Down-regulation of DNA mismatch repair proteins in human and murine tumor spheroids: implications for multicellular resistance to alkylating agents

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Abstract

Similar to other anticancer agents, intrinsic or acquired resistance to DNA-damaging chemotherapeutics is a major obstacle for cancer therapy. Current strategies aimed at overcoming this problem are mostly based on the premise that tumor cells acquire heritable genetic mutations that contribute to drug resistance. Here, we present evidence for an epigenetic, tumor cell adhesion–mediated, and reversible form of drug resistance that is associated with a reduction of DNA mismatch repair proteins PMS2 and/or MLH1 as well as other members of this DNA repair process. Growth of human breast cancer, human melanoma, and murine EMT-6 breast cancer cell lines as multicellular spheroids in vitro, which is associated with increased resistance to many chemotherapeutic drugs, including alkylating agents, is shown to lead to a reproducible down-regulation of PMS2, MLH1, or, in some cases, both as well as MSH6, MSH3, and MSH2. The observed down-regulation is in part reversible by treatment of tumor spheroids with the DNA-demethylating agent, 5-azacytidine. Thus, treatment of EMT-6 mouse mammary carcinoma spheroids with 5-azacytidine resulted in reduced and/or disrupted cell-cell adhesion, which in turn sensitized tumor spheroids to cisplatin-mediated killing in vitro. Our results suggest that antiadhesive agents might sensitize tumor spheroids to alkylating agents in part by reversing or preventing reduced DNA mismatch repair activity and that the chemosensitization properties of 5-azacytidine may conceivably reflect its role as a potential antiadhesive agent as well as reversal agent for MLH1 gene silencing in human tumors. [Mol Cancer Ther 2005;4(10):1484–94]

Introduction

Resistance to anticancer drugs continues to be a major problem, limiting the success of cancer therapy. Recent studies defining point mutations in the bcr-abl oncogene or c-kit receptor tyrosine kinase encoding gene, which underlie acquired resistance to imatinib (STI571/Gleevec) in chronic myelogenous leukemia or gastrointestinal stromal tumors (1), have, if anything, reinforced the perceived importance of mutation-driven mechanisms responsible for drug resistance. However, in contrast to molecularly targeted drugs, similar mutational mechanisms that are unambiguously responsible for clinical forms of intrinsic or acquired resistance to conventional cytotoxic anticancer agents, such as DNA-damaging agents, have proven much more elusive to uncover and validate. In part, this may be a reflection that resistance to such drugs may be mediated in many instances by epigenetic, environmentally mediated mechanisms, such as hypoxia, low tumor cell growth fractions (population kinetics), and high intratumoral interstitial fluid pressures that act to impede drug delivery (2, 3). Some of these physiologic mechanisms of resistance operate at the multicellular (tissue) level and can be detected and studied in vitro when tumor cells are grown as three-dimensional multicellular tumor spheroids rather than as dispersed monolayer cell cultures (2). Spheroids are also more intrinsically resistant to the cytotoxic effects of most chemotherapeutic drugs, a phenomenon we have termed “multicellular resistance” (2, 4).

Most studies of chemotherapeutic drug resistance using tumor spheroid models deal with intrinsic, as opposed to acquired, resistance. However, there are some notable exceptions. For example, Teicher et al. reported the isolation in vitro of alkylating agent–resistant variants of the EMT-6 mouse mammary carcinoma, which lost their relative resistance properties when cultured in vitro as monolayers; however, the drug-resistant properties of these variants could be reexpressed by regrowth in vitro as solid tumors, thus giving rise to the concept of a new form of drug resistance “operative only in vivo” (5). Subsequently, we showed that the drug resistance properties of the EMT-6 variants could be reexpressed in vitro but only if the cells...
were cultured as three-dimensional spheroids (2, 4). Furthermore, it was found that EMT-6 spheroid disruption with preparations of bovine testicular hyaluronidase could sensitize the cells in vitro to 4-hydroperoxycyclophosphamide and in vivo (where they were grown as ascites in the form of clumps or in vitro spheroids) to cyclophosphamide (6). With respect to examples of intrinsic resistance, operationally similar chemosensitization results were obtained by spheroid disruption using p27kip antisense oligonucleotides (7). Similarly, using E-cadherin-positive HT29 in human colorectal cancer cells, spheroid disruption could be achieved using an anti-E-cadherin antibody (SHE-78-7) as an anti-adhesive agent, which also caused sensitization to alkylating agents (8, 9). Thus, some forms of both acquired and intrinsic resistance to such drugs may be epigenetic in nature and operate at the multicellular or tissue level. These observations suggest that a therapeutic strategy directed toward disruption of cell-cell adhesion/communication could represent a potentially new approach for the sensitization of tumors that are resistant to alkylating agents (4, 6).

With respect to potential molecular mechanisms underlying or contributing to multicellular resistance, we have reported previously the down-regulation of the PMS2 DNA mismatch repair protein in EMT-6 spheroids compared with monolayers (10). Moreover, this down-regulation was more marked in spheroids of drug-resistant variants compared with EMT-6 parental cells. Analysis of EMT-6 and drug-resistant tumors, by Western blotting, showed that three of four drug-resistant variants grown in vitro expressed lower levels of PMS2 compared with the parental tumor. We also confirmed a role for loss of PMS2 in resistance to alkylating agents by showing that genetically deficient PMS2−/− mouse embryo fibroblasts were significantly more resistant to cisplatin than wild-type mouse embryo fibroblasts using in vitro colony formation assays. PMS2 down-regulation in spheroids was associated with a reduced level of DNA mismatch repair (which is the prime known function of PMS2) compared with monolayer cultures. Finally, transfection of a dominant-negative PMS2 construct in EMT-6 cells resulted in increased resistance to cisplatin. Loss of PMS2 is thought to allow cells to bypass the detection of base mismatches, which are typically caused by alkylating agents (11–14). This could directly result in relative resistance to DNA-damaging agents as well as facilitating indirect mechanisms of resistance due to secondary mutations caused by increased genetic instability. We therefore proposed that a reduction (as opposed to loss) of PMS2 might allow for reduced detection of DNA mismatches and increased cell survival following treatment with alkylating agents, as is observed in spheroid cultures, when compared with monolayers (10). Thus, the purpose of this study was to probe and analyze further the role of PMS2 and other enzymes involved in DNA mismatch repair with respect to the resistance properties of tumor spheroids to alkylating agents. We now report analysis of PMS2 expression in spheroids of both murine EMT-6 and human cancer cell lines. Analysis of the PMS2-binding protein MLH1 revealed in several human breast and melanoma cell lines a striking down-regulation of this protein in spheroids compared with monolayers. In addition, we also observed the down-regulation of one or more components of this process (MSH3, MSH6, or MSH2) in some tumor spheroids compared with monolayers. Treatment with 5-azacytidine (a methylation inhibitor that has been used in trials for the treatment of leukemias; refs. 15, 16) reversed MLH1 down-regulation in EMT-6-resistant spheroids. Together, the results reinforce the hypothesis that a generalized deficiency in DNA mismatch repair, which is not reversible and mutational in nature, may underlie some forms of multicellular resistance, especially to alkylating agents.

Materials and Methods

Materials

5′-Azacytidine, cisplatin [cis-platinum(II)-diaminedichloride], 2′-deoxy-5′-azacytidine, and trichostatin A were all purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Hyaluronidase was obtained from Worthington (Freehold, NJ). The primary antibodies used for Western blotting were anti-MLH1 (clone G168-15, BD PharMingen, Mississauga, Ontario, Canada), anti-PMS2 (clone A16-6, BD PharMingen), anti-MSH6/GETBP (clone 44, BD Transduction Laboratories, Franklin Lakes, NJ), anti-MSH3 (clone 52, BD Transduction Laboratories), anti-MSH2 (Oncogene Research Products, San Diego, CA), anti-(pan)extracellular signal-regulated kinase (ERK; clone 16, BD Transduction Laboratories), anti-PTEN (MMAc1 Ab-2, Medicorp, Montreal, Quebec, Canada), anti–glyceraldehyde-3-phosphate dehydrogenase (clone 6C5, Ambion, North York, Ontario, Canada), and anti-β-actin (clone AC-15, Sigma, Oakville, Ontario, Canada).

Cell Culture

The EMT-6 mouse mammary tumor cell line and the EMT-6 variants used in this study were grown in Waymouth’s medium. Human RKO (colon), SW626 (ovarian), CaOV3 (ovarian), PA-1 (ovarian), WM35 (melanoma), WM1341B (melanoma), MDA-MB-435 (breast), MDA-MB-435.T0.1 (breast), and MDA-MB-231 (breast) carcinoma cells were grown in DMEM (17). Human HT29 (colon), LNCaP (prostate), WM239 (melanoma), WM9 (melanoma), SkOv3 (ovarian), A2780 (ovarian), and A2780DDP (ovarian) cell lines were grown in RPMI. All media were supplemented with 10% fetal bovine serum and 4 mmol/L l-glutamine. Cells were maintained in a 37°C humidified incubator with an atmosphere of 5% CO2. Single-cell suspensions were prepared by treatment with trypsin-EDTA (Life Technologies, Inc., Carlsbad, CA) and resuspension in complete medium before setting up monolayer or spheroid cultures. For some of the cell lines employed [e.g., MDA-MB-231 (18), EMT-6 (2, 6), and HT29 and CaOV3 (4, 9)], the level of resistance in spheroids (to alkylating agents) has been reported. It should be noted that although multicellular resistance is not a commonly observed phenomenon in epithelial tumor cells (4, 19) not all cell lines used in this study were tested for resistance in spheroids compared with monolayer.
Spheroid Culture
Multicellular tumor spheroids were generated as described previously (2) using the liquid overlay technique. Briefly, 24-well culture plates (Nunc, Rochester, NY) were coated with 0.2 mL of 1% Seaplaque agarose (FMC, Bioproducts, Rockland, ME). Cells (1 × 10^6) from a single-cell suspension were added per well in a total volume of 1 mL. Spheroids were allowed to form over 24 hours for EMT-6 cells (and EMT-6 variants) or 48 hours for the human cell lines as described previously (8). Where indicated, 1 × 10^5 cells were plated in 96-well plates for smaller-scale experiments.

Treatment with 5-Azacytidine, Trichostatin A, and Hyaluronidase
5-Azacytidine was made up as a 4 mmol/L stock solution in PBS, sterilized by filtering, and frozen; treatment in vitro was at a final concentration of 2 μmol/L by addition of 5-azacytidine to the spheroid medium. As indicated in the text, in some experiments, cells were pretreated as monolayers for 10 hours in complete medium containing 2 μmol/L 5-azacytidine before replating as spheroids in complete medium containing fresh 5-azacytidine. Trichos- tatin A was made up as a 3.33 mmol/L solution in DMSO and diluted in culture medium to 300 or 100 mmol/L. DMSO alone (a 1:11,000 dilution in medium; i.e., <0.01% DMSO) or medium without any DMSO were used as negative controls. Hyaluronidase was used at 2 mg/mL in Waymouth’s medium (6).

Generation of Alkylating Agent–Resistant Cell Lines
In vitro selected drug-resistant variants of murine EMT-6 and human H69 lung cancer cell lines were originally obtained from Dr. P. Twentyman (Medical Research Council Centre, Cambridge, United Kingdom). PC3 and other tumors resistant to metronomic cyclophosphamide were obtained from tumors relapsing on therapy after showing an initial and extended response; PC3-3R and PC3-3LL, variants of the PC3 human prostate cancer cell line, were obtained by mechanical and enzymatic dissec- tion from relapsed, end-stage tumors under a regimen of low-dose metronomic cyclophosphamide as described by Man et al. (20) and frozen after three passages until further use. EMT-6P cells were grown as monolayers in the presence of 50 nmol/L cisplatin for 1 week followed by escalating doses (200, 400, 600, and 800 nmol/L) of continuous cisplatin in the growth medium for a total of 6 weeks of continuous selection. Two in vitro cisplatin-resistant cell populations were thus obtained: one selected up to a final concentration of 1 μmol/L cisplatin, called EMT-6/P (1-CDDP), and a second population selected up to a final concentration of 2 μmol/L cisplatin, called EMT-6/P (2-CDDP). An aliquot of the surviving cells was frozen after 2 weeks of selection in 1 or 2 μmol/L cisplatin treatment (which typically causes 100% death of the parental line). To date, these variants have been studied up to passage 10, without loss of cisplatin resistance.

Western Blotting
Protein lysates and RNA were obtained by the Trizol method following the manufacturer’s instructions. The organic phase (3 volumes) of Trizol lysate was mixed with ethanol (1 volume) and precipitated by adding 3 volumes of acetone to 1 volume ethanol/Trizol solutions. Protein concentration was evaluated using the Bio-Rad (Hercules, CA) protein assay. Equal amounts of protein were loaded onto a 8% polyacrylamide gel and resolved by standard SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with blocking buffer [5% nonfat dry milk, 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 0.1% Tween 20]. Primary antibody was added in blocking buffer (without Tween 20) at concentrations recommended by the manufacturer. Membranes were washed with 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20. Rabbit anti-mouse horseradish peroxidase–conjugated secondary antibody was added in blocking buffer. Membranes were washed and detection was carried out using enhanced chemiluminescence (Amersham, Piscataway, NJ) and exposure to film. ERK was used as a loading control as reported previously (9). Expression from the human β-actin prom-oter was reported previously to be suppressed in spher- oids (21), although our limited analysis showed no differential expression between ERK and β-actin in analysis of monolayer and spheroid cell lysates.

Human MLH1 Promoter Methylation Analysis
For each sample, genomic DNA (25 μg) was digested with EcoRV and XbaI. Samples were then divided in three equal parts, which were digested with MspI, HpaII, or no enzyme. Southern blot analysis was then done using a MLH1 promoter probe as originally described by Stratthdee et al. (22). In addition, for each sample, DNA (5 μg) was analyzed using the MLH1 Transignal Promoter Methyla- tion Detection kit (Panomics, Redwood City, CA) following the manufacturer’s instructions.

In vitro Colony Formation Assays
Monolayer cultures pretreated with or without 5-azacyti-dine (2 μmol/L in growth medium for 10 hours) followed by exposure to various micromolar concentrations of cisplatin for 1 hour. Thereafter, cells were rinsed, trypsinized, and plated at various dilutions in a colony formation assays (2). Surviving fractions were calculated using the formula: Surviving fraction = (plating efficiency of untreated control) / (plating efficiency of untreated control). Statistical analysis was done with PRISM version 4.00 software (GraphPad, San Diego, CA). Results are reported as mean ± SD. The level of significance was set at P < 0.05 (two tailed).

Results
Down-Regulation of PMS2 and MLH1 in Tumor Spheroids
Analysis of human cancer cell lines (Figs. 1 and 2) showed that some (e.g., MDA-MB-231 and MDA-MB-435.TO.1; see Fig. 1) expressed lower levels of PMS2 protein in spheroids compared with the same cells grown as monolayers. One possible explanation for this observed down-regulation of PMS2 comes from the work of Prolla et al. (23), as well as others (24, 25), on cells lacking MLH1. Thus, cancer cell lines that have lost MLH1 expression (through mutation or
MLH1 promoter hypermethylation), or established cell lines from MLH1 knockout mice, also show down-regulation or loss of PMS2 protein. Although this MLH1-PMS2 association was not directly implied in these aforementioned reports, it occurred to us that down-regulation of PMS2 might reflect loss or down-regulation of MLH1. Hence, we tested an antibody to MLH1 for this possibility.

Our initial analysis showed some modest down-regulation in spheroids, compared with monolayers, of either PMS2 or MLH1 in some cell lines tested (Fig. 1). Further analysis showed marked down-regulation of PMS2, MLH1, and, in some cases, both in human breast, prostate, and ovarian cancer cell lines when grown as spheroids (Fig. 1; data not shown). However, we also observed that some cell lines, such as WM35 (melanoma), LNCaP (prostate), and HT29 (colon), showed no change in either protein when spheroid cultures were compared with monolayers (data not shown). Of considerable interest, we also observed down-regulation of MSH6, MSH3, and, in some cases, MSH2 in several human tumor spheroids, again compared with their respective monolayer counterparts (Fig. 1). Thus, suppression of PMS2 protein expression is

![Figure 1](image1.png)

**Figure 1.** A, Western blot analysis of DNA mismatch repair proteins in human tumor spheroids (S) and subconfluent monolayer (M) cultures. Cells were grown for 1 to 4 d and lysed, and proteins from each sample were blotted for PMS2, MLH1, PTEN, ERK, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin. ERK was subsequently used as a loading control in Western blots. B, examples of Western blotting analysis of DNA mismatch repair proteins in human cancer cell lines grown as spheroids or monolayers (Mono). Cells were lysed at the indicated times, and for each sample, proteins were tested for the expression of PMS2 and MLH1 as well as other DNA mismatch repair proteins, such as MSH6, MSH3, and MSH2. MDA-MB-435.TO.1 is a Taxol-resistant variant of the MDA-MB-435 breast cancer cell line. C and D, Western blot analysis of DNA mismatch repair proteins in human tumor spheroids and subconfluent monolayer cultures. Cells were grown for 1 to 4 d and lysed, and protein lysates were blotted for PMS2, MLH1, and MSH-2. ERK was used as a loading control.

![Figure 2](image2.png)

**Figure 2.** Growth of EMT-6/P spheroids and Western blotting analysis of EMT-6/P as monolayers or spheroids treated with or without 5-azacytidine (5-Aza). Cells were plated in 96-well plates precoated with agarose. For some samples, after growth as spheroids for 2 d, cells were treated with 5-azacytidine (1 μmol/L final concentration) or PBS (control) and left for 2 more days. At the indicated times (in days), cells were photographed under ×40 magnification and lysed, and protein from each sample was probed for expression of PMS2, MLH1, MSH6, and MSH2. Bar, 0.5 mm. ERK was used as a loading control.
definitely not restricted to the EMT-6 mouse tumor spheroid model (10). Moreover, down-regulation of MSH6, MLH1, and MSH2 was also observed in EMT-6 spheroids (Fig. 2). Therefore, growth of several human tumor spheroids is associated with reduced expression of one or more components of the DNA mismatch repair system.

Possible Role of DNA Methylation: Effect of Demethylating Agents

Because the best known mechanism of deregulating DNA mismatch repair protein expression is the DNA methylation–mediated silencing of MLH1, we tested the effect of the demethylating agent 5-azacytidine on EMT-6 tumor spheroids. The parental EMT-6/P cells were treated with 5-azacytidine, and this induced a gradual reduction in spheroid compaction (Fig. 2) accompanied by up-regulation of DNA mismatch repair proteins (Fig. 2). However, we were unable to affect spheroid compaction, in the absence of alkylating agent treatment, of the drug-resistant variants of the EMT-6 tumor; an example is shown for EMT-6/Thio (an EMT-6 variant selected in vivo for resistance to Thiotepa; Fig. 3A, i). Because these highly compact/adhesive spheroids have significantly reduced cell cycle kinetics (6, 7), we hypothesized that 5-azacytidine might not be sufficiently incorporated into the cells to cause any effect. We therefore first exposed drug-resistant variants to 5-azacytidine for 10 hours as monolayers (to allow incorporation of the drug) and then replated the cells as spheroids. The pretreatment with 5-azacytidine inhibited spheroid compaction of EMT-6 drug variants (EMT-6/Thio and EMT-6/CTX, which are variants selected in vivo for resistance to Thiotepa and cyclophosphamide respectively, are shown as examples; Fig. 3A, ii and iii) compared with controls pretreated with PBS. Interestingly, spheroid disruption by 5-azacytidine was distinct and not as extensive as that observed by treatment of spheroids with hyaluronidase, which we noted that although spheroid formation was disrupted by trichostatin A (i.e., the edges of spheroids are rough and uneven compared with DMSO-treated (used at 1:1,000 dilution in medium) controls that were indistinguishable from spheroids grown in normal medium), the effect is markedly different from that of 5-azacytidine treatment, with no obvious decreased in cell-cell adhesion in trichostatin-treated spheroids. Therefore, these observations further distinguish the down-regulation of mismatch repair protein in spheroids from that described for the same cells grown under hypoxic conditions by Mihaylova et al. (26), suggesting that at least two independent pathways can lead to suppression of DNA mismatch repair protein expression in EMT-6 cells.

PMS2 and MLH1 Expression in Other Tumor Systems/Models Involving Resistant to Alkylating Agents

We next sought to analyze DNA mismatch repair protein expression in other models of drug resistance to alkylating agents. Of particular interest to us was the evolution of resistance to metronomic low-dose cyclophosphamide in which the drug is administered to human tumor xenograft-bearing mice via the drinking water on a daily basis at low doses for extended periods (20, 27). This form of therapy is considered to target mainly endothelial cells via an antiangiogenic mechanism (27), but emergence of resistance to this therapy eventually occurs (20). However, in contrast to the EMT-6 model of Teicher et al., where tumors are exposed repeatedly to maximum tolerated doses of drugs, cell lines derived from tumors that have become resistant in vivo to low-dose daily metronomic cyclophosphamide therapy do not show any increased spheroid compaction compared with the parental cell line (Fig. 6A). Analysis of PC3 parental tumors and PC3 tumors relapsing on metronomic cyclophosphamide therapy showed no difference in their expression of PMS2 or MLH1 (Fig. 6B).

Next, we generated in vitro cisplatin-resistant populations of the parental EMT-6 tumor (by continuous exposure to cisplatin in tissue culture). The emerging resistant population showed reduced expression of MLH1 protein compared with the parental (untreated) cell line (Fig. 6C). Previously derived EMT-6 in vitro drug-resistant variants also showed a down-regulation of MLH1 (Fig. 6C), whereas no obvious change in PMS2 or MLH1 was observed in
previously derived in vitro drug-resistant variant of the human lung cancer cell line H69 (Fig. 6C). Other drug-resistant models (shown in Fig. 6) show suppressed PMS2 and MLH1 expression, but only in spheroids does this effect seem readily reversible. Therefore, although the down-regulation of PMS2 or MLH1 is not completely restricted to multicellular (spheroid) drug resistance phenotype, other in vivo and in vitro models of tumor resistance to alkylating agents would seem to arise independently of the mechanism studied here (i.e., down-regulation of DNA mismatch repair enzymes), emphasizing once again the highly pleiotropic and complex nature of drug resistance in cancer. However, the mechanism by which such suppression occurs remains to be elucidated.

**Analysis of MLH1 Promoter Methylation in the Down-Regulation of MLH1**

Our results with 5-azacytidine, as well of those of Mihaylova et al. (26) in tumor cells grown under hypoxic conditions, strongly suggested a role for MLH1 promoter methylation in the observed down-regulation of MLH1 protein expression. We therefore decided to investigate MLH1 promoter methylation in spheroids. Strathdee et al. (22) have described an elegant method for analysis of MLH1 promoter methylation for human samples. To do so, we studied two human melanoma cell lines (WM9 and WM239), which show different levels of down-regulation of MLH1 (Figs. 1 and 7) when grown as spheroids, to study the effect, if any, on the MLH1 gene promoter. Genomic DNA was extracted from monolayers and 4-day-old spheroids and a MLH1 promoter fragment was obtained by digestion with EcoRV and XhoI as described by Strathdee et al. (22). Next, each DNA sample was divided into three parts, which were treated with no enzyme, HpaII enzyme (which only cuts if the target site is not methylated), or with MspI enzyme (which cuts at

![A](image1.png)

**Figure 3.** A, morphology of EMT-6 spheroids in the absence of alkylating agent treatment. Examples of EMT-6/P and drug-resistant variants grown as spheroids following treatment with 5-azacytidine or hyaluronidase. i, spheroids of EMT-6/P (parental) and Thio-resistant variants after 24 h in tissue culture with 5-azacytidine (5-Aza, 2 μmol/L final concentration). ii, EMT-6/Thio-resistant variants were pretreated as monolayer cultures with PBS (control) or 5-azacytidine (2 μmol/L final concentration), trypsinized, and replated as spheroids in medium supplemented with PBS (control) or 5-azacytidine (2 μmol/L final concentration). Control EMT-6/Thio cells were also plated as spheroids in medium containing hyaluronidase (HA, 2 mg/mL final concentration) as a positive control for spheroid disruption. iii, EMT-6/CTX-resistant variants were pretreated as monolayer cultures with PBS (control), 5-azacytidine (2 μmol/L final concentration), or 6-azacytidine (6-Aza, 2 μmol/L final concentration), trypsinized, and replated as spheroids in medium supplemented with PBS (control), 5-azacytidine (2 μmol/L final concentration), or 6-azacytidine (2 μmol/L final concentration). Cells were photographed after 24 h of plating spheroid cultures. Magnification, ×40. Bar, 0.5 mm. B, Western blotting analysis of DNA mismatch repair proteins in EMT-6 drug-resistant variants (EMT-6/Thio and EMT-6/CTX) grown as monolayers or spheroids (Sph) in the presence or absence of 5-azacytidine, 6-azacytidine, or hyaluronidase (2 mg/mL) as described above. Lysates of monolayer cultures were included as a control. For 5-azacytidine- and 6-azacytidine-treated groups, cells were first pretreated as monolayers for 10 h with either 5-azacytidine or 6-azacytidine (2 μmol/L final concentration) and replated as spheroids in medium containing 5-azacytidine or 6-azacytidine (2 μmol/L final concentration). After 24 h of spheroid culture, cells were lysed and protein lysates were blotted for expression of PMS2 and MLH1. ERK was used as a loading control.

![B](image2.png)

**Figure 4.** EMT-6 spheroid compaction and Western blotting analysis of EMT-6/P-resistant (parental) and EMT-6/cisplatin-resistant (EMT-6/DDP) variants. Cells were plated as spheroids for 24 h in 96-well plates precoated with agarose. Numbers in brackets, approximate number of cells plated per well. Cells were photographed after 1 to 4 d of plating. Magnification, ×40. EMT-6/DDP cells formed highly cohesive spheroids compared with the parental cell line. Bar, 0.5 mm. For Western blotting, EMT-6/DDP monolayer cultures were lysed and compared with lysates of EMT-6/DDP spheroids grown for 1 to 4 d. After 4 d of spheroid culture, several EMT-6/DDP spheroids were dispersed by trypsin treatment, replated for 48 h as monolayers (Mono*), lysed, and included in the Western blotting analysis of PMS2 and MLH1. ERK was used as a loading control.
Figure 5. Effect of 5’-azacytidine and trichostatin A on EMT-6 spheroids. A, trichostatin A (Tricho) did not cause loss of spheroid compaction in EMT-6/DDP cells compared with medium alone or DMSO controls (diluted 1:11,000 in medium). No effect was observed in DMSO alone compared with medium alone. B, in contrast, 5’-azacytidine or 2’-deoxy-5’-azacytidine (2-deoxyAza) both caused the edge of EMT-6/DDP spheroids to grow in a loose fashion. C, trichostatin A treatment of EMT-6/P cells led to increased spheroid compaction in contrast to the relatively loose spheroids observed in cells treated with 5’-azacytidine or controls.

**Discussion**

The principal new findings to emerge from our studies is that the DNA mismatch repair enzyme expression is effectively suppressed at the protein level in several human and murine tumor spheroids and involves not only PSM2 but also a large and diverse family of such enzymes. Thus, our results clearly show that down-regulation of PMS2 in mouse EMT-6 spheroids (10) is not a unique or highly esoteric phenomenon, which is either restricted to this cell line, the murine species, or PMS2. Instead, it seems a more general process involving multiple DNA mismatch repair proteins, which we believe is important to document, including cell lines in which the process is not observed (e.g., HT29 human colon). Furthermore, spheroid compaction (which positively correlates with drug resistance; refs. 2, 6) and MLH1 down-regulation can be reversed by 5’-azacytidine treatment, which sensitizes EMT-6 spheroids to cisplatin. The

the same site as HpaII regardless of methylation status of the target sites). Figure 8 shows the excision of the expected MLH1 promoter fragment with EcoRV and XhoI (no further enzyme treatment; lanes 1-4) from all samples. Treatment with either HpaII or MspI resulted in full cleavage with a resulting fragment of ~350 bp. The identical result obtained with MspI or HpaII cuts indicates no obvious methylation of the target sites detected by this assay. In a repeat experiment, DNA from RKO human colon cancer cell lines was also analyzed. RKO cells have been shown to lack MLH1 expression as a consequence of methylation of the MLH1 promoter (28). The MLH1 promoter fragment from RKO cells was not digested by HpaII treatment (Fig. 8B), as expected from the reported methylation status of this region. On the other hand, the same region of DNA from WM239 cells could be digested by HpaII irrespective of whether the cells had been grown as monolayers or spheroids for 4 days. We sought to independently test for methylation of the MLH1 promoter using a methylation-specific PCR strategy provided by a Transignal Methylation kit from Panomics. As shown in Fig. 8C, following bisulfite modification of DNA, RKO cells were found to produce a PCR product only with the methylated MLH1 promoter PCR conditions. On the other hand, only unmethylated MLH1 promoter PCR products were obtained for WM239 and WM9 cells grown as either monolayers or spheroids. Thus, we were unable to detect any methylation of the MLH1 promoter to explain the down-regulation of this gene product in spheroid, although we cannot exclude methylation of other regions of the MLH1 promoter.

**5’-Azacytidine Treatment as a Chemosensitizing Strategy**

We have established previously a clear positive correlation between spheroid compaction and relative drug resistance to alkylating agents in the EMT-6 spheroid model (6). Furthermore, disruption of tumor spheroids by enzymatic (hyaluronidase), molecular (p27kip antisense oligo treatment), or antibody (e.g., anti-E-cadherin antibody treatment of human HT29 spheroids) methods was shown in each case to result in chemosensitization of spheroids to alkylating agents (6, 7, 9). We therefore sought to determine the effect of 5-azacytidine on EMT-6/DDP spheroids treated with cisplatin. To ensure incorporation of 5-azacytidine, cells were pretreated as monolayers with this drug (or PBS only for controls) as described in Materials and Methods. By colony formation assay, we noted increased cisplatin killing in EMT-6/DDP spheroids disrupted by 5-azacytidine (Fig. 9A) compared with PBS (control)–treated spheroids (Fig. 9B). Therefore, 5-azacytidine sensitizes EMT-6/DDP tumor spheroids to cisplatin in vitro.
suppression of DNA mismatch repair proteins in spheroids seems to occur in the absence of heritable inactivating mutations in either PMS2 or MLH1. Instead, an epigenetic down-regulation of DNA mismatch repair is implicated, which is experimentally reversible in at least two ways: either by treatment with the demethylating agent 5-azacytidine or by replating as monolayers (e.g., Figs. 4 and 7).

An alternative and intriguing interpretation of our results is not necessarily to consider the notion that spheroids have a “suppressed” DNA mismatch repair system but rather that monolayer culture systems abnormally overexpress the DNA mismatch repair machinery, which would be a distortion of the situation in vivo. If so, drugs such as cisplatin would be much more effective, perhaps artificially so, at cell killing when tested on monolayer (unicellular) tissue culture conditions than they might be in a three-dimensional tumor mass whether in vitro or in vivo.

Unfortunately, we have not yet been able to determine the molecular mechanism(s) leading to the down-regulation of DNA mismatch repair proteins, as our limited analysis of the MLH1 promoter failed to detect any role in the methylation of the promoter of this gene. This result is not entirely surprising because methylation of the MLH1 promoter sites we analyzed is associated with MLH1 gene silencing (ref. 22; as opposed to down-regulation). Although we cannot yet rule out promoter methylation (e.g., in other regions of the MLH1 promoter), the mechanism(s) for suppression of DNA mismatch repair proteins in tumor spheroids remains to be elucidated.

With respect to precedents for our results, Mihaylova et al. (26) reported recently a down-regulation and suppression of the DNA mismatch repair system in the EMT-6 cell line model when the cells were grown under hypoxic conditions. However, in contrast to our previous findings showing a down-regulation of PMS2 mRNA in tumor spheroids compared with monolayers (10), Mihaylova et al. only observed translational (but not transcriptional) down-regulation of PMS2. In addition, although in agreement with our data, these investigators observed inhibition of MLH1 and PMS2 down-regulation when cells were treated with 5-azacytidine, our observations differs from theirs with regard to the histone deacetylase inhibitor, trichostatin A. Thus, this agent was reported to inhibit the hypoxia-induced down-regulation of MLH1 and PMS2 (26), but such an effect was not observed in our study with EMT-6 tumor spheroids. Finally, Mihaylova et al. found no change in MSH6 or MSH2 in EMT-6 grown under hypoxic conditions, whereas we observed a down-regulation of these proteins in several spheroid models. Taken together, these observations suggest that three-dimensional tumor growth and hypoxia independently contribute to the down-regulation of the DNA mismatch repair system.

To assimilate recent findings by Mihaylova et al. as well as our own observation into a testable model, we propose the scheme shown in Fig. 10. We presume that following cell transformation leading to a population capable of growing as a three-dimensional (multicellular) mass (e.g., by escaping from anoikis; ref. 29), cell-cell adhesion interactions (4, 6), as well as hypoxia, lead to relative suppression of the DNA mismatch repair mechanism. This in turn contributes to increased DNA damage, eventually leading to mutational changes that contribute to tumor progression. In this simple model, we therefore consider the Teicher et al. EMT-6 alkylating agent–resistant model (5) to be the result of an enrichment of a subpopulation with increased cell-cell adhesion and down-regulation of the DNA mismatch repair system. The model of Fig. 10
may also help to explain the phenomenon of low-level microsatellite instability as described by Tomlinson et al. (30) and Halford et al. (ref. 31; i.e., a subset of human tumors that show reduced mismatch repair activity yet seem to maintain a functional, albeit suppressed, DNA mismatch repair system). Clearly, more work on the mechanisms involved in the suppression of DNA mismatch repair (e.g., MLH1 promoter methylation status and microsatellite stability measurements) will be necessary to test the validity of this hypothesis. Interestingly, another mechanism of suppression of a DNA mismatch repair protein, MSH2, has been described (i.e., as a consequence of Bcl-2 expression; ref. 32). It is also imperative to note that other mechanisms, independent of the DNA mismatch repair machinery (e.g., low tumor cell growth fraction or high intratumoral interstitial fluid pressure; refs. 2, 3), act in concert to produce the resulting levels of drug resistance and genetic instability (33) observed at the tumor tissue level.

Demethylating agents, such as 5-azacytidine and 2'-deoxy-5'-azacytidine (Decitabine in clinical trials), have been shown to effectively target methylated genes in human tumors (34) and to reverse drug resistance to alkylating agents in some preclinical models of ovarian and colorectal cancer (35). Therefore, demethylating agents are being considered as potential chemosensitizers.
for human tumors that have silenced MLH1 expression as a consequence of hypermethylation of the MLH1 promoter (34, 35). Our results suggest that of 5-azacytidine may also be of use as a chemosensitizer for tumors that express positive but low levels of DNA mismatch repair activity, such as microsatellite instability–low tumors.

One particular example of how the current dogma of complete loss or inactivation of two copies of a tumor suppressor gene (“two-hit hypothesis”) is required for tumor development and progression might have some notable exceptions comes from the work of Nicolaides et al. (36). Analysis of one patient with a single inherited mutant PMS2 allele showed microsatellite instability in normal healthy tissue (37). Because microsatellite instability has been associated with loss or silencing of both alleles, this patient’s condition seemed to contradict the two-hit hypothesis (i.e., healthy tissues had one copy of the wild-type allele yet exhibited microsatellite instability). Genetic and biochemical analyses were used to show that the mutant PMS2 allele from this patient acted in a “dominant-negative” fashion, reducing the level of DNA mismatch repair in experimental models. This reduction presumably contributed to the observed microsatellite instability in normal healthy tissues (36). The apparently unique case presented by Nicolaides et al. (36) may have other similar examples, were it not for the current dogma to classify microsatellite instability as merely present or absent; for example, several observations led Tomlinson et al. to ask the question whether low-level microsatellite instability exists (30), which was recently addressed by the same group through analysis of clinical studies (31). One possible mechanism to explain low microsatellite instability levels could be a reduction (but not necessarily compete loss) of DNA mismatch repair. In this respect, we have shown previously the down-regulation of PMS2 in EMT-6 drug-resistant tumor spheroids compared with the relative drug-sensitive monolayer cultures (10). Moreover, this reduction was associated with reduced DNA mismatch repair in EMT-6 tumor spheroids compared with monolayers. Because loss of PMS2 was shown to increase survival after cisplatin exposure (10), consistent with data by others showing a correlation between loss of PMS2-mediated DNA mismatch repair and increased resistance to alkylating agents (23, 24), we proposed that reduced PMS2 and DNA mismatch repair in tumor spheroids might contribute to their relative resistance to alkylating agents. Our results reported herein are consistent with this possibility.

In summary, our results suggest a generalized deficiency of DNA mismatch repair protein expression in both murine and human tumor spheroids (in vitro models of solid tumors in vivo) when compared with conventional monolayer cell cultures. As such, this may represent a common mechanism to help explain relative resistance of solid tumors to alkylating agents in addition to other mechanisms, such as reduced drug penetration, hypoxia, and low tumor cell growth fractions.
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References

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