

Relationship between Protein Levels and Gene Expression of Dihydropyrimidine Dehydrogenase in Human Tumor Cells during Growth in Culture and in Nude Mice

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Protein levels and gene expression of dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme for degradation of 5-fluorouracil, were studied in two human tumor cell lines (fibrosarcoma HT-1080 and pancreatic carcinoma MIAPaCa-2) in various growth phases of the cultured cells and of tumor xenografts implanted into nude mice. DPD catalytic activity and DPD protein content in cytosolic preparations were determined by means of radioenzymatic assay and western blot analysis, respectively. Relative DPD mRNA expression was determined by using a semi-quantitative reverse transcription-polymerase chain reaction in which glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal standard. DPD activity and protein content in cultures of both cell lines increased in proportion to cell density (DPD activities ranged from undetectable to 84 pmol/min/mg protein in the HT-1080 cells and from undetectable to 335 pmol/min/mg protein in the MIAPaCa-2 cells). DPD mRNA levels, on the other hand, tended to decrease slightly during cell growth. DPD activity and protein content in HT-1080 tumor xenografts increased during growth in proportion to tumor weight (DPD activities ranged from 7 to 131 pmol/min/mg protein), but DPD mRNA levels did not correlate with tumor weight. DPD activity and protein content in MIAPaCa-2 tumor xenografts did not change much, and seemed to have already plateaued, since the tumors were small (weighing about 30 mg). These findings suggest that DPD protein expression during tumor growth is controlled at the post-transcriptional level.

Key words: Dihydropyrimidine dehydrogenase — 5-Fluorouracil sensitivity — Tumor growth — Semi-quantitative RT-PCR — Post-transcriptional control

5-Fluorouracil (5-FU) is widely used in the treatment of solid tumors, but its clinical effect is unsatisfactory from the standpoint of survival. More than 80% of an administered 5-FU dose is degraded within 24 h after a bolus injection¹⁾ in a three-step pathway that is initially catalyzed by the rate-limiting enzyme dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2), and this may be one of the factors limiting the efficacy of 5-FU.

Recently, determination of tumoral DPD has become of clinical interest, because elevated intratumoral DPD can influence the tumor response to 5-FU therapy as a result of increased inactivation. Etienne *et al.*²⁾ determined DPD activity in tumor biopsy specimens from head and neck cancer patients before administration of 5-FU-based therapy and found that the tumoral/non-tumoral DPD activity ratio in the 52 patients assessable for clinical response was higher in the non-responding patients than in those with a partial or complete response. DPD activity in tumors appears to be a promising parameter for predicting 5-FU sensitivity. Moreover, certain biochemical modulations to enhance the antitumor activity of 5-FU by inhibiting intratumoral DPD activity have been tried in recent years. Milano *et al.* demonstrated³⁾ that 5-ethynyluracil

(EU), which is a potent, irreversible DPD inhibitor,^{4,5)} enhanced 5-FU cytotoxicity in 5 human cancer cell lines expressing high basal DPD activity. We also demonstrated a similar effect of uracil⁶⁾ and 5-chloro-2,4-dihydropyridine⁷⁾ (CDHP), which is a potent, reversible DPD inhibitor,⁸⁾ in two human cancer cell lines. As a result of the increasing interest in tumoral DPD, alternative methods of DPD determination that are more accurate and convenient than the conventional radioenzymatic assay and can be applied to measurements in small specimens, such as those obtained by biopsy, are needed.

The DPD activity in human tumors seems to be highly variable, since it was found to range from 13 to 193 pmol/min/mg protein in biopsy specimens of 63 head and neck tumors²⁾ and from 28 to 207 pmol/min/mg protein in surgical specimens of 60 colorectal tumors.⁹⁾ However, data on tumoral DPD activity are still limited, and nothing is known about the regulatory mechanisms determining DPD activity in human tumors.

It is interesting that analysis of mouse neuroblastoma cells showed an increase in DPD activity as the duration of growth in culture increased.¹⁰⁾ We also observed the same phenomenon in human fibrosarcoma HT-1080 cells

and human pancreatic carcinoma MIA PaCa-2 cells during growth in culture in preliminary experiments. These observations have raised the possibility that DPD activity is controlled by mechanism(s) related to tumor growth. If so, it would be important to clarify at what level, i.e., the transcriptional level or the post-transcriptional level, DPD activity is controlled.

In the present study, catalytic activity, protein content, and mRNA levels of DPD were measured in two human tumor cell lines in the various growth phases of cultured cells and of tumor xenografts implanted into nude mice. We found interesting interrelationships among tumor growth, DPD protein expression, and DPD mRNA expression, which suggest the existence of a mechanism that controls DPD protein expression at the post-transcriptional level.

MATERIALS AND METHODS

Tumor cell lines HT-1080 human fibrosarcoma cells¹¹⁾ were kindly provided by Dr. T. Sasaki (Cancer Research Institute, Kanazawa University, Kanazawa) and were maintained in RPMI 1640 (ICN Biomedicals Inc., Aurora, OH) containing 10% fetal bovine serum (FBS). MIA PaCa-2 human pancreatic carcinoma cells¹²⁾ were purchased from Dainippon Pharmaceutical Co. (Osaka) and were maintained in Dulbecco's modified Eagle's medium (ICN Biomedicals Inc.) containing 10% FBS and 2.5% horse serum.

Cultured cell samples Various numbers of tumor cells (HT-1080, 0.5×10^6 to 4×10^6 cells per flask; MIA PaCa-2, 1×10^6 to 8×10^6 cells per flask) were seeded in 75-cm² flasks. After incubation for 48 h, cells were harvested by trypsinization and counted with a hemocytometer. The cells were then washed once in phosphate-buffered saline, immersed in liquid nitrogen, and stored at -80°C until analysis.

Tumor samples Tumor cells grown in 175-cm² flasks were collected before reaching confluence. Cells (HT-1080, 1×10^6 cells per mouse; MIA PaCa-2, 5×10^6 cells per mouse) suspended in 0.1 ml of saline were implanted subcutaneously into BALB/c nude mice (CLEA Japan Inc., Tokyo). On each day after implantation, as indicated in Figs. 5 and 6, four mice were killed and the tumors were collected. The tumors were immersed in liquid nitrogen and stored at -80°C until analysis.

Enzyme assay The enzyme assay, using a modification of the method of Naguib *et al.*,¹³⁾ has been described in detail previously.⁶⁾ Briefly, tumor cells were freeze-thawed in 2 volumes of homogenization buffer [20 mM potassium phosphate (pH 8.0) containing 1 mM EDTA and 1 mM mercaptoethanol (β -ME)], while tumor tissues were sonicated in 4 volumes of homogenization buffer. Each homogenate was centrifuged at 105,000g for 1 h at 4°C , and

the supernatant fluid (cytosol) was collected as the enzyme source. The enzyme reaction mixture, which contained 10 mM potassium phosphate (pH 8.0), 0.5 mM EDTA, 0.5 mM β -ME, 2 mM dithiothreitol (DTT), 5 mM MgCl_2 , 20 μM [6-¹⁴C]5-FU (56 mCi/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO), 100 μM NADPH (Sigma Chemical Co., St. Louis, MO), and 25 μl of cytosol in a final volume of 50 μl , was incubated at 37°C for 30 min. DPD activity was determined by measuring the sum of the products, i.e., dihydrofluorouracil (DHFU), 2-fluoro- β -ureidopropionate, and 2-fluoro- β -alanine, formed from [6-¹⁴C]5-FU. After addition of 25 μl of 0.36 mM KOH, the reaction mixture was allowed to stand at room temperature for at least 30 min to hydrolyze the DHFU formed. The solution was mixed with 25 μl of 0.36 mM HClO_4 to neutralize it, and then centrifuged at 14,000 rpm for 5 min. A 5 μl aliquot of the supernatant was applied to a thin-layer chromatography plate (silica gel 60 F254, Merck, Darmstadt, Germany), and developed with a mixture of ethanol and 1 M ammonium acetate (5:1, v/v), according to the method of Ikenaka *et al.*¹⁴⁾ Each product was visualized and quantified using an imaging analyzer (BAS-2000, Fujix, Tokyo).

Western blot analysis Aliquots of the cytosol described above were heated for 2 min in a boiling water bath and loaded on 7.5% polyacrylamide gel. After electrophoresis, the proteins were electrically blotted onto a polyvinylidene fluoride membrane at 4°C overnight. The membranes were then incubated with anti-DPD serum obtained from rabbits immunized with a total of 500 μg (100 μg 5 times at 2-week intervals) of recombinant human DPD proteins that we had prepared (data to be published elsewhere). The membranes were washed, incubated with secondary antibody conjugated to alkaline phosphatase, washed again, and reacted with CDP-Star (Triopix, Bedford, MA). The DPD proteins in the membrane were detected with a chemiluminescence imaging system (AE-6930 Densitograph Lumino-CCD, Atto, Tokyo) and quantified by image analysis using Image Master 1D Version 2.01 (Pharmacia Biotech, Tokyo). A preliminary experiment using various human tumor xenografts revealed that DPD activity measured by radioenzymatic assay correlated well with DPD protein content determined by this western blot analysis (data not shown).

Semi-quantitative RT-PCR Lysis buffer contained in the RNeasy mini kit (Qiagen Inc., Chatsworth, CA) with 1/100 volume of β -ME was added to the tumor cells (0.5×10^6 to 2×10^6 cells) or tumor tissues (10 to 50 mg). Cells were homogenized with QIAshredder (Qiagen Inc.) and tumors were homogenized with a Polytron homogenizer. Total RNA in each homogenate was isolated using the RNeasy mini kit as outlined by the manufacturer. The yield and purity of total RNA were determined spectrophotometrically by measuring the absorbances of an ali-

Table I. Primers for DPD and GAPDH Amplification

mRNA	Bases	Sequence (5'-3')	Product size
DPD	2516-2536	TCCTCCAGGTATGCAGTGCCA	514 bp
	3029-3009	GTTATGGTGGGCAGGTGGGTT	
GAPDH	483-494	CAACAGCCTCAAGATCATCAGC	328 bp
	810-790	TTCTAGACGGCAGGTCAGGTC	

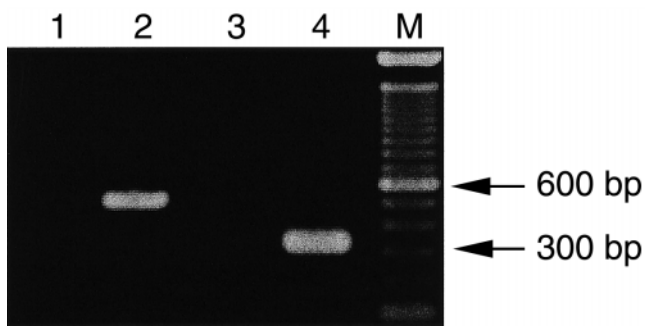


Fig. 1. Specificity of DPD/GAPDH primers for human mRNA. Reverse-transcribed RNA (100 ng) derived from mouse liver (lanes 1 and 3) or MIAPaCa-2 (lanes 2 and 4) was amplified for 30 cycles, with the primers for DPD (lanes 1 and 2, 40 pmol each) and GAPDH (lanes 3 and 4, 40 pmol each). Lane M contains a 100-bp DNA ladder. The PCR products were separated on 2% agarose gel, which was then stained with ethidium bromide and photographed under UV light.

quot at 260 and 280 nm. RNA was checked by agarose gel electrophoresis in the presence of formaldehyde to evaluate the integrity of the rRNA bands.

Reverse transcription with up to 10 µg of total RNA was carried out in a total volume of 100 µl containing 250 pmol of oligo(dT)₁₈, 80 U of rRNasin ribonuclease inhibitor (Promega, Madison, WI), and 500 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 0.5 mM dNTPs. Initially, the total RNA solution mixed with oligo(dT)₁₈ was heated at 70°C for 10 min and immediately chilled on ice, then the other reagents were added. First-strand cDNAs were obtained after 15 min at 30°C and 60 min at 42°C.

PCR primers, summarized in Table I, were designed based on the sequences of human DPD mRNA¹⁵⁾ and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.¹⁶⁾ Primer sequences on separate exons^{17,18)} were chosen in order to detect mRNA rather than contaminating genomic DNA. The melting temperatures of all 4 primers are the same, 66°C, as estimated by the rule-of-thumb calculation.¹⁹⁾ The specificity of the

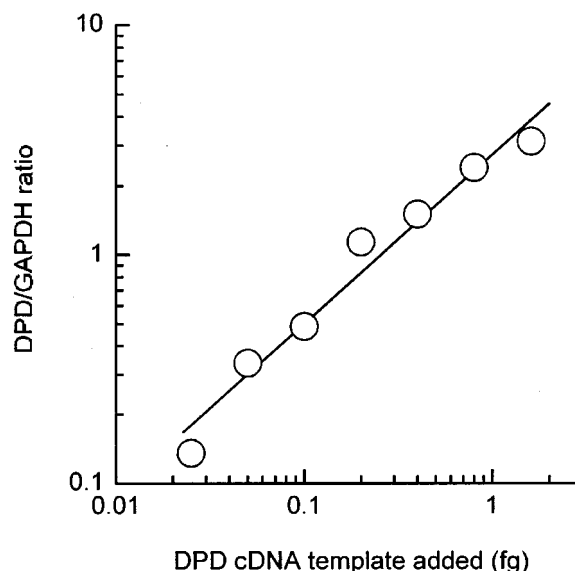


Fig. 2. Relationship between the actual cDNA template ratio and the signal intensity ratio of PCR products. DPD cDNA (PCR product) within the 0.025-1.6 fg range was added to a constant amount of GAPDH cDNA (PCR product, 10 fg), and co-amplification was performed for 30 cycles, with the primers for DPD (40 pmol each) and GAPDH (2 pmol each). The PCR products were separated on 2% agarose gel, which was then stained with ethidium bromide, photographed under UV light, and quantified with Image Master 1D (Pharmacia Biotech). $y=2.78x^{(0.75)}$, $r=0.98$, $P=0.00013$.

DPD primers and GAPDH primers was confirmed by DNA sequencing of the PCR products amplified with them.

PCR was carried out in a final volume of 50 µl containing reverse-transcribed total RNA (1 ng for analysis of cultured cells and 2 ng for analysis of tumor xenografts), 40 pmol of each DPD primer, 2 pmol of each GAPDH primer, and 1.25 U of Ex Taq (Takara, Shiga) in 5 µl of 10× Ex Taq buffer (Takara) and 0.2 mM dNTPs, using a thermal cycler (PC-800, Astec, Tokyo). The PCR profile consisted of a 3-min initial denaturation at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of polymerization at 72°C, and finally a 10-min extension at 72°C.

PCR products were separated by 2% agarose gel electrophoresis. Gels were stained with ethidium bromide, visualized on a UV transilluminator and photographed on Type 667 films (Polaroid, Cambridge, MA). The positives were scanned with an image scanner (JX-330, Sharp, Mahwah, NJ) and analyzed with Image Master 1D (Pharmacia Biotech). The relative amount of DPD mRNA was expressed as the ratio of DPD to GAPDH.

MTT assay Various numbers of tumor cells (1,000 to 4,000 cells/well) in the exponential growth phase were seeded in 96-well microtitration plates on day 0. At 24 h after plating, cells were exposed for 3 days to various concentrations of 5-FU (8 concentrations, from 0.03 to

100 $\mu\text{g/ml}$). The growth phase of the cells (i.e., whether confluent or not) was examined every 24 h. The cytotoxic effect of 5-FU was measured on day 4 by using the tetrazolium-based colorimetric assay (MTT assay).²⁰ Results are expressed as follows: growth inhibition (%) = $[1 - (\text{the relative percentage of absorbance detected in treated cells}) / (\text{the relative percentage of absorbance detected in untreated control cells})] \times 100$. The 5-FU concentration causing 50% growth inhibition as compared to the control (IC_{50}) was calculated from the regression line.

Statistics Linear regression analysis was performed on Microsoft Excel for Windows 95, version 7.0.

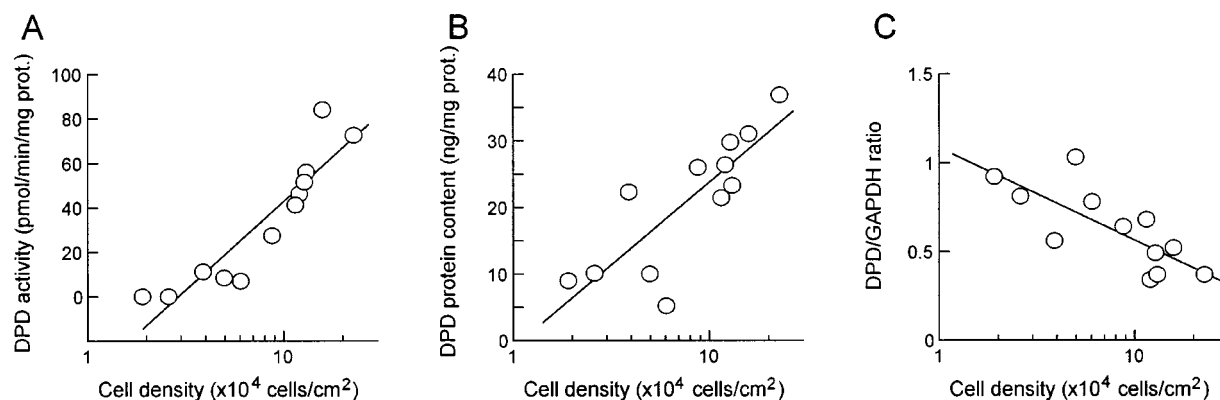


Fig. 3. Relationship of DPD protein/mRNA levels to cell growth in cultured HT-1080 cells. Cells in various growth phases were separately harvested, and cytosol and total RNA were prepared from the cells for the following analysis. Each plot represents the correlation of cell density with DPD activity (A) measured by radioenzymatic assay, DPD protein content (B) by western blotting, and DPD/GAPDH mRNA ratio (C) by semi-quantitative RT-PCR. Each method is described in detail in "Materials and Methods." A: $y = -36.86 + 79.88 \log x$, $r = 0.92$, $P = 0.000029$, B: $y = -1.05 + 24.84 \log x$, $r = 0.82$, $P = 0.0011$, C: $y = 1.08 - 0.51 \log x$, $r = -0.76$, $P = 0.0041$.

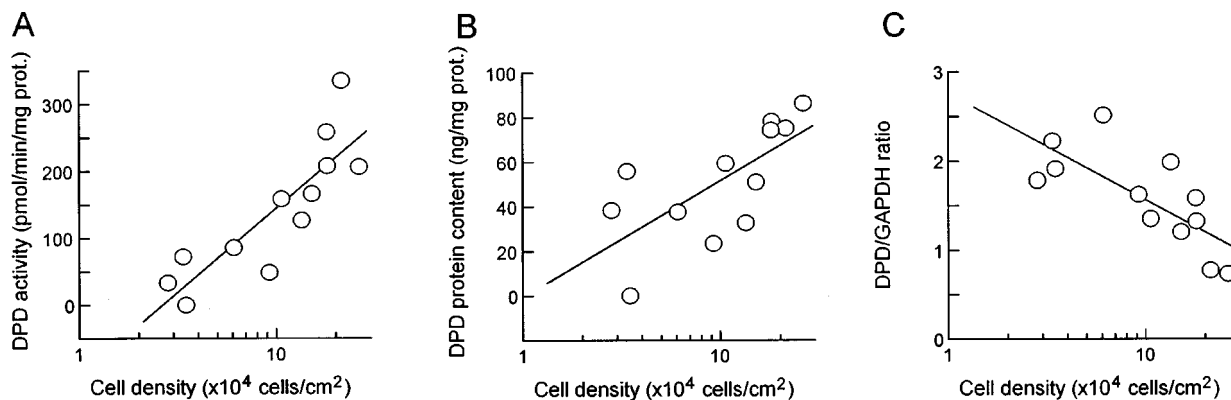


Fig. 4. Relationship of DPD protein/mRNA levels to cell growth in cultured MIA PaCa-2 cells. Cells in various growth phases were separately harvested, and cytosol and total RNA were prepared from the cells for the following analysis. Each plot represents the correlation of cell density with DPD activity (A) measured by radioenzymatic assay, DPD protein content (B) by western blotting, and DPD/GAPDH mRNA ratio (C) by semi-quantitative RT-PCR. Each method is described in detail in "Materials and Methods." A: $y = -106.2 + 250.7 \log x$, $r = 0.85$, $P = 0.00048$, B: $y = -0.69 + 52.25 \log x$, $r = 0.69$, $P = 0.013$, C: $y = 2.75 - 1.18 \log x$, $r = -0.74$, $P = 0.0063$.

RESULTS

Development of semi-quantitative RT-PCR One of the objectives in this study was to measure *human* DPD mRNA levels with *human* GAPDH mRNA as the internal standard. One possible problem is that mouse DPD/GAPDH mRNA (e.g., DPD/GAPDH mRNA in mouse fibroblasts) might be present as a contaminant in total RNA solution extracted from the human tumor xenografts. We therefore designed primers specific to human DPD/GAPDH mRNA sequences, by comparing with the mouse DPD/GAPDH mRNA sequences. The mouse GAPDH mRNA sequence was in agreement with published data,²¹⁾ and a part of the mouse DPD mRNA sequence was determined in our laboratory by sequencing the RT-PCR product derived from total RNA extracted from mouse liver. Every primer sequence was different

from the corresponding mouse DPD/GAPDH mRNA sequence in 4 or 5 bases (data not shown), and only human DPD/GAPDH mRNA was amplified with these specific primers, as shown in Fig. 1.

To minimize tube-to-tube variation in the efficiency of PCR, we developed a semi-quantitative RT-PCR co-amplified with GAPDH mRNA. As reported previously,^{22,23)} co-amplification of mRNAs initially present at widely different levels can not achieve the optimal condition of PCR linearity. A preliminary experiment revealed that GAPDH mRNA was more highly expressed than DPD mRNA in the cells used in this study. We therefore determined the optimal concentration of each set of primers according to the relative amount of each mRNA amplified, following the method of Dukas *et al.*²³⁾ As a result, uniform co-amplification was achieved using a 20/1 ratio of DPD/GAPDH specific primers. Under these

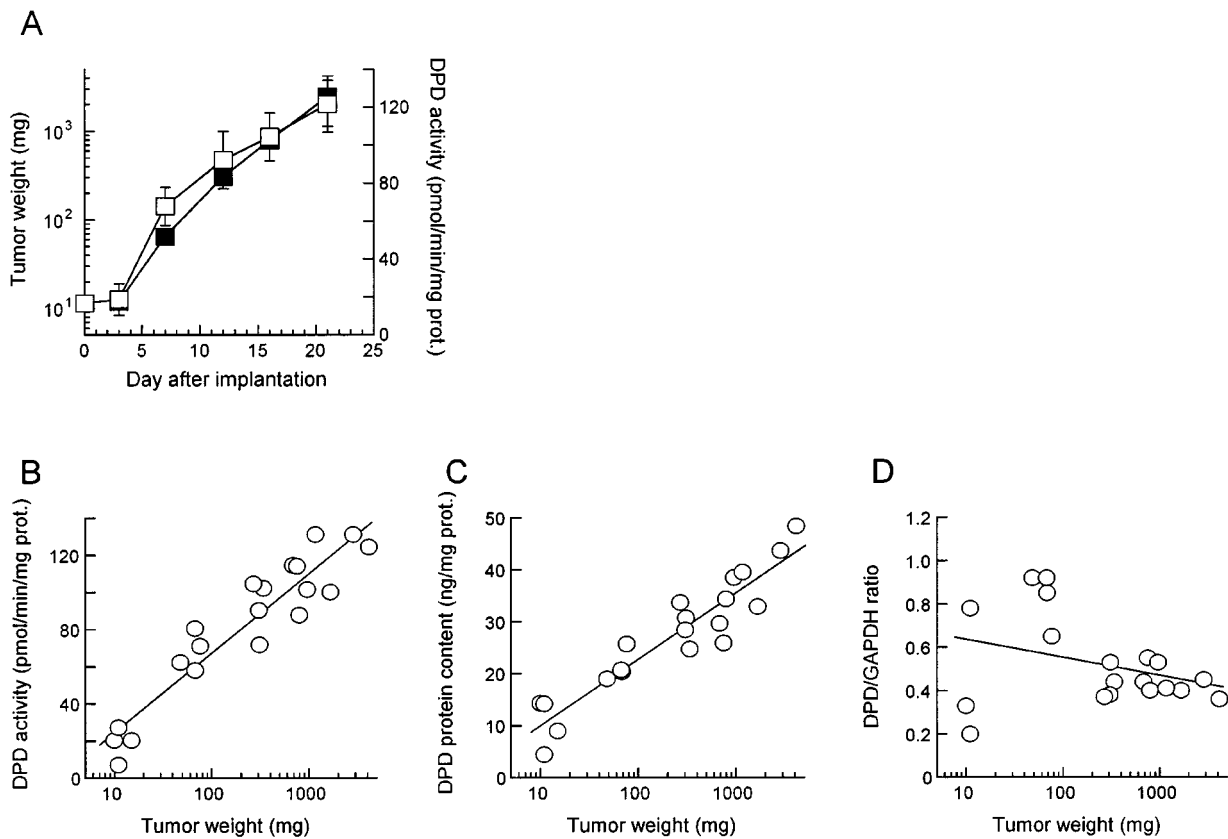


Fig. 5. Relationship of DPD protein/mRNA levels to tumor growth in HT-1080 tumor xenografts. HT-1080 cells were harvested for implantation on day 0, and tumors of various sizes that had formed were collected from 4 mice each on days 3, 7, 12, 16, and 21. Cytosol and total RNA were prepared from the cells and the individual tumors for the following analysis. Plots show tumor growth (closed squares; mean±SD) and the elevation of DPD activity in tumors (open squares; mean±SD) after implantation (A), and the correlation of tumor weight with DPD activity (B) measured by radioenzymatic assay, DPD protein content (C) by western blotting, and DPD/GAPDH mRNA ratio (D) by semi-quantitative RT-PCR. Each method is described in detail in "Materials and Methods." B: $y = -18.85 + 43.08 \log x$, $r = 0.94$, $P = 1.02 \times 10^{-9}$; C: $y = -2.85 + 12.83 \log x$, $r = 0.93$, $P = 1.72 \times 10^{-9}$; D: $y = 0.72 - 0.082 \log x$, $r = -0.32$, $P = 0.19$.

conditions, namely, 30 cycles of PCR using 40/2 pmol of DPD/GAPDH primers in each reaction, 0.5 to 4 ng of reverse-transcribed total RNA (cDNA) was sufficient to observe a quantifiable signal within the linear range of amplification (data not shown). Moreover, the relationship between the actual cDNA template ratio and the signal intensity ratio was examined following the method of Iijima *et al.*,²⁴ with slight modifications. As shown in Fig. 2, the DPD cDNA (PCR product) added within a 64-fold range lay within the linear range of the signal intensity ratio of DPD to GAPDH. Using this method, DPD/GAPDH mRNA could be determined even in 10-mg tumor samples.

Correlation of DPD mRNA/protein levels with cell density The growth phase of each cell line varied from exponential growth to confluence at the time when the

cells were harvested: the cell density range of the HT-1080 line was $1.9\text{--}22.7 \times 10^4$ cells/cm² and the cell density range of the MIAPaCa-2 line was $2.8\text{--}26.3 \times 10^4$ cells/cm². As shown in Fig. 3A, DPD activity in the HT-1080 cells varied over a 12-fold range (7–84 pmol/min/mg protein in 10 cultures and undetectable in 2 cultures), and increased in proportion to cell density. DPD protein content also increased in proportion to cell density (Fig. 3B), and a good correlation was found with DPD activity ([DPD protein content]= $10.66 + 0.30$ [DPD activity], $r = 0.87$, $P = 0.00021$). On the other hand, DPD mRNA levels varied within only a 3-fold range (0.34–1.03 DPD/GAPDH ratio in 12 cultures), and tended to decrease slightly with cell growth (Fig. 3C). Similarly, DPD activity in the MIAPaCa-2 cells varied over a 10-fold range (33–335 pmol/min/mg protein in 11 cultures and

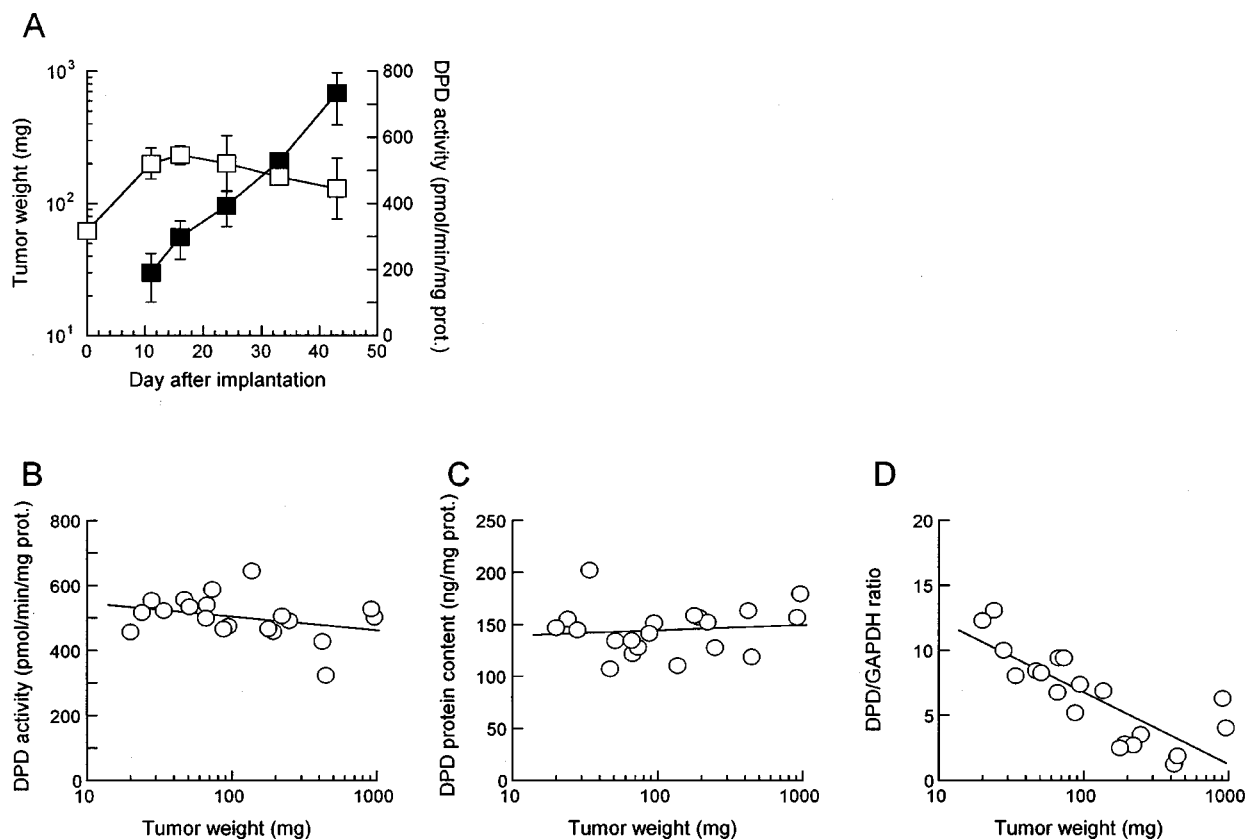


Fig. 6. Relationship of DPD protein/mRNA levels with tumor growth in MIAPaCa-2 tumor xenografts. MIAPaCa-2 cells were harvested for implantation on day 0, and tumors of various sizes that had formed were collected from 4 mice each on days 11, 16, 24, 33, and 43. Cytosol and total RNA were prepared from the cells and the individual tumors for the following analysis. Plots show tumor growth (closed squares; mean \pm SD) and the change of DPD activity in tumors (open squares; mean \pm SD) after implantation (A), and the correlation of tumor weight with DPD activity (B) measured by radioenzymatic assay, DPD protein content (C) by western blotting, and DPD/GAPDH mRNA ratio (D) by semi-quantitative RT-PCR. Each method is described in detail in "Materials and Methods." B: $y = 590.1 - 42.47 \log x$, $r = -0.32$, $P = 0.17$, C: $y = 132.5 + 5.80 \log x$, $r = 0.13$, $P = 0.60$, D: $y = 17.83 - 5.52 \log x$, $r = -0.80$, $P = 0.000024$.

Table II. Response of Tumor Cells to 5-FU in Various Growth Phases in Culture

Tumor cells	Initial cell number ($\times 1000$ cells/well)	IC ₅₀ of 5-FU ($\mu\text{g/ml}$)		Days until confluence after plating
		Exp. 1	Exp. 2	
HT-1080	1	3.5	1.7	day 3
	2	8.9	7.9	day 3
	4	13.7	21.6	day 2
MIAPaCa-2	1	1.6	1.2	— ^{a)}
	2	4.3	5.5	day 4
	4	20.3	13.7	day 3

The growth inhibition rate was assessed by using the MTT test. The 5-FU concentration causing 50% growth inhibition as compared to the control (IC₅₀) was calculated from the regression line.

a) Sub-confluence on day 4.

undetectable in 1 culture), and increased in proportion to cell density, as shown in Fig. 4A. DPD protein content also increased in proportion to cell density (Fig. 4B), and showed a good correlation with DPD activity ([DPD protein content] = $20.57 + 0.21$ [DPD activity], $r=0.83$, $P=0.00073$). On the other hand, DPD mRNA levels varied within only a 3-fold range (0.73–2.51 DPD/GAPDH ratio in 12 cultures), and tended to decrease slightly with cell growth (Fig. 4C).

Correlation of DPD mRNA/protein levels with tumor mass The HT-1080 cells collected for implantation into nude mice on day 0 were in the exponential growth phase: the cell density was 2.5×10^4 cells/cm². During the 21 days after implantation, HT-1080 tumors grew to 2.45 ± 1.31 g, and the DPD activity in tumors increased 7-fold (17 pmol/min/mg protein in cultured cells just before implantation and 122 ± 15 pmol/min/mg protein in tumors on day 21), as shown in Fig. 5A. Both DPD activity and DPD protein content increased in proportion to tumor weight (Fig. 5, B and C), and there was a high correlation between activity and protein content ([DPD protein content] = $5.18 + 0.27$ [DPD activity], $r=0.90$, $P=9.2 \times 10^{-8}$). DPD mRNA levels, on the other hand, did not correlate with tumor weight (Fig. 5D).

The MIAPaCa-2 cells collected for implantation into nude mice on day 0 were in a sub-confluent state: the cell density was 15.8×10^4 cells/cm². During the 43 days after implantation, MIAPaCa-2 tumors grew to 0.68 ± 0.29 g (Fig. 6A). While the tumor was growing, neither the DPD activity nor the DPD protein content in the tumors changed much (within a 2-fold range), as shown in Fig. 6, A–C. DPD mRNA levels tended to decrease with tumor growth (Fig. 6D).

Response of tumor cells to 5-FU in various growth phases in culture To confirm that the sensitivity of both tumor cells to 5-FU actually decreases during tumor growth in culture, the MTT test was performed at various cell densities. Naturally, the greater the number of cells of

both tumors plated initially, the faster they reached confluence (Table II). The IC₅₀ values of 5-FU for both tumor cell lines increased in proportion to the initial cell density (Table II).

DISCUSSION

The present study has demonstrated an increase of DPD protein expression with tumor growth in human tumor cell lines HT-1080 and MIAPaCa-2. Contrary to expectation, DPD protein expression in MIAPaCa-2 tumors did not change much during growth (Fig. 6, A–C). It is suspected that DPD protein expression in the MIAPaCa-2 tumors had already plateaued when the tumors were very small (about 30 mg or less), because the initial level of DPD activity in the cells (on day 0: just before implantation) was already quite high (Fig. 6A).

Many reports have described other examples of correlations between protein levels and the cell growth phase: increases in 7 lysosomal hydrolases in human fibroblasts,²⁵⁾ decreases in topoisomerase II in HT-29 human colon cancer cells,²⁶⁾ decreases in thymidylate synthase (TS) in 3 kinds of human cells,²⁷⁾ and so on. While rat liver was differentiating, DPD activity in the liver increased 2.7-fold from the level in newborn rats to the level in adults.²⁸⁾ In rat hepatomas, DPD activity decreased in parallel with the increase in growth rate.²⁸⁾ DPD activity in mouse neuroblastoma cells increased during culture, a finding consistent with our results, while thymidine kinase activity decreased markedly.¹⁰⁾ Increases in DPD protein expression during tumor growth may occur frequently in human tumors, since we observed increases in other human tumor cell lines, such as HT-1080 and MIAPaCa-2 (data not shown). McLeod *et al.* suggested the possibility of down-regulation of DPD in culture, since the DPD activity in 5 strains of colorectal cancer cells in culture was significantly lower than in colorectal tumor specimens.⁹⁾ Since DPD activity and pro-

tein content were independent of mRNA levels, as shown in Figs. 3–5, it appears that DPD protein expression may be controlled at the post-transcriptional level. DPD activity in exponentially growing cells was very low, sometimes undetectable, while DPD mRNA levels were consistently high (Figs. 3, 4), suggesting that translation of DPD mRNA may be suppressed by some mechanism that acts at low cell density, and that the suppression is released by some signal related to cell growth or differentiation.

Generally, proliferating cells are much more sensitive to anticancer agents than nonproliferating cells. When the cell population reached confluence in culture, HT-29 human colon cancer cells were resistant to several major anticancer agents, including doxorubicin, etoposide, cisplatin and 5-FU,²⁶⁾ and DLD-1 human colon cancer cells were resistant to several anticancer agents including 5-FU.²⁹⁾ These findings are consistent with the response of HT-1080 and MIAPaCa-2 (Table II). The mechanism of this confluence-dependent resistance is thought to involve a decrease in drug accumulation in the cells, an increase in the intrinsic resistance of the DNA to the drug-induced damage, and induction of expression of specific enzymes, such as topoisomerase II.^{26,30)} Similarly, growth of the tumor mass is generally associated with a decrease in therapeutic efficacy via mechanisms such as decreased drug accessibility, metastases with altered characteristics, and development of heterogeneity in the tumor itself.^{31,32)} In the case of 5-FU treatment, the antitumor activity of 5-FU has been found to be lower in large tumors than in small tumors in the cases of murine colon carcinomas, colon 26 and colon 38.³³⁾ However, the mechanisms by which resistance to 5-FU is related to tumor growth are not yet fully understood. The results of the present study may provide a partial explanation: increased DPD protein

expression during tumor growth may allow tumors to acquire resistance to 5-FU. Inhibiting DPD activity and suppressing 5-FU degradation in the tumor would probably be effective in overcoming this resistance to 5-FU. Therefore, combined therapy consisting of 5-FU plus a DPD inhibitor such as EU, uracil, or CDHP, which enhanced 5-FU cytotoxicity to human tumor cells in culture,^{3,6,7)} is expected to be more effective in tumors that show little response to 5-FU alone because of high basal DPD activity.

Determination of DPD levels in tumors provides a means of predicting the clinical therapeutic efficacy of 5-FU, like other predictive parameters, such as TS^{34,35)} and p53.³⁶⁾ Radioenzymatic assay is not always convenient for DPD determination because it involves the use of a radioisotope. Our semi-quantitative RT-PCR method for determination of DPD mRNA expression should be more useful for measurement in small specimens, such as biopsy specimens, without the need for a radioisotope.

However, our findings indicate that DPD protein levels may not always parallel DPD mRNA levels in clinical specimens. Moreover, tumoral DPD protein levels may change in a circadian rhythm, as in peripheral mononuclear cells.^{37–39)} In such cases, it would be important to clarify which parameter is more significant in predicting 5-FU-responsiveness. Since DPD protein levels in tumors change with time, DPD mRNA levels may be a better parameter than DPD activity or DPD protein content. In any event, it is necessary to uncover the regulatory mechanisms of DPD protein expression and to investigate further the correlation between clinical response to 5-FU and DPD protein or DPD mRNA levels.

(Received July 1, 1998/Revised September 1, 1998/Accepted September 5, 1998)

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