Advances in Brief

Overexpression of Ribonucleotide Reductase as a Mechanism of Resistance to 2,2-Difluorodeoxycytidine in the Human KB Cancer Cell Line[1]

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Abstract

In this study, human oropharyngeal epidermoid carcinoma KB cells that were resistant to 2,2-difluorodeoxycytidine (dFdCyd) were selected and designated the KB-Gem clone. The KB parental cell line IC_{50} was 0.3 μM dFdCyd, as compared with the KB-Gem clone IC_{50} of 32 μM dFdCyd. The KB-Gem clone demonstrated overexpression of ribonucleotide reductase (RR) M2 subunit mRNA (9-fold) and overexpression of M2 protein (2-fold); RR activity was 2.3-fold higher than the KB parental cell line. Both the dATP and dCTP pools of the KB-Gem clone increased 2-fold over the parental cell line, with no change in the dGTP and dTTP pools. Reverse transcriptase-PCR was used to clone the cDNA of deoxycytidine kinase (DCK). Resulting sequences revealed two silent mutations in the KB-Gem clone. The amino acid sequence of the DCK protein and mRNA expression remained unchanged. The KB-Gem clone's DCK enzyme activity was 56% of that of the parental cell line. After the endogenous dNTPs were removed with a G-25 column, no difference was evident between the enzyme activities of the KB-Gem clone and parental cells. Thus, contrary to previous hypotheses, DCK deficiency does not play the primary role in the resistance mechanism of dFdCyd, accepting a secondary role to the overexpression of the target gene, RR, and pool expansion.

Introduction

dFdCyd[3] (gemcitabine) is a novel deoxycytidine analogue with antitumor activity that bears a resemblance, structurally and metabolically, to Ara-C (1). Pharmacologically, DCK phosphorylates dFdCyd to dFdCMP, and other kinases phosphorylate dFdCMP intracellularly to its di- and triphosphate forms (2, 3). RR is inhibited by dFdCDP. The incorporation of the active metabolite dFdCTP triphosphate molecule into the DNA strand halts DNA elongation because only one more dNTP molecule can be joined to the DNA strand (1–3). Gemcitabine diphosphate reduces competition with deoxycytidine by directly inhibiting RR, thereby lowering deoxynucleotide production (1–3). RR is an S phase-specific, rate-limiting enzyme of the DNA synthesis pathway (4). RR is mainly responsible for the conversion of ribonucleoside diphosphate to deoxyribonucleoside triphosphate, which is essential for DNA synthesis (4, 5). RR consists of two subunits, M1 and M2. The M1 subunit possesses a binding site for enzyme regulation, and the M2 subunit is involved with RR activity, as it is related to DNA synthesis and cell proliferation and metastatic potential (6, 7). Gemcitabine has been used widely in the treatment of lung, pancreatic, and head and neck cancer (8, 9). Understanding the mechanism of resistance to gemcitabine is important because of its

potentially widespread clinical applications. Previous reports cited the DCK mutation/deficiency as the main mechanism for gemcitabine resistance (2, 10–13). These observations were based mainly on studies performed on Ara-C-resistant clones. Because gemcitabine has different targets than Ara-C, the resistance mechanisms to gemcitabine may differ from those to Ara-C. Moreover, because gemcitabine targets RR as a mechanism of cytotoxicity, we believe the alteration of RR may play a role in gemcitabine resistance. In this study, gemcitabine resistance in a human oropharyngeal cancer KB cell line was induced, and the expressions of RR and DCK were analyzed. The molecular properties of the gemcitabine-resistant clones confirmed our hypothesis.

Materials and Methods

Cells. Human oropharyngeal carcinoma KB cells (American Tissue Culture Collection, Manassas, VA) were incubated in plastic tissue culture plates in RPMI 1640 supplemented with 10% fetal bovine serum at 5% CO_2 and 37°C. HU-resistant clones (HURS) were incubated and maintained in the presence of 1 mM HU as a control for this study. A gemcitabine-resistant clone (KB-Gem) was selected from stepwise exposure to increasing concentrations of gemcitabine. This cloned cell line was selected and maintained in the presence of 8 μM gemcitabine.

IC_{50} Determination. Cells in logarithmic growth were plated at a density of 25,000 cells/ml in 24-well plates to determine the effects of drugs delivered in varying dosages. Methylene blue assays were performed, and IC_{50} values were determined by interpolation of the plotted data to show the drug concentration that produced 50% cell death (14).

Probes, Primers, and RT-PCR. The human cDNA sequences of DCK and the M1 and M2 subunits of RR have been published (14, 15). To construct a probe of human DCK, we performed RT-PCR technique on total RNA taken from wild-type KB cells. Primers were designed from the cDNA sequences of two enzymes, as described previously (14, 15).

mRNA and Northern Blot Analyses. Total RNA was extracted from logarithmically grown cells, as described previously. The RNA was electrophoresed in a formaldehyde-agarose gel and blotted onto a Hybond-N membrane (Amersham, Arlington, IL). Radioactive probes of DCK, M1, and M2 were prepared as described previously (14, 15). Hybridizations were performed under stringent conditions.

Protein and Western Blot Analyses. Protein extracts were separated by 7.5% SDS-PAGE, loaded with equal amounts of protein. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane (Amersham Life Sciences). Antirat M2 polyclonal and antimouse M1 monoclonal antibodies purchased from Accurate Chemical and Scientific Co. (Westbury, NY) were used for Western blot analysis (14, 15).

RR Enzyme Assay. RR activity was measured using the [^{14}C]CDP-assay method published previously (16, 17). The assay mixture had a final volume of 75 μl. The [^{14}C]CDP was converted to [^{14}C]dCDP by RR. The incubation time for the reaction was 20 min. The amount of [^{14}C]dCDP was determined after enzymatic dephosphorylation by high-performance liquid chromatography with the use of a C18 ion exchange column.

Determination of dNTP Pools. This assay was conducted according to a previously described method (16). The total reaction volume was 50 μl. The mixture was allowed to react at room temperature for 20 min. After incubation,

Table 1 *Effects of gemcitabine, HU, and Ara-C on KB-Gem clones*

	IC$_{50}$[a]		
	Gemcitabine (μM)	HU (mM)	Ara-C (μM)
KB wild-type	0.30 ± 0.06	0.43 ± 0.07	0.90 ± 0.11
KB-Gem	32.30 ± 1.97	2.36 ± 0.3	134.84 ± 11.7
HURS	44.81 ± 1.33	12.5 ± 1.3	150.77 ± 13.7

[a] IC$_{50}$ was determined as described in "Materials and Methods." Values are means ± SD calculated from three separate experiments, each performed in duplicate.
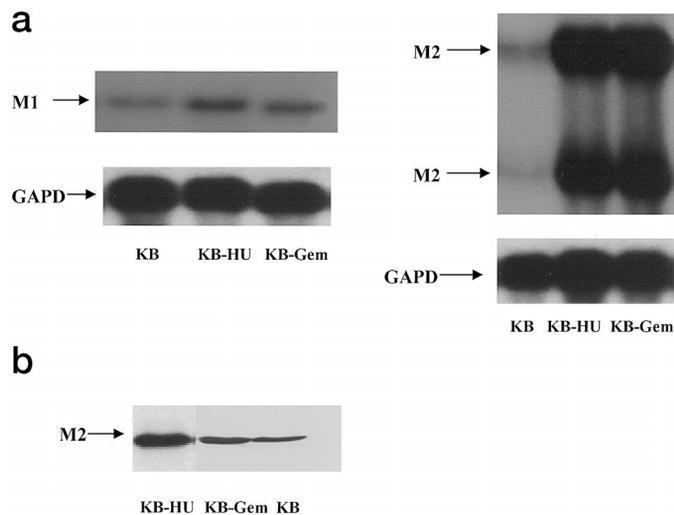

Fig. 1. *a*, Northern blot of KB-Gem clone, HURS clone, and KB wild-type cells. Twenty μg of total RNA were isolated from all cell lines. Blots were hybridized with random-primed ^{32}P-labeled RR M1 or M2 full-length cDNA probe. *b*, Western blot of protein extracts from the cells with M2 antibodies. Crude cell extracts (30 μg of protein) were loaded in each lane. The M2 subunit is represented by the 44,000 band (monomer of subunit).

40-μl aliquots were spotted onto Whatman DE81 paper. After adequate washing, tritium-labeled dNTPs were counted by a liquid scintillation counter.

DCK Enzyme Assay. Enzyme activity was measured according to a procedure derived from the protocol of Ivies *et al.* (18). Cell extracts were prepared with a cold DCK buffer (50 mM Tris-HCl, pH 8.0). The protein contents of the cell extracts were measured with the Bio-Rad Bradford protein assay (19). In each assay, the reaction substrate mixture was combined with an equal volume of enzyme. The final concentrations of the DCK assay components were: 10 mM ATP, 10 mM MgCl$_2$, 50 mM Tris-HCl (pH 8.0), 0.02 mM [^{3}H]CdR, and 15 mM NaF. This reaction mixture was incubated for 30 min at 37°C. The reaction was terminated by heating in a boiling water bath for 2 min. Adequate aliquots were pipetted onto DE-81 paper discs and allowed to stand for 10 min at room temperature. The discs were washed twice with 1 mM ammonium formate for 20 min each. Then the radioactive discs were placed into counting vials, and 1-ml aliquots of 0.1 M HCl-0.2 M KCl were added to elute labeled nucleotides from the discs. After elution, 5 ml of trito-toluene scintillation solvent were added into each vial. The samples were counted in a Beckman LS-100 scintillation counter (18, 19).

DCK cDNA Analysis by PCR. Total RNAs were extracted from the KB wild-type, KB-Gem, and HURS cell lines. RT-PCR was used to yield the cDNAs of all three cell lines. The cDNA products of all cell lines were then cloned with a general contractor DNA cloning system (5 Prime→3 Prime, Inc.). After the cloned DNAs were purified, the cDNAs were sequenced at the Sequencing Facility Laboratory of the City of Hope National Medical Center (Duarte, CA).

Results

Establishment of a KB Gemcitabine-resistant Clone. Resistance to gemcitabine was successfully induced in the human oropharyngeal epidermoid carcinoma KB cell line via stepwise exposure to increasing concentrations of dFdCyd. The sensitivities of the KB wild-type,

HURS, and KB-Gem cells to gemcitabine, HU, and Ara-C are shown in Table 1. The IC$_{50}$s of gemcitabine for KB wild-type, HURS, and KB-Gem clone were 0.3, 32.3, and 44.8 μM, respectively. The KB-Gem clone was cross-resistant to both HU and Ara-C, although the clone was 5-fold more resistant to HU and 150-fold more resistant to Ara-C than the KB wild-type (Table 1). The HURS clone was used as a control and demonstrated the same range of resistance to HU, as reported previously.

Molecular Characterization of RR Expression in the KB-Gem Clone. Total RNA was extracted from each cell line and analyzed via Northern blot to detect M1 and M2 mRNA expressions. The cDNA probes of M1 and M2 were prepared as reported previously. β-Actin was studied as an internal control.

The Northern blot of M1 and M2 mRNA expressions is shown in Fig. 1*a*. A 3.3-kb RNA was detected with the M1 probe, and 3.4- and 1.6-kb RNAs were detected with the M2 probe. Overall, there was no

Table 2 *Molecular characterization of RR expression in the KB-Gem clone[a]*

	M1		M2	
Cells	Relative amounts of M1[b] mRNA	Relative amounts of M1[c] protein	Relative amounts of M2[b] mRNA	Relative amounts of M2[c] protein
KB wild-type	1.0	1.0	1.0	1.0
KB-Gem	1.2	1.0	9.0	2.0
HURS	1.5	1.2	10.0	3.0

[a] Quantitative values are the means of three experiments.
[b] Determined from phosphoimaging measurements of the band hybridizing with M1 or M2 probes with mRNA from KB-Gem clone and HURS clones, relative to measurement of the band hybridizing with RNA from wild-type cells. Results were corrected according to a fixed control value of β-actin mRNA.
[c] Determined the densitometric measurements of the crude extracted protein blot with M1 or M2 antibodies from KB-Gem clone and HURS clones, relative to protein from wild-type cells.

Table 3 *Relative changes in RR activity and dNTP pools in the KB-Gem clone*

		dNTP pools[b]	
	Relative RR enzyme activity[a]	dATP	dCTP
KB wild-type	1	1	1
KB-Gem	2.3×	2×	2×
HURS	6.7×	7×	15×

[a] Enzyme activity was determined as described in "Materials and Methods" and was expressed as activity relative to the KB wild-type. Enzyme activity is expressed as nmol CDP reduced/h/mg protein.
[b] Relative values represent the averages of data from three assays, each performed in duplicate.
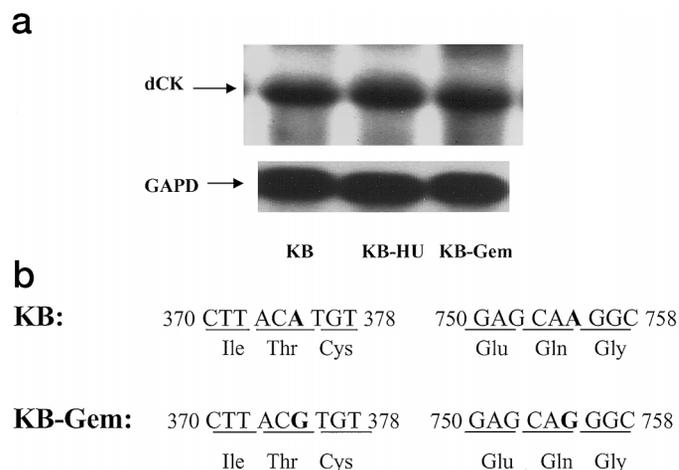

Fig. 2. *a*, Northern blot of total RNA (20 μg) isolated from each cell. Blots were hybridized with random-primed ^{32}P-labeled DCK full-length cDNA probes. *b*, comparison of amino acid sequence and cDNA sequences of DCK in KB-Gem clone and KB wild-type.
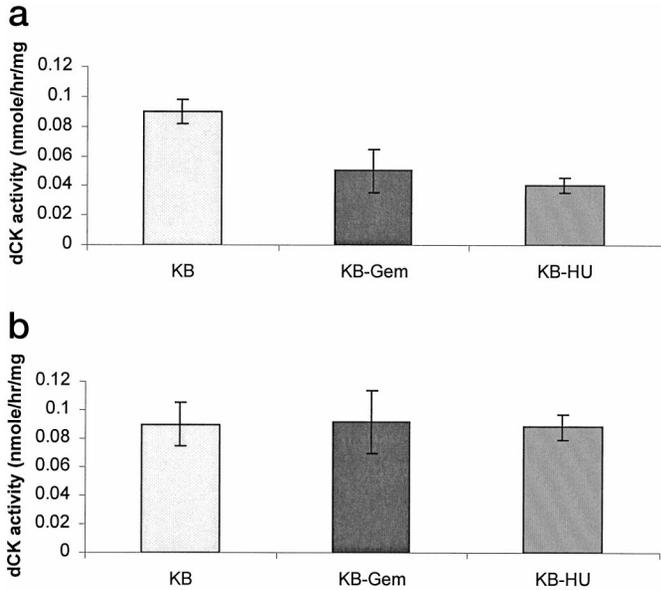
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Fig. 3. *a*, DCK enzyme activity in KB-Gem, HURS clone, and KB wild-type cells in a crude extraction study. *b*, DCK enzyme activity after G-25 column purification.

Molecular Characterization of DCK Expression in KB-Gem Clone. To test whether a gemcitabine resistant phenotype of a KB-Gem clone is associated with DCK deficiency or mutation, we performed molecular characterization tests.

A Northern blot was used with a DCK probe that was obtained from the RT-PCR products of total RNAs. The DCK probe detected a 1.5-kb RNA band. The results from the Northern blot of DCK mRNA are shown in Fig. 2*a* and reveal no difference in the expressions of DCK mRNA among the three cell lines. Primers were designed, and RT-PCR was used to study further the DCK cDNA sequences of the KB-Gem clone and KB wild-type cells (Fig. 2*b*). Full-length cDNAs from KB parental cells and the KB-Gem clone were sequenced, revealing ^{375}A→G and ^{755}A→G point mutations in the KB-Gem clone, as compared with the KB parental cells. These mutation sites were confirmed in three experiments and were not the results of PCR artifact. However, there were no changes in the amino acids (Thr and Gln, respectively).

DCK Activity of KB-Gem Clone. The crude enzyme extracts from KB-Gem, HURS clones, and KB wild-type were studied. The DCK activity of the KB-Gem clone was 56% of that of the KB parental cells. The DCK activity of the HURS clone was 43% of the parental KB cells (Fig. 3*a*). Crude extracted proteins were then passed

significant difference in M1 mRNA expression among the KB-Gem clone, HURS clone, and KB wild-type. The expression of M2 mRNA was higher in the KB-Gem and HURS clones than it was in the KB wild-type. The quantitative data were summarized from the means of the data gathered from the three experiments and are shown in Table 2. KB-Gem demonstrated a level of M2 mRNA expression that was 9-fold higher than that of the KB wild-type. The M2 mRNA expression in HURS clone increased 10-fold, as reported previously. To determine the M1 and M2 protein quantities of the KB-Gem clone, we extracted equal amounts of total protein from the KB wild-type and HURS and KB-Gem clones and analyzed them via Western blot (Fig. 1*b*). The molecular weight of the M2 protein was 88,000, and that of the M1 was 85,000 (monomer). The M1 proteins from each cell demonstrated equal levels of expression (data not shown). The M2 protein in the KB-Gem clone and HURS showed 2- and 3-fold increases, respectively, as compared with the wild-type cells. The relative quantitative results from the means of the three experiments are summarized in Table 2.

Determination of RR Enzyme Activity and dNTP Pools. The crude RR enzyme extracts from the KB wild-type and KB-Gem and HURS clones were studied to confirm enzyme activity. Table 3 shows the relative ratios of the RR activities of the cell lines. The RR activity of the KB-Gem clone was 2.3-fold higher than the RR activity of KB wild-type cell lines. The HURS clone exhibited a 6-fold elevated level of RR, comparable with data from previous reports. The concentrations of dNTP pools were calculated in pmol per 1 million cells and were presented as relative ratios in which KB-Gem was compared with the KB wild-type cells. The dTTP and dGTP pools revealed no significant changes and are not shown in this study. The dATP and dCTP pools of each cell line are shown in Table 3. The results reveal that the dATP pool of KB-Gem cell line was 2-fold higher than that of the KB parental cell line. The dCTP pool of the KB-Gem clone was 2-fold higher than that of the parental cell line. The HURS clone demonstrated a 7-fold increase in dATP and a 15-fold increase in dCTP over that of the KB wild-type. This remarkable increase has been reported previously.
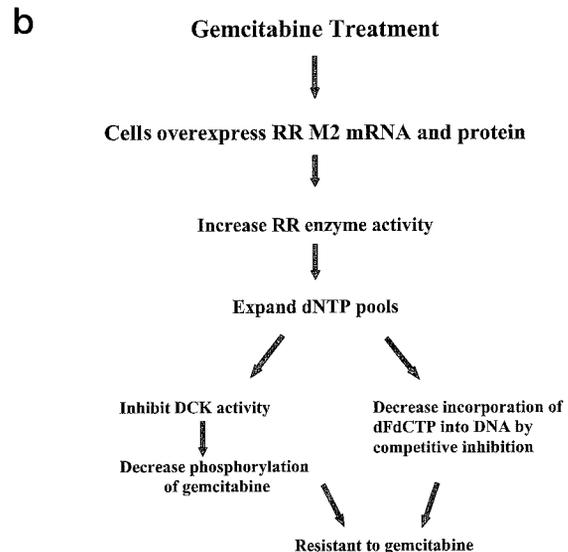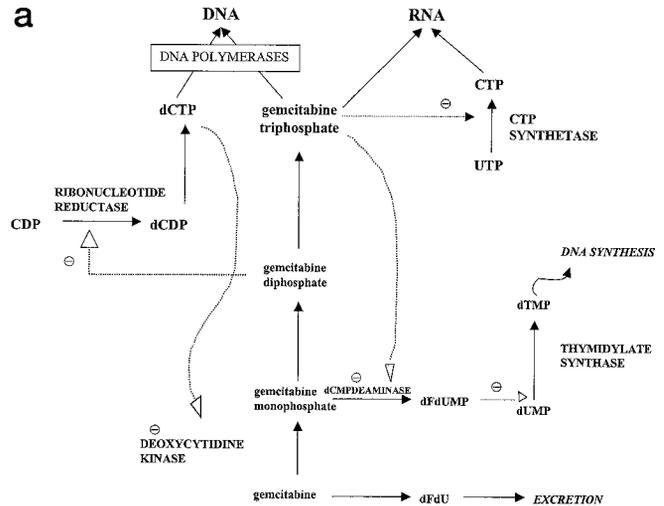
Fig. 4. *a*, the schematic diagram of the metabolism of gemcitabine (modified from Peters *et al*. Semin. Oncol., *23:* 16–24, 1996). *b*, the hypothesis of the role of ribonucleotide reductase in gemcitabine resistance.

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through a G-25 column to remove the endogenous dNTP pools. The results are shown in Fig. 3*b*. DCK enzyme activities were relatively similar among the three cell lines.

Discussion

The mechanism of resistance to gemcitabine was previously believed to have been based on a mutation or deficiency of DCK (10). These observations resulted from studies performed on Ara-C-resistant clones (11–13). The first acquired gemcitabine resistant cell line was reported by Ruiz van Haperen *et al.* (10). They showed that their gemcitabine-resistant cell line was cross-resistant not only to Ara-C but also to several other unrelated anticancer agents (10). Their findings suggested that low DCK activity plays an important role in the mechanism of gemcitabine resistance. Whether DCK deficiency is secondary to inhibition by a dNTP pool or simply a partial mechanism of a larger process remained undetermined. Moreover, studies of the murine colon 26-A cell in animal models have failed to show that DCK deficiency is the mechanism of gemcitabine resistance. In these studies, thymidine kinase or other enzymes were suggested as the mechanisms of gemcitabine resistance (20). Because gemcitabine targets RR, we hypothesized that the resistance mechanism may be based on RR alteration.

Our current findings support the possibility that increased RR activity plays an important role in the mechanism of gemcitabine resistance of the KB-Gem clone (Fig. 4). Still, other mechanisms of gemcitabine resistance should be considered, such as: increased deoxycytidine deaminase levels, which metabolize the gemcitabine; increased CTP synthetase, which increases endogenous CTP pools; and further alterations of the binding site of the DNA polymerase (10). Other resistance phenotypes, such as decreased membrane transport or altered DNA excision/repair, also need to be considered (10).

In this study, the HURS clone was used as a positive control. The characteristics of our HURS clone was thoroughly reported in a previous paper (6, 14–16). Interestingly, the M2 mRNA expression of the KB-Gem clone was 9-fold higher than that of the KB wild-type cells, but with only a 2-fold increase in M2 protein. This phenomenon has been demonstrated in the HURS clone as well. This finding suggests that posttranslational and posttranscriptional regulation may play a role in the RR expression of the KB-Gem clone. Therefore, the KB-Gem clone may be a useful tool in studying the regulation of RR expression.

In summary, we reported the overexpression of RR but not DCK as a mechanism of resistance to gemcitabine. Increased RR activity expands the size of the dNTP pools, which competitively inhibits the incorporation of dFdCTP into DNA. The expanded dNTP pools further down-regulate the activity of DCK via negative feedback pathways, reducing the phosphorylation of gemcitabine. Finally, excess dCTP may be a positive feedback mechanism of dCMP deaminase that results in increased gemcitabine metabolism. However, other mechanisms may be involved, and further study is required.

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