Deletion Mutants of Human Deoxycytidine Kinase mRNA in Cells Resistant to Antitumor Cytosine Nucleosides

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We studied mutational events in deoxycytidine (dCyd) kinase mRNA expression, focusing on aberrant dCyd kinase mRNA, which has been frequently observed in established cell lines resistant to antitumor dCyd nucleoside analogues such as 1-β-D-arabinofuranosyl cytosine (Ara-C), gemcitabine (dFdC) and 2′-C-cyano-2′-deoxy-1-β-D-arabinofuranosylcytosine (CNDAC). We describe here the expression of aberrant dCyd kinase mRNAs identified as splicing mutants. These mutants included deletions of the fifth exon in CNDAC-resistant cells (originating from HT-1080 cells), of the third exon in Ara-C-resistant cells (originating from SK-MEL-28 cells) and of the fourth exon in 2′-deoxy-2′-methylidenecytidine (DMDC)-resistant cells (originating from SK-MEL-28 cells). Various nucleoside-resistant cells originating from the same parental HT-1080 cells were established. The resulting cells expressed the same mRNA with deletion of the fifth exon, and the location of splicing was independent of the type of nucleosides used for the establishment of resistant cells. The deletion of the fifth exon in dCyd kinase seems to be a target for acquisition of resistance to antitumor cytosine nucleosides. However, distinct mutations in the *dCyd kinase* gene seem to be associated with acquisition of resistance to different antitumor cytosine nucleosides.

Key words: 2′-C-Cyano-2′-deoxy-1-β-D-arabinofuranosylcytosine — Acquisition of resistance — Deoxycytidine kinase — Mutation

2′-Deoxy-2′-methylidenecytidine (DMDC) and 2′-C-cyano-2′-deoxy-1-β-D-arabinofuranosylcytosine (CNDAC) are novel antitumor cytosine nucleosides with unique mechanisms of action similar in part to those of gemcitabine (dFdC), which has been shown to be an effective therapy against solid tumors. These antimetabolites are incorporated into the cells and phosphorylated by enzymes including deoxycytidine (dCyd) kinase (EC 2.7.1.74). The tri-phosphates of these nucleosides inhibit DNA polymerase, and tri-phosphorylated CNDAC incorporated into DNA induces a DNA self-strand-break.[1, 2] These compounds are reported to be highly effective against various types of solid tumors in animals,[3–6] although their parent compound, 1-β-D-arabinofuranosylcytosine (Ara-C), is mainly effective against leukemia. We have previously observed that deficiency of dCyd kinase activity is the main mechanism of CNDAC resistance and that dCyd kinase plays a key role in the acquisition of resistance.[7]

The CNDAC-resistant cell line CN20 originated from human fibrosarcoma cell line HT-1080. These cells express aberrant dCyd kinase mRNA which has a deletion of the fifth exon. dCyd kinase activity is markedly decreased in these cells. This fifth exon deletion has been reported in Ara-C-resistant human T-lymphoblast cells[8] and dFdC-resistant human ovarian carcinoma cells.[9] Point mutations have been reported in Ara-C-resistant cells[8] and dFdC-resistant cells.[10] In the clinic, the *dCyd kinase* gene in some patients with acute myeloid leukemia (AML) has been reported to have point mutations and exon deletion.[11, 12] In most cases, dCyd kinase activity is significantly decreased as a result of *dCyd kinase* gene mutation. In this paper, we report dCyd kinase mRNA with novel mutations expressed in cells resistant to antitumor cytosine nucleosides. Various mutations in dCyd kinase were associated with acquisition of resistance to antitumor cytosine nucleosides. Therefore, we studied mutational events in dCyd mRNA expression, focusing on aberrant dCyd kinase mRNA which has been frequently observed in established cell lines resistant to antitumor dCyd nucleoside analogues such as Ara-C, dFdC and CNDAC. These data provide additional information on the highly complex mechanism of the acquisition of resistance to antitumor cytosine nucleosides.

MATERIALS AND METHODS

Drugs and tumor cells CNDAC and DMDC were synthesized as described earlier.[13, 14] Ara-C was purchased from Yamasa Co. (Chiba). [5-³H]dCyd (26.9 Ci/mmol)

was purchased from NER Life Science Products (Boston, MA).

Human fibrosarcoma HT-1080 cells were obtained from the American Type Culture Collection (Rockville, MD). CNDAC-resistant cells (CN20) originating from HT-1080 cells were established as reported previously.[7] CNDAC-, DMDC- and Ara-C-resistant cells originating from HT-1080 cells were newly established. Human melanoma SK-MEL-28 cells and DMDC-resistant cell line SK-MEL-28 (DC-10) were kindly provided by Dr. Yamagami (Yoshitomi Pharmaceutical Co., Osaka). 28-A, 28-C and 28-D cells (Ara-C-, CNDAC- and DMDC-resistant cells, respectively) were newly established from SK-MEL-28 cells.

Cytotoxicity assay Cytotoxicity was examined *in vitro* using a modified tetrazolium-based semi-automatic colorimetric assay (MTT assay) as reported previously.[15] The percentage of cell growth inhibition was calculated by applying the following formula: % of cell growth inhibition = $(1 - T/C) \times 100$, where C is the mean A_{540} of the control group and T is that of the treated group. The 50%-inhibitory drug concentration (IC_{50} value) was measured graphically from the dose-response curve with at least three drug concentration points.

dCyd kinase activity The dCyd kinase enzyme assay was performed according to our previous report.[7] Briefly, [5-^{3}H]dCyd (0.5 μCi) and 2 mg protein/ml of crude cell extract was incubated for 30 min at 37°C. Mouse dCyd kinase used as a positive control was purified according to our previous report.[16] The reaction solution was applied to

a flexible PEI Cellulose plate (Merck, Darmstadt, Germany) and separated using distilled water as the first developing solvent and 1 M acetic acid/1 M lithium chloride (1:1) as the second developing solvent. Each spot (dCyd and each phosphate form) was cut out and measured for radioactivity.

RT-PCR analysis for dCyd kinase mRNA RT-PCR analysis was performed as described in our previous report.[7] Total RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo) according to the manufacturer's protocol. The RT-PCR product was amplified using specific primers of human dCyd kinase (5′-TGTCT-CAGTCGAATAAGAGCT-3′ for the sense primer and 5′-CATCCAGTGTTAAGATAGGCACCTCTTGAAGATAA-TCGAA-3′ for the antisense primer).[17] The expected size of the normal amplified fragment is 579 bp. The RT-PCR products were electrophoresed in TAE buffer [40 mM Tris-HCl (pH 8.0), 20 mM acetic acid and 2 mM EDTA] on a 1% agarose gel.

Restrictive digestion analysis and sequencing The segment including the full-length coding region of the dCyd kinase cDNA was amplified and the PCR products were purified using a QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany). Primers used were 5′-ACAAGAC-TAAGGAATGGCCA-3′ for the sense primer and 5′-AGATCACAAAGTACTCAAAAACT-3′ for the antisense primer (fragment size, 799 bp). Purified PCR fragments were digested using *Sac*I, *Eco*T22I or *Hin*dIII in universal buffer for 2 h at 37°C. The digested products

Table I. Comparison of IC_{50} Values and dCyd Kinase Activities among the Cell Lines Resistant to Various Antitumor Cytosine Nucleosides

Cell line	Selection nucleoside[a]	IC_{50} (μg/ml)			dCyd kinase activity (%)[b]
		Ara-C	DMDC	CNDAC	
HT-1080		0.25	0.95	1.3	79
CN20	CNDAC	>1000	980	>1000	9
HT-1080/Ara-C	Ara-C	>1000	>1000	>1000	ND[c]
HT-1080/DMDC	DMDC	>1000	>1000	>1000	ND
HT-1080/CNDAC	CNDAC	>1000	>1000	>1000	ND
SK-MEL-28		1.1	0.89	2.7	80
DC-10	DMDC	>1000	>1000	>1000	19
28-A	Ara-C	>1000	285	>1000	14
28-C	CNDAC	170	65	830	ND
28-D	DMDC	710	380	>1000	ND

In vitro chemosensitivity was evaluated by the MTT assay. In the MTT assay, the cells were incubated for 72 h in medium containing various concentrations of each nucleoside.
a) Each resistant cell line was established by using Ara-C, DMDC or CNDAC.
b) dCyd kinase activity was evaluated by using [5-^{3}H]dCyd, as described in "Materials and Methods," and is shown as the percentage of the nucleotide produced. Under these conditions, 75% of [5-^{3}H]dCyd as a positive control was converted to its 5′-mono-phosphate form by mouse dCyd kinase.
c) ND, not done.

were electrophoresed on a 2.0% agarose gel. To sequence *dCyd kinase* gene, the purified PCR fragments were sequenced by the cycle-sequencing method using Gene Rapid (Amersham Pharmacia Biotech, Uppsala, Sweden) or TaKaRa Cycle Sequencing Kit (TaKaRa Shuzo Co., Kyoto).

RESULTS

Sensitivity to antitumor cytosine nucleosides   The IC_{50} values of the resistant cells were compared with those of the corresponding parental cells. Table I shows the IC_{50} values in the parental and derived resistant cells. The IC_{50} values of all resistant cells were over 300 μg/ml against each selection nucleoside and the cells showed cross-resistance against all of the antitumor cytosine nucleosides tested. We newly established three resistant cell lines originating from the same parental HT-1080 cells against Ara-C, DMDC or CNDAC. These resistant cells showed very low sensitivity (IC_{50} value of over 1000 μg/ml) and high cross-resistance to the other nucleosides tested.

dCyd kinase activity   [5-^{3}H]dCyd was incubated with crude extract from the parental or the resistant cells for 30 min. In the parental cells (HT-1080 and SK-MEL-28 cells), 80% of [5-^{3}H]dCyd was phosphorylated to its 5′-mono-, 5′-di- and 5′-tri-phosphate forms. However, all extracts from the resistant cells phosphorylated less than 20% of [5-^{3}H]dCyd. Thus, the main resistance mechanism in these resistant cells was a marked decrease of dCyd kinase activity (Table I).

Analysis of dCyd kinase fragment in resistant cells   dCyd kinase mRNA expressed in the resistant cells was
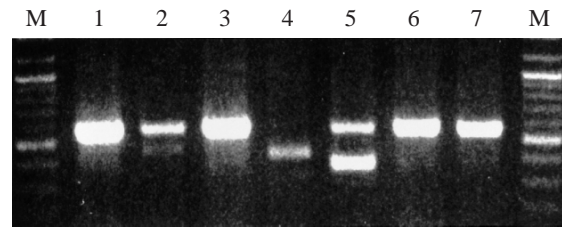

Fig. 1.   Total RNAs from cells of various cell lines resistant to antitumor cytosine nucleosides were analyzed by RT-PCR for *dCyd kinase* gene. The amplified products were electrophoresed on a 1.0% agarose gel. Lane 1, HT-1080 cells; lane 2, CN20 cells; lane 3, SK-MEL-28 cells; lane 4, DC-10 cells; lane 5, 28-A cells; lane 6, 28-C cells; lane 7, 28-D cells, lane M, DNA size marker (100 bp DNA ladder).

compared with mRNA expressed in the parental cells by RT-PCR analysis (Fig. 1). Two bands of long-size RT-PCR products (L fragment) with the expected fragment size (579 bp) and a shorter size (S fragment) were detected in CN20 cells. The products from 28-A cells were also separated into L and S fragments. In contrast, only a single band of the S fragment was observed in DC-10 cells.

To analyze the S fragments from dCyd kinase mRNA, the segment including the full-length coding region of the dCyd kinase cDNA was amplified and S fragments were purified by agarose gel electrophoresis. The S fragments were digested with restriction enzymes [*Hin*dIII (restriction site at the fifth exon), *Eco*T22I (restriction site at the fourth exon) or *Sac*I (restriction site at the third exon)], and the digested products were analyzed on 2% agarose gel (Fig. 2). The L fragments from HT-1080 cells were
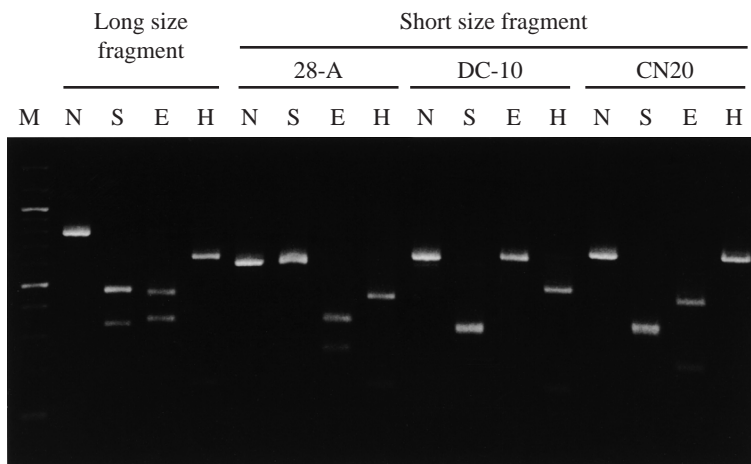

Fig. 2.   The purified S fragments of dCyd kinase were digested using *Sac*I, *Eco*T22I or *Hin*dIII in universal buffer for 2 h at 37°C. The digested products were electrophoresed on a 2.0% agarose gel. The normal fragment was prepared from HT-1080 cells. Lane M, 100 bp ladder; lane N, not treated; lane S, *Sac*I digestion; lane E, *Eco*T22I digestion; lane H, *Hin*dIII digestion.

Table II. dCyd Kinase mRNA Mutations in Cell Lines Resistant to Antitumor Cytosine Nucleosides

Cell line	Fragment size[a]	cDNA sequence change[b]		Amino acid sequence change[b]
CN20	L	797–799, AAA to AA one base Del	(within exon 5)	213, FS (221, stop)
	S	709–824, 116 bp Del	(exon 5)	195, stop
HT-1080/Ara-C	L	797–799, AAA to AA one base Del	(within exon 5)	213, FS (221, stop)
	S	709–824, 116 bp Del	(exon 5)	195, stop
HT-1080/CNDAC	L	797–799, AAA to AA one base Del	(within exon 5)	213, FS (221, stop)
	S	709–824, 116 bp Del	(exon 5)	195, stop
HT-1080/DMDC	L	797–799, AAA to AA one base Del	(within exon 5)	213, FS (221, stop)
	S	709–824, 116 bp Del	(exon 5)	195, stop
DC-10	S	561–708, 148 bp Del	(exon 4)	135, FS (157, stop)
28-A	L	610, G to C	(within exon 4)	151, Glu to Gln
	S	367–560, 194 bp Del	(exon 3)	74, stop
28-C	L	574, TCA Ins	(within exon 4)	139, Ser Ins
28-D	L	698, C to A	(within exon 4)	180, Ala to Asp

a) L, long fragment; S, short fragment.
b) Del, deletion; Ins, insertion; FS, frame shift.

digested by all of the restriction enzymes tested. However, the S fragments from CN20, DC-10 and 28-A cells could not be digested by *Hin*dIII, *Eco*T22I and *Sac*I, respectively. This result demonstrates that these S fragments from CN20, DC-10 and 28-A cells have deletions in the sequences corresponding to the fifth exon, the fourth exon and the third exon, respectively. These results were confirmed by cycle-sequencing analysis (Table II).

Resistant HT-1080 cells expressing aberrant dCyd kinase mRNA dCyd kinase RT-PCR products from these resistant cells were separated into two bands (L and S fragments) as observed in CN20 cells. DNA sequencing was performed on the S fragments of dCyd kinase from the resistant cells. The sequences showed that all the dCyd kinase mRNAs expressed in these resistant cell lines had the same deletion of the fifth exon as seen in CN20 cells (Table II).

Mutations in various nucleoside-resistant cells All the RT-PCR products from dCyd kinase mRNAs expressed in the resistant cells were sequenced and the results are summarized in Table II. Only a single S fragment was detected in DC-10 cells. In 5 other resistant cells both the L and the S fragments were detected (Fig. 1 and Table II). The S fragment was observed in 6 out of 8 resistant cell lines that we have established.

All resistant cells from HT-1080 cells expressed the L and S fragments due to a deletion of the fifth exon of the *dCyd kinase* gene. The L fragments expressing in resistant cells were amplified and sequenced to confirm whether the L fragment codes a completely normal sequence. A one-base deletion at the nucleoside position 797–799 (AAA to AA) within the fifth exon was observed in the L fragment from resistant HT-1080 cells. The L fragment from 28-A

cells had a one-base substitution (G to C) at the nucleoside position 610. This point mutation could not be found in the S fragment of the same 28-A cells, which had a splicing mutation in the third exon. In 28-C and 28-D cells only the L fragments were recognized. 28-C cells had a TCA insertion at nucleoside position 573 and 28-D cells had a one-base substitution (C to A) at nucleoside position 698. The 28-C and 28-D cells without exon deletion were deficient in the enzyme activity of dCyd kinase, like the other resistant cells with deletion.

DISCUSSION

The chemosensitivity and dCyd kinase activity of all of the resistant cells showed a marked decrease when compared with those of the parental cells (Table I). dCyd kinase, which phosphorylates dCyd, is considered to be the key target molecule involved in drug resistance to and activation of CNDAC.[7] Our results indicate that the decrease of dCyd kinase activity plays a major role in the acquisition of resistance to antitumor cytosine nucleosides.

To elucidate why dCyd kinase activity was decreased, we analyzed the dCyd kinase mRNA of resistant cells. Previously, we reported that aberrant mRNA expression of dCyd kinase in CN20 cells is due to deletion of the fifth exon.[7] The same deletion is also observed in Ara-C-resistant human T-lymphoblast leukemia cells,[8] dFdC-resistant human ovarian carcinoma cells[9] and leukemic blast samples from patients with Ara-C-resistant AML.[12] We identified novel splicing mutants with a deletion of the third exon in 28-A cells and the fourth exon in DC-10 cells (Fig. 2 and Table II). The nature of the deletion mutant seems to be dependent on the nucleoside which was used

for establishing resistant cells, although the chemical structures and the molecular targets of these nucleosides are similar.

Furthermore, we studied whether the type of nucleoside used for establishing resistant cells affects the location of splicing. Newly established resistant cells from HT-1080 cells had the same deletion of the fifth exon (Table II) and the location of splicing was independent of the type of antitumor cytosine nucleoside used in the establishment. The fifth exon in the *dCyd kinase* gene is considered to be a target for the acquisition of resistance to antitumor cytosine nucleosides. In contrast, there were different splicing mutants between DMDC- and Ara-C-resistant cells (DC-10 and 28-A cells, respectively) derived from the same parental SK-MEL-28 cells. It is possible that the differences in splicing mutation depend on the nucleosides used to establish the resistant cells. Further investigation of splicing mutations of other nucleoside-resistant cells and analysis of resistant cell genomic DNA are required. In a preliminary experiment, genomic DNA from 28-A cells showed a point mutation at an upstream position (acceptor junction of the fourth intron) from the fourth exon in the *dCyd kinase* gene. It seems that this point mutation caused aberrant splicing during the RNA processing reaction.

The RT-PCR products (Fig. 1) of the *dCyd kinase* gene in all resistant cells except DC-10 cells showed the normal size (L fragment), but contained some mutations (Table II). Although these mutations were located in various positions, they were concentrated in the fourth or the fifth exon. The fourth and fifth exons appear to be hot spots for mutation in the *dCyd kinase* gene. Other point mutations have been reported in Ara-C-resistant cells (G to A substitution at nucleoside position 242)[8] and in dFdC-resistant cells (A to G substitution at nucleoside positions 375 and 755).[10] In clinical specimens, *dCyd kinase* genes from some patients with AML have point mutations[11] and exon deletion.[12] However, the point mutations we describe here are novel and differ from those previously reported. The most important amino acid motif in dCyd kinase, a putative nucleotide-binding domain (ATP-binding domain), is located at amino acid positions 28–34 within the first to the second exon.[17] The splicing mutation and the point mutation that we newly identified in the resistant cells are located in an unrelated position within the ATP-binding domain. However, the third exon to the fifth exon may be essential for high enzyme activity, because all resistant cells with these deletions were deficient in dCyd kinase enzyme activity.

Interestingly, the point mutation (G to C substitution at the nucleoside position 610) in the L fragment of 28-A cells with the third exon deletion could not be found in the S fragment of the same resistant cells. In 28-A cells, the splicing mutation and the point mutation may be generated independently, and each mutation seems to appear in a different allele of the genome DNA. Further, these mutations were recognized in the later stage of resistant cells with advanced resistance, not in the early stage with resistance index ratios of approximately 10 (data not shown).

In conclusion, we have described novel deletions/mutations of dCyd kinase mRNA in antitumor cytosine nucleoside-resistant cell lines. Various mutations in the *dCyd kinase* gene seem to be associated with the acquisition of resistance to antitumor cytosine nucleosides. A better understanding of the acquisition of resistance to antitumor cytosine nucleosides may be useful in the design of future clinical trials.

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