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Short communication

Development and initial characterization of a mitomycin C-resistant colon cancer cell line variant

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Abstract. Resistance may limit the clinical usefulness of a variety of chemotherapeutic drugs, including mitomycin C (MMC). In order to study resistance to MMC, a variant of the HT-29 human colon cell line was isolated by exposure to repeated doses of MMC. The 95% inhibitory concentration of MMC for this isolate (HT-29R13) was found to be approximately twice that for the parent line. The level of resistance did not increase with additional drug exposure, and resistance was stable for at least 6 months in the absence of drug exposure. HT-29R13 cells exhibit cross-resistance to melphalan and 5-FU but not to doxorubicin, cis-platinum, or etoposide. HT-29R13 cells are characterized by slightly decreased plating efficiency and slightly increased total protein compared with the parent line. This model of stable, low-level MMC resistance with an unusual cross-resistance pattern may prove useful for the study and characterization of MMC resistance mechanisms.

Introduction

The initial presence or subsequent emergence of drug resistance limits the usefulness of chemotherapeutic drugs. Mitomycin C (MMC) is one of the few active drugs against gastrointestinal malignancy, including colorectal cancer [11]. This drug has several interesting properties, including apparent increase in activity under hypoxic conditions [7]. Resistance to MMC has been described as part of the multidrug resistance (MDR) phenotype modulated by P-glycoprotein [12]. Other mechanisms, including deficiency of DT-diaphorase, an enzyme thought to be important in the reduction of MMC to its active form, have also been shown to be responsible for resistance in certain situations [10, 14].

However, there are only a few models available in which MMC resistance was specifically induced and selected. Much of the work thus far has been performed in the HCT116 human colon cancer cell line and several variants [9]. Resistance in this system has been attributed to increased drug efflux and decreased drug activation [2]. There remains a great deal of controversy regarding the relationship of various mechanisms to the actual development of MMC resistance [10]. In order to learn more about MMC resistance, we established a new MMC-resistant variant of the human colon cancer cell line HT-29. This variant, which we have designated HT-29R13, is not cross-resistant to doxorubicin and has several interesting characteristics.

Materials and methods

Cell line and culture techniques. The HT-29 cell line was obtained from American Tissue Culture Collection (ATCC, Rockville, Md.). Cells were maintained in Ham's F-12 medium supplemented with 20% newborn calf serum, glutamine, penicillin, and streptomycin. The cells were incubated at 37° C in a 95% air/5% CO_2 environment.

Drugs and biochemicals. MMC was kindly provided by Daniel Elliott of Bristol-Myers Co. (Evansville, Ind.). Fresh solutions were prepared on the day of each experiment. All other chemotherapeutic drugs and chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.).

Selection of resistant variant. To select for resistance to MMC, 5×10^5 HT-29 cells in logarithmic growth phase were exposed in T-75 flasks for 1 h to 2 μM MMC. The treated cells were cultured in fresh medium which was changed twice weekly until the survivors resumed logarithmic growth phase. The cells were subsequently passaged and the MMC treatment repeated weekly whenever possible.

Clonogenic assay. This was performed as previously described [13] with minor modifications. Briefly, 3×10^5 cells in logarithmic growth phase were plated in 60-mm petri dishes 24 h prior to drug exposure for 1 h or 24 h. After completion of drug treatment, the cells were trypsinized,

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Table 1. Cell line characteristics

	HT-29	HT-29R13
$TD^1/_2$ (h)	28.0 ±0.5	28.7 ±0.7
PE	0.78±0.02	0.70±0.02*
Volume (fl)	1.64±0.09	1.66±0.13
Protein (mg/10^6 cells)	0.41±0.03	0.50±0.02*
DNA content	1.0	1.0
% G_0/G_1	53.8 ±4.2	55.6 ±3.8
% S	29.2 ±7.0	23.3 ±1.9
% G_2-M	17.0 ±3.2	21.2 ±5.6

All results mean ± SEM $TD^1/_2$, doubling time; PE, plating efficiency
* P <0.01, Wilcoxon rank-sum test

Table 2. Sensitivity of HT-29 and HT-29R13 to cytotoxic drugs

Drug	IC_{95}(μM)	
	HT-29	HT-29R13
MMC	2.04	4.25*
Doxorubicin	2.61	2.76
Cis-platinum	72.6	78.3
Etoposide	386	395
Melphalan	46.2	79.8*
5-FU	634	8122*

IC_{95} values were obtained from clonogenic assays after a 1-h drug treatment in vitro
* P <0.05, Wilcoxon rank-sum test

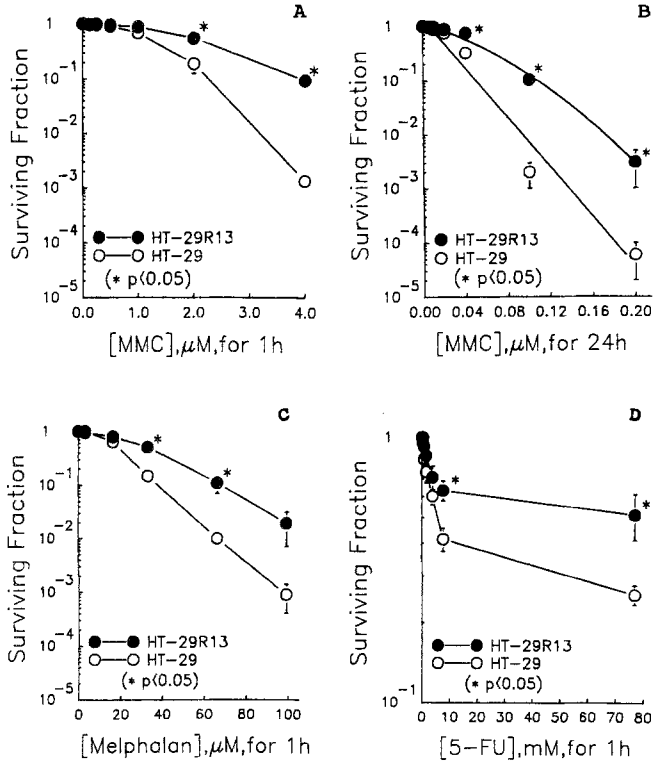

Fig. 1. Clonogenic cell survival comparing sensitivity of HT-29 cells and HT-29R13 cells with **A** 1-h and **B** 24-hour MMC treatments. Note the significant MMC resistance of the R13 variant compared with the parental HT-29 line with either treatment interval. The HT-29R13 variant was cross-resistant **C** to melphalan and **D** to 5-FU

washed, and counted. Serial dilutions were performed at each experimental point and each dilution was plated in triplicate. Ten days later, the plates were fixed with methanol : acetic acid and stained with crystal violet. Macroscopic colonies greater than 50 cells were counted, and survival curves were constructed by plotting the surviving fractions against drug concentrations. Each survival curve experiment was performed at least three times.

Other assays. To determine cell cycle kinetics, single cell preparations stained with propidium iodide were analyzed on a Coulter EPICS 753 flow cytometer (Coulter Electronics, Hialeah, Florida) as previously described [13]. Cell volume was determined using a Coulter ZBI particle counter after calibrating the instrument using 10-μm spheres. Cell protein was measured using the Bradford method [1].

Results

After a decline in the HT-29 surviving fraction with each of the first four MMC treatments, the surviving fraction began to return to baseline with subsequent treatments (data not shown). After six MMC treatments over a period of 8 weeks, the sensitivity of the cells to subsequent drug treatment did not change appreciably. Aliquots of cells were saved and not subjected to further drug treatment, with the HT-29R13 (13 treatments over 18 weeks) variant being used in subsequent experiments. The basic characteristics of the R13 variant compared to the parental HT-29 line are shown in Table 1. There were no significant differences in doubling time or cell cycle kinetics, but modest differences in plating efficiency and protein content were noted. There were no significant morphologic differences, with both the R13 variant and the parent line consisting of small round to oval cells which form tightly packed colonies.

The MMC resistance of the R13 variant compared with the parent line is shown in Fig. 1. In addition to a 1-h MMC treatment (Fig. 1A), a 24-h treatment was examined (Fig. 1B) because of the importance of treatment duration on MMC cytotoxicity [13]. The MMC resistance of the R13 variant was modestly but significantly greater with either duration of drug exposure. Thus far we have not been able to induce higher levels of MMC resistance in this model system (data not shown). HT-29R13 demonstrated significant cross-resistance to melphalan (Fig. 1C) and 5-FU (Fig. 1D). No significant cross-resistance was demonstrated to doxorubicin, cis-platinum, or etoposide (Table 2). Even in the absence of additional drug exposure, the R13 variant has shown continued resistance to MMC for a period of more than 6 months (data not shown).

Discussion

This study has shown the HT-29R13 subline to be significantly resistant to MMC compared with the parental HT-29 line, with cross-resistance to melphalan and 5-FU but not to doxorubicin, cis-platinum, or etoposide. This model is particularly interesting in that the R13 variant possesses a low level of resistance to MMC which was not found to increase with additional exposure to the drug. Equally significant is that once MMC resistance is induced, the re-

sistant phenotype persists, even in the absence of drug exposure for more than 6 months. The UV-2237 ADM murine fibrosarcoma line, which exhibits cross-resistance to MMC, shows significant reversion towards normal drug sensitivity when cultured in the absence of doxorubicin for 4 months [5]. However, HCT116 variants which are MMC resistant are characterized by stable resistance [4].

The stability of the MMC resistance in HT-29R13 suggests that an underlying genetic change has been induced. Recently, a human gene has been identified which can confer resistance to MMC-sensitive Chinese hamster ovary (CHO) cells [3]. The identity of this gene is not yet known, but it appears to be unrelated to P-glycoprotein [3].

HT-29R13 cells differ in several respects from previously described MMC resistance models. P388/MMC [16] and CHO-MMC [6] cells show much higher levels of resistance and different cross-resistance patterns than HT-29R13. Several HCT116 variants exhibit resistance to MMC twice as great as that in the parent strain [4], similar to HT-29R13 in our study. However, with additional drug exposure, MMC resistance in HCT116 cells was noted to increase, unlike our study, where additional treatment did not result in any further increase in resistance.

A variety of mechanisms have been associated with MMC resistance, including reduced drug concentration or decreased drug activation [2], glutathione-*S*-transferase [2], cell surface protein alterations [17], cytosolic protein and phosphoprotein changes [15], and P-glycoprotein-mediated MDR [5, 12]. Thus, multiple mechanisms may be responsible for MMC resistance, similar to the situation with doxorubicin resistance, where P-glycoprotein and glutathione-*S*-transferase have been shown to be important [8]. It will be interesting to determine which of these resistance mechanisms are present in the model described in this paper, or whether in fact other mechanisms are responsible.

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