Jpn. J. Cancer Res. **93**, 825–833, July 2002

Sensitivity of Human Cancer Cells to the New Anticancer *Ribo*-nucleoside TAS-106 Is Correlated with Expression of Uridine-cytidine Kinase 2

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TAS-106 [1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine] is a new anticancer *ribo*-nucleoside with promising antitumor activity. We have previously presented evidence suggesting that the TAS-106 sensitivity of cells is correlated with intracellular accumulation of the triphosphate of TAS-106, which may be affected both by cellular membrane transport mechanisms and uridine-cytidine kinase (UCK) activity. Since the presence of a UCK family consisting of two members, UCK1 and UCK2, has recently been reported in human cells, we investigated the relation between expression of UCK1 and UCK2 at both the mRNA and protein levels and UCK activity (TAS-106 phosphorylation activity) in a panel of 10 human cancer cell lines. Measurement of UCK activity in these cell lines revealed that it was well correlated with the cells' sensitivity to TAS-106. In addition, the mRNA or protein expression level of UCK2 was closely correlated with UCK activity in these cell lines, but neither the level of expression of UCK1 mRNA nor that of protein was correlated with enzyme activity. We therefore compared the protein expression level of UCK2 in several human tumor tissues and the corresponding normal tissues. Expression of UCK2 protein was barely detectable in 4 of the 5 human tumor tissues, but tended to be high in the pancreatic tumor tissue. It could not be detected at all in any of the normal tissues. Thus, expression of UCK2 appeared to be correlated with cellular sensitivity to TAS-106, and it may contribute to the tumor-selective cytotoxicity of TAS-106.

Key words: TAS-106 — Anticancer *ribo*-nucleoside — Uridine-cytidine kinase 1 — Uridine-cytidine kinase 2

1-(3-*C*-Ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd, TAS-106) is a new *ribo*-nucleoside analogue of cytidine that has been found to possess significant cytotoxicity and antitumor activity in preclinical therapeutic models.[1–3] As shown in Fig. 1, TAS-106 taken up by the cells is rapidly phosphorylated to the monophosphate by uridine-cytidine kinase (UCK, EC 2.7.1.48), and this product is subsequently phosphorylated to the di- and the triphosphate.[4] The triphosphate of TAS-106, ECTP, is an active metabolite and inhibits RNA synthesis by nonspecifically blocking RNA polymerases I, II, and III.[5–8] Thus, intracellular accumulation of ECTP seems critical for TAS-106 to exert cytotoxic activity. In fact, we have reported that the sensitivity of cells to TAS-106 is correlated with inhibition of cellular RNA synthesis by TAS-106 and that inhibition of cellular RNA synthesis by TAS-106 is correlated with intracellular accumulation of ECTP. We have also shown that the amount of TAS-106 transported into cells and the cells' UCK activity are decreased in TAS-106-resistant cell variants in comparison with their parental cells.[4] These results suggest that both cellular membrane transport by nucleoside transporters (NTs) and initial phosphorylation by UCK may be factors limiting the display of antitumor activity of TAS-106.

Most mammalian cells possess NTs that mediate the cellular membrane transport of physiologic purine and pyrimidine nucleosides for incorporation into cellular nucleotides and nucleic acids.[9] Nucleoside transport processes in mammalian cells have been categorized into seven types: *es* (equilibrative and nitrobenzylmercaptopurine ribonucleoside [NBMPR]-sensitive), *ei* (equilibrative and NBMPR-insensitive), *cit* (concentrative, NBMPR-insensitive and thymidine-selective), *cib* (concentrative, NBMPR-insensitive and broadly selective), *cif* (concentrative, NBMPR-insensitive and formycin B-selective), *csg* (concentrative, NBMPR-sensitive and guanosine-selective), and *cs* (concentrative and NBMPR-sensitive). The tissue and tumor distribution of NTs has not been fully defined, but since equilibrative NTs are widely distributed among mammalian cell types, while concentrative NTs are found in specialized cell types, including intestinal and

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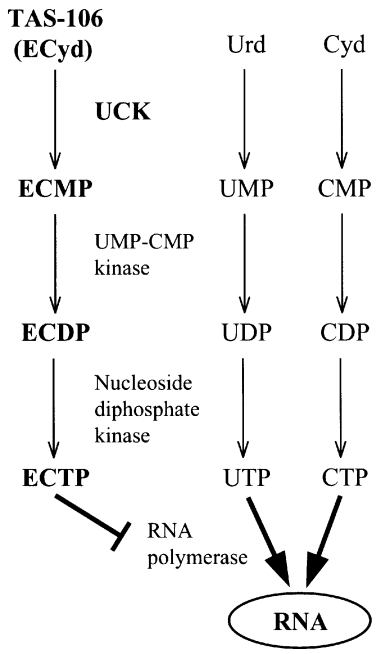

Fig. 1. Metabolic activation pathways of TAS-106 (ECyd). ECMP, ECyd 5′-monophosphate; ECDP, ECyd 5′-diphosphate; ECTP, ECyd 5′-triphosphate.

renal epithelia and the liver, the major mediators of nucleoside analogue transport in human tissues are probably equilibrative NTs. However, pyrimidine nucleoside analogues, such as 2′-deoxy-5-fluorouridine (floxuridine) and 2′-deoxy-2′,2′-difluorodeoxycytidine (gemcitabine), have been reported to be good substrates for *cit* NT in *in vitro* experiments.[10–13] Although Tsuji *et al.* reported that TAS-106 is transported by *es* NT in HT-1080 human fibrosarcoma cells,[14] little is known about the cellular membrane transport mechanism of TAS-106, and further study of TAS-106 is required.

UCK is the rate-limiting enzyme in the pyrimidine salvage pathway of all mammalian cells. It catalyzes the phosphorylation of uridine and cytidine to uridine 5′-monophosphate (UMP) and cytidine 5′-monophosphate (CMP), respectively, and is also responsible for the initial phosphorylation of TAS-106 (Fig. 1). We previously reported that UCK activity in human tumor xenografts is higher than in various normal tissues of nude rats[7] and demonstrated that the excellent antitumor activity of TAS-106 observed in nude rat models bearing human tumors reflects the tumor-selective distribution of TAS-106.[3] We therefore suspected that the antitumor activity and pharmacodynamic features of TAS-106 may be attributable to a higher level of phosphorylation of TAS-106 by UCK in tumor tissue than in normal tissue. Since UCK activity in human tumor tissues is well known to be relatively high compared to its activity in normal tissues,[15–20] tumor-selective cytotoxicity of TAS-106 could therefore be expected clinically. To investigate UCK status as a limiting factor for TAS-106 sensitivity in greater detail, we previously isolated a cDNA for UCK from human cancer cells, and sequenced and characterized it.[21] The isolated cDNA for human UCK was found to contain an open reading frame of 786 bp, and translation of the open reading frame yielded a protein sequence of 261 amino acids. Nucleotide and amino acid sequences of two isoforms of human UCK, UCK1 (GenBank No. AF237290, AAK28324) and UCK2 (GenBank No. AF236637, AAK14053), were recently submitted to GenBank and subsequently reported by Van Rompay *et al.*[22] Based on the sequence data reported, the UCK we had isolated and cloned appeared to be UCK2. As reported previously, it seems obvious that the cells' sensitivity to TAS-106 is affected by UCK activity,[4, 5] but the relations between UCK activity and levels of expression of UCK1 and UCK2 have never been defined. Accordingly, in the present study we investigated the relation between expression of UCK1 and UCK2 at both the mRNA and protein levels, and the UCK activity (TAS-106 phosphorylation activity) in a panel of 10 human cancer cell lines, including TAS-106-sensitive cell lines and less sensitive cell lines.

MATERIALS AND METHODS

Chemicals TAS-106 was synthesized by Taiho Pharmaceutical Co., Ltd. (Tokyo). [Cytosine-5-^{3}H]TAS-106 (5.4 Ci/mmol) was synthesized by Amersham International plc (Buckinghamshire, UK). [5-^{3}H]Uridine (Urd; 11 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). All other chemicals were of analytical grade, purchased from commercial sources.

Cell lines and cell culture The HT-1080 human fibrosarcoma cell line and its 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)uracil (EUrd)[1, 23]-resistant variant, HT-1080/EUrd,[5] were generous gifts from Professor Takuma Sasaki of the Cancer Research Institute, Kanazawa University (Kanazawa). The NCI-H630 human colorectal carcinoma cell line was kindly provided by Dr. Keisuke Aiba of the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo). The AZ-521 human gastric cancer cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo). The DLD-1 human colorectal, MCF7 human breast, and BxPC-3 human pancreatic adenocarcinoma cell lines were from the American Type Culture Collection (Manassas, VA) and were purchased through Dainippon Pharmaceutical Co., Ltd. (Osaka). A TAS-106-resistant variant of HT-1080, HT-1080/ECyd, and two variants of DLD-1, TAS-106-resistant DLD-1/ECyd and EUrd-resistant DLD-1/EUrd, were

established in our laboratory.[4] AZ-521 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS; ICN Biomedicals, Inc., Aurora, OH) at 37°C and 5% CO_2. MCF7 cells were cultured in Eagle's minimum essential medium supplemented with non-essential amino acids, 1 m*M* sodium pyruvate, and 10% FCS. HT-1080, HT-1080/ECyd, HT-1080/EUrd, DLD-1, DLD-1/ECyd, NCI-H630, and BxPC-3 cells were cultured in RPMI 1640 medium supplemented with 10% FCS. DLD-1/EUrd cells were maintained *in vitro* in RPMI 1640 medium supplemented with 10% FCS and 1 μM EUrd, however, all further experiments were performed using the cells grown in drug-free medium for 7 days.

Sensitivity of cells to TAS-106 The growth-inhibitory effect of TAS-106 on human cancer cells was determined by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma Chemical Co., St. Louis, MO),[24] as previously described.[4] Cells in the exponential growth phase were seeded onto 96-well plates at a density of 500–4000 cells/well 24 h prior to drug exposure. The cells were then exposed to various concentrations of TAS-106 (at least 3 wells at each concentration) for up to 72 h. After exposure to the drug, MTT reagent was added to each well and the plates were incubated at 37°C for 4 h. The medium was carefully removed, and DMSO was added to each well to dissolve the formazan that had formed. The absorbance of the reaction mixture was measured at 540 nm, and IC_{50} was determined as the drug concentration required to reduce the absorbance to 50% as compared to the control. Two or three individual experiments were conducted on each cell line to confirm reproducibility.

Preparation of cell cytosol fractions Test cancer cells were collected during logarithmic growth, and cell pellets were stored frozen at −135°C. Immediately before use, the pellets were thawed, supplemented with two volumes of 10 m*M* potassium phosphate buffer (pH 7.5) corresponding to their wet weights, and then sonicated. The homogenates were centrifuged at 105 000*g* for 1 h at 4°C, and the supernatants (cytosol fractions) were collected. The cytosol fractions were divided into small portions and stored frozen at −80°C until use for enzyme assay or western blot analysis. The protein concentration in these cell cytosols was measured by the Bradford method[25] using a solution of Protein Assay Dye (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin serving as the reference protein.

Human tissue proteins Human breast, lung, pancreas, colon, and rectum tumor cytoplasmic proteins and corresponding normal tissue cytoplasmic proteins, except for normal rectum tissue (5 or 10 mg protein/ml each), were obtained from Biochain Institute, Inc. (Hayward, CA).

UCK activity UCK activity in human cancer cells was measured with Urd or TAS-106 as the substrate according to the method of Ikenaka *et al.*,[26] with a slight modification, as previously described.[4] The volume of each enzyme solution (cell cytosol) was 50 μl, and the total volume of the reaction mixture was 125 μl. In addition to the enzyme solution, the reaction mixture contained 50 m*M* Tris-HCl buffer (pH 8.0), 10 m*M* ATP (pH 7.5), 5 m*M* $MgCl_2$, 10 m*M* NaF, and 0.6 m*M* (0.25 μCi/tube) [^{3}H]Urd or [^{3}H]TAS-106. The reaction mixture was incubated at 37°C, and the reaction was stopped by heating the solution on a heating block at 105°C for 3 min. The sample was then centrifuged at 3000 rpm for 10 min at 4°C, and 10 μl of the supernatant was spotted onto a polyethyleneimine-cellulose F thin-layer chromatography plate (2.5×10 cm, Merck KGaA, Darmstadt, Germany) and developed with water. The phosphorylated Urd or the phosphorylated TAS-106 remaining at the point of origin was harvested into a vial and combined with 0.5 ml of 1 *M* HCl. The mixture was then combined with 10 ml of liquid scintillator ACS-II (Amersham) to measure the radioactivity with a Wallac 1414 WinSpectral liquid scintillation counter (Wallac Berthold Japan Co., Ltd., Tokyo). All measurements were performed in duplicate. The reaction rates were linear with respect to time and enzyme concentration under the conditions used. Two or three individual experiments were conducted on each cell line to confirm reproducibility.

Real-time PCR The UCK1 and UCK2 mRNA levels of human cancer cells were quantified by the real-time PCR method with an ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA). Total RNA was extracted from approximately 1×10^7 cells of each cell line with an RNeasy RNA Purification Kit (QIAGEN GmbH, Hilden, Germany), and first-strand cDNAs were synthesized from 5 μg of total RNAs with a Superscript Preamplification System for First Strand cDNA Synthesis (GIBCO BRL, Gaithersburg, MD). The PCR solution (25 μl) was composed of 10 μl of cDNA solution, 2.5 μl of primer/probe mixture (containing 5 pmol of each of the forward and reverse primers, 2.5 pmol of internal probe), and 12.5 μl of TaqMan Universal PCR Master Mix (PE Applied Biosystems). The internal probes were labeled with a reporter dye, 6-carboxyfluorescein (FAM), at the 5′-end, and a quencher dye, 6-carboxytetramethylrhodamine (TAMRA), at the 3′-end. The primer and internal probe sequences for UCK1 and UCK2 are shown in Table I. The PCR reaction conditions were 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the PCR products of UCK1 and UCK2 were generated by using serial dilutions of RNA in the assay, and the data were analyzed according to the manufacturer's instructions. The PCR assay was performed in duplicate, and the mean value was used for cal-

Table I. Primers and Probes

UCK1	forward primer	5′-GCCGACAAAGAAGTATGCCG-3′
	reverse primer	5′-GCCATTTGCAGATGTCACCA-3′
	internal probe	5′-FAM-TGCCATCAACCTGATCGTGCAGC-TAMRA-3′
UCK2	forward primer	5′-GTGATCATCCCTAGAGGTGCAGATA-3′
	reverse primer	5′-GGCCCTCCATTCAGGATGT-3′
	internal probe	5′-FAM-TCTGGTGGCCATCAACCTCATCGTG-TAMRA-3′

Table II. Sensitivity to TAS-106 and UCK Activity of a Panel of Human Cancer Cell Lines

Cell line	Sensitivity to TAS-106 (IC_{50}, μM)[a]	UCK activity (nmol/min/mg protein)		
		Substrate[b]		Ratio[c]
		Urd	TAS-106	
AZ-521	0.017	19.087	10.074	1.89
HT-1080	0.027	12.269	4.624	2.65
DLD-1	0.058	10.147	4.777	2.12
BxPC-3	0.081	1.454	1.538	0.95
NCI-H630	0.150	8.091	3.836	2.11
MCF7	0.532	3.720	2.064	1.80
DLD-1/EUrd	2.387	2.043	0.882	2.32
DLD-1/ECyd	6.088	1.038	0.466	2.23
HT-1080/ECyd	8.299	4.011	1.536	2.61
HT-1080/EUrd	29.76	0.269	0.240	1.12

Cell lines are listed in descending order of sensitivity to TAS-106. IC_{50} values and enzyme activities are means of data from three individual experiments or from single representative experiments.
a) The IC_{50} values, defined as the concentration of TAS-106 causing 50% growth inhibition, at 72 h exposure, were determined by MTT assay.
b) UCK activity was measured by using Urd or TAS-106 as the substrate.
c) Urd phosphorylation activity:TAS-106 phosphorylation activity rates.

culations. The amounts of the PCR products of UCK1 and UCK2 were normalized with respect to the GAPDH level. The experiments were repeated three times, and the average of the data obtained from the three measurements is reported as the final value.

Antibodies UCK1 and UCK2 polyclonal antibodies were developed by using synthetic peptides corresponding to C-terminal amino acids 259 to 277 (PGMLTSGKRSH-LESSSRPH) of the human UCK1 protein (GenBank No. AAK28324) and 243 to 261 (NGYTPSRKRQASESSS-RPH) of the human UCK2 protein,[21] and these peptides (+cysteine) were coupled to keyhole limpet hemocyanin (KLH) to prepare the immunogens. Each immunogen was injected into two pathogen-free rabbits (8 injections per rabbit), and anti-UCK1 and anti-UCK2 sera were used as primary antibodies for western blotting.

Western blotting Samples containing 50 μg protein (human cancer cell cytosols or human tissue cytoplasmic proteins) were subjected to SDS-polyacrylamide gel electrophoresis on a 10–20% gradient gel (Daiichi Pure Chemicals Co., Ltd., Tokyo), and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Atto Co., Tokyo). The membranes were then immunoblotted with anti-UCK1 or anti-UCK2 antibody, and the immune complexes were visualized with a Western-Star Immunodetection System (Tropix, Inc., Foster City, CA) and quantified with a LAS-1000 Lumino image analyzer (Fuji Film, Tokyo).

RESULTS

TAS-106 sensitivity of human cancer cells Chemosensitivity to TAS-106 was determined in a panel of 10 human cancer cell lines, and their IC_{50} values, defined as the concentration of TAS-106 causing 50% growth inhibition, at the end of 72 h of exposure are shown in Table II. The cell lines are listed in descending order of sensitivity to TAS-106. The IC_{50} value of the most resistant cell line, HT-

1080/EUrd (29.76 μM), was 1750-fold higher than that of the most sensitive cell line, AZ-521 (0.017 μM).

UCK activity of human cancer cells The UCK activity of the panel of 10 human cancer cell lines was measured by using Urd or TAS-106 as the substrate, and the enzyme activities are shown beside the IC_{50} values in Table II. With the exception of lines BxPC-3 and HT-1080/ECyd, the order of Urd phosphorylation activity (measured with Urd as the substrate) of the cell lines paralleled their sensitivity to TAS-106. The ratio of Urd phosphorylation activity to TAS-106 phosphorylation activity (measured with TAS-106 as the substrate) of each cell line was approximately 2, except for BxPC-3 and HT-1080/EUrd. The TAS-106 phosphorylation activity of these 10 human cancer cell lines ranged from 0.240 (HT-1080/EUrd) to 10.074 (AZ-521) nmol/min/mg protein, with a 42-fold difference between the highest and lowest values. While the difference in their TAS-106 phosphorylation activity range was much smaller than in their TAS-106 sensitivity range, the sensitivity of these cell lines to TAS-106 appeared to be well correlated with their TAS-106 phosphorylation activity (Fig. 2).

Levels of expression of UCK1 and UCK2 mRNA in human cancer cells We examined the levels of expression of UCK1 and UCK2 mRNA in the panel of 10 human cancer cell lines by real-time PCR using primers specific for the transcript. The cell lines tested are listed from left to right in Fig. 3 in descending order of sensitivity to TAS-106. The level of expression of UCK2 mRNA was almost twice as high as that of UCK1 mRNA in 5 of the 10 human cancer cell lines, while expression of UCK1 mRNA was fairly high in only 2 of the 10 cell lines and relatively low in the others. The level of expression of UCK1 mRNA did not appear to correlate with the sensitivity of these 10 human cancer cell lines to TAS-106. The level of expression of UCK2 mRNA, on the other hand, tended to correlate with their TAS-106 sensitivity, although there was only approximately a 6-fold difference between the cell line with the highest expression, AZ-521 (UCK2/GAPDH=0.0426), and that with the lowest expression, HT-1080/ECyd (UCK2/GAPDH=0.0071). The level of expression of UCK2 mRNA by HT-1080/EUrd (UCK2/GAPDH=0.0115), which had the strongest resistance to TAS-106 and the lowest UCK activity in this study, was almost the same as that of HT-1080/ECyd.

Levels of expression of UCK1 and UCK2 protein in human cancer cells We performed western blot analysis with anti-UCK1 and anti-UCK2 polyclonal antibodies to detect UCK1 and UCK2 protein expression. As shown in Fig. 4, although anti-UCK1 antibody seemed to weakly recognize UCK2 protein in addition to UCK1 protein, anti-UCK2 antibody appeared to selectively recognize UCK2 protein. The levels of expression of UCK1 and UCK2 protein in the panel of 10 human cancer cell lines were quantitated by western blotting using these anti-UCK1 and anti-UCK2 antibodies, respectively, and the

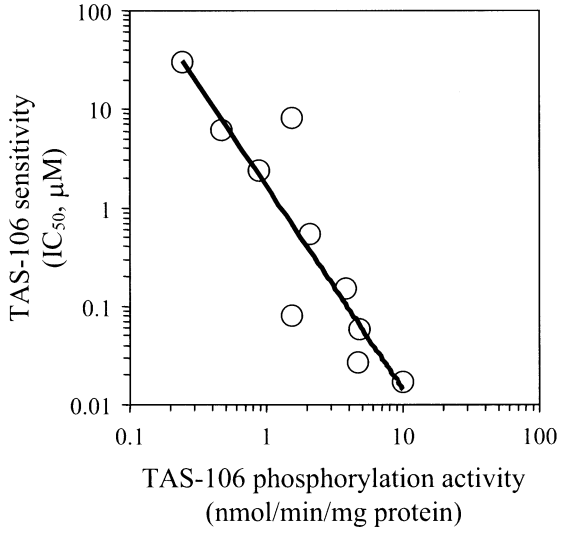

Fig. 2. Relation between the TAS-106 sensitivity and TAS-106 phosphorylation activity of a panel of 10 human cancer cell lines. The plots show the correlations between IC_{50}, defined as the concentration of TAS-106 causing 50% growth inhibition, at the end of 72 h of exposure, and UCK activity measured with TAS-106 as the substrate. $y=1.6553x^{-2.0655}$, $r=0.8983$.

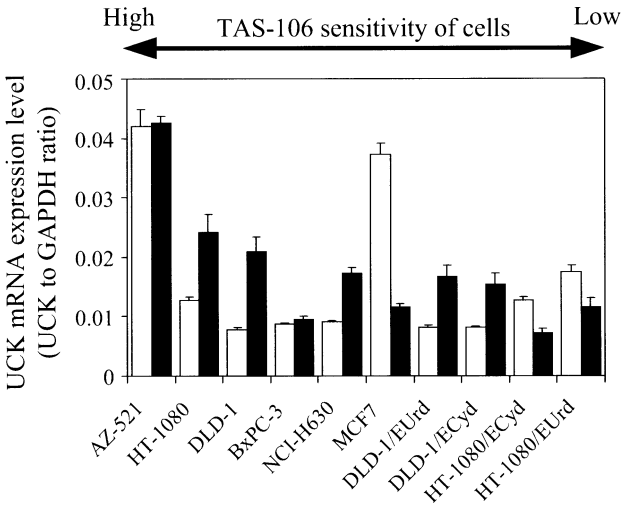

Fig. 3. Levels of expression of UCK mRNA in a panel of 10 human cancer cell lines. Cell lines are listed in descending order of sensitivity to TAS-106 from left to right. The amounts of UCK PCR products were normalized with respect to the GAPDH levels. UCK1 (□) and UCK2 (■). The columns represent the means of data from three individual experiments; bars represent the SD.

chemiluminescence signal intensity values are shown in Fig. 5. While the expression levels of UCK1 protein in these cell lines appeared to be almost the same (Fig. 5A), expression of UCK2 protein tended to correlate with the TAS-106 sensitivity (Fig. 5B). The volume of the UCK2 protein band of the cell line with the highest expression, DLD-1 (band volume=21.8×10^4), was 16-fold higher than that of the cell line with the lowest expression, HT-1080/EUrd (band volume=1.36×10^4). The UCK2 protein expression level of AZ-521 (band volume=20.6×10^4), which was the most sensitive cell line and had the highest UCK activity in this study, was almost the same as that of DLD-1.

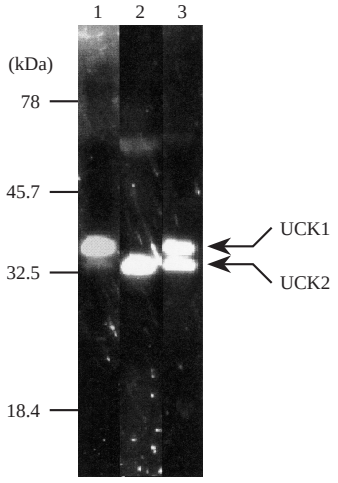

Fig. 4. Detection of UCK1 and UCK2 protein expression by western blotting. Equal amounts of cytosols from AZ-521 cells (containing 50 μg protein) were subjected to analysis, and band volume (chemiluminescence signal intensity) was quantified with a Lumino image analyzer, as described in "Materials and Methods." The antibodies used for immunodetection were anti-UCK1 serum (lane 1), anti-UCK2 serum (lane 2), and a mixture of anti-UCK1 and anti-UCK2 sera (lane 3).

Relation between the UCK expression level and UCK activity of human cancer cells The relation between the UCK expression level and UCK activity of the panel of 10 human cancer cell lines is shown in Fig. 6. Fig. 6A shows the relation between the level of expression of UCK2 mRNA and TAS-106 phosphorylation activity, and Fig. 6B shows the relation between the levels of expression of UCK2 protein and TAS-106 phosphorylation activity. These results indicate that both the levels of expression of UCK2 mRNA and protein of human cancer cells were closely correlated with the TAS-106 phosphorylation activity (UCK activity).

Levels of expression of UCK1 and UCK2 protein in human tissues The levels of expression of UCK1 and UCK2 protein were compared by western blotting in 5 human tumor tissues and corresponding normal tissues (except rectum). Expression of UCK1 protein was undetectable in both human tumor and normal tissues (data not shown). In contrast, while expression of UCK2 protein could not be detected in human normal tissues, it was detected in 4 of the 5 human tumor tissues (Fig. 7), and was particularly high in the pancreatic tumor tissue.

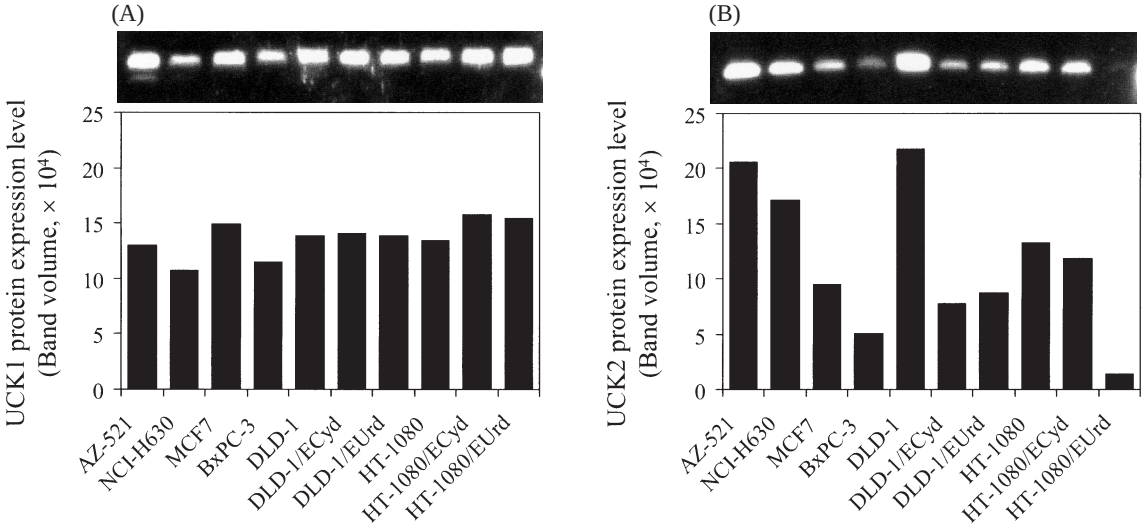

Fig. 5. Levels of expression of UCK1 (A) and UCK2 (B) protein in a panel of 10 human cancer cell lines. Equal amounts of cytosols (containing 50 μg protein) were subjected to analysis, and band volume was quantified with a Lumino image analyzer.

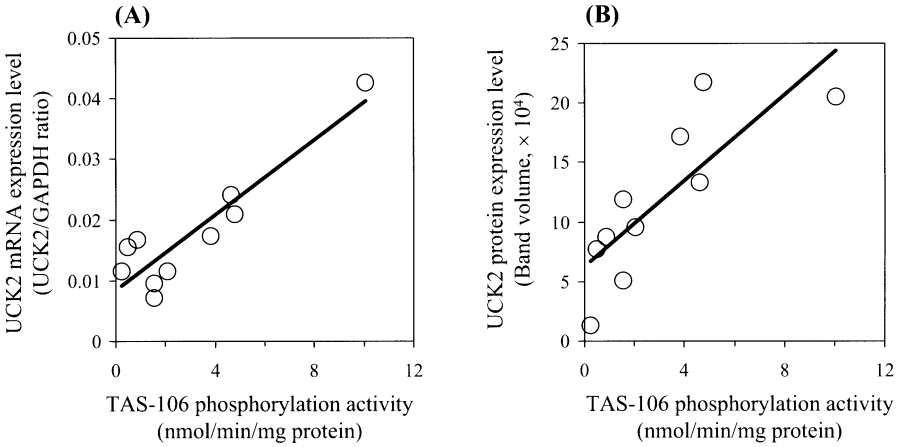

Fig. 6. Relation between the UCK2 expression level and TAS-106 phosphorylation activity of a panel of 10 human cancer cell lines. (A) Correlation between the UCK2 mRNA expression level and TAS-106 phosphorylation activity. $y=0.0031x+0.0083$, $r=0.9144$. (B) Correlation between the UCK2 protein expression level and TAS-106 phosphorylation activity. $y=1.7947x+6.3138$, $r=0.8113$.

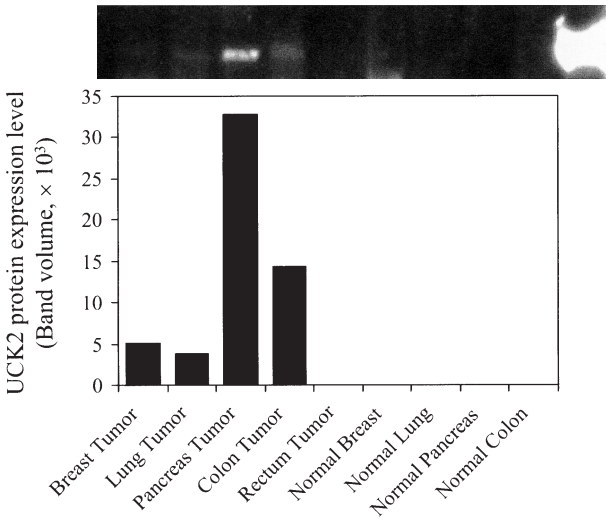

Fig. 7. Levels of expression of UCK2 protein in human tumor and normal tissues. Equal amounts of tissue cytoplasmic proteins (containing 50 μg protein) were subjected to analysis, and band volume was quantified with a Lumino image analyzer. AZ-521 cell cytosol (containing 50 μg protein) was loaded on the far right lane as a positive control.

DISCUSSION

UCK has been considered to be an important target for chemotherapy ever since it was shown to be required for the intracellular activation of certain pyrimidine nucleoside analogues to cytotoxic nucleotides. The first step in the phosphorylation of TAS-106 is catalyzed by UCK and is thought to be rate-limiting, and the level of UCK activity affects the sensitivity of cells to TAS-106.[4, 5] More recently, a UCK family consisting of at least two members, UCK1 and UCK2, has been found to be present in human cells.[22] We therefore decided to define the relationship between cellular sensitivity to TAS-106, UCK activity, and expression of UCK1 and UCK2 at both mRNA and protein levels in human cancer cells. In preliminary experiments, incubation with our anti-UCK1 or anti-UCK2 antiserum failed to neutralize the respective enzyme activity in HT-1080 cell cytosols (data not shown), and thus the UCK activity in this study represents the total activity of UCK1 and UCK2. When we used Urd or TAS-106 as the substrate to measure UCK activity in a panel of 10 human cancer cell lines, the ratio of Urd phosphorylation activity to TAS-106 phosphorylation activity was found to be almost constant. These results suggested that the TAS-106 phosphorylation efficiency of UCK1 and UCK2 tends to be similar to their efficiency in regard to Urd. Since Urd has been reported to be an approximately 20-fold more efficient substrate for UCK2 than for UCK1,[22] TAS-106 may also be a more efficient substrate for UCK2 than for UCK1. Needless to say, the kinetic properties of both UCK1 and UCK2 for TAS-106 phosphorylation must be investigated to corroborate this. The UCK1 and UCK2 protein content of human cells is also still unknown. We therefore do not know how much UCK1 or UCK2 participates in the TAS-106 catalytic activity of UCK in human cells. The UCK activity of human cancer cell lines tested, however, was clearly well correlated with the sensitivity to TAS-106, except for 2 cell lines, BxPC-3 and HT-1080/ECyd, whose sensitivity is probably dependent on other factors, such as cellular membrane transport.

The level of expression of UCK1 mRNA was generally low in 8 of the 10 human cancer cell lines, with only 2 cell lines, AZ-521 and MCF7, expressing relatively high levels of UCK1 mRNA. AZ-521 was the most sensitive cell line to TAS-106 and had the highest UCK activity in this study, while MCF7 had moderate levels of both TAS-106 sensitivity and UCK activity. The level of expression of UCK1 protein was almost the same in the 10 human cancer cell lines. As might be expected, the level of expression of UCK1 protein was not correlated with the mRNA expression level in the human cancer cell lines tested, and neither protein nor mRNA expression level was correlated with the UCK activity. On the other hand, the level of expression of UCK2 protein roughly correlated with the mRNA expression level in the human cancer cell lines tested, and both protein and mRNA expression levels were closely correlated with the UCK activity. The difference in UCK2 expression between the highest and lowest cell lines was 6-fold at the mRNA level and 16-fold at the protein level, and thus was not very large. However, the cell lines with high expression of UCK2 at both the mRNA and protein levels, i.e., AZ-521, DLD-1, HT-1080, and NCI-H630, had clearly higher UCK activity and TAS-106 sensitivity than did the other cell lines.

Measurement of the UCK1 and UCK2 protein levels in several human tumor and normal tissue cytoplasmic proteins by western blotting revealed much lower levels of expression than in human cancer cell cytosols. We could not detect UCK1 expression in either the human tumor or the normal tissue proteins tested, and could not detect UCK2 expression among human normal tissue proteins. However, UCK2 expression was detected in 4 of the 5 human tumor tissue proteins. The UCK activity (Urd phosphorylation activity) of the 10 human cancer cell lines ranged from 0.269 to 19.087 nmol/min/mg protein. The UCK activity levels reported in human tumor and normal tissues range from 0.031 to 0.86 nmol/min/mg protein and 0.006 to 0.37 nmol/min/mg protein, respectively.[15–20] The lower levels of expression of UCK protein in the human tissues compared with the human cancer cell lines seemed to correspond to the enzyme activity of UCK. UCK activity in human tumor tissues, however, has been reported to be several-fold higher than in human normal tissues. Thus, the UCK2 protein expression may have been responsible for the higher UCK activity in the human tumor tissues.

The results of this study indicated that UCK2 expression at both the mRNA and protein levels is correlated with the UCK activity and sensitivity to TAS-106 of human cancer cells, and the higher UCK2 expression in human tumor tissues compared with normal tissues may contribute to the tumor-selective cytotoxicity of TAS-106. Since the UCK2 protein expression was relatively high in the pancreatic tumor tissue compared with the other tumor tissues tested, TAS-106 shows promise as a therapeutic agent for pancreatic cancer. Although we could not detect UCK1 and UCK2 protein in any of the human normal tissues tested, Van Rompay *et al.* reported ubiquitous UCK1 mRNA expression in the normal human tissues they investigated, but noted that UCK2 mRNA was only detected in the placenta.[22] The status of UCK appears to be closely related to the antitumor activity of TAS-106, but the physiological roles, tissue-specific expression, and subcellular localization of UCK1 and UCK2 are still unclear. Characterization of UCK1 and UCK2 in greater detail will be important for further development of TAS-106.

(Received February 8, 2002/Revised April 10, 2002/Accepted April 15, 2002)

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