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Resistance of a Human Ovarian Cancer Line to 5-Fluorouracil Associated with Decreased Levels of 5-Fluorouracil in DNA

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SUMMARY

Two human ovarian cancer cell lines were established from a patient before (PEO1) and after (PEO4) the onset of resistance to 5-fluorouracil (5-FU)/cisplatin-based chemotherapy. Using growth inhibition assays, we determined that the PEO4 line was almost 5-fold more resistant to 5-FU than the PEO1 line. The addition of either 1 or 20 μM leucovorin did not enhance the growth-inhibitory effects of 5-FU against the resistant PEO4 line. In characterizing the potential mechanisms of 5-FU resistance, we found no differences in thymidylate synthase activity between the two lines using both the 5-fluoro-2′-deoxyuridine-5′-monophosphate-binding and catalytic assays. A 4-hr exposure to 1 μM 5-FU resulted in greater ternary complex formation in the resistant line, and we observed no differences between the two lines in 5-FU incorporation into RNA. However, a 4-hr exposure to 1 μM [^{3}H]5-FU resulted in a 3-fold decrease in 5-FU accumulation in the DNA of the resistant PEO4 line. Cesium sulfate gradient centrifugation was used to more accurately separate and analyze for DNA-incorporated 5-FU metabolites and confirmed that the absolute level of 5-FU in the DNA of the PEO4 cells was markedly decreased (6.5-fold) compared with that of the sensitive PEO1 cell line. Moreover, time course studies demonstrated that the accumulated 5-FU in the DNA of the PEO4 cells was more rapidly removed compared with that in the PEO1 cells. Our findings suggest that decreased 5-FU levels in DNA, in part due to an enhanced removal from DNA, represent a mechanism by which the human ovarian cancer PEO4 line expresses decreased sensitivity to 5-FU.

The fluoropyrimidines 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine (FdUrd) are important antineoplastic agents and continue to be widely used clinically in the treatment of solid tumors of the gastrointestinal tract, breast, and head and neck (1–3). The cytotoxic effects of these compounds have been traditionally ascribed to (a) conversion to the metabolite FdUMP, an inhibitor of thymidylate synthase (TS), leading to reduced thymidylate formation and subsequent inhibition of DNA synthesis, (b) incorporation of drug in the form of FUTP into RNA with disruption of RNA synthesis and function, and (c) incorporation of drug in the form of FdUTP into DNA with disruption of DNA synthesis and function (1–11). More recently, Lönn and colleagues (12, 13) have shown that the 5-fluoropyrimidines can induce DNA strand breaks without being directly incorporated into DNA. However, the exact mechanism by which this occurs has not been well defined.

The development of resistance to 5-FU has become one of the major obstacles in its clinical utility. It is clear, given the multiple sites of action of 5-FU, that various mechanisms of resistance to 5-FU within a given tumor might develop. Clearly, an enhanced understanding of the underlying biochemical and/or molecular events leading to resistance might result in treatments that circumvent resistance and, ultimately, lead to an improved clinical use of 5-FU.

Several mechanisms of resistance to fluoropyrimidine chemotherapy have been well characterized (1, 3, 5, 14–22) in various experimental tumors. They include (a) decreased incorporation of 5-FU into RNA, (b) changes in the target enzyme, TS, including altered binding of FdUMP or enhanced intracellular enzyme activity, (c) decreased levels of anabolic enzyme activity resulting in decreased formation of the active metabolites, (d) enhanced activities of acid and alkaline phosphatase leading to decreased intracellular accumulation of FdUMP, and (e) relative deficiency of the intracellular folate substrate 5,10-methylene-H_4PteGlu.

The present study was undertaken to investigate further the underlying mechanisms important in the development of resistance to 5-FU in a human ovarian PEO4 cell line. Our results have particular clinical relevance since we studied two human ovarian cell lines, PEO1 (sensitive) and PEO4 (resistant), both established from the same patient prior to and after the devel-

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ABBREVIATIONS: 5-FU, 5-fluorouracil; FUR, 5-fluorouridine; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUTP, 5-fluoro-2′-deoxyuridine-5′-triphosphate; FUTP, 5-fluorouridine-5′-triphosphate; dUMP, 2′-deoxyuridine-5′-monophosphate; dUTP, 2′-deoxyuridine-5′-triphosphate; TS, thymidylate synthase; TdR, thymidine; 5,10-methylene-H_4PteGlu, 5,10-methylenetetrahydrofolate; CDDP, cisplatin.

opment of resistance to 5-FU/cisplatin combination chemotherapy. The results of this study suggest that decreased accumulation of 5-FU in DNA may explain the resistance of the PEO4 line to 5-FU. Moreover, the decreased level of 5-FU in the DNA appears to result from an enhanced DNA repair enzyme activity within the resistant PEO4 line.

Materials and Methods

Chemicals. 5-FU, FdUrd, FUR, dextran (clinical grade), dUMP, bovine serum albumin fraction V, and acid-washed activated charcoal were purchased from Sigma Chemical Co. (St. Louis, MO). [5-^{3}H]dUMP (20 Ci/mmol), [6-^{3}H]FdUMP (18 Ci/mmol), [2-^{14}C]thymidine (56 mCi/mmol), [6-^{3}H]FdUrd (20 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) was obtained from New England Nuclear (Boston, MA). Cesium sulfate (ultracentrifugation grade) was obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals were of reagent grade and were obtained from Sigma.

Cell culture. The origin and characteristics of the human ovarian cancer PEO1 and PEO4 cell lines have been well described previously (23, 24). Cells were grown in 75-cm^2 plastic tissue culture flasks (Falcon Labware, Oxnard, CA) in growth medium consisting of RPMI 1640 with 10% dialyzed fetal bovine serum, 2 mM glutamine, and 0.25 units of regular insulin per ml. All media components were obtained from Biofluids Co. (Rockville, MD).

***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 either PEO1 or PEO4 and incubated at 37°. After a 24-hr incubation, 0.1 ml of 5-FU or FdUrd at various concentrations was added to each flask. Sterile water (0.1) was added to control flasks. All experiments were carried out in duplicate. After a 12-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 (IC_{50}) was estimated from the plot of percent control growth (cell number) versus the logarithm of drug concentration.

The doubling times for the PEO1 and PEO4 cell lines were 36 and 40 hr, respectively.

TS-FdUMP-folate complex formation. Human ovarian cancer PEO1 and PEO4 cells were exposed to 10^{-6} M 5-FU for 4 hr at 37°. Cells were then harvested and suspended 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, and the supernatants were immediately assayed. The formation of a ternary complex consisting of TS-FdUMP-5,10-methylene-H_4PteGlu was determined by measuring total FdUMP sites (TS_{total}) and unoccupied FdUMP binding sites (TS_F) by previously described methods (25). The difference between these parameters was taken to represent TS-binding sites occupied by FdUMP (TS_B).

The apparent TS_F was assayed in a total volume of 200 μl containing 75 μM 5,10-methylene-H_4PteGlu, 3 pmol of [6-^{3}H]5-F-dUMP (specific activity, 18 Ci/mmol), 100 mM 2-mercaptoethanol, and 50 mM KH_2PO_4, pH 7.2. The binding was initiated by adding 50 μl of cytosolic extract. Samples were incubated at 37° for 30 min, after which 1 ml of a buffered charcoal slurry (prepared by mixing 10 g of acid-washed activated charcoal with 2.5 g of bovine serum albumin, 0.25 g of dextran, and 100 ml of ice-cold water) was added. The mixtures were vortexed, allowed to stand at room temperature for 10 min, and then centrifuged at 10,000 × *g* for 30 min. An 800-μl sample of the supernatant was counted by liquid scintillation. The results were corrected for exchange of [^{3}H]FdUMP with bound cold FdUMP present in the cytosols. We have previously found that 7% of FdUMP bound to TS was exchanged during 30 min under the assay conditions used (25).

Total TS (TS_T) was determined experimentally by allowing TS_B present in the cytosol to fully exchange with the labeled FdUMP at pH 8.0 in 0.6 M ammonium bicarbonate buffer over a 3-hr period. A 3-hr incubation results in greater than 95% release of bound FdUMP and its replacement by [^{3}H]FdUMP. TS_T was assayed in a total volume of 200 μl containing 75 μM 5,10-methylene-H_4PteGlu, 0.6 M NH_4CO_3 buffer, and 3 pmol of [6-^{3}H]5-FdUMP. The reaction was initiated by adding 50 μl of cytosolic extract, and the mixture was incubated for 3 hr at 37°. The remainder of the procedure is as described above.

TS catalytic assay. Human ovarian cancer PEO1 and PEO4 cells in the logarithmic phase of growth were harvested and suspended in 0.1 M KH_2PO_4, pH 7.4. Cell lysis was accomplished by sonication using three 2- to 3-sec bursts. The extracts were centrifuged at 10,000 × *g* for 30 min, and the supernatants were immediately assayed. The assay was performed in a total volume of 200 μl containing 10^{-5} M [5-^{3}H]dUMP (specific activity, 20 Ci/mmol), 100 mM 2-mercaptoethanol, 50 mM KH_2PO_4, pH 7.2, 75 μM CH_2-H_4PteGlu → 5,10-methylene-H_4-PteGlu and 50 μl of cytosolic extract. Samples were incubated at 37° for 30 min. The reaction was stopped by adding 100 μl of ice-cold 20% trichloroacetic acid. Residual [5-^{3}H]dUMP was removed by adding 200 μl of an albumin-coated activated charcoal solution. The samples were vortexed and allowed to stand at room temperature for 10 min. The charcoal was removed by centrifugation at 10,000 × *g* for 30 min. A 250-μl sample of the supernatant was then assayed for [^{3}H]H_2O radioactivity by liquid scintillation counting.

Incorporation of 5-FU into nucleotide pools, RNA, and DNA. Exponentially growing human ovarian cancer cells, PEO1 and PEO4, were treated with [^{3}H]5-FU (1 μM; final specific activity, 40 μCi/mmol) for 4 hr at 37°. The cells were then washed three times with ice-cold phosphate-buffered saline and fractionated for cold acid-soluble, RNA, and DNA fractions as outlined previously by Chu and Fischer (26, 27) and Harkrader *et al.* (28).

The cold acid-soluble extract was fractionated by reverse-phase high-performance liquid chromatography as initially described by Pogolotti *et al.* (29) and modified by Grem and Fischer (30). The isolation of FdUTP was performed using the method of Garrett and Santi (31). In brief, to 1 ml of the neutralized (pH 7–7.5) cold acid-soluble fraction was added 40 μl of 0.5 M sodium periodate, followed within 5 min by 50 μl of a 4 M solution of methylamine that had been slowly brought to pH 7.5 with phosphoric acid. After mixing, the reaction was incubated at 37° for 30 min. Ten microliters of 1 M rhamnose was then added to destroy the remaining periodate. The samples were immediately placed on ice and were then ready for high-performance liquid chromatography analysis. Fluoropyrimidine metabolites were identified by their retention times with respect to standard compounds and quantitated using an in-line scintillation counter (Flow-One Beta; Radiomatic Inc., Tampa, FL).

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 cocktail, and tritium radioactivity was measured in a Packard Tricarb liquid scintillation counter.

Metabolizing enzyme assays. Enzyme activities were measured in extracts prepared from freshly isolated cells or from cell pellets frozen at −80°. Extracts were prepared by suspending the cell pellet in 50 mM Tris·HCl buffer containing 1 mM EDTA (pH 7.4). Cell suspensions were sonicated using three 3-sec bursts each at maximal output and then centrifuged at 10,000 × *g* for 30 min at 4°. The supernatant fraction was removed and used as the enzyme source.

The enzymes important for 5-FU anabolism include thymidine phosphorylase, thymidine kinase, uridine phosphorylase, uridine kinase, and orotate phosphoribosyltransferase. They were assayed for as previously described by Peters *et al.* (32). All enzyme assays were performed for 30 min at 37° in a shaking water bath. The reactions were stopped by heating the tubes for 3–5 min at 95°. The tubes were chilled on ice, and 50 μl of the suspension was placed onto a Whatman DE-81 filter disc (Whatman Int. Ltd., Maidstone, England) to separate the product from the substrate by adherence of the nucleotide to the DEAE disc (33). After allowing the filters to air-dry for 30 min, they were washed three times with distilled water. They were then placed into

scintillation vials to which 1 ml of 0.1 M HCl/0.1 M KCl solution was added to elute the nucleotide product. After 10 min, 10 ml of 3a70B counting cocktail was added, and tritium radioactivity was measured by liquid scintillation counting.

The assay mixture for pyrimidine nucleoside phosphorylase included 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM ATP, 1 mM [6-^{3}H]5-FU (4.5 mCi/mmol), substrate (2.5 mM ribose-1-phosphate or 2.5 mM deoxyribose-1-phosphate), and 20 μl of enzyme extract in a final volume of 120 μl.

Pyrimidine nucleoside kinase activity was measured in a reaction mixture containing 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM ATP, 1 mM [6-^{3}H]FdUrd (4.5 mCi/mmol) or 1 mM [6-^{3}H]FUR (4.5 mCi/mmole), and 20 μl of cell extract in a final volume of 120 μl.

The assay mixture for orotate phosphoribosyltransferase activity consisted of 50 mM Tris·HCl (pH 7.4), 2.5 mM 5-phosphoribosyl-1-pyrophosphate, 10 mM $MgCl_2$, 10 mM ATP, 1 mM [6-^{3}H]5-FU (4.5 mCi/mmole), and 20 μl of cell extract.

Acid and alkaline phosphatase activities were determined according to the method of Fernandes and Crawford (18). Reactions were carried out in a final volume of 120 μl and contained 1 mM [6-^{3}H]FdUMP (2.42 mCi/mmol), 25 mM sodium acetate (pH 5.8), and 20 μl of cell extract for the acid phosphatase assay. Alkaline phosphatase activity was assayed under the same conditions as above, except that the buffer used was 25 mM ammonium bicarbonate (pH 9.2). The assay mixture was incubated for 30 min at 37°. The reaction mixtures were placed on DE-81 Whatman paper discs and allowed to air-dry for 30 min. The filters were washed with two aliquots of distilled water (10 ml/disc), placed in scintillation vials, and covered with 1 ml of 0.1 M KCl/0.1 M HCl to elute the radiolabeled nucleotide from the filters. Tritium radioactivity was measured in 10 ml of 3a70B scintillation fluid.

Isolation of total RNA. Human ovarian cancer cells PEO1 and PEO4 were harvested from 150-cm^2 tissue culture flasks with a rubber policeman, and their total RNA was isolated according to the method of Chomczynski and Sacchi (34). Briefly, the cell pellet was lysed by vortexing in 3 ml of a lysing solution (4 M guanidinium thiocynate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) for 2–3 min. In sequence, 3 ml of phenol (water-saturated), 0.3 ml of 3 M sodium acetate, and 0.6 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate with thorough mixing by inversion after addition of each reagent. The suspension was vigorously shaken for 10 sec and cooled on ice for 15 min. Samples were then centrifuged at 10,000 × *g* for 20 min at 4°. After centrifugation, RNA present in the upper aqueous phase was transferred to a fresh tube to which 3 ml of isopropanol was added. This mixture was placed at −20° for 1 hr to precipitate RNA. Centrifugation at 10,000 × *g* for 20 min at 4° was repeated. The resulting RNA pellet was dissolved in 0.3 ml of the original lysing solution, transferred into a 1.5-ml Eppendorf tube, and precipitated with an equal volume of isopropanol at −20° for 1 hr. The sample was then centrifuged for 10 min at 4°, and the RNA pellet was suspended in 75% ethanol, sedimented, vacuum-dried for 15 min, and dissolved in 150 μl of diethylpyrocarbonate (DEPC) water (5 Prime → 3 Prime Inc., West Chester, PA). At this point, the RNA was ready for Northern blot analysis and stored at −80 for future use.

Northern blot hybridization. For Northern transfer analysis, 30 μg per sample of total cellular RNA (isolated by procedure outlined above) was denatured, fractionated on a 1% formaldehyde agarose gel, and transferred to a Nytran filter membrane (Schleicher & Schuell Inc., Keene, NH) by a modification of the method of Ayusawa et al. (35). The membrane was baked for 2 hr at 80° in a vacuum oven. Prehybridization of the membrane for 2 hr at 42° in 50% formamide, 5× SSC, 5× Denhardt's solution, 20 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4), 0.5 mg of yeast transfer RNA per ml, and 0.1% sodium dodecyl sulfate was then followed by hybridization for 24 hr in the same solution with 10^7 cpm/ml of a ^{32}P-labeled nick-translated TS cDNA probe. The cDNA clone for human thymidylate synthase was a generous gift of Dr. T. Seno (Saitama Cancer Center Research Institute, Saitama-ken,

TABLE 1

Growth inhibitory effect (IC_{50}) of 5-FU, FdUrd, and CDDP against human ovarian carcinoma cells

Human ovarian carcinoma cells in the exponential phase of growth were incubated with 5-FU (10^{-4} to 10^{-7} M), FdUrd (10^{-7} to 10^{-10} M), and CDDP (10^{-4} to 10^{-7} M) at 37° for 120 hr. Concentrations causing 50% inhibition (IC_{50}) were determined as described in Materials and Methods. Results shown are mean ± standard error of at least four separate experiments in duplicate. The PEO4 line was significantly more resistant to 5-FU ($p_2 = 0.0001$) and to FdUrd ($p_2 = 0.0001$) than the PEO1 line. Statistical analysis was performed using a two-tailed Student's *t* test.

Cell line	5-FU	FdUrd	CDDP
	μM	nM	μM
PEO1	1.5 ± 1.2	2.3 ± 0.1	0.21 ± 0.1
PEO4	7.0 ± 1.6	9.2 ± 1.0	1.60 ± 0.5

TABLE 2

Thymidylate synthase activity levels in human ovarian cancer cells

Human ovarian cancer cells at equal cell density and in the exponential phase of growth were harvested, and cytosolic extracts as enzyme source were prepared. TS activity by using both the FdUMP-binding and catalytic assays was determined as described in Materials and Methods. Results shown are mean ± standard error of at least four separate experiments in duplicate.

Cell line	Binding	Catalytic
	pmol/mg of protein	pmol/min/mg of protein
PEO1	0.1 ± 0.01	26 ± 2.0
PEO4	0.1 ± 0.02	20 ± 4.2

TABLE 3

Formation of FdUMP and FUTP in human ovarian cancer cells

Human ovarian cancer cells in the exponential phase of growth were incubated at 37° for 4 hr with either [6-^{3}H]5-FU (1 μM; final specific activity, 40 μCi/mmol) or [6-^{3}H]FdUrd (1 nM; final specific activity, 40 μCi/mmol). The cold acid-soluble fraction was isolated as outlined in Materials and Methods. Results shown are mean ± standard error of three separate experiments.

Cell line	FdUMP Formation		FUTP Formation	
	FU	FdUrd	FU	FdUrd
	pmol/mg of protein			
PEO1	0.25 ± 0.02	1.35 ± 0.4	2.0 ± 0.30	ND[a]
PEO4	0.09 ± 0.01	1.40 ± 0.1	0.7 ± 0.03	ND

[a] ND, Not detectable.

TABLE 4

Activities of enzymes involved in 5-FU metabolism in human ovarian cancer cells

Human ovarian cancer cells in the exponential phase of growth were harvested, and cytosolic extracts as enzyme source were prepared. Enzyme activities were determined as described in Materials and Methods. Results shown are mean ± standard error of three to four separate experiments, each done in duplicate.

Enzyme	Activity in:	
	PEO1	PEO4
	pmol/min/mg of protein	
Thymidine phosphorylase/thymidine kinase	1.53 ± 0.20	0.45 ± 0.10
Uridine phosphorylase/uridine kinase	1.44 ± 0.20	1.86 ± 0.30
Thymidine kinase	1.36 ± 0.10	2.10 ± 0.10
Uridine kinase	1.40 ± 0.20	1.45 ± 0.30
Orotate phosphoribosyltransferase	2.20 ± 0.40	2.00 ± 0.30
Acid phosphatase	0.40 ± 0.20	0.25 ± 0.04
Alkaline phosphatase	0.44 ± 0.10	0.22 ± 0.02

TABLE 5

Incorporation of 5-FU into RNA and DNA of PEO1 and PEO4 human ovarian cancer cells

Human ovarian carcinoma cells, PEO1 and PEO4, in the exponential phase of growth were incubated at 37°C for 4 hr with [6-^{3}H]5-FU (1 μM, final specific activity, 40 μCi/mmol). Cells were harvested and fractionated for RNA and DNA as described in Materials and Methods. Results shown are mean ± standard error of at least five separate experiments. The PEO4 line was associated with a significantly decreased level of 5-FU incorporation into DNA (p_2 = 0.0034): Statistical analysis was performed using a two-tailed Student's *t* test.

Cell line	5-FU incorporation into:	
	RNA	DNA
	pmol/mg of RNA	*pmol/mg of DNA*
PEO1	16.0 ± 3.5	0.80 ± 0.20
PEO4	19.8 ± 3.8	0.26 ± 0.01

TABLE 6

Incorporation of FdUrd into RNA and DNA of PEO1 and PEO4 human ovarian cancer cells

Human ovarian cancer cells, PEO1 and PEO4, in the exponential phase of growth were incubated at 37°C for 4 hr with [6-^{3}H]FdUrd (1 nM; final specific activity, 40 μCi/mmol). The RNA and DNA fractions were determined as described in Materials and Methods. Results shown are mean ± standard error of three separate experiments.

Cell line	FdUrd incorporation into:	
	RNA	DNA
	pmol/mg of RNA	*pmol/mg of DNA*
PEO1	11 ± 1.7	0.88 ± 0.03
PEO4	10 ± 0.7	0.24 ± 0.07

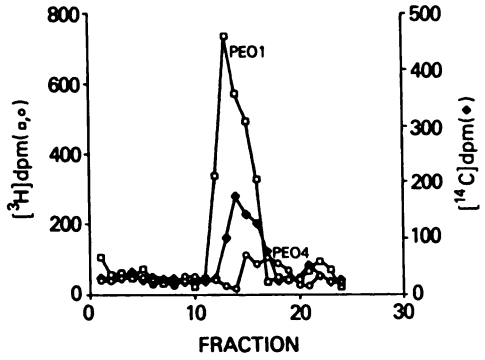

Fig. 1. Incorporation of 100 μM [^{3}H]5-FU and [^{14}C]TdR into the DNA of the PEO1 and PEO4 human ovarian cancer cell lines. PEO1 (□) and PEO4 (○) human ovarian cancer cells in the exponential phase of growth were incubated with 10^{-4} M [^{3}H]5-FU (final specific activity, 200 μCi/mmol) and 1 μCi of [^{14}C]TdR (♦) per ml for 4 hr. The DNA was purified and analyzed by cesium sulfate gradient centrifugation as outlined in Materials and Methods.

Japan) (35). The hybridized filter was initially washed twice with 2× SSC at room temperature, followed by a 1-hr wash at 65° with 2× SSC and 1% sodium dodecyl sulfate, and autoradiographed with Kodak XAR-5 film and an intensifying screen at −70°.

Cesium sulfate density gradient centrifugation. Exponentially growing cells were incubated with 100 μM [^{3}H]5-FU (final specific activity, 200 μCi/mmol) for 4 hr, washed twice with ice-cold phosphate-buffered saline, and then treated overnight at 37° with a nucleic acid extraction buffer containing 1% sodium lauroyl sulfate, 0.4 M NaCl, 10 mM EDTA, 10 mM Tris·HCl (pH 7.6), and proteinase K (150 μg/ml). After phenol-chloroform extraction and ethanol precipitation, nucleic acids were treated with RNAase (100 μg/ml) at 37° for 1 hr. A repeat phenol-chloroform extraction and ethanol precipitation were performed, after which the DNA was dissolved in 2 mM Tris·HCl (pH 8), 1 mM disodium EDTA. The DNA was then subjected to cesium sulfate (refractive index, 1.375) density gradient centrifugation in a Ti 50 rotor

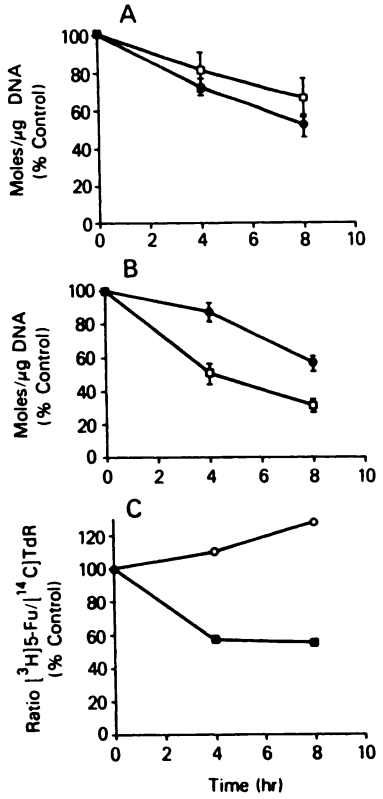

Fig. 2. Time course study of removal of incorporated [^{3}H]5-FU from the DNA of human ovarian cancer cells. PEO1 and PEO4 human ovarian cancer cells in the exponential phase of growth were incubated with 10^{-4} [^{3}H]5-FU (final specific activity, 200 μCi/mmol) and 1 μCi of [^{14}C]TdR per ml for 4 hr. The cells were then washed and replaced with drug-free medium. Cells were harvested at 0, 4, and 8 hr after drug removal. The DNA was extracted and analyzed as outlined in Materials and Methods. In panel A, the amount of [^{3}H]5-FU in the DNA of PEO1 (□) and the amount of [^{14}C]TdR in the PEO1 DNA (●) are shown. In panel B, [^{3}H]5-FU in the DNA of PEO4 (□) and [^{14}C]TdR in the PEO4 DNA (●) are shown. In panel C, the true rate of repair is expressed as the ratio of [^{3}H]5-FU/[^{14}C]TdR for the PEO1 (○) and PEO4 (■) lines.

at 40,000 rpm, for 40 hr at 20° using a Beckman ultracentrifuge (9–12). After centrifugation, 0.25-ml fractions were collected from bottom to top. DNA was precipitated with 10% ice-cold trichloroacetic acid, washed twice with ice-cold 5% trichloroacetic acid, and washed once with ethanol. The tritium radioactivity was measured by scintillation counting with a Packard liquid scintillation counter.

Results

***In vitro* cytotoxicity studies.** The PEO1 and PEO4 ovarian cancer lines were developed from tumor samples taken from a patient before and after treatment with combination chemotherapy including cisplatin, 5-FU, and chlorambucil. The relative sensitivities of these two lines to cisplatin have been well described previously (23), and various assay systems have shown that the PEO4 line is consistently 3- to 4-fold more resistant to cisplatin. As shown in Table 1, using the growth inhibition assay, the cisplatin IC_{50} for the PEO4 line was almost 8-fold higher than that for the PEO1 line. When tested against 5-FU in the same growth-inhibition assay, the PEO4 line was 4.6-fold more resistant to 5-FU than the PEO1 line. Neither 1 nor 20 μM leucovorin enhanced the cytotoxicity of 5-FU, suggesting that the intracellular concentration of reduced folates within the PEO4 line does not limit 5-FU cytotoxicity com-

pared with that of the PEO1 line (data not shown). The cytotoxic effects of FdUrd against these two cell lines were also determined. As shown in Table 1, the PEO4 line was 4-fold more resistant to FdUrd compared with the PEO1 line.

Thymidylate synthase as a determinant of resistance. One of the principal mechanisms of resistance to 5-FU is increased expression of its target enzyme, TS (1, 3, 19, 20). TS activities in these two cell lines, as measured by the FdUMP binding and the catalytic assays (Table 2), were equivalent. In addition, Northern blot analysis revealed no differences between the two cell lines in the level of TS mRNA expression (data not shown). Although there were no apparent alterations in basal activity of TS that could account for the 5-FU resistance of the PEO4 line, alterations in the affinity of TS for the active 5-FU metabolite FdUMP or in the amount of ternary complex formation might be more biologically relevant. After a 4-hr incubation with 1 μM 5-FU, the PEO4 line effectively formed the FdUMP-TS-reduced folate ternary complex, and the percent TS inhibition was almost 2-fold higher in the 5-FU-resistant line (79%) than in the sensitive PEO1 line (40%). When the two cell lines were incubated for 24 hr with 1 μM 5-FU, the extent of ternary complex formation was exactly the same for both the PEO1 and PEO4 lines (data not shown).

Formation of intracellular 5-FU metabolites. We next investigated other potential mechanisms that might explain the resistance of PEO4 to 5-FU. Since the levels of FdUMP and FUTP are important determinants of 5-FU activity, the intracellular levels of these metabolites were measured. As shown in Table 3, when PEO4 cells were incubated for 4 hr with 1 μM 5-FU, almost 3-fold less FdUMP was present compared with the amount of FdUMP measured in the PEO1 line. The resistant PEO4 also had 3-fold less FUTP formation than the PEO1 line. When both the PEO1 and PEO4 cells were treated with 1 nM FdUrd for 4 hr, there were no measurable differences in FdUMP levels between the two cell lines. However, there were no detectable levels of FUTP in either the PEO1 or the PEO4 cell line with FdUrd exposure.

Activities of the enzymes involved in 5-FU metabolism. In an attempt to determine the basis for the differences in the levels of FdUMP and FUTP between these two cell lines, we compared the activities of various enzymes involved in the metabolism of 5-FU. The enzyme activities investigated were uridine phosphorylase, orotate phosphoribosyltransferase, uridine kinase, thymidine phosphorylase, thymidine kinase, acid phosphatase, and alkaline phosphatase. As shown in Table 4, there is a 3.4-fold decrease in the activity of the coupled thymidine phosphorylase-thymidine kinase reaction in the PEO4 line compared with that in PEO1. Since the activities of thymidine kinase are the same in the two lines, the thymidine phosphorylase reaction appears to be rate limiting and, thus, 3.4-fold less in the resistant PEO4 line. There were no significant differences noted in the activities of the other metabolic enzymes.

Net accumulation of 5-FU in RNA and DNA. Since accumulation of 5-FU in both RNA and DNA has been correlated with cytotoxicity in a number of cell lines, we determined the levels of 5-FU in both of these nucleic acids. Table 5 presents the results of studies on the accumulation of 1 μM 5-FU in nucleic acids after a 4-hr incubation. The amount of 5-FU metabolites in RNA was the same for both sensitive and resistant lines. However, the total amount of 5-FU in DNA was different, with the resistant PEO4 line having 3-fold lower amounts than the PEO1 line. This difference was statistically significant (p_2 = 0.0034). We also determined the extent of fluorinated nucleotide accumulation in RNA and DNA following FdUrd treatment. As shown in Table 6, the levels in RNA were the same for the two cell lines. However, as in the case of 5-FU, the level of fluoropyrimidine metabolites in DNA was 3.6-fold less in the resistant PEO4 line compared with the PEO1 sensitive line.

To confirm these findings, cesium sulfate density centrifugation was used to more accurately determine 5-FU metabolite levels in DNA. As seen in Fig. 1, there is a significantly greater level of 5-FU incorporation in the DNA of the PEO1 line compared with that in the PEO4 line. In this experiment, a concentration of 100 μM 5-FU was used to increase the amount of 5-FU incorporation into DNA and facilitate its measurement. When the absolute amount of fluorinated metabolites per microgram DNA was determined, there was 6.5-fold more metabolite accumulation in the PEO1 line than in the resistant PEO4 line. Initially, concentrations of 1 μM and 10 μM 5-FU were used in these experiments, but no accumulation in the DNA of the PEO4 line was detected (data not shown).

These levels of 5-FU in the DNA are a function of both the rate of incorporation into DNA and the rate of excision of the incorporated fluoropyrimidine. We were unable to measure the intracellular levels of FdUTP, the 5-FU metabolite that serves as a substrate in the incorporation reaction, in these two cell lines (data not shown). Thus, we next determined the rate of removal of incorporated 5-FU from the DNA.

As seen in Fig. 2B, there was significant removal (50%) of the incorporated 5-FU from the PEO4 DNA 4 hr after drug washout. By 8 hr after drug removal, only 30% of the original 5-FU tritium radioactivity remained in the DNA. In contrast, Fig. 2A shows that there was greater retention of radioactive 5-FU metabolites in the DNA of the PEO1 cells, with only 20% and 30% being removed at 4 and 8 hr, respectively. These values, however, do not account for the radioisotope dilution resulting from ongoing semisynthetic replication in these two lines. To correct for this, we determined the effect of 100 μM 5-FU on DNA that had been prelabeled with [^{14}C]TdR prior to 5-FU treatment. When the rate of semisynthetic replication for each cell line is taken into account, a true rate of excision for the incorporated fluoropyrimidine can then be determined. As seen in Fig. 2C, there was a significant net removal of incorporated 5-FU from the DNA of the PEO4 cells. However, there was no apparent removal of 5-FU from the DNA of the PEO1 line; instead, the data suggest ongoing incorporation of 5-FU into the DNA at both 4 and 8 hr after drug removal. A true rate of repair can be calculated using the ratio of the absolute values of [^{3}H]5-FU/[^{14}C]TdR over the 8-hr experimental period. When the values for these two lines were determined, the PEO1 line had a negative rate of excision, i.e., incorporation of 19 attomol/mg/hr, compared with the PEO4 line, which had a rate of repair of 6.3 attomol/mg/hr.

With 100 μM 5-FU exposures, the net level of fluorinated metabolites in the DNA of the PEO1 line was much greater (6.5-fold) compared with that of the PEO4 line. No accumulation was detectable in the PEO4 line with lower 5-FU concentrations of 1 and 10 μM. Because the measured rate of excision may be influenced by this difference in absolute levels, we determined the rate of removal of 5-FU metabolites from the

PEO1 DNA following a 4-hr exposure to 1 μM 5-FU in an attempt to measure removal of equivalent levels of accumulated fluorinated metabolites compared with the PEO4 cells. Using this lower 5-FU concentration, there remained 3-fold more 5-FU metabolites in the PEO1 DNA compared with that of the PEO4 line treated with 100 μM (data not shown). Measurements of the rate of removal of incorporated 5-FU from the DNA of the PEO1 cells under these conditions were similar to those for 100 μM 5-FU exposures, in that they revealed ongoing incorporation of 5-FU into the PEO1 DNA at both 4 and 8 hr after drug washout (data not shown).

Discussion

The results of our study show that the PEO4 human ovarian cancer cell line is 4- and 5-fold more resistant to the growth inhibitory effects of FdUrd and 5-FU, respectively, than the PEO1 line. We have examined a number of potential mechanisms to explain this resistance to the fluoropyrimidines. With the use of two independent assay systems, our studies reveal a significantly decreased level of fluoropyrimidine metabolites in the DNA of the resistant PEO4 line. In our initial studies looking at gross incorporation of 5-FU into DNA, we used a concentration of 1 μM that we felt to be clinically and biologically relevant. There was a significant 3-fold decrease in 5-FU accumulation into the DNA of PEO4 cells compared with PEO1 cells. When cesium sulfate density gradient centrifugation studies were subsequently performed using a much higher dose of 5-FU (100 μM) to more precisely determine DNA incorporation, a similar 6.5-fold decrease in 5-FU accumulation in the DNA of the PEO4 cells was observed. Furthermore, when FdUrd was used as the substrate, there was also 4-fold less incorporated fluoropyrimidine metabolites in the DNA of the resistant PEO4 line.

The net level of fluoropyrimidine metabolites in the DNA is dependent on both the rate of synthesis and incorporation of 5-FU moieties into DNA as well as on the rate of removal or excision of the incorporated fluoropyrimidine nucleotides from the DNA. As seen in Table 3, the levels of both FdUMP and FUTP are 3-fold less in the resistant PEO4 line compared with the PEO1 line. Both of these 5-FU metabolites are metabolized to the nucleotide FdUTP, which is subsequently incorporated into DNA. Thus, it is conceivable that some of the observed difference in absolute levels of accumulated fluorinated metabolites in DNA may be attributed to decreased formation and subsequent incorporation of FdUTP. Given the importance of this nucleotide in DNA incorporation, we attempted to measure the intracellular pools of FdUTP within these two cell lines. However, the level of this metabolite in these cell lines fell below the detection limits of the available assay systems. Previous studies examining the presence of FdUTP or FdUMP residues in DNA were also unable to detect these moieties in 5-FU-treated cells (35, 36). In a study using cell-free extracts from HeLa 53 and KB cell lines, Caradonna and Cheng (36) determined that FdUMP is phosphorylated to FdUTP, which subsequently serves as a substrate for the isolated enzymes α-DNA polymerase, dUTPase, and uracil-DNA glycosylase. They showed that the activities of the two repair enzymes uracil-DNA glycosylase and dUTP nucleotidohydrolase contributed to the markedly decreased levels of FdUTP found in the DNA of KB cells.

In addition to the role of the absolute amount of FdUTP in determining the rate of synthesis and incorporation of fluoropyrimidine nucleotides into DNA, the intracellular pools of dTTP also need to be considered. Since dTTP directly competes with FdUTP for the enzyme α-DNA polymerase, it is theoretically possible that elevated intracellular levels of dTTP in the PEO4 cells could contribute to the overall decreased incorporation of FdUTP into the DNA. However, our results showing a 2-fold greater inhibition of TS enzyme in the PEO4 cells compared with the PEO1 cells after a 4-hr exposure to 5-FU suggest that the levels of dTTP are likely to be lower in the resistant line.

Given the present limitations in measuring FdUTP levels and the rate of synthesis and incorporation of 5-FU moieties into DNA, we have focused our attention on comparing the rates of removal of the incorporated 5-FU metabolites from the DNA of these two cell lines. As shown in Fig. 2C, there is no net removal of incorporated 5-FU from the DNA of the PEO1 cells. Instead, there is continued incorporation of 5-FU into DNA up to 8 hr after drug removal. In comparison, there is rapid removal of incorporated 5-FU from the DNA of the PEO4 cells. Even when the PEO1 cells were exposed to a 100-fold lower concentration of 5-FU (1 μM), there was no change in the rate of removal of 5-FU from the PEO1 DNA. This finding suggests that the inability to remove fluoronucleotides from the DNA may be an important factor in determining the absolute levels within the DNA. Furthermore, the decreased levels of 5-FU in the DNA of the PEO4 cells may, in part, be the result of an enhanced DNA excision process.

Since multiple mechanisms of resistance may occur within a given cell line, we examined other commonly described resistance processes to 5-FU. In sum, there were no deficiencies in the intracellular level of 5,10-methylene-H_4PteGlu, no differences in levels of TS activity, and no alteration in ternary complex formation. In addition, the levels of incorporated 5-FU in the RNA were the same for the two cell lines. The intracellular metabolites FdUMP and FUTP were each 3-fold lower in the resistant PEO4 line compared with the sensitive PEO1 line. Previous studies have suggested that these two metabolites are important determinants of 5-FU cytotoxicity. However, when the final endpoints of these metabolites are considered, i.e., TS inhibition and RNA incorporation, the decreased levels of FdUMP and FUTP in the PEO4 line do not appear to explain its relative resistance to the fluoropyrimidines.

The decreased formation of FdUMP is the result of a 3.4-fold decreased level of thymidine phosphorylase activity in the resistant PEO4 line. However, we have shown that a 4-hr exposure to FdUrd results in a similar level of FdUMP in the PEO1 and PEO4 cells. Since FdUrd bypasses the enzymatic reaction catalyzed by thymidine phosphorylase, this finding suggests that the relative deficiency of this enzyme is not a critical determinant in the development of resistance of PEO4 to fluoropyrimidine chemotherapy. Measurement of the other metabolic enzymes did not reveal an explanation for the decreased formation of FUTP in the PEO4 cells. One possibility is that the levels of other nucleotidases or phosphatases, not measured in this study, are increased. In addition, differences in the availability of substrate sugar donors may contribute to the lower levels of FUTP in the resistant line.

When cells are exposed to 5-FU, significant increases in the intracellular levels of dUMP result due to the inhibition of TS

by the metabolite FdUMP. This increase in dUMP pools can then lead to an increase in dUTP pools. Moreover, it has been suggested that dUTP incorporation into DNA may result in the cellular cytotoxicity due to 5-FU (36, 37). In the face of a potentially enhanced repair enzyme activity in the resistant PEO4 cells involving either dUTP nucleotidohydrolase or uracil-DNA glycosylase, there would be decreased levels of dUTP in their DNA, similar to their decreased level of DNA-incorporated 5-FU. This potential inability to incorporate dUTP into DNA may represent an additional factor in the decreased sensitivity of the PEO4 line to fluoropyrimidine compounds.

The development of the human ovarian cancer cell lines PEO1 and PEO4 has been important in our understanding of the process of drug resistance. These lines were obtained from the same patient pre- and postresistance to 5-FU/cisplatin combination chemotherapy. Therefore, the resistant PEO4 line represents a stable cell line selected for an *in vivo* clinical setting. As a result, the earlier studies investigating the effects of cisplatin against these cell lines and our own studies examining the effects of 5-FU should have particular clinical and biological relevance.

In conclusion, our results suggest that resistance to the fluoropyrimidines 5-FU and FdUrd in the PEO4 line is a DNA-mediated event and appears to be associated with a significant decrease in the level of fluoropyrimidine metabolites in the DNA. An enhanced removal of 5-FU from the DNA appears to be one mechanism by which the PEO4 cell line accumulates less 5-FU moieties in its DNA. These findings contribute to a growing understanding of the significance of fluoropyrimidine incorporation into DNA as well as the potential role of DNA repair in mediating cellular resistance to 5-FU. Additional studies are needed to define more clearly the repair process and to identify the specific DNA repair enzymes involved. Recent work by Lai and co-workers (38) has shown that the PEO4 line is also resistant to cisplatin. They concluded that an enhanced DNA repair enzyme activity was responsible for the resistance of this cell line. Preliminary studies by Parker *et al.* (39) suggest that human repair genes may be expressed at higher levels in patients who show clinical resistance to platinum compounds. A number of other studies have now shown enhanced DNA repair to mediate resistance to adriamycin (40, 41), bleomycin (42, 43), and a variety of alkylating agents (44–47). Thus, our findings and those of other investigators suggest that DNA repair processes may be critical elements in determining resistance to a number of antineoplastic agents in current clinical use.

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