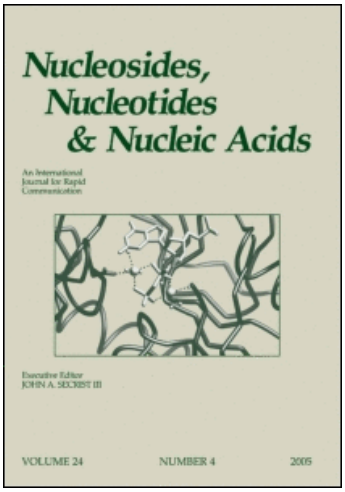

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Micro-Array Analysis of Resistance for Gemcitabine Results in Increased Expression of Ribonucleotide Reductase Subunits

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MICRO-ARRAY ANALYSIS OF RESISTANCE FOR GEMCITABINE RESULTS IN INCREASED EXPRESSION OF RIBONUCLEOTIDE REDUCTASE SUBUNITS

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□ *To study in detail the relation between gene expression and resistance against gemcitabine, a cell line was isolated from a tumor for which gemcitabine resistance was induced in vivo. Similar to the in vivo tumor, resistance in this cell line, C 26-G, was not related to deficiency of deoxycytidine kinase (dCK). Micro-array analysis showed increased expression of ribonucleotide reductase (RR) subunits M1 and M2 as confirmed by real time PCR analysis (28- and 2.7-fold, respectively). In cell culture, moderate cross-resistance (about 2-fold) was observed to 1-β-D-arabinofuranosylcytosine (ara-C), 2-chloro-2'deoxyadenosine (CdA), LY231514 (ALIMTA), and cisplatin (CDDP), and pronounced cross-resistance (> 23-fold) to 2',2'-difluorodeoxyuridine (dFdU) and 2',2'-difluorodeoxyguanosine (dFdG). Culture in the absence of gemcitabine reduced resistance as well as RRM1 RNA expression, demonstrating a direct relationship of RRM1 RNA expression with acquired resistance to gemcitabine.*

Keywords: Gemcitabine; Drug resistance; Ribonucleotide reductase; Micro-array

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INTRODUCTION

In most in vitro studies, the main resistance mechanism against gemcitabine (2′,2′-difluorodeoxycytidine, dFdC, Gemzar) is decreased activity of dCK,[1,2] which enzyme is responsible for its initial phosphorylation, whereafter DNA incorporation and growth inhibition occurs. However, resistance to gemcitabine may include other mechanisms like increased activity of deoxycytidine deaminase (dCDA) and DNA polymerase or decreased gemcitabine triphosphate (dFdCTP) accumulation. Also, RR is reported to be related to gemcitabine resistance, but in a nonconsistent way. Some in vitro acquired resistance models associate gemcitabine resistance with increased RR activity,[3,4] others with increased RRM1 expression,[5], or increased[3] or decreased RRM2[6] expression. One study suggests that RRM1 expression is a crucial predictive marker of survival in gemcitabine/cisplatin-treated non-small cell lung cancer (NSCLC) patients.[7] Although a special R2 protein is induced by p53,[8] this has not yet been related to gemcitabine resistance. However, no studies have been reported on acquired resistance in in vivo model systems nor in patients.

We derived cell lines from a previously developed in vivo resistance model,[9] in which we found an increased RRM1 expression using micro-array technology. We isolated cell lines from these tumors that subsequently were tested for cross-resistance patterns to other drugs, which was not possible in vivo. Next, the cells were analyzed by expression micro-array and LC-PCR, demonstrating a direct relationship between RRM1 and acquired resistance to gemcitabine in this cell line as well.

MATERIALS AND METHODS

Gemcitabine, dFdU, and dFdG were supplied by Eli Lilly Inc. (Indianapolis, IN, USA). All other chemicals were of analytical grade and commercially available.

Resistance to gemcitabine was induced by treating Colon 26A tumor bearing mice as described previously.[9] The C 26-A cell line and gemcitabine resistant variant C 26-G were derived from the parent tumor Colon 26A and the gemcitabine resistant tumor Colon 26G, respectively, by mechanical dissociation followed by incubation with DNAse/collagenese and ex vivo monolayer culture. Resistance was sustained by weekly 480 nM gemcitabine for 4 hours.

Chemosensitivity, dFdCTP pools and enzyme assays were performed as described previously,[10] and PCR was performed as described before.[9] Micro-array analysis was performed as described before.[9] Basically, a mouse oligoLibrary (Compugen/Sigma-Aldrich Chemie B.V., Zwijndrecht, NL) representing 7230 separate genes was spotted. After printing the CodeLink activated 3D-Link slides were processed according to the manufacturers

protocol (Amersham, Roosendaal, NL). Single-stranded cDNA was synthesized from total RNA using aminoallyl-labeled dUTP according to de Risi et al.[11]

RESULTS

After initial isolation, resistance to gemcitabine and other cytostatics, including deoxynucleoside analogs, was determined in C 26-G cells (Table 1). Omission of gemcitabine gradually decreased the resistance factor (RF) after 72 hours drug treatment from 7.7- to 1.5-fold resistance. No or moderate cross-resistance was found for the DNA-polymerase inhibitor aphidicolin, the antifolate LY231514 (ALIMTA), the dCK substrates ara-C and CdA, and the alkylating agent CDDP, while marked cross-resistance was found for the catabolite dFdU and the purine analog dFdG.

Initial analysis focussed on deoxynucleoside metabolizing enzymes. No major differences were found in dCK and dCDA enzyme activity. Also, deoxyguanosine kinase (partially) responsible for dFdG activation was not changed. Only thymidine kinase 2 (TK2), which poorly phosphorylates gemcitabine, was substantially decreased in C 26-G cells (Table 2).

C 26-G cells accumulated about 2-fold more overall dFdCTP after 4- and 24-hour exposure to gemcitabine (Figure 1). This was surprising, since we previously observed a significant relationship between gemcitabine sensitivity and dFdCTP accumulation.[12] After incubation in drug free medium, this dFdCTP accumulation was even more pronounced.

TABLE 1 RF- and IC50 Values of the Gemcitabine Resistant C 26-G Cell Line

Exposure time (hr)	Drug	IC50 (nM) C 26-A	IC50 (nM) C 26-G	RF
4[a]	dFdC	85.1 ± 24.6	482 ± 71	5.7
24[a]	dFdC	5.1 ± 2.2	32.8 ± 6.9	6.4
72	dFdC	3.4 ± 1.0	26.2 ± 4.1	7.7
72	dFdG	0.6 ± 0.3	95.0 ± 13.2	158
	dFdU	20.0 ± 1.7	468.8 ± 78.6	23
	CdA	41.8 ± 9.6	115 ± 23	2.8
	ara-C	48.8 ± 4.3	125.0 ± 39.4	2.6
	CDDP	1.0 ± 0.2	2.5 ± 0.3	2.5
	LY231514	49.3 ± 6.8	85.0 ± 6.5	1.7
	Aphidicolin	250.0 ± 0.0	273.3 ± 39.3	1.2

Average IC50 values ± standard error mean (SE) of at least 3 experiments. Average RF of at least 3 experiments is defined as IC50 ratio of C 26-G and C 26-A with the latter set at 1.

[a]Drug exposure was 4 or 24 hours followed by culture in drug free medium for 68 or 48, respectively.

Culture in the absence of gemcitabine for 9 months reduced the IC50 for gemcitabine in C 26-G to 7.5 nM; challenging the cell line for 7 weeks weekly for 4 hours to the IC50 for 4 hours, increased the IC50 (72 hours) to 104 nM. Subsequent culture in drug-free medium again reduced the resistance factor to 1.2.

TABLE 2 Enzyme Activities in C 26-A and Gemcitabine Resistant Variant C 26-G

Enzyme	Substrate	C 26-A	C 26-G
dCK+TK2	dCyd	0.27 ± 0.04	0.31 ± 0.04
dCK	dCyd (+TdR inhibition)	0.22 ± 0.01	0.22 ± 0.03
TK1+TK2	TdR	0.71 ± 0.08	0.18 ± 0.08
TK1	TdR (+dCTP inhibition)	0.21 ± 0.03	0.07 ± 0.02
dCDA	dCyd	0.50 ± 0.50	0.20 ± 0.10
dGK	dGuano	1.88	3.15
DNA polymerase	dTTP	0.059 ± 0.023	0.094 ± 0.032
TS	FdUMP	384 ± 26	460 ± 18
	dUMP	965 ± 124	1180 ± 72

Average enzyme activities ± SEM of at least 3 experiments in nmol/hr/10^6 cells. For measuring deoxycytidine (dCyd) phosphorylation by dCK, assayed at 230 μM dCyd, TK2 was inhibited by thymidine (TdR) and for measuring TdR phosphorylation by TK1, TK2 was inhibited by dCTP. DNA polymerase in fmol/hr/10^6 cells. Thymidylate synthase (TS) levels were assayed as FdUMP binding (fmol/10^6 cells) and TS catalytic activity was assayed with 10 μM dUMP (pmol/hr/10^6 cells).

In the micro-array analysis, the average log2 RNA expression for RRM2 and RRM1 were substantially higher in C 26-G versus C 26-A: 0.816 ± 0.016 and 1.008 ± 0.046 respectively compared to the average of all genes: 0.017 ± 0.385 (Figure 2). Also, dCK expression was increased: 0.610 ± 0.047. All increases were significant as determined by students T-test ($p < 0.02$). The 4.5-fold decreased TK2 enzyme activity might be related to the decreased TK2 expression being −0.195 ± 0.146.

Real time LC-PCR, used to verify the micro-array data, revealed a 28-fold difference in the RRM1 RNA expression between C 26-A and C 26-G (RRm1/ß-actin ratio of 1.42 and 39.6, respectively), which decreased to only

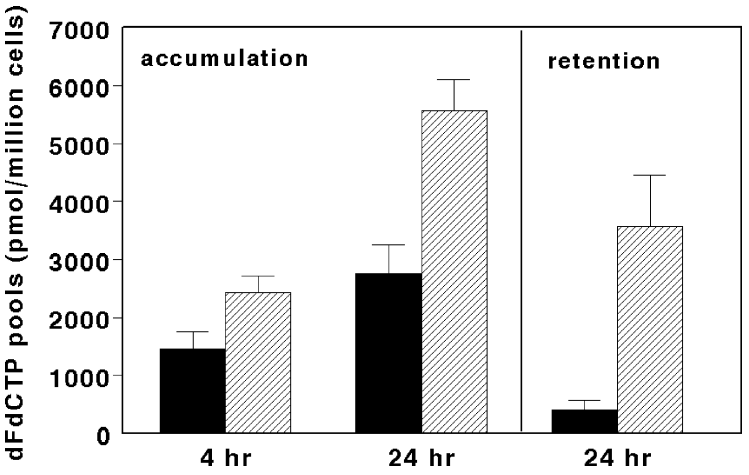

FIGURE 1 Accumulation of dFdCTP in C 26-A (solid bars) and C 26-G (striped bars) cells after 4- and 24-hour exposure to 10 μM gemcitabine and 24-hour retention after 24-hour exposure to 10 μM gemcitabine. Values represent means ± SEM of at least 3 experiments.

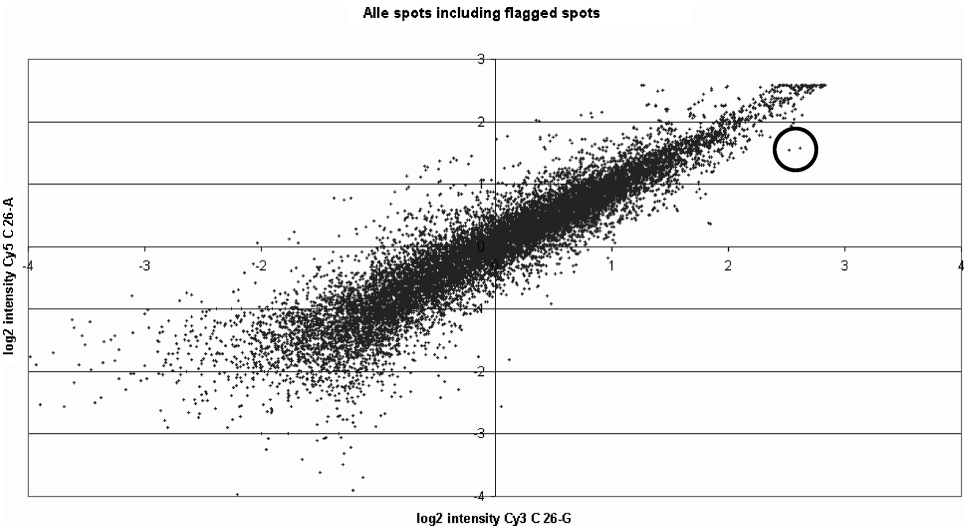

FIGURE 2 A representative micro-array analysis of C 26-A and C 26-G cells. All flagged spots on the array are included and no corrections were performed. Array elements (probes) were spotted in duplicate, GEO accession # GSM43964. The spots of ribonucleotide reductase subunit M1 are circled.

2.2-fold when RRM1 was analyzed in cells cultured without gemcitabine. The difference in RRM2/ß-actin ratio between C 26-A and C 26-G was only 2.7-fold (0.61 and 1.63, respectively).

DISCUSSION

Bergman et al.[9] described the first in vivo gemcitabine resistant model, which was developed by treating mice bearing Colon 26A tumors for about one year. The derived C 26-G cell line was resistant, but to a lesser extent than cell lines made resistant to gemcitabine.[1,10] Although a decrease in dCK activity often is linked to acquired resistance to gemcitabine, the dCK enzyme activity did not differ between C 26-A and C 26-G. Both TK2 expression and enzyme activity were decreased in C 26-G; the relevance is not clear since gemcitabine is a poor TK2 substrate,[13] while C 26-G cells had even an increased dFdCTP accumulation. However, the decreased TK2 is in line with the cross-resistance for dFdU, which is a good substrate for TK2.[13]

Although C 26-G cells were cross-resistant to dFdG, phosphorylated by both dCK and dGK, these enzyme activities were not decreased.

The most striking increase in RNA expression in C 26-G cells was that of RRM1. This also was found in the original Colon 26G tumor.[9,14] However, when gemcitabine treatment of the cell line was omitted, both resistance and RRM1 expression decreased dramatically, demonstrating a direct relationship with acquired resistance. A role for RRM1 in intrinsic

resistance to gemcitabine was suggested in patients with metastatic NSCLC. Patients with a low pretreatment RRM1 mRNA had a significantly longer median survival than those with a high expression[7] when treated with a gemcitabine containing regimen. Also RRM2 expression, which was reported to be related to gemcitabine resistance previously,[3] was increased in the C 26-G cells. Since ara-C, in contrast to gemcitabine, can not inhibit RR, this might explain the only moderate cross-resistance to ara-C in the C 26-G cells.

The C 26-G cells had increased levels and retention of dFdCTP. This lack of an effect of dFdCTP, which is normally associated with gemcitabine sensivity,[12] might be explained by the molecular sink theory as proposed by Davidson et al.,[5] implicating that at an increased RRM1 expression, dFdCDP might be trapped in this "sink," resulting in increased dFdCTP levels.

In conclusion, we isolated a C 26-G cell line derived from a tumor with in vivo induced gemcitabine resistance. Micro-array profiling revealed a marked increase in RRM1 expression, which is in line with in an acquired in vitro resistance model,[5] suggesting a key role for acquired in vivo gemcitabine resistance.

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