUGC-3 cells. In contrast, the thymidine kinase activity of NUGC-3/5FU/L cells was rather higher than that of NUGC-3 cells, and thymidine phosphorylase activities were similar in the two cell lines.

Of the cosubstrates involved in 5-FU anabolism, only the PRPP level in the cells was measured. The PRPP level was lower in NUGC-3/5FU/L cells than NUGC-3 cells (Table IV).

Effect of dIno on 5-FU resistance Cells were cultured with various concentrations of 5-FU in the presence or absence of dIno for 96 h, followed by a colony assay. The lowest cytotoxic concentration of dIno alone was 150 *μM* for NUGC-3 and 500 *μM* for NUGC-3/5FU/L cells. As shown in Fig. 6, sensitivity to 5-FU of NUGC-3/5FU/L cells was markedly enhanced by 500 *μM* dIno, but that of NUGC-3 cells was not significantly affected. Leucovorin,

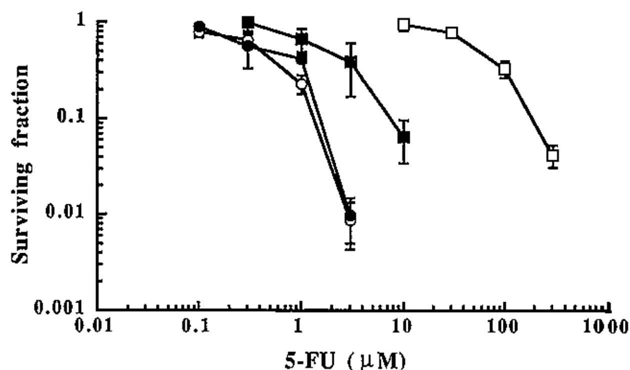

Fig. 6. Effect of dIno on sensitivities to 5-FU of NUGC-3 and NUGC-3/5FU/L cells. Cells were cultured with various concentrations of 5-FU in the presence or absence of dIno for 96 h and the cell-killing effects were assessed by a colony assay. ○, NUGC-3 (5-FU alone); ●, NUGC-3 (5-FU+150 *μM* dIno); □, NUGC-3/5FU/L (5-FU alone); ■, NUGC-3/5FU/L (5-FU+500 *μM* dIno).

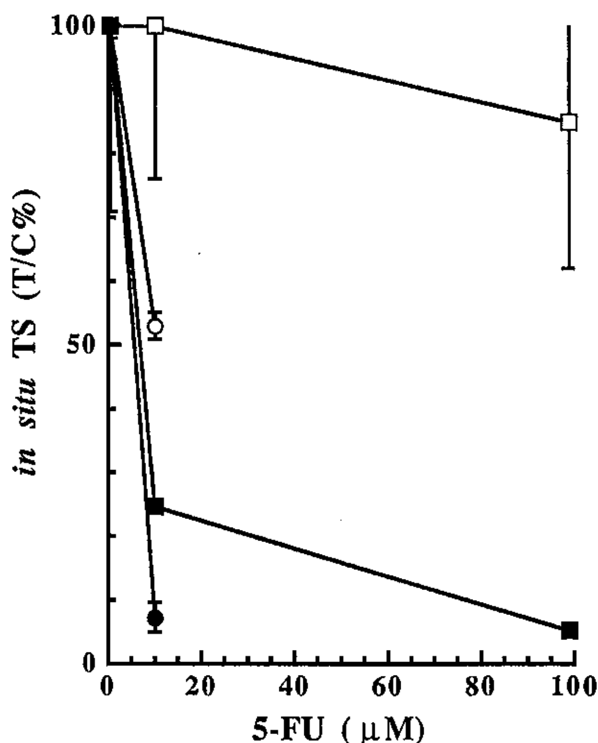

Fig. 7. Effect of dIno on *in situ* TS inhibition by 5-FU in NUGC-3 and NUGC-3/5FU/L cells. Cells were incubated with 3 concentrations of 5-FU in the presence or absence of dIno, and *in situ* TS activities were measured. ○, NUGC-3 (5-FU alone); ●, NUGC-3 (5-FU+150 *μM* dIno); □, NUGC-3/5FU/L (5-FU alone); ■, NUGC-3/5FU/L (5-FU +500 *μM* dIno).

interferon-α and -γ influenced neither NUGC-3 nor NUGC-3/5FU/L cells. We also found that *in situ* TS was inhibited by 5-FU in NUGC-3/5FU/L cells in the presence of dIno (Fig. 7).

DISCUSSION

5-FU is transformed into two major active metabolites, FdUMP and FUTP. FdUMP forms a covalent ternary complex with TS and 5,10-CH_2-FH_4, thereby inhibiting TS activity and finally DNA synthesis. FUTP is incorporated into RNA, interfering with the processing and function of RNA (see Ref. 12 for review). The biochemical features of target cells and the treatment schedules seem to determine which effect 5-FU exerts upon a given tumor. Based on a kinetic analysis of the cell killing actions of 5-FU, we reported that 5-FU tends to show a cell cycle phase-specific action that corresponds to its DNA-directed effect when cells are continuously exposed to it for a relatively long time, and it exerts a cell cycle phase-nonspecific action, which probably corresponds to its RNA-directed effect, when cells are exposed to it for a relatively short time.[21, 31]

NUGC-3/5FU/L was highly resistant to long and short exposures to 5-FU (Fig. 1), suggesting that this subline simultaneously acquired resistance to both the DNA- and RNA-directed actions of 5-FU. Therefore, we first compared the incorporation of 5-FU into RNA and the inhibition of *in situ* TS by 5-FU between the two cell lines under the same experimental conditions. The difference in 5-FU incorporation into RNA between the two lines after 1-h exposure became remarkable after a subsequent 24-h incubation in the absence of 5-FU (Fig. 2). This suggested that the ribonucleotide species of 5-FU produced during a 1-h exposure to 5-FU remains in the cells even after the removal of 5-FU, that the FUTP incorporation into RNA potentially continued thereafter, and that there is a considerable difference in the metabolic production of FUTP between the two lines.

NUGC-3/5FU/L was definitely resistant to the inhibitory effect of 5-FU on *in situ* TS as compared with the parent line (Fig. 3). As major causes of such resistance to TS inhibition, quantitative and qualitative alterations of TS are quite likely. Copur *et al.* have found elevated TS levels and amplified TS DNA in 5-FU-resistant sublines of human colon cancer.[14] Although qualitative alterations of TS have never been found in 5-FU-resistant cells, Barbour *et al.* have identified a T-to-C transition at base position 97, resulting in tyrosine-to-histidine replacement at residue 33 of TS in a human colon cancer line naturally resistant to FdUrd.[9] Houghton *et al.* have also reported two mutations of G-to-A and C-to-T at base positions 652 and 766, resulting in Asp-to-Asn and His-to-Tyr substitutions at residues 218 and 256, respectively, in a human colon cancer line with a TS-negative phenotype.[32] Therefore, to determine the mechanism(s) underlying the decreased inhibition of *in situ* TS in the resistant cells, we assessed the basal levels of TS by a FdUMP ligand binding assay and mutation(s) of the TS gene by a PCR-SSCP analysis. The results showed neither higher levels of TS nor a mutation of TS gene in the resistant cells (Table I and Fig. 5), indicating that neither quantitative nor qualitative alteration of TS is associated with decreased TS inhibition by 5-FU in NUGC-3/5FU/L cells. Although no alterations in the coding region of TS DNA were found in this study, PCR-SSCP and subsequent base sequence analysis of another DNA segment, including exon 1 and some of the introns before and after exon 1 revealed a loss of 28 bases (base position -42 to -15) in the non-coding region of exon 1 in the resistant cells. In addition, a G-to-C transition at base position -58 was found in both NUGC-3 and NUGC-3/5FU/L cells.

Based on the above results of TS, we investigated the intracellular levels of two active metabolites by an HPLC assay, demonstrating lower levels of both FUTP and FdUMP in the resistant cells (Table II). It is possible that this caused a lower incorporation of 5-FU into RNA and decreased inhibition of *in situ* TS by 5-FU in NUGC-3/5FU/L cells. The higher level of bound TS (Table II) than its basal value (Table I) in NUGC-3 seemed to be due to induction by incubating the cells with 5-FU for 3 h, although the TS level was not elevated in NUGC-3/5FU/L cells. Since a decreased production of active metabolites in the resistant cells should result from a reduction of 5-FU-anabolizing enzyme activities and/or the cosubstrate pool, we measured and compared them between two cell lines. The activities of 3 out of 5 enzymes measured, uridine phosphorylase, uridine kinase and OPRT, were significantly lower in the resistant cells (Table III). We assessed only the level of PRPP among the cofactors, because measurement of R-1-P and dR-1-P requires a large number of cells. The PRPP level was also significantly lower in NUGC-3/5FU/L cells. The results suggested that two major pathways of FUMP production, a one-step pathway by OPRT with PRPP and a two-step pathway by uridine phosphorylase with R-1-P and uridine kinase, have a markedly reduced capacity in NUGC-3/5FU/L cells, although quantitative analysis based on these data is difficult. Suppression of the two routes would effectively inhibit the production of FUMP. It is very likely that this decreased production of FUMP results in the reduction of not only FUTP, but also FdUMP, because the pathway from 5-FU to FdUMP via FdUrd seems practically nonfunctional, probably due to the extremely low level of dR-1-P. Since the enzyme activities of thymidine phosphorylase and kinase were not lower in the resistant than in the parent cells (Table III),

if there is enough dR-1-P, the production of FdUMP would not be decreased in the resistant cells. Therefore, it is very likely that sublines with lower 5-FU-anabolizing enzyme activity exhibit resistance to both the RNA- and DNA-directed action of 5-FU.

We studied the effects of some modifiers on 5-FU sensitivity of the resistant subline. Coexposure to leucovorin affected neither 5-FU sensitivity nor the inhibition of *in situ* TS by 5-FU in the resistant cells (data not shown), indicating that the reduced folate pool is not responsible for the resistance. In contrast, as shown in Fig. 6, dIno markedly sensitized the resistant cells to 5-FU. We also found that dIno enhanced the inhibitory effect of 5-FU on *in situ* TS in the resistant cells (Fig. 7). These findings seemed to support our speculation that FdUMP is produced via FUMP but not FdUrd. It is plausible that dIno supplies dR-1-P in the cells, thus enabling them to produce FdUMP via FdUrd. This effect seems remarkable in the resistant cells, because the metabolic flow of 5-FU to FUMP is more severely restricted. The dR-1-P level does not seem to play an important role in the mechanism of resistance in NUGC-3/5FU/L cells even if it can reverse 5-FU resistance. The therapeutic application of 5-FU and dIno in combination seems difficult, because the effective concentration of dIno is as high as 500 μM. However we are planning an *in vivo* study.

We think this is the first report on acquired resistance to 5-FU in a human stomach cancer cell line. We found that significantly low activities of three 5-FU-anabolizing enzymes and a reduced PRPP level are possible mechanisms of resistance in NUGC-3/5FU/L cells. To understand the general mechanism(s) of 5-FU resistance in human gastrointestinal cancers, we are planning more studies using other sublines resistant to 5-FU.

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