Expression of CYP2A6 in Tumor Cells Augments Cellular Sensitivity to Tegafur

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To examine the role of cytochrome P450 2A6 (CYP2A6) in the cellular sensitivity to an anti-tumor prodrug, tegafur (FT), a CYP2A6 cDNA construct was transfected into cells of a colon cancer cell line, DLD-1. CYP2A6-expressing cells (DLD-1/CYP2A6 cells) more efficiently catalyzed the conversion of FT to 5-fluorouracil (5-FU) (2.6-fold) and the 7-hydroxylation of coumarin (7.9-fold) than cells transfected with a null construct (DLD-1/null cells). These results indicated that the expressed CYP2A6 was functionally active. The extent of growth inhibition of the DLD-1/CYP2A6 cells by FT was greater than that of DLD-1/null cells; the difference between the DLD-1/CYP2A6 and DLD-1/null cells was statistically significant at the concentrations of 250, 500 and 1000 μM. 5-FU, an active metabolite of FT, inhibited the growth of both types of cells to the same extent. Thus, intracellular expression of CYP2A6 can sensitize cells to FT.

Key words: Tegafur — 5-FU — Prodrug — CYP2A6 — Tumor cells

Tegafur (FT) is a component of UFT (uracil and tegafur, Taiho Pharamaceutical Co., Ltd.), an anti-cancer combination prodrug that is widely used for the treatment of stomach, breast, and colon cancers. FT must be metabolized to 5-fluorouracil (5-FU) to exert its anti-tumor actions.[1, 2] S-1, another combination oral anti-tumor drug containing gimestat, otastat, and FT,[3] has recently been developed and approved for the treatment of stomach cancer in Japan. Recently, cytochrome P450 2A6 (CYP2A6) has been shown by different research groups to catalyze the conversion of FT to 5-FU.[4–6] Those investigators extensively studied the role of individual CYPs in FT activation by using recombinant CYP forms and evaluating the microsomal conversion of FT to 5-FU in the presence of specific CYP inhibitors. These results prompted us to test whether cDNA-mediated expression of CYP2A6 in cultured human colon cancer cells makes them more sensitive to FT as compared with cells transfected with a CYP2A6-null construct. Such a system would be useful for assessing the role of CYP2A6 in exerting the anti-tumor effects of fluoropyrimidine prodrugs through bioactivation. In the present study, we report that expression of CYP2A6 in DLD-1 cells (cells of a human colon cancer cell line) augments cellular sensitivity to FT, as assessed by cell growth inhibition.

MATERIALS AND METHODS

Reagents   5-FU and FT were obtained from Sigma

Chemical (St. Louis, MO). Coumarin was obtained from WAKO Pure Chemical Industries (Osaka). Umbelliferone was obtained from DOJIN (Kumamoto). NADP$^+$ glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co. (Tokyo). Mouse anti-human CYP2A6 monoclonal antibody was purchased from GENTEST Corp. (Woburn, MA) through Daiichi Pure Chemicals (Tokyo). All other chemicals and reagents used were of the highest grade available.

Plasmid construction   Construction of the pcDNA3.1/CYP2A6 plasmid for human CYP2A6 expression was achieved using cDNA synthesized from human liver poly(A)$^+$ RNA (Clontech, Palo Alto, CA) by reverse transcriptase-polymerase chain reaction (RT-PCR). The primers used were: 2A6F1, 5′-GCTACCACCATGCTGGCCTCAGGG-3′ and 2A6R1, 5′-CCAGACCTGCACCGGCACAG-CCCTCGCTCA-3′. The resultant PCR product was ligated into the pCR3.1 vector to generate pCR3.1/CYP2A6 using a TA cloning kit (Invitrogen, Carlsbad, CA). A 1.5-kb fragment was excised from pCR3.1/CYP2A6 by digestion with *Eco*RI and ligated into pcDNA3.1 (Invitrogen), a plasmid that had been cleaved with *Eco*RI, to generate pcDNA3.1/CYP2A6.

Human CYP2A6 expression in DLD-1 cells (transient expression with pcDNA3.1/CYP2A6 plasmids in DLD-1 cells)   DLD-1 cells were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO$_2$ and 95% air at 37°C. DLD-1 cells were seeded into a 100-mm culture dish and grown to 70–90% confluence. Cells were rinsed with serum-free OPTI-MEM medium before transfection. pcDNA3.1/CYP2A6 plasmid DNA (6 μg), 24 μl of PLUS reagent (Life Technologies Inc., Rockville, MD), and 24 μl of Lipo-

fectAMINE reagent (Life Technologies Inc.) were used for transfection. Twenty-four hours after transfection, fresh RPMI 1640 media containing 5 mg/ml geneticin was added, and the cells were cultured for another 2 days. The adhered cells were harvested by trypsinization and cultured for another 24 h for western blot analyses and enzyme activity measurements. For determination of drug sensitivity, the cells were re-plated in 96-well tissue culture plates at a density of 5×10^4 cells/well. Drug sensitivity assays were performed for 72 h from the next day.

Western blot analysis The transfected cells were homogenized by repeated sonication on ice for 15 s. Microsomal protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). The cell lysate (25 μg protein) was dissolved in SDS-sample buffer, electrophoresed on 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose sheets (Bio-Rad).[7] For immunostaining, mouse anti-human CYP2A6 monoclonal antibody (GENTEST Corp.) as the primary antibody, and rabbit anti-mouse IgG (Bio-Rad) conjugated with horseradish peroxidase as the second antibody were used, and CYP2A6 was visualized with the Enhanced Chemiluminescence kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK).[8] CYP2A6 content in the DLD-1/CYP2A6 cells was determined by comparison with that (66 pmol/mg protein) in human liver microsomes (GENTEST Corp.).

Formation of 5-FU from FT Microsomes from lysates of the transfected cells were prepared by differential centrifugation and resuspended in distilled water. 5-FU formation from FT was determined using a slight modification of a previously described method.[9] The incubation mixture (250 μl) contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM FT, an NADPH-generating system (0.8 mM NADP$^+$, 8 mM glucose 6-phosphate, 1 unit/ml glucose 6-phosphate dehydrogenase and 6 mM MgCl$_2$), and the microsomal fraction (0.2 mg protein/ml) of DLD-1 cells with or without CYP2A6 expression. Incubation was carried out at 37°C for 60 min and terminated by addition of 1 ml of ethyl acetate. 5-Bromouracil was added as an internal standard. After centrifugation, the organic phase was evaporated to dryness under a gentle nitrogen gas stream. The residue was dissolved in a mixture of the mobile phase used for HPLC. The concentration of 5-FU was determined by HPLC with an analytical reverse-phase C$_8$ (5 μm) column (150×4.4 mm i.d., Senshu Pak PEGASIL C$_8$, Senshu Scientific Co., Ltd., Tokyo). The elution conditions were as follows: solvent A (10 mM potassium phosphate buffer, pH 5.5) for 10 min, followed by a linear gradient to 90% solvent B (methanol) in 20 min at a flow rate of 1.0 ml/min. The chromatogram was monitored by measuring the UV absorbance at 268 nm.

Coumarin 7-hydroxylation[10] Hydroxylation of coumarin was assessed by using 250 μg of microsomal protein in a 500-μl reaction system containing 100 mM Tris-HCl (pH 7.4), 10 μM coumarin, and an NADPH-generating system. The enzyme reaction was initiated by addition of the NADPH-generating system, and the mixtures were incubated at 37°C. The reaction was stopped after 60 min by addition of 500 μl of 5% trichloroacetic acid. The concentration of 7-hydroxycoumarin was determined fluorometrically (λ_{ex}=380 nm, λ_{em}=460 nm) with a spectrofluorometer (Hitachi 650-40 fluorescence spectrophotometer, Hitachi Co., Ltd., Tokyo).

Inhibition of cell growth by anti-tumor agents The effect of the anti-cancer agents (5-FU and FT) on cell growth was assessed using a crystal violet assay in 96-well micro-titer plates. The cells (5×10^4 cells/well) were treated for 72 h with various concentrations of drugs in 200 μl of fresh medium. The cells were then washed with PBS and stained with 100 μl of crystal violet (0.4% in methanol) as previously described.[11] Data were expressed as percentage inhibition of cell growth. Statistical significance was evaluated using Student's t test.

RESULTS

Expression of CYP2A6 in DLD-1 cells As illustrated in Fig. 1, CYP2A6 was expressed in CYP2A6-transfected cells, but not in vector-transfected cells (DLD-1/null cells). The level of the immunoreactive CYP2A6 protein on the western blots was approximately 17 pmol/mg microsomal protein, which was about one-fourth of that found in human liver microsomes.

Microsomal 5-FU formation from FT and hydroxylation of coumarin Microsomal 5-FU formation from FT was measured in CYP2A6-expressing cells and vector-transfected cells (Fig. 2A). The microsomal fraction from the CYP2A6-expressing cells converted FT to 5-FU at a rate of 145±49 pmol/min/mg protein, which was 2.6-fold higher than that from the DLD-1/null cells.
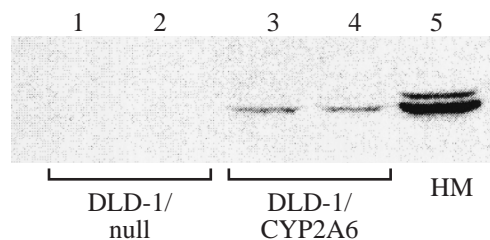

Fig. 1. Western blot detection of CYP2A6. The CYP2A6 proteins were analyzed in vector-transfected cells (DLD-1/null cells), CYP2A6 cDNA-transfected cells (DLD-1/CYP2A6 cells), and human liver microsomes. Microsomal proteins (25 μg) were electrophoresed and blotted as described in "Materials and Methods." Lanes 1 and 2, DLD-1/null cells; lanes 3 and 4, DLD-1/CYP2A6 cells; lane 5, microsomes from pooled human livers.

Microsomal coumarin 7-hydroxylase activity was determined in CYP2A6-expressing cells and vector-transfected cells (Fig. 2B). The microsomes from DLD-1/CYP2A6 cells actively catalyzed the 7-hydroxylation of coumarin, a typical CYP2A6 substrate, at a rate of 6.38 pmol/min/mg protein. In contrast, the microsomes from the vector-transfected cells catalyzed it at a rate of only 0.807 pmol/mg protein.

Increase in sensitivity of DLD-1/CYP2A6 cells to FT
Growth inhibition by FT and 5-FU was measured in DLD-1/CYP2A6 and DLD-1/null cells. DLD-1/CYP2A6 cells

showed a higher overall sensitivity to FT than DLD-1/null cells, whereas the CYP2A6-expressing and CYP2A6/null cells showed comparable sensitivity to 5-FU (Fig. 3). The differences in cell growth inhibition between the CYP2A6-expressing and CYP2A6/null cells were statistically significant at 250 μM (48%±7% vs. 23%±10%, $P<0.02$), 500 μM (52%±8% vs. 36%±6%, $P<0.02$), and 1000 μM FT (74%±15% vs. 40%±17%, $P<0.01$), respectively. No statistical significance was obtained for the difference in 5-FU sensitivity between the DLD-1/CYP2A6 and DLD-1/null cells at any concentration used in this study.

DISCUSSION

In the present study, we succeeded in sensitizing DLD-1 cells (human colon cancer cells) to FT by engineering the cDNA-mediated intracellular expression of CYP2A6. No significant difference in 5-FU sensitivity was observed between cells with and without CYP2A6 expression.

The ability of the CYP2A6-expressing cells to convert FT to 5-FU was 2.6-fold higher than that of the DLD-1/null cells. Furthermore, microsomal coumarin 7-hydroxylase activity, a typical CYP2A6-dependent activity, was 7.9-fold higher in the cells expressing CYP2A6 than in the vector-transfected cells. These results indicated that the intracellular expression of enzymatically active CYP2A6 made the cells more sensitive to FT, but not to 5-FU. It has been documented that the prodrug FT must be activated metabolically to 5-FU, a process catalyzed by hepatic drug-metabolizing enzymes including microsomal CYP2A6 as well as CYP1A2 and CYP2C, and a cytosolic
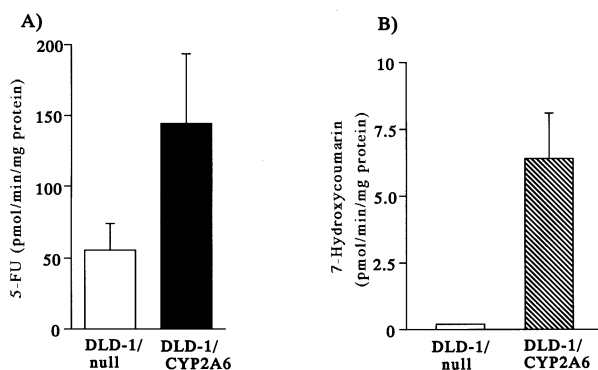

Fig. 2. Microsomal 5-FU formation from FT (A) and microsomal coumarin 7-hydroxylase activity (B) in DLD-1/CYP2A6 cells. The rates of 5-FU formation from FT and microsomal coumarin 7-hydroxylation were determined as described in "Materials and Methods." Bars represent the mean enzymatic activities with standard deviations (SD) ($n=3$).
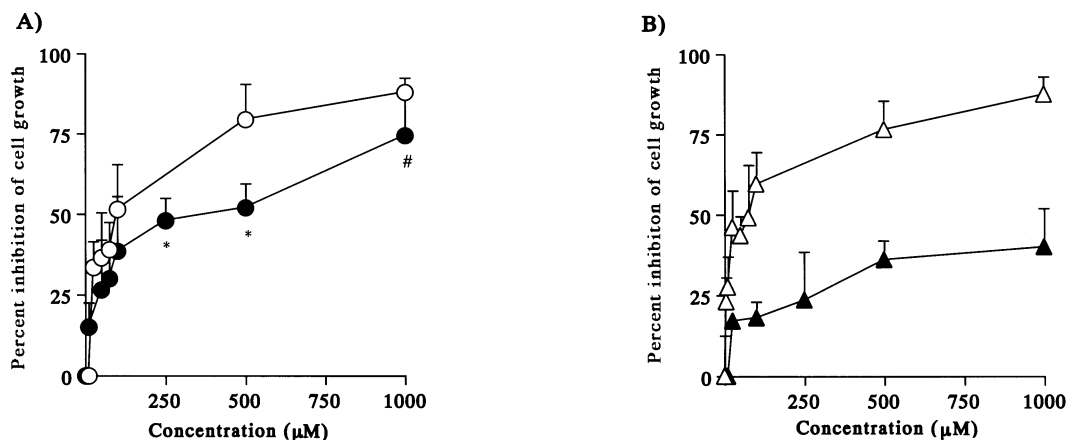
Fig. 3. Inhibition of cell growth by FT and 5-FU of DLD-1/CYP2A6 (A) and DLD-1/null (B) cells. Inhibition of cell growth by FT and 5-FU was determined by staining the cells with crystal violet as described in "Materials and Methods." The extent of cell growth inhibition was determined using micro-titer plates and was derived from five to eight wells per drug concentration. ∗, $P<0.02$; #, $P<0.01$. A) ○, 5-FU; ●, FT. B) △, 5-FU; ▲, FT.

thymidine phosphorylase.[1, 4–6, 9, 12] We have also expressed CYP3A4 in DLD-1 cells (DLD-1/CYP3A4 cells), whose CYP3A4 content was comparable to or higher than the CYP2A6 content in the DLD-1/CYP2A6 cells. The DLD-1/CYP3A4 cells did not, however, show a remarkable increase in sensitivity to FT as compared with DLD-1/null cells (data not shown). As shown in Fig. 3B, FT itself seemed to have growth-inhibitory effects on tumor cells. DLD-1/null cells exhibited some 5-FU production from FT using a microsomal fraction, as shown in Fig. 2A. The 5-FU production unrelated to CYP2A6 in DLD-1 cells may be the cause of the growth inhibition, although the mechanism of 5-FU production is not clear at present (i.e. spontaneous or enzymatic). The soluble enzyme pathway should also be considered. Thus, all of our present results are consistent with previous findings on the capability of CYP enzymes to catalyze the bioactivation of FT. CYP2A6 has been shown to be expressed mainly in liver, but not in tumor cells. Dihydropyrimidine dehydrogenase and thymidine phosphorylase are known factors affecting tumor cell sensitivity to 5-FU.[13] CYP2A6 may play a role in releasing the 5-FU generated from FT into the circulation for transport to the target organ.[14]

Increased expression of hepatic CYP2A6 has been reported for a subpopulation of human hepatocellular carcinomas (HCC).[15] Levels of CYP2A6 within HCC may be an important factor for the efficacy of UFT, an FT-containing anti-tumor drug that is used to treat HCC.

In the Japanese population, deletion of the whole CYP2A6 gene has been reported.[16–18] Deletion of CYP2A6 may result in decreased efficacy of FT, as intracellular CYP2A6 expression renders the cDNA-transfected cells sensitive to FT.

Our newly developed mammalian cell system of CYP2A6 expression should be useful in the evaluation of the role of CYP2A6 in the intracellular activation of anti-tumor prodrugs.

ACKNOWLEDGMENTS

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