Advances in Brief

Induction of Resistance to Fluorodeoxyuridine Cytotoxicity and DNA Damage in Human Tumor Cells by Expression of *Escherichia coli* Deoxyuridinetriphosphatase[1]

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Abstract

Recent studies from our laboratory suggested that, in some human colorectal tumor cell lines, sensitivity to fluorodeoxyuridine may depend upon the extent of dUTP accumulation that occurs following drug treatment and that elevation of dUTPase activity might be the basis for some instances of resistance to fluoropyrimidines. To test this model, we expressed *Escherichia coli* dUTPase in an established human tumor cell line (HT29) and measured the effect of this manipulation on response to fluorodeoxyuridine. As predicted, HT29 derivatives containing dUTPase activity 4–5-fold higher than controls were protected from fluorodeoxyuridine-induced loss of clonogenicity and from formation of DNA double strand breaks. These data provide the first direct evidence that alteration in a component of the uracil misincorporation/misrepair pathway can confer resistance to fluoropyrimidines in human tumor cells.

Introduction

The fluoropyrimidines, fluorouracil and FdUrd,[4] provide significant therapeutic benefit in the treatment of gastrointestinal and breast cancers. One of the major difficulties in the use of these drugs is the variability of response to them among individual tumors. Studies aimed at understanding the basis for this variation have revealed several mechanisms of intrinsic or acquired fluoropyrimidine resistance, most of which lead to reduced inhibition of TS by the common active metabolite, FdUMP. These include decreased anabolism of fluoropyrimidines to FdUMP, lowered affinity of target enzyme for FdUMP, decreased availability of reduced folate cofactor, and elevated intracellular TS levels (1). Although differences in overall cellular response to fluoropyrimidines can often be attributed to differences in susceptibility to TS inhibition, in some cases it appears that the response is limited by events occurring after this blockade. One such example is the SW620 cell line. Our initial studies showed that SW620 cells are significantly resistant to FdUrd-induced cytotoxicity and DNA double strand break formation compared to another human colorectal tumor cell line (HT29), despite the fact that the two lines are equally sensitive to TS inhibition by FdUrd (2). Because it

has been proposed that one consequence of TS inhibition that can lead to DNA damage and cytotoxicity is the accumulation of dUTP and its incorporation into (and excision from) DNA (3–6), we hypothesized that the observed SW620 resistance might be due to attenuation of such a uracil misincorporation/misrepair process. We subsequently found that SW620 cells contain about 5-fold more dUTPase activity than HT29 cells and that FdUrd-induced expansion of dUTP pools and single strand break formation were much less in SW620 cells than in HT29 cells (7), suggesting the more specific hypothesis that elevation of dUTPase activity can confer resistance to FdUrd in human tumor cells. In the present paper, we test this hypothesis directly by engineering the expression of the *Escherichia coli* dUTPase gene in HT29 cells.

Materials and Methods

Construction of an Expression Vector Containing the *E. coli* dUTPase Gene. The wild-type *E. coli* dUTPase gene was amplified by polymerase chain reaction from the plasmid pWB30 (8) using primers containing flanking *Eco*RI and *Bam*HI restriction sites (sense, 5'-GCCGAATTCGGATCCAC-CATGAAAAAAATCGACGTTAAGATT; antisense, 5'-CGGGAATTCG-GATCCTTATTACTGACGACCAGAGTGAC). The resulting polymerase chain reaction product was first cloned into pUC18 at the *Eco*RI site and sequenced. Functionality of the dUTPase gene was tested through complementation of BW286 (9), an *E. coli* strain containing both an allele (*dut-1*) that encodes a temperature-sensitive dUTPase protein and a deletion of the major apyrimidinic/apurinic endonuclease gene (*xth*). Only recombinant molecules containing dUTPase inserted in the correct orientation for transcription complemented at the restrictive temperature. The dUTPase gene was then subcloned from pUC18 into pCMV-Neo-Bam (10) at the *Bam*HI restriction site. The resulting construct, pCMVdutE, also complemented BW286 at the restrictive temperature.

Cell Culture and DNA Transfection. HT29 cells were obtained from the American Type Tissue Collection and grown as monolayers in McCoy's 5A medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO) at 37°C. For clonogenicity assays, treated cells were diluted and plated into 6-well dishes in medium containing 10 μM thymidine to provide immediate and uniform cessation of the thymidylate-deprived state. The plating efficiency of untreated cells was typically 0.6 to 0.8.

HT29 cells were transfected with pCMVdutE or pCMV-Neo-Bam (as a control) using DOTAP transfection reagent (Boehringer Mannheim, Indianapolis, IN). After a 2-day recovery period, cells were split into 100-mm dishes and selected with 1 mg/ml Geneticin (GIBCO) for 12–14 days. Geneticin was then removed, and Geneticin-resistant cells were allowed to establish colonies which were then isolated through the use of cloning cylinders. Geneticin-resistant clones were assayed for dUTPase activity as described (11). Southern blot analysis confirmed the presence and integrity of the *E. coli* dUTPase gene in the colonies derived using the pCMVdutE construct (data not shown).

Analysis of DNA Damage by Pulsed Field Gel Electrophoresis. Cells were resuspended (10^7 cells/ml) in low melting point agarose (BRL, Grand Island, NY; 0.7% final concentration) and lysed according to standard procedures (12). Pulsed field gel electrophoresis was performed using a CHEF

Table 1 *Resistance to various FdUrd treatments conferred by expression of E. coli dUTPase*

Values are given for the surviving fraction observed after treatment with the indicated concentrations of FdUrd for 24 or 48 h (mean ± SE; N = 3–11 except for con3, 10 nM/24 h, for which N = 2).

Cell line	24 h			48 h		
	10 nM	100 nM	1000 nM	10 nM	100 nM	1000 nM
con2	0.70 ± 0.13	0.33 ± 0.06	0.03 ± 0.01	0.18 ± 0.06	0.035 ± 0.011	0.008 ± 0.003
con3	1.08	0.33 ± 0.05	0.16 ± 0.07	0.15 ± 0.07	0.036 ± 0.010	0.023 ± 0.010
dutE1	0.77 ± 0.18	0.67 ± 0.07[a]	0.23 ± 0.04	0.26 ± 0.06	0.069 ± 0.012	0.003 ± 0.002
dutE7	0.84 ± 0.11	0.71 ± 0.10[a]	0.21 ± 0.09	0.51 ± 0.09[a]	0.095 ± 0.024	0.011 ± 0.010

[a] Conditions under which either of the dutE cell lines was significantly resistant compared to both control cell lines (Student's t test, $P < 0.05$).

DR-II apparatus (Bio-Rad, Richmond, CA). Blocks containing $2–3 \times 10^5$ cells were loaded onto a 0.7% agarose gel and electrophoresed at 1.9 volts/cm in 0.045 M Tris-borate, 1 mM EDTA at 10°C. The switching interval was ramped linearly from 30 to 120 s for 30 h followed by 2–42 min for 51 h (13). Under these conditions, both the chromosomes of *Schizosaccharmyces pombe* and *Saccharomyces cerevisiae* are resolved (0.2–6 megabases). After electrophoresis, the gel was stained with ethidium bromide and photographed.

Results

Following transfection with the pCMVdutE vector, we isolated two clones of HT29 cells expressing elevated dUTPase activity, designated dutE1 and dutE7. dUTPase activities measured in cellular extracts were 26.6 ± 1.5 (dutE1) and 23.8 ± 2.9 (dutE7) pmol dUMP formed/min/μg protein (n = 4). Cellular extracts obtained from HT29 cells transfected with the control vector, pCMV-Neo-Bam (clones con2 and con3), were found to contain dUTPase activity of 6.9 ± 2.8 and 5.4 ± 1.2 pmol/min/μg protein (n = 4), respectively. These values are slightly less than we had reported previously for parental HT29 cells (7). Therefore, the two dutE clones contain approximately 4- to 5-fold higher dUTPase activities as compared to the control transfectants.

To determine whether this increase in dUTPase activity could modulate sensitivity to FdUrd-induced cytotoxicity, we measured the loss in clonogenicity following treatment with various concentrations of FdUrd for 24 or 48 h (Table 1). Survival of the dutE cell lines was greater than the control cell lines under all conditions tested except for 24 h/10 nM (which was not substantially toxic to any of the cell lines) and 48 h/1000 nM. This difference reached statistical significance in three of the groups shown. In additional studies not shown here, survival of all four cell lines was measured after exposure to 100 nM FdUrd for 16, 32, or 40 h. At all time points, survival of the dutE cell lines exceeded that of the control cell lines, typically by 2–3-fold.

The increased survival in dutE1 and dutE7 correlated with attenuated DNA double strand break formation as measured by pulsed field gel electrophoresis. Following treatment with 100 nM FdUrd, con2 and con3 cells displayed a similar rate of DNA double strand break formation as reported previously for the parental HT29 cell line (Fig. 1; Refs. 2 and 7). DNA fragmentation was detectable following 16 h of exposure in control transfectants, whereas it was only detectable after 40 h of treatment in dutE1 and dutE7 cells, very similar to that previously demonstrated in DNA damage-resistant SW620 cells (2, 7). Therefore, similar elevations in dUTPase activity correlate with similar delays in the induction of DNA fragmentation in dutE HT29 transfectants and SW620 cells.

Discussion

A substantial body of evidence suggests that DNA strand breakage caused by TS inhibitors is, in some cell lines, mediated through an increase in intracellular dUTP levels that leads to uracil misincorporation and misrepair (3, 4, 14). dUTPase has been postulated to play a protective role in this process by opposing dUTP pool expansion, thereby mitigating DNA damage and cytotoxicity. Here we demon-

strate directly that elevating dUTPase activity through gene transfer greatly diminishes the extent of DNA fragmentation and delays cytotoxicity in HT29 human colorectal tumor cells.

The finding that dUTPase overexpression can confer resistance to fluoropyrimidine treatment *in vitro* does not necessarily mean that this enzyme is an important determinant of drug response *in vivo*. Thus, if dUTPase activities were found to be similar in many tissue types or tumors, then it would be unlikely that differences in expression of this enzyme would be responsible for heterogeneity of drug sensitivity to any great extent. However, activities of dUTPase measured in extracts from human cells have been found to vary significantly (15, 16), and Beck *et al.* (16) reported an inverse relationship between intracellular dUTPase levels and methotrexate cytotoxicity among several different cell lines. Also, McIntosh *et al.* (17) recently described several different dUTPase mRNA species whose levels of expression vary among different tissues. Furthermore, dUTPase activity appears to be regulated through protein phosphorylation (18, 19). It is therefore apparent that dUTPase activities do vary widely enough so that the level of expression of this enzyme could be a critical determinant of intrinsic fluoropyrimidine sensitivity.

In addition to possibly having a role in naturally occurring fluoropyrimidine resistance, dUTPase may also be useful in the protection of host tissues from fluoropyrimidine cytotoxicity. One obvious strategy for using gene transfer to protect cells from fluoropyrimidines is to elevate expression of the initial target enzyme, TS. Although this approach would be expected to raise the maximum tolerated drug concentration roughly in proportion to the level of TS expression obtained, very high drug concentrations (such as those found after bolus injection of 5-fluorouracil) might still produce complete TS inhibition for some time period. However, if dUTPase were concurrently overexpressed in these cells, the impact of a relatively brief TS blockade might be considerably reduced.

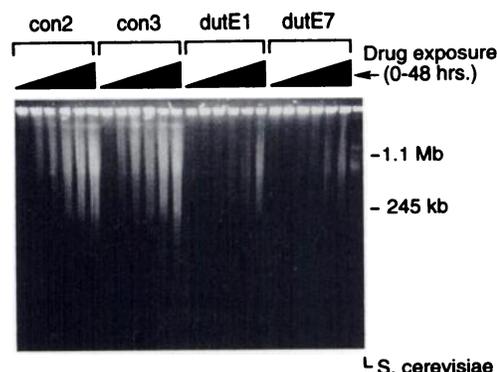Although our data clearly show that dUTPase expression prevents

Fig. 1. Attenuation of FdUrd-induced DNA double strand break formation by expression of *E. coli* dUTPase. HT29 cells transfected with either the control vector pCMV-Neo-Bam (con2 and con3) or the dUTPase expression vector pCMVdutE (dutE1 and dutE7) were exposed to 100 nM FdUrd for 0, 16, 24, 32, 40, or 48 h and then harvested and analyzed by pulsed field gel electrophoresis.

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fluorodeoxyuridine-induced DNA damage and cytotoxicity in one cell line, several aspects of this phenomenon remain to be investigated. Whereas some cell lines (such as HT29) respond to TS inhibition with one pattern of genomic DNA fragmentation, the fragmentation observed in other lines appears to be a stereotyped response, common to a variety of cellular insults, that may represent a cell death program (2, 20). It is not known if elevation of dUTPase would affect response to fluoropyrimidines in the latter cell type. In regard to therapeutics, it would also be important to determine if the magnitude of the dUTPase effect is similar for all TS inhibitors; because FdUTP incorporation may contribute to fluoropyrimidine cytotoxicity in some cases (5, 21, 22), it is possible that dUTPase expression (which would prevent accumulation of FdUTP as well as dUTP) may be more effective at protecting cells from fluoropyrimidines than from other kinds of TS inhibitors. In addition, the quantitative relationship between dUTPase expression and protection from fluoropyrimidine effects is unknown. Although the level of fluoropyrimidine resistance induced in HT29 cells is significant, it would be useful to know if higher levels of dUTPase expression can suppress fluoropyrimidine actions even further. Finally, because drug resistance is usually a multifactorial process, a thorough evaluation of the role of dUTPase in cellular response to fluoropyrimidines should include an evaluation of whether the effects of modulating this enzymatic activity depends on the level of other activities involved in fluoropyrimidine-induced DNA damage and cytotoxicity, such as TS and uracil-DNA glycosylase.

Acknowledgments

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