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THYMIDYLATE SYNTHASE GENE AMPLIFICATION IN HUMAN COLON CANCER CELL LINES RESISTANT TO 5-FLUOROURACIL

SITKI COPUR,* KEISUKE AIBA,† JAMES C. DRAKE,* CARMEN J. ALLEGRA* and EDWARD CHU*‡

*NCI-Navy Medical Oncology Branch, National Cancer Institute, Bethesda, MD 20889-5105, U.S.A.; and †Japanese Foundation for Cancer Research, Cancer Chemo Center, Tokyo, Japan

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Abstract—A series of 5-fluorouracil (5-FU)-resistant human colon H630 cancer cell lines were established by continuous exposure of cells to 5-FU. The concentration of 5-FU required to inhibit cell proliferation by 50% (IC_{50}) in the parent colon line (H630) was 5.5 μM. The 5-FU IC_{50} values for the resistant H630-R1, H630-R10, and H630-R cell lines were 11-, 29-, and 27-fold higher than that for the parent H630 cell line. Using both the radioenzymatic 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) binding and catalytic assays for measurement of thymidylate synthase (TS) enzyme activity, there was significantly increased TS activity in resistant H630-R1 (13- and 23-fold), H630-R10 (37- and 40-fold), and H630-R (24- and 34-fold) lines, for binding and catalytic assays, respectively, compared with the parent H630 line. The level of TS protein, as determined by western immunoblot analysis, was increased markedly in resistant H630-R1 (23-fold), H630-R10 (33-fold), and H630-R (26-fold) cells. Northern analysis revealed elevations in TS mRNA levels in H630-R1 (18-fold), H630-R10 (39-fold), and H630-R (36-fold) cells relative to parent H630 cells. Although no major rearrangements of the TS gene were noted by Southern analysis, there was significant amplification of the TS gene in 5-FU-resistant cells, which was confirmed by DNA slot blot analysis. These studies demonstrate that continuous exposure of human colon cancer cells to 5-FU leads to TS gene amplification and overexpression of TS protein with resultant development of fluoropyrimidine resistance.

Key words: thymidylate synthase; gene amplification; 5-FU resistance

The fluoropyridine 5-FU§ remains presently the single most active agent for the treatment of human colorectal cancer [1–3]. Since few other agents have been identified for the treatment of this disease, considerable attention has focused on understanding both the mechanisms of action of 5-FU and the mechanisms by which malignant cells develop resistance. The cytotoxic effects of 5-FU have been well characterized and include: (1) inhibition of the target enzyme TS by the 5-FU metabolite FdUMP with subsequent inhibition of thymidylate and DNA biosynthesis, (2) incorporation of 5-FU into RNA with subsequent alterations in RNA processing and function, and (3) incorporation of 5-FU into DNA with resultant DNA damage [1–11]. However, the relative biological significance of each of these events in determining 5-FU cytotoxicity remains to be defined.

‡ Corresponding author: Edward Chu, M.D., NCI-Navy Medical Oncology Branch, National Cancer Institute, Bethesda Naval Hospital, 8901 Wisconsin Ave., Bldg. 8, Rm. 5101, Bethesda, MD 20889-5105. Tel. (301) 402-1841; FAX (301) 496-0047.

§ Abbreviations: dUTP, 2′-deoxyuridine-5′-triphosphate; dUTPase, dUTP nucleotidohydrolase; DHFR, dihydrofolate reductase; 5-FU, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUrd, 5-fluoro-2′-deoxyuridine; FdUTP, 5-fluoro-2′-deoxyuridine-5′-triphosphate; FUTP, 5-fluorouridine-5′-triphosphate; MTX, methotrexate; PDDF, 10-propargyl-5,8-dideazafolate; and TS, thymidylate synthase.

One of the principal obstacles to the clinical efficacy of 5-FU chemotherapy has been the rapid emergence of cellular resistance. Various mechanisms of resistance to fluoropyrimidine chemotherapy have been well characterized in a number of *in vitro* and *in vivo* experimental systems, and they include [1, 3, 5, 12–25]: (1) increased levels of target enzyme TS, (2) alterations in binding affinity of TS for the 5-FU metabolite FdUMP, (3) decreased incorporation of 5-FU into RNA, (4) decreased incorporation of 5-FU into DNA, (5) decreased intracellular pools of the reduced folate substrate 5,10-methylene-H_4PteGlu, (6) increased activity of catabolic enzymes such as acid and alkaline phosphatase leading to a decreased accumulation of the active 5-FU metabolites, (7) decreased levels of anabolic enzymes with subsequent decreased formation of the active 5-FU metabolites, and (8) increased levels of dUTPase preventing accumulation of FdUTP and dUTP in cellular DNA. Recent studies have also suggested the importance of schedule of 5-FU administration in determining the eventual resistance mechanism to 5-FU [26]. When human colon cancer HCT-8 cells were exposed to high-dose (1 mM) 5-FU for 4 hr, sublines were established that were resistant to 5-FU on the basis of decreased incorporation of 5-FU metabolites into cellular RNA. In contrast, when this same cell line was exposed to low-dose (15 μM) 5-FU for 7 days, sublines were selected that were insensitive to 5-FU as a result of impaired polyglutamation of the reduced folate 5,10-methylenetetrahydrofolate.

Gene amplification as a mechanism of drug resistance was first observed in Chinese hamster ovary cells and subsequently in murine and human leukemic cell lines following their exposure *in vitro* to increasing concentrations of MTX [27]. The gene encoding for DHFR was shown to be present at levels several hundred-fold higher than in the parent wild-type cells. Further investigation revealed that clones with amplification of the DHFR gene were highly resistant to MTX. More recently, this phenomenon of DHFR gene amplification has been observed in clinical specimens taken from patients treated with MTX [28, 29]. TS gene amplification has been described in cultured neoplastic cells treated either with FdUrd [17, 30] or with the specific antifolate TS inhibitor, PDDF [31, 32]. Clark *et al.* [18] observed a 4- to 6-fold increase in the TS gene copy number in a tumor sample obtained from a patient with colon cancer following prolonged therapy with 5-FU. Although a pre-therapy biopsy specimen was unavailable for determination of the baseline TS gene copy number, the findings from this study suggested that the process of TS gene amplification may have direct clinical relevance in the development of resistance to 5-FU chemotherapy.

In this study, we have established 5-FU-resistant human colon cancer H630 cell lines following continuous exposure to 5-FU. Our studies show that these cell lines are resistant to 5-FU due to a significant increase in the level of expression of TS protein. We present further evidence demonstrating that the increased level of TS protein expression results from amplification of the TS gene.

MATERIALS AND METHODS

Chemicals. 5-FU, dextran (clinical grade), dUMP, bovine serum albumin (fraction V), and acid-washed activated charcoal were purchased from the Sigma Chemical Co. (St. Louis, MO). [5-^{3}H]dUMP (20 Ci/mmol), [6-^{3}H]FdUMP (18 Ci/mmol), and [6-^{3}H]5-FU (20 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). 5′-[α-^{32}P]dCTP (3000 Ci/mmol) and 5′-[α-^{32}P]dGTP (3000 Ci/mmol) were obtained from New England Nuclear (Boston, MA). All other chemicals were obtained from Sigma.

Cell culture. The origin and characteristics of the human colon cancer H630 cell line have been described previously [33]. Resistant sublines were selected *in vitro* for resistance to 5-FU by continuous exposure of the parental H630 cell line to medium supplemented with 5-FU. The resistant H630-R1 and H630-R10 colon cancer cells were grown and maintained in medium containing 1 and 10 μM 5-FU, respectively. The H630-R subline was derived from the 5-FU-resistant H630-R10 subline by maintaining the H630-R10 line in the absence of 5-FU. All cell lines were grown in 75-cm^2 plastic tissue culture flasks (Falcon Labware, Oxnard, CA) in growth medium containing RPMI 1640 with 10% dialyzed fetal bovine serum and 2 mM glutamine. Dialyzed fetal bovine serum was purchased from the Grand Island Biological Co. (Grand Island, NY). All other medium components were obtained from the Biofluids Co. (Rockville, MD). In all experiments, resistant colon cancer cell lines were grown in the absence of 5-FU for a minimum of 2 weeks before study.

In vitro *cytotoxicity studies.* Plastic 25-cm^2 tissue culture flasks (Falcon Labware) were seeded with 4.9 mL suspensions of 5×10^4 cells/mL of H630, H630-R1, H630-R10, and H630-R and were then incubated at 37°. After a 24-hr incubation, 0.1 mL of 5-FU, at various concentrations (10^{-4} to 10^{-8} M) was added to each flask. Sterile water (0.1 mL) was added to control flasks. All experiments were carried out in duplicate. After a 72-hr incubation at 37°, cells were trypsinized and counted using a ZBI Coulter counter (Coulter Electronics Inc., Hialeah, FL). The concentration of drug that produced 50% inhibition of cell growth was determined from the plot of percent control growth (cell number) versus the logarithm of drug concentration.

The doubling times for the H630, H630-R1, H630-R10, and H630-R lines were 25, 32, 37, and 28 hr, respectively.

Measurement of DNA synthesis. Human colon cancer cells, in the logarithmic phase of growth, were incubated with 1 μCi/mL [*methyl*-^{3}H]thymidine (specific activity 60 Ci/mmol) for 30 min. The cells were then processed as previously described [22].

Incorporation of 5-FU into nucleic acids. Exponentially growing human colon cancer cells were treated with [^{3}H]5-FU (1 μM; final specific activity 40 μCi/mmol) for 4 hr at 37°. Cells were then washed three times with ice-cold PBS and fractionated for RNA and DNA, as previously described [22]. Cellular RNA was hydrolyzed in 0.2 M NaOH, and DNA was hydrolyzed in 1 M perchloric acid. The 200-μL samples of the hydrolysates were added to scintillation vials containing 10 mL of 3a70B counting fluid, and tritium radioactivity was measured in a Packard Tricarb Liquid scintillation counter.

TS binding and catalytic assay. Human colon cancer cells, in the logarithmic phase of growth, were washed three times with ice-cold PBS, harvested, and resuspended in 0.1 M KH_2PO_4, pH 7.2. Cell lysis was accomplished by sonication using three 2- to 3-sec bursts. The extracts were centrifuged at 10,000 *g* for 30 min at 4°, and the supernatants were then assayed for both TS binding and catalytic activity, as previously described [22].

FdUMP binding affinity determination (K_d). Cell extracts from the H630 and H630-R10 cell lines were obtained as described above. The assay was performed in a final volume of 200 μL containing 75 μM 5,10-methylene-H_4PteGlu, [6-^{3}H]FdUMP at concentrations ranging from 40 pM to 40 nM, 100 μM 2-mercaptoethanol, 50 mM KH_2PO_4, and aliquots of cytosolic extract (50 μL). The reaction mixtures were incubated for 30 min at 37°, enzyme-bound and free [6-^{3}H]FdUMP were determined, and affinity calculations were performed as previously described by Lockshin and Danenberg [34].

Western immunoblot analysis. Human colon cancer cells were harvested and extracted as described above. Protein concentrations were determined by the Bio-Rad protein assay [35], and equivalent amounts of protein (200 μg) from each cell line were resolved by SDS–PAGE, using 12.5% acrylamide,

Table 1. Growth-inhibitory effect (IC_{50}) of 5-FU against human colon cancer cells

Cell line	IC_{50} (μM)
H630	5.5 ± 1.0
H630-R1	61 ± 8.5
H630-R10	160 ± 10
H630-R	153 ± 11

Human colon cancer cells in the logarithmic phase of growth were incubated with 5-FU (10^{-4} to 10^{-8} M) at 37° for 72 hr. The IC_{50} values (concentration producing 50% growth inhibition) were determined as described in Materials and Methods. The H630 line represents the parent cell line, and the resistant H630-R1 and H630-R10 lines were maintained in 5-FU concentrations of 1 and 10 μM, respectively. The H630-R line is a subline of H630-R10 that was maintained in the absence of 5-FU. Results are the means ± SEM from five separate experiments, each done in duplicate.

Table 2. Incorporation of 5-FU into RNA and DNA of human colon cancer cells

	5-FU incorporation	
Cell line	RNA (pmol/mg RNA)	DNA (pmol/mg DNA)
H630	1.4 ± 0.9	0.8 ± 0.05
H630-R10	1.8 ± 1.9	1.1 ± 0.10
H630-R	1.9 ± 0.8	1.4 ± 0.20

Human colon cancer cells in the logarithmic phase of growth were incubated with [6-^{3}H]5-FU (1 μM; specific activity 40 μCi/mL) at 37° for 4 hr. Cells were extracted and fractionated for RNA and DNA as described in Materials and Methods. Results are the means ± SEM from at least three separate experiments.

according to the method of Laemmli [36]. The gels were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Antibody staining was performed with the use of a TS polyclonal primary antibody (1/1000 dilution) and horseradish peroxidase-conjugated secondary antibody (1/1000 dilution) (Bio-Rad Laboratories, Richmond, CA).

Isolation of total RNA and RNA blot hybridization (Northern) analysis. Human colon cancer cells were harvested from 150-cm^2 plastic tissue culture flasks and washed three times with ice-cold PBS. Total RNA was isolated according to the method of Chomczynski and Sacchi [37]. After extraction, 30 μg/sample of total cellular RNA was denatured, resolved on a 1% formaldehyde-agarose gel, and transferred onto a Nytran filter membrane (Schleicher & Schuell). The membrane was hybridized to a ^{32}P-radiolabeled human TS cDNA insert probe after the human TS cDNA was labeled by the random primer method of Feinberg and Vogelstein [38]. The cDNA for human TS was a gift of Dr. T. Seno (Saitama Cancer Center Research Institute, Saitama-ken, Japan). The filters were washed as previously described [22], and autoradiography was performed using Kodak XAR-5 film exposed for 3–5 days at −70°.

Extraction and analysis of DNA. Cellular DNA from human colon cancer cells was isolated as previously described [22]. Southern analysis was performed by digestion of 15 μg of the isolated genomic DNA with *Hin*dIII restriction endonuclease, fractionation of the DNA fragments on a 0.5% agarose gel followed by transfer onto a Nytran filter membrane (Schleicher & Schuell). The membrane was hybridized to a ^{32}P-radiolabeled human TS cDNA insert probe and then processed as previously described [22].

Slot blot hybridization analysis was performed according to previously described methods [39]. Serial dilutions of known concentrations of genomic DNA from each cell line were prepared and spotted onto nitrocellulose filters by means of a 24-well vacuum manifold (Bethesda Research Laboratories). The filters were air-dried and vacuum-baked at 80° for 2 hr, and then hybridized to a ^{32}P-radiolabeled human TS cDNA probe as outlined above.

Densitometric analysis. Quantitation of signal intensities on autoradiographs and on Western blot filter membranes was performed by densitometry using a Scan Jet Plus scanner (Hewlett-Packard) and analyzed using NIH Image 1.36 software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD).

RESULTS

In vitro *cytotoxicity studies.* The H630-R1 and H630-R10 human colon cancer cell lines were selected by *in vitro* exposure of the parent H630 cell line to stepwise increases in 5-FU concentration and were then maintained in medium containing 1 and 10 μM 5-FU, respectively. As shown in Table 1, the 5-FU IC_{50} values for the H630-R1 and H630-R10 lines were 61 and 160 μM, respectively. These lines were 11- and 29-fold more resistant to 5-FU compared with the parent H630 line (IC_{50} = 5.5 μM). H630-R cells, which were derived from the most resistant H630-R10 line, were still resistant to 5-FU (IC_{50} = 153 μM) despite being grown in drug-free medium for up to 28 weeks. Moreover, the stability of the resistance phenotype was confirmed when H630-R cells passaged out of 5-FU for greater than 52 weeks were resistant to 5-FU to nearly the same degree as H630-R10 cells.

Additional growth experiments revealed that the presence of 10 μM thymidine completely protected against the growth-inhibitory activity of 5-FU in both the parent H630 and resistant H630-R10 cells (data not shown). These results suggest that inhibition of the target enzyme TS and/or involvement of a DNA-mediated event represent two possible mechanisms through which 5-FU exerts its cytotoxic effects in these cell lines.

Measurement of DNA synthesis. To measure the relative rates of DNA synthesis in each of the H630 cell lines, the level of thymidine incorporation was determined. Incorporation of [^{3}H]thymidine into DNA was lower in each of the 5-FU-resistant cell

Table 3. TS activity in human colon cancer cells

Cell line	Binding assay (pmol/mg protein)	Fold increase	Catalytic assay (pmol/min/mg protein)	Fold increase
H630	0.17 ± 0.03	1	1.3 ± 0.2	1
H630-R1	0.82 ± 0.10	13	31 ± 5	23
H630-R10	6.27 ± 0.96	37	52 ± 10	40
H630-R	4.10 ± 0.50	24	42 ± 5	34

Human colon cancer cells in the logarithmic phase of growth were harvested, and cytosolic extracts served as the source of enzyme. TS enzyme activity was determined using the FdUMP radioenzymatic binding and catalytic assays as described in Materials and Methods. Results are the means ± SEM of at least five separate experiments. The H630-R1 ($P < 0.005$), H630-R10 ($P < 0.005$) and H630-R ($P < 0.005$) lines were associated with a significantly increased level of TS, as determined by the binding and catalytic assays (two-sided Student's *t*-test).

lines [H630-R1 (0.36 ± 0.1 nmol/mg protein), H630-R10 (0.38 ± 0.3 nmol/mg protein), and H630-R (0.34 ± 0.2 nmol/mg protein)] when compared with the parent H630 cell line (0.62 ± 0.2 nmol/mg protein). These differences in thymidine incorporation were not statistically significant as determined by a two-sided Student's *t*-test.

Incorporation into nucleic acids. Incorporation into RNA and DNA has been correlated with 5-FU cytotoxicity in various *in vitro* and *in vivo* model systems. Given these observations, we determined the level of 5-FU incorporation into each of these nucleic acid fractions. As shown in Table 2, there were no significant differences in the extent of incorporation of 5-FU nucleotide metabolites into either RNA or DNA between parent H630 and the resistant H630-R10 and H630-R cell lines.

Measurement of thymidylate synthase. Increased expression of TS has been documented to be an important mechanism of resistance to 5-FU. Using the radioenzymatic FdUMP binding assay, TS activity was increased 13- and 37-fold, respectively, in the H630-R1 and H630-R10 lines when compared with the parent H630 line (Table 3). In the H630-R line, the level of TS enzyme remained 24-fold elevated even though these cells were grown in drug-free medium for 28 weeks (Table 3). TS activity, as determined by the catalytic assay, was similarly increased in the 5-FU-resistant human colon cancer cells. The H630-R1 and H630-R10 cell lines had 23- and 40-fold higher TS enzyme activity relative to the parent H630 line (Table 3). The H630-R line demonstrated a 34-fold increased TS catalytic activity relative to parent H630 cells (Table 3).

The tightness of binding of FdUMP to TS extracted from both sensitive H630 and 5-FU-resistant H630-R10 colon cancer cells was determined by measuring the respective dissociation constants, K_d, for FdUMP. There was no difference in TS binding affinity, inasmuch as the K_d values for FdUMP binding to TS of sensitive H630 and resistant H630-R10 cells were 0.7 ± 0.4 and 0.6 ± 0.3 nM, respectively.

The level of expression of TS protein in each of these cell lines was subsequently analyzed by Western immunoblot to determine whether differences in TS enzyme activities corresponded to changes in the amount of immunoreactive protein. A representative

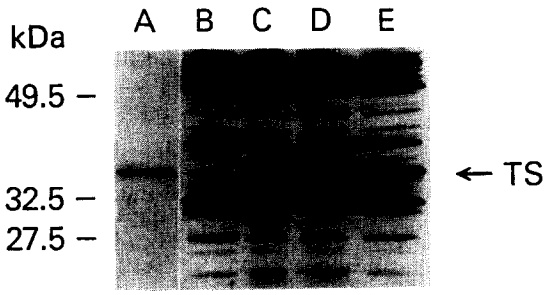

Fig. 1. Western immunoblot analysis of TS from parent and 5-FU-resistant H630 cells. Cytosolic extracts from human colon cancer cells were prepared as described in Materials and Methods. Equal amounts of protein (200 μg) were loaded onto each lane and resolved on a 12.5% SDS-polyacrylamide gel. Human recombinant TS protein (50 ng) was applied to the gel to verify the position of TS staining (lane A). Proteins were from parent H630 (lane B), H630-R (lane C), H630-R10 (lane D), and H630-R1 (lane E) cells.

blot is presented in Fig. 1. Equal amounts of total cytosolic protein (200 μg) were loaded onto each lane. The primary TS antiserum employed in this analysis was polyclonal, and, consequently, stained a number of proteins. However, the molecular weight of the denatured monomeric form of TS is 35,000, and the correct position of the TS band was confirmed by immunostaining of human recombinant TS protein (Fig. 1, lane A). Parent H630 cells displayed minimal staining for TS (Fig. 1, lane B). In contrast, resistant H630-R10 (Fig. 1, lane D), H630-R (Fig. 1, lane C), and H630-R1 (Fig. 1, lane E) cells all demonstrated significantly increased levels of TS. Densitometric scanning of the immunoblots revealed a 23-, 33-, and 26-fold increased expression of TS in H630-R1, H630-R10, and H630-R lines, respectively, when compared with the parent H630 line.

RNA blot hybridization analysis. Analysis of TS mRNA levels by Northern blot hybridization revealed a single 1.5-kb mRNA species that hybridized with the radiolabeled human TS cDNA

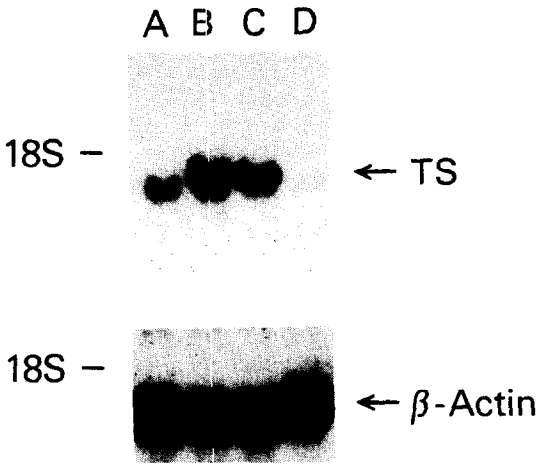

Fig. 2. Northern blot analysis of TS mRNA in parent and 5-FU-resistant cells. Total RNA (30 μg) from each cell line was fractionated on a 1% formaldehyde–agarose gel, transferred onto a Nytran membrane, and hybridized with a ^{32}P-radiolabeled TS cDNA insert. The filters were stripped of the TS probe and rehybridized with a human β-actin probe to control for RNA loading. RNAs were from resistant H630-R1 (lane A), H630-R10 (lane B), and H630-R (lane C) cells, and parent H630 (lane D) cells.

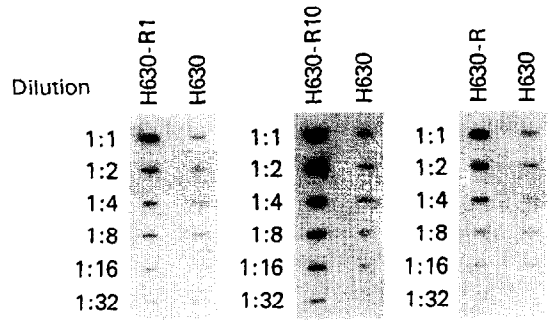

Fig. 3. Slot blot analysis of DNA from parent and resistant H630 cell lines. Serial dilutions of genomic DNA were spotted onto nitrocellulose membranes and probed for TS sequences. An estimate of the relative hybridization intensity is made by determining the dilution of DNA from resistant cell lines that generates a hybridization signal equivalent to the 1:1 dilution of parent H630 DNA. A 1:1 dilution corresponds to a concentration of 15 μg genomic DNA.

probe. The levels of TS mRNA were elevated 18- and 39-fold, respectively, in the H630-R1 (Fig. 2, lane A) and H630-R10 (Fig. 2, lane B) lines compared with the parent H630 line (Fig. 2, lane D). There was 36-fold more TS mRNA in the H630-R line (Fig. 2, lane C) than that expressed in the parent H630 line. The RNA from each of these cell lines appeared intact, equally loaded and transferred, based on ethidium bromide staining of the gel and

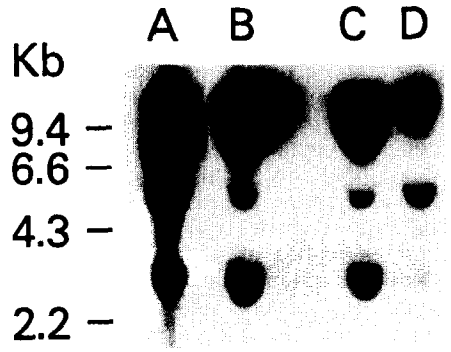

Fig. 4. Southern blot analysis of the TS gene from parent and 5-FU-resistant H630 cells. Genomic DNAs were digested with *Hin*dIII and resolved on a 0.5% agarose gel. The products were transferred onto a Nytran filter membrane and hybridized to ^{32}P-radiolabeled TS cDNA insert as described in Materials and Methods. DNAs were from H630-R1 (lane A), H630-R10 (lane B), H630-R (lane C), and parent H630 (lane D) cells.

on hybridization analysis of the membrane filter with a radiolabeled human β-actin probe (Fig. 2).

Slot blot analysis and Southern blot analysis. The process of TS gene amplification in the H630 5-FU-resistant sublines was first analyzed by quantitative slot blot hybridization. Serial dilutions of genomic DNA extracted from parental H630 cells and each of the 5-FU-resistant lines were spotted onto nitrocellulose filters and then probed for TS. As shown in Fig. 3, the increase in the TS gene copy number in these cell lines correlated with their relative resistance to 5-FU. There was an approximately 8-, 16-, and 32-fold increase, respectively, in intensity of hybridization of the H630-R1, H630-R, and H630-R10 resistant cells when compared with control H630 cells.

To confirm that the increased hybridization observed in the slot blot experiments was the result of amplified TS-specific sequences, a Southern blot hybridization analysis was performed. There were no alterations in the size of the DNA fragments generated by restriction digestion of parent (Fig. 4, lane D) and resistant (Fig. 4, lanes A–C) genomic DNAs, suggesting no major rearrangement of TS gene structure. This analysis demonstrated significantly more intense DNA bands in the resistant cells than in the parent wild-type cells, confirming that there was amplification of TS-specific sequences (Fig. 4).

DISCUSSION

In this study, we demonstrated that H630 human colon cancer cells grown in the continuous selective pressure of 5-FU become resistant to its cytotoxic effects. The resistance phenotype is remarkably stable as evidenced by the fact that resistant H630-R10 cells grown in drug-free medium for up to 1 year retain the same high level of insensitivity to 5-FU. The process of 5-FU selection was associated with a significant increase in the level of TS enzyme

activity and TS protein expression, as determined by biochemical techniques, including the radioenzymatic FdUMP binding and TS catalytic assays, and a Western immunoblotting analysis. Of note, the relative differences in TS expression in parent and resistant human colon cancer cell lines were equivalent using any of these biological assays. Scatchard analyses of TS protein from parent, wild-type H630 and resistant H630-R10 cells revealed no alteration in binding affinity of TS for FdUMP. These studies, taken together, suggest that TS protein in 5-FU-resistant cell lines is qualitatively identical to native TS protein expressed in wild-type H630 cells.

The increase in immunoreactive TS in 5-FU-resistant colon cancer cells was associated with corresponding changes in the steady-state levels of both the TS gene and TS mRNA. Southern blot analysis revealed significant amplification (approximately 32-fold) of TS-specific genomic sequences without any apparent rearrangement of the amplified gene. Moreover, the elevation in TS gene copy number remained stable given that there was, at most, a 2-fold decrease in the level of TS gene amplification despite resistant H630-R10 cells being maintained in drug-free medium for nearly 7 months. Previous studies characterizing amplification of the DHFR gene demonstrated that the amplified gene can be localized to either homogeneously staining regions (HSRs) or to double-minute chromosomes [27]. In the absence of the selective pressure of drug, HSR-mediated gene amplification confers stable resistance in contrast to amplification resulting from double-minute chromosomes whereby resistance is unstable with rapid reversion to the original genotype [27]. Our results are similar to those of Imam *et al.* [31], who identified TS gene amplification in murine leukemic L1210 cells resistant to the antifolate TS inhibitor CB3717. When CB3717-resistant L1210 cells were grown in the absence of the antifolate inhibitor for 375 generations, drug resistance remained stable with only a minimal 2- to 3-fold decrease in the TS gene copy number.

Given the multiple sites of 5-FU cytotoxic action, it was important to rule out alternative mechanisms that might contribute to the development of cellular resistance to 5-FU in these 5-FU-resistant colon cancer cells. Earlier studies have shown that resistance to 5-FU can develop as a result of decreased incorporation of 5-FU metabolites into either total cellular RNA or DNA [5, 23, 26]. Thus, we determined the level of 5-FU incorporation in the specific RNA and DNA fractions of parent H630 and resistant H630-R10 cells, and, as shown in Table 2, there were no significant quantitative differences between parent and resistant cells. Although the levels of the individual 5-FU nucleotide metabolites such as FUTP or FdUTP were not measured, the fact that RNA and DNA incorporation, the target endpoints of these respective metabolites, remained unchanged suggests that direct measurement of these metabolites may not be of further value. Recently, a novel resistance mechanism has been identified whereby resistance to 5-FU is mediated by enhanced DNA repair enzyme activity [23–25]. Increased DNA repair activity has been associated with decreased incorporation of 5-FU-specific metabolites and/or dUTP into cellular DNA. Our observation that incorporation of 5-FU into DNA is nearly identical in wild-type parent H630 and resistant H630-R10 cells suggests that there can be no biologically significant difference in levels of DNA enzyme repair activity such as dUTPase in either of these cell lines.

Earlier studies described elevations in TS in cells resistant to CB3717 [31, 32], an antifolate analog that directly targets TS, and FdUrd [17, 30], a fluoropyrimidine analog that exerts its cytotoxic action through inhibition of TS by the metabolite FdUMP. Selection with either agent resulted in an increase in TS enzyme levels that was associated with amplification of the TS gene. A recent study reported TS gene amplification in 5-FU-resistant HT-29 human colon cancer grown in the selective pressure of 5-FU [40]. However, this report did not attempt to examine other mechanisms through which resistance to 5-FU might be mediated. For this reason, the present study is the first to specifically identify increased expression of TS with TS gene amplification as the principal mechanism by which human colon cancer cells chronically exposed to 5-FU develop fluoropyrimidine resistance.

Significant research efforts are now ongoing to identify novel antifolate and/or non-antifolate-based inhibitors of TS. ZD1694 [41, 42] and LY231514 [43, 44] are two antifolate-based compounds that are presently undergoing clinical testing. One issue raised by this study is whether cancer patients previously treated with a 5-FU-based regimen can be treated subsequently with a TS-directed antineoplastic agent. Our findings suggest that this patient population would be refractory to such therapy and that alternative therapeutic approaches should be considered. Further work using tumor biopsy specimens from patients will be required to determine the clinical relevance of TS gene amplification and TS overexpression as a mechanism of 5-FU resistance. Such studies may identify patients who will most benefit from non-TS-directed therapeutic approaches.

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