Role of Estrogen Receptor Gene Demethylation and DNA Methyltransferase-DNA Adduct Formation in 5-Aza-2'-deoxycytidine-induced Cytotoxicity in Human Breast Cancer Cells*

(Received for publication, May 19, 1997, and in revised form, September 3, 1997)

Anne T. Ferguson‡§, Paula M. Vertino†§, Jeffrey R. Spitzner‡, Stephen B. Baylin‡, Mark T. Muller‡, and Nancy E. Davidson‡**

From the Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, the Division of Cancer Biology, Department of Radiation Oncology, Emory University School of Medicine, Atlanta, Georgia 30335, and the Department of Molecular Genetics, Ohio State University, Columbus, Ohio 43210

The cytosine analog 5-aza-2'-deoxycytidine is a potent inhibitor of DNA methyltransferase. Its cytotoxicity has been attributed to several possible mechanisms including reexpression of growth suppressor genes and formation of covalent adducts between DNA methyltransferase and 5-aza-2'-deoxycytidine-substituted DNA which may lead to steric inhibition of DNA function. In this study, we use a panel of human breast cancer cell lines as a model system to examine the relative contribution of two mechanisms, gene reactivation and adduct formation. Estrogen receptor-negative cells, which have a hypermethylated estrogen receptor gene promoter, are more sensitive than estrogen receptor-positive cells and underwent apoptosis in response to 5-aza-2'-deoxycytidine. For the first time, we show that reactivation of a gene silenced by methylation, estrogen receptor, plays a major role in this toxicity in one estrogen receptor-negative cell line as treatment of the cells with anti-estrogen-blocked cell death. However, drug sensitivity of other tumor cell lines correlated best with increased levels of DNA methyltransferase activity and formation DNA-DNA methyltransferase adducts as analyzed in situ. Therefore, both reexpression of genes like estrogen receptor and formation of covalent enzyme-DNA adducts can play a role in 5-aza-2'-deoxycytidine toxicity in cancer cells.

Current studies suggest that DNA methyltransferase (DNA MTase), the enzyme that methylates cytosines that are 5' to guanosines, plays a role in human carcinogenesis. In general, the level of DNA MTase activity is elevated significantly in neoplastic cells compared with normal cells (1). Moreover, increased enzyme activity is characteristic of the progression of both colon and lung cancer (2, 3). Studies demonstrate that overexpression of DNA MTase leads to the tumorigenic conversion of NIH3T3 cells (4), whereas decreasing the levels of DNA MTase through a combination of genetic and pharmacologic means drastically reduces the incidence of colonic adenomas in the Apcmin mouse model of colon carcinogenesis (5). These studies provide substantial evidence for the involvement of DNA MTase in oncogenesis.

There are at least two potential mechanisms by which DNA MTase may influence oncogenicity. Elevated levels of DNA methylation may lead to increased frequency of C to T transition mutations derived from deamination of methylcytosine (6). Alternatively, increased DNA MTase may play a role in the establishment of altered patterns of methylation at CpG island sequences found in the 5' region of genes involved in growth control and tumor progression (7). For example, aberrant hypermethylation of CpG islands in cancer cells has been implicated in the transcriptional inactivation of the Rb, p16, estrogen receptor (ER), E-cadherin, and glutathione S-transferase Pi genes (8–12).

These studies have sparked a renewed interest in the use of DNA MTase inhibitors such as the cytosine analogs 5-aza-2'-deoxycytidine and 5-aza-2'-deoxycytidine (5-aza-dC) in the treatment of human cancers. In vitro studies on the mechanism of action of 5-aza-dC indicate that the interaction of cytosine methyltransferases with the 5-aza-dC-substituted DNA in the presence of S-adenosylmethionine results in the irreversible binding of the cysteine in the catalytic center of the enzyme to the 6-position of the cytidine ring (Fig. 1 and Refs. 13 and 14). Consequently, 5-aza-dC-treated cells are depleted of active DNA MTase through sequestration of the enzyme to azacytosine residues in DNA, resulting in genome-wide demethylation.

5-Azacytidine and 5-aza-dC have shown some clinical utility in the treatment of human hemoglobinopathies and malignancies (15–17). At least part of their success in these settings has been attributed to induction of cellular differentiation resulting from hypomethylation and changes in gene expression (17). For example, in patients with sickle cell anemia or β-thalassemia, 5-aza-dC treatment leads to reexpression of the developmentally silenced fetal hemoglobin gene (15). Furthermore, 5-aza-dC can induce the differentiation of immature blasts to more mature cells in patients with myelodysplastic and acute myeloid leukemias (16). However, the extent to which the clinical efficacy of 5-aza-dC is related to alteration of gene expression is still in question.

Recently, it has been suggested that the cellular effects of 5-aza-dC are a direct result of the formation of stable adducts between DNA MTase and 5-aza-dC-substituted genomic DNA rather than the ensuing DNA hypomethylation. Such adducts could sterically inhibit DNA replication, transcription, and DNA repair and may play a role in 5-aza-dC-induced mutagenesis (18–21). If DNA MTase-DNA adducts are responsible for...
5-aza-dC-mediated cytotoxicity, then cells with lower DNA MTase levels would be expected to produce fewer protein-DNA adducts and be less sensitive to the drug. In support of this hypothesis, embryonic stem cells with reduced levels of DNA MTase caused by a targeted disruption of the DNA MTase gene are less sensitive to 5-aza-dC (22).

The present study was designed to examine the relative roles of hypomethylation-induced changes in gene expression and DNA MTase-DNA adduct formation in 5-aza-dC-mediated cytotoxicity using a panel of human breast cancer cell lines as a model system. Previously we have shown that high levels of DNA MTase activity correlate with the more aggressive ER-negative tumor phenotype in a series of established breast cancer cell lines (23). The absence of ER gene expression in these cells is associated with hypermethylation of CpG island sequences in the promoter and first exon of the ER gene, and treatment of cells with 5-aza-dC leads to hypomethylation and reexpression of ER suppresses the growth of ER-negative breast cancer cell lines (23). The absence of ER gene expression in these cells correlates with hypermethylation of CpG island sequences in the promoter and first exon of the ER gene, and treatment of cells with 5-aza-dC leads to hypomethylation and production of a functional ER protein (24). Because introduction of ER suppresses the growth of ER-negative breast cancer cells in an estrogen-dependent manner, it is possible that 5-aza-dC-induced reactivation of ER may inhibit the growth of ER-negative cells selectively (25–27). The data reported here indicate that both reexpression of ER and intracellular DNA MTase levels contribute to 5-aza-dC-induced growth inhibition of ER-negative breast cancer cells. In particular, we provide direct evidence that a major mechanism of action of 5-aza-dC involves the formation of stable complexes between cellular DNA and DNA MTase.

**MATERIALS AND METHODS**

**Cell Culture, Reagents, Growth Curves, and IC₅₀ Determinations**—Human breast cancer cell lines were maintained in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum (T47D, MCF-7, Hs578t, and MDA-MB-435 and MDA-MB-468). For drug treatments, exponentially growing cells were seeded at a density of 3–6 × 10⁵ cells/100-mm-diameter dish. Cells were allowed to attach overnight before the addition of the appropriate concentration of freshly prepared 5-aza-dC (Sigma), cytosine arabinoside (araC, Sigma), or ICI 182,780 (Zeneca). At the indicated time points, cells were trypsinized and quantitated using a Coulter counter. For the growth curves shown in Fig. 3, the number was assayed in duplicate, and each growth curve represents the mean of at least two independent experiments. For the data shown in Table I, the 50% growth inhibitory concentrations (IC₅₀) were extrapolated from a plot of the percent of control cell growth (triplicate determinations) versus drug concentration after 4 days of treatment.

**Detection of Poly (ADP-ribose) Polymerase Cleavage**—Exponentially growing cells seeded at approximately 0.6–1.2 × 10⁶ cells/100-mm-diameter dish were either not treated or were treated with 0.75 μM 5-aza-dC as described above. At the indicated time points, cells were washed twice with phosphate-buffered saline and collected by centrifugation at 1,000 rpm for 10 min at 4 °C. Cells were resuspended at a concentration of 1 × 10⁶ cells/0.030 ml in reducing loading buffer (62.5 mM Tris, pH 6.8, 6 μM urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% freshly added β-mercaptoethanol) and disrupted by sonication for 10 s. Proteins from 10⁶ cell equivalents were resolved by electrophoresis in an 8% denaturing polyacrylamide gel, transferred to nitrocellulose, and analyzed by immunoblot using a mouse monoclonal anti-poly(ADP-ribose) polymerase antibody C2–10 (Enzyme Systems Products, Dublin, CA). Primary immunocomplexes were detected with a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody and chemiluminescence (ECL; Amersham).

**DNA Fragmentation Analysis**—Cells were treated and collected as described for poly(ADP-ribose) polymerase cleavage analysis. Cell pellets were resuspended in agarose plugs, and high molecular weight DNA was analyzed by pulse field gel electrophoresis as described previously (28).

**Creation of Ha578t Sense ER Cell Line**—The vector pSAR-MT-ER sense-neo is described elsewhere.² Ha578t cells were stably transfected with 10 μg of this plasmid as described previously (30). G418-resistant clones were selected using medium containing 400 μg/ml Geneticin (G418, Life Technologies, Inc.).

**Western Blot Analyses**—To analyze changes in the levels of Bcl-2 and Bax proteins, cells were grown in the presence of 0.75 μM 5-aza-dC and harvested at 0, 3, and 5 days. 50 μg of total cellular protein from each time point was resolved by electrophoresis in a 12% denaturing polyacrylamide gel. Immunoblot analysis was performed with anti-Bcl-2 monoclonal antibody (clone 124, DAKO) and anti-Bax polyclonal antibody (N-20, Santa Cruz Biotechnology) using standard protocols. Western blot analyses were performed using an enhanced chemiluminescence-based photoblot system (ECL; Amersham). Western blots were stained with fast green to demonstrate that there were equivalent amounts of protein in each lane. ER protein in cell lysates was detected by Western blot analysis as previously described (24). For analysis of DNA MTase protein, cells grown for 2 days in the presence or absence of 0.75 μM 5-aza-dC were harvested by trypsinization, washed with phosphate-buffered saline, and lysed on ice in 50 mM Tris, pH 7.5, 1 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. In some cases, NaCl was added to the lysis at a final concentration of 0.5 M. Protein from the soluble fraction (300 μg) was separated on an SDS-polyacrylamide gel (6.5%), and immunoblot analysis was performed as described previously (7).

**DNA MTase Activity**—Cells grown for 2 days in the presence or absence of 0.75 μM 5-aza-dC were harvested by trypsinization, washed with phosphate-buffered saline, and lysed on ice for 10 s. Proteins from 10³ cells/100-mm-diameter dish and treated with a dose of

² R. G. Lapidus, A. T. Ferguson, and N. E. Davidson, submitted for publication.
5-aza-dC equal to 50 times the calculated IC_{50} concentration. After 24 h, the cells were lysed in situ with 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl plus 1% Sarkosyl, and the lysates were loaded onto discontinuous CsCl gradients as described previously (31). Fractions were analyzed for DNA content by diphenylamine binding and for DNA MTase protein by slot-blot analysis using a 1:1,000 dilution of the human DNA MTase antisera HMT 1147. After locating DNA in each gradient, bound DNA MTase was quantified by pooling the DNA-containing fractions and loading the equivalent of 5.0 μg of DNA from each fractionated cell lysate on a second slot blot on which the DNA-containing fractions from each treated cell line were loaded for comparison. Slot blots were then incubated with anti-DNA MTase antibody and {^{125}}I-labeled protein A and exposed to film. The relative levels of DNA MTase were determined by densitometric scanning of the resulting autoradiograph.

### RESULTS

**Sensitivity of Breast Cancer Cell Lines to 5-Aza-dC**—Dose-response studies for 5-aza-dC were performed on a panel of six different human breast cancer cell lines, two that are unmethylated at the ER gene and actively express the ER gene, T47D and MCF-7, and four that are hypermethylated at the ER gene and lack ER gene expression, Hs578t, MDA-MB-231, MDA-MB-435, and MDA-MB-468. Comparison of the concentration of 5-aza-dC necessary to inhibit the growth of the different cell lines indicated that the four ER-negative cell lines necessary to inhibit the growth of the different cell lines were 4–70-fold more sensitive than the two ER-positive cell lines (Table I). To characterize further the response of the breast cancer cell lines to 5-aza-dC, we investigated the possibility that 5-aza-dC treatment caused programmed cell death. A dose of 0.75 μM 5-aza-dC was chosen for these studies because it had been shown previously that this concentration was sufficient to restore physiological levels of functional ER protein in ER-negative cells after 5 days in culture (24). Commitment to cell death involves activation of proteases, as indicated by cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase from an 118-kDa protein to an 85-kDa peptide in the four ER-negative cell lines within 3–5 days but not in the ER-positive cell lines after 7 days of drug treatment. Pulse field gel electrophoresis demonstrates degradation of genomic DNA to high molecular weight 50-kilobase pair fragments in the four ER-negative cell lines within 3–5 days but not in the ER-positive cell lines after 7 days of drug treatment.

### Table I

| Phenotype and cell line | IC_{50}^{a} (nM) | IC_{50} (nM) |
|------------------------|-----------------|-------------|
| Estrogen receptor-positive  | 500             | 10          |
| MCF-7                  | 200             | 10          |
| Estrogen receptor-negative | 50              | 20          |
| Hs578t                 | 20              | 10          |
| MDA-MB-231             | 20              | 10          |
| MDA-MB-435             | 7               | 30          |

#### Notes

- Concentration of drug which inhibited growth by 50% after a 4-day exposure.

Fig. 2. ER-negative cells undergo apoptosis in response to 5-aza-dC. Cells were grown for the indicated period of time in the presence (+) or absence (−) of 0.75 μM 5-aza-dC. A, Western blot analysis indicates cleavage of poly(ADP-ribose) polymerase from an 118-kDa protein to an 85-kDa peptide in the four ER-negative cell lines within 3–5 days but not in the ER-positive cell lines after 7 days of drug treatment. B, pulse field gel electrophoresis demonstrates degradation of genomic DNA to high molecular weight 50-kilobase pair fragments in the four ER-negative cell lines within 3–5 days but not in the ER-positive cell lines after 7 days of drug treatment. C, Western blot analysis of Bcl-2 and Bax proteins extracted from cells treated with 0.75 μM 5-aza-dC for 0, 3, and 5 days.

Changes in the relative levels of the antiapoptotic protein Bcl-2, and the proapoptotic protein Bax, may be important determinants of the response of breast cancer cells to apoptotic stimuli such as chemotherapeutic agents (34, 35). Because the ER-negative cells underwent programmed cell death in response to 0.75 μM 5-aza-dC, we examined whether this was associated with changes in the levels of Bcl-2 and Bax. As indicated in Fig. 2C, the levels of these two proteins did not fluctuate significantly in response to 5-aza-dC in the ER-positive cell lines. In contrast, within 5 days of drug treatment, there was a dramatic increase in the level of Bax protein and a concomitant decrease in the level of Bcl-2 protein in the three ER-negative cell lines, Hs578t, MDA-MB-231, and MDA-MB-435, which precedes the onset of proteolysis and nucleic acid fragmentation. These results suggest that 5-aza-dC-induced apoptosis of the ER-negative cell lines is mediated through the Bcl-2/Bax signal transduction pathway.

5-Aza-dC exerts its effects on DNA MTase only after incorporation into DNA. One possible explanation for the differences in sensitivity between the ER-positive and ER-negative cell lines could be differences in cellular uptake of cytosine analogs, activation by cytosine kinases, and/or inactivation by cytosine deaminases (36). To test these possibilities, the breast cancer negative breast cancer cells are more sensitive and undergo apoptosis in response to 5-aza-dC.
cell lines were examined for their sensitivity to another cytosine analog, araC. AraC is subject to the same uptake, activation, and degradation pathways as 5-aza-dC, but it does not interact specifically with DNA MTase (37). Comparison of the IC<sub>50</sub> values for araC in various breast cancer cell lines indicated that both ER-negative and ER-positive cell lines responded similarly to araC treatment (Table I). Therefore, the differential sensitivity between ER-positive and ER-negative cell lines to 5-aza-dC is unlikely to be related to differences in the ability to metabolize the drug.

**Role of ER Reactivation in 5-Aza-dC-induced Cytotoxicity**—Because ER-negative cells were more sensitive to 5-aza-dC than ER-positive cells, we examined the role of 5-aza-dC-induced reactivation of ER gene expression in ER-negative cell toxicity. Several studies indicate that exogenous ER expression inhibits the growth of ER-negative cell lines grown in the presence of estrogen (25–27). This growth inhibitory effect is dependent on the presence of estrogen and can be suppressed in the presence of an anti-estrogen. Our previous studies indicated that 5 days of treatment with 0.75 μM 5-aza-dC caused partial demethylation of the ER CpG island and production of functional ER protein in two ER-negative cell lines, Hs578t and MDA-MB-231 (24). Because those experiments were performed under estrogen-containing conditions, it is possible that 5-aza-dC-induced ER protein may activate downstream signaling events that lead to cell death. Western blot analyses confirmed that 5-day 5-aza-dC treatment led to the synthesis of the 67-kDa ER protein in the four ER-negative cell lines, whereas similar treatment did not alter significantly the levels of ER protein in the two ER-positive cell lines (Fig. 3A).

Having established treatment conditions that result in ER protein synthesis, we compared the growth of the four ER-negative cell lines in the presence of 5-aza-dC alone or in combination with the anti-estrogen ICI 182,780 (38). We anticipated that if ER is an important factor in 5-aza-dC-mediated cytotoxicity, inhibition of ER function with an anti-estrogen such as ICI 182,780 will rescue ER-negative cells from growth inhibition. As expected, treatment with ICI 182,780 alone had no effect on cell growth, whereas treatment with 5-aza-dC alone inhibited the growth of the ER-negative cell lines significantly (Fig. 3B). The addition of ICI 182,780 did not alter significantly 5-aza-dC-induced cytotoxicity in the three ER-negative cell lines MDA-MB-231, MDA-MB-435, and MDA-MB-468. These data suggest that reactivation of ER was not a major determinant of 5-aza-dC-mediated apoptosis in these cell lines. In contrast, the addition of 400 nM ICI 182,780 restored the growth rate of 5-aza-dC-treated Hs578t cells to nearly control levels after 5 days of drug treatment (Fig. 3B). These data suggest that reactivation of ER, which occurs within 5 days of 5-aza-dC treatment, can contribute to 5-aza-dC-mediated growth inhibition in the Hs578t cell line.

Because 5-aza-dC may have effects on cell growth which are unrelated to reexpression of ER, Hs578t cells were transfected with a zinc<sup>2+</sup>-inducible ER expression vector to determine the direct effect of ER on cell growth. 12-h exposure of Hs578t sense ER cells to 100 μM ZnSO<sub>4</sub> led to production of functional ER protein (as determined by its ability to activate PR gene expression; Ref. 39 and data not shown) and a net loss in cell number indicative of cell death (Fig. 3C). To determine whether ZnSO<sub>4</sub>-induced growth suppression in Hs578t sense ER cells was caused specifically by estrogen-mediated stimulation of ER, cells were grown in the presence of 100 μM zinc and the anti-estrogen ICI 182,780. Whereas Hs578t sense ER cells exhibited cell death upon ZnSO<sub>4</sub> induction of ER, cells treated simultaneously with ICI 182,780 grew normally (Fig. 3C). Combined, these data show a protective effect of ICI 182,780 on both 5-aza-dC-induced and ER-induced growth inhibition and support a role for ER gene reactivation in 5-aza-dC-mediated cell death in the ER-negative cell line Hs578t.

**Cytotoxicity of 5-Aza-dC Is Mediated Primarily by the Cellular Levels of DNA MTase and the Formation of DNA Adducts**—The above studies indicated that there was a differential effect of 5-aza-dC on ER-positive and ER-negative cells and that...
TABLE II
DNA MTase activity and DNA cross-link formation in breast cell lines treated with 5-aza-dC

| Phenotype and cell line | Treatment | DNA MTase activity<sup>a</sup> | % control | Cross-link formation |
|------------------------|-----------|-------------------------------|-----------|---------------------|
| **Immortalized epithelial** | | | | |
| MCF-10A                | None      | 129 ± 27                      | ND        | ND                  |
|                        | 5-Aza-dC  | ND                           | ND        | ND                  |
| **ER-positive cancer** | | | | |
| T47-D                  | None      | 638 ± 102                     | ND        | ND                  |
|                        | 5-Aza-dC  | 73 ± 20 (11)                 | 0         | 0                   |
| MCF-7                  | None      | 1697 ± 105                    | ND        | ND                  |
|                        | 5-Aza-dC  | 166 ± 57 (10)                | 345       | 345                 |
| **ER-negative cancer** | | | | |
| Hs578t                 | None      | 1,459 ± 354                   | ND        | ND                  |
|                        | 5-Aza-dC  | 241 ± 39 (11)                | 454       | 454                 |
| MDA-MB-435             | None      | 1,744 ± 120                   | ND        | ND                  |
|                        | 5-Aza-dC  | 99 ± 13 (6)                  | 407       | 407                 |
| MDA-MB-231             | None      | 3,015 ± 292                   | ND        | ND                  |
|                        | 5-Aza-dC  | 261 ± 20 (9)                 | 753       | 753                 |
| MDA-MB-468             | None      | 6,334 ± 383                   | ND        | ND                  |
|                        | 5-Aza-dC  | 432 ± 85 (7)                 | 2016      | 2016                |

<sup>a</sup> DNA MTase activities were determined in cell lysates after growth for 48 h in the absence or presence of 0.75 µM 5-aza-dC. Data represent the mean ± S.D. of triplicate cultures assayed in duplicate.

<sup>b</sup> Cells were grown for 24 h in the absence or presence of a dose of 5-aza-dC which was 50 times the deduced IC<sub>50</sub> concentration shown in Table I, i.e. T47D, 10 µM; MCF-7, 5 µM; Hs578t, 0.5 µM; MDA-MB-435, 1 µM; MDA-MB-231, 1 µM; MDA-MB-468, 350 nM. DNA containing fractions from discontinuous CsCl gradient centrifugation were pooled, and 5 µg was blotted to nitrocellulose and probed with DNA MTase antibody. Complexes were detected with 125I-labeled protein A and analyzed by densitometric scanning. Data shown are the results from a representative experiment. Fractionation of duplicate cell lysates showed similar results.

<sup>c</sup> ND, not determined.

Reactivation of ER played a significant role in 5-aza-dC-induced cell death in only one ER-negative cell line. This result suggests that the sensitivity of the breast cancer cells to 5-aza-dC is not solely a result of changes in gene expression. Therefore, we next investigated the role of DNA MTase in the cytotoxicity of 5-aza-dC to the breast cancer cells. DNA MTase activity levels were measured during log phase growth in six breast cancer cell lines and a spontaneously immortalized, nontumorigenic mammary epithelial cell line, MCF-10A. The tumor-derived cell lines had between 5- and 50-fold higher levels of DNA MTase activity than MCF-10A (Table II). Both ER-positive and ER-negative cell lines exhibited a >90% inhibition of DNA MTase activity after a 2-day exposure to 0.75 µM 5-aza-dC (Table II). Therefore, it is unlikely that the differential response of the two cell types results from a differential ability of the drug to incorporate into DNA and interact with DNA MTase. Instead, examination of the enzyme activity levels indicated that, in general, the ER-negative cell lines had higher DNA MTase activity than the ER-positive cell lines (Table II). Comparison of DNA MTase levels with the IC<sub>50</sub> values reported in Table I demonstrated a direct correlation between enzyme activity and sensitivity to 5-aza-dC (as indicated by the inverse of the IC<sub>50</sub> value; Fig. 4A).

Several previous in vitro studies suggest that DNA MTase is likely to exist as a stable complex with 5-aza-dC-substituted cellular DNA in 5-aza-dC-treated cells, but the existence of such complexes in whole cells has not been demonstrated previously (40, 41). To examine the fate of DNA MTase in 5-aza-dC-treated breast cancer cells, untreated or treated cells were extracted under moderate lysis conditions (150 mM NaCl and 1% Nonidet P-40), and the proteins that were soluble after 2,000 x g separation were analyzed by Western blot using an antibody that recognizes human DNA MTase. If DNA MTase is bound covalently to DNA, it will pellet with insoluble chromatin and be lost from the soluble protein fraction. As shown in Fig. 4B, the 200-kDa DNA MTase protein was readily detectable in the fraction of soluble proteins from untreated cells, and the relative amounts among the cell lines were consistent with the DNA MTase activity levels shown in Table II. In contrast, 2-day treatment with 0.75 µM 5-aza-dC led to an almost complete loss of soluble DNA MTase protein in all of the cell lines. Identical results were obtained when cells were extracted with lysis buffer containing 0.5 M NaCl and/or 1% SDS. Furthermore, DNA MTase must exist as a very stable complex with DNA as the protein could not be recovered from the insoluble material despite extraction with high salt (0.5 M NaCl), detergent (2% SDS or 1% Sarkosyl), or high temperature (boiling for 10 min in lysis buffer or 1 x Laemmli sample buffer) (not shown). We found no evidence of DNA MTase proteolytic cleavage using either the DNA MTase antibody HMT 1147, which recognizes the amino-terminal domain of the enzyme, or the antibody HMT 1509, which recognizes the more carboxyl-terminal catalytic domain (7). These data suggest that proteolytic degradation of DNA MTase is not responsible for its loss from the soluble fraction.
The data suggest that DNA MTase forms a stable complex with 5-aza-dC-substituted cellular DNA. To test this directly, whole cell lysates from untreated and 5-aza-dC-treated cells were subjected to discontinuous CsCl gradient centrifugation (31). This method allows the separation of genomic DNA and interacting proteins from free protein. Slot-blot analysis of CsCl gradient fractions with the HMT 1147 antibody identified DNA MTase in the DNA-containing fractions of lysates from 5-aza-dC-treated but not of lysates from untreated cells (representative fractionation shown in Fig. 4C). The response to 5-aza-dC appeared to be specific for DNA MTase because there was no reactivity in the DNA-containing fractions from either treated or untreated cells when the same blots were probed with an antibody to human topoisomerase I (data not shown).

Furthermore, when human cells treated with camptothecin in vitro were similarly fractionated and analyzed, the DNA-containing fractions reacted positively only with the topoisomerase I antibody (as shown previously; Ref. 31) and not with the DNA MTase-specific antibody HMT 1147. These results indicate that the DNA MTase antibody did not react nonspecifically with DNA or other protein-DNA complexes (data not shown).

Similar findings were obtained with all six breast cancer cell lines; that is, in CsCl gradients, DNA MTase protein copurified with genomic DNA only in cells treated with 5-aza-dC. Next, the relative levels of DNA MTase complexed to the DNA after 5-aza-dC treatment were determined for the breast cancer cell lines. After fractionation of cell lysates on CsCl gradients, DNA-containing fractions for each cell line were identified and pooled, and 5.0 μg of DNA was blotted directly onto nitrocellulose to be probed with antiserum to human DNA MTase. Comparison of the relative DNA MTase associated per μg of cellular DNA in 5-aza-dC-treated breast cancer cell lines indicated a correlation between DNA MTase-DNA adducts and the endogenous levels of DNA MTase protein and activity (Table II). Taken together, these results indicate that the difference in sensitivity between ER-negative and ER-positive cells is not caused by an inability of ER-positive cells to form stable complexes with 5-aza-dC-substituted DNA per se. Instead, those cells with higher levels of DNA MTase have the potential to form more protein-DNA complexes, and it is this lesion that plays a direct role in the sensitivity of these cell lines to 5-aza-dC.

**DISCUSSION**

The cytosine analog 5-aza-dC is a potent inhibitor of DNA MTase, which has been widely used in vitro as a demethylating agent and has undergone clinical trials in the treatment of some leukemias and hemoglobin disorders (15–17). The mechanism of DNA MTase inhibition by 5-aza-dC suggests that the cellular response to 5-aza-dC may be caused either by the direct effects of cross-link formation between DNA MTase and 5-azacytosine-substituted cellular DNA or by the indirect effect of DNA MTase inhibition on DNA methylation and altered gene expression/chromatin structure (Fig. 1). Our findings indicate that both of these mechanisms contribute to 5-aza-dC-mediated cytotoxicity in human breast cancer cells.

We have shown previously in a series of established breast cancer cell lines that the ER-negative phenotype is associated with increased levels of DNA MTase activity and hypermethylation of the CpG island in the 5′ region of the ER gene (10, 23). Likewise, hypermethylation of the ER gene CpG island is associated with loss of ER gene expression in intestinal neoplasias and a majority of human hematopoietic malignancies, indicating that inactivation of ER occurs in the development of a number of human cancers (42, 43). Indeed, ER can act as an estrogen-dependent growth suppressor when expressed in ER-negative tumor cell lines of several origins including breast (25–27). Therefore, demethylation of the ER CpG island that has become aberrantly methylated during carcinogenesis has the potential to limit tumor cell growth by allowing the reexpression of a functional growth suppressor gene. Our finding that the anti-estrogen ICI 182,780 can partially block the growth inhibitory effect of 5-aza-dC in at least one ER-negative cell line, Hs578t, supports this hypothesis.

Across both ER-negative and ER-positive cell lines, sensitivity to 5-aza-dC was related directly to endogenous levels of DNA MTase. These results are consistent with previous studies showing that sensitivity to 5-aza-dC correlated with endogenous DNA MTase levels in cells with a targeted disruption of one or both copies of the DNA MTase gene (22). In that study, the authors hypothesized that cells with higher levels of DNA MTase can form more cross-links between DNA MTase and 5-azacytosine residues in DNA, and this lesion accounted for the cytotoxic effects of 5-aza-dC (22). Indeed, several in vitro findings indicated the probable existence of such complexes. For example, studies of bacterial cytosine methyltransferases indicate formation of covalent complexes when 5-azacytosine is substituted for cytosine within the recognition sequence of the enzyme (14). Data from Christman et al. (40) and Michalowsky and Jones (29) demonstrated that nuclear proteins form salt and Sarkosyl-resistant complexes with DNA isolated from 5-aza-dC-treated mammalian cells. These complexes copurified with DNA MTase enzyme activity (40). Now, we show conclusively that in cells treated with 5-aza-dC, soluble DNA MTase is lost and appears in a complex with intracellular DNA. Furthermore, the amount of complex formed correlates with endogenous DNA MTase activity.

As predicted by Gabbbara and Bhagwat (13), the DNA MTase-DNA complexes that formed intracellularly in the presence of the cofactor S-adenosylmethionine were essentially irreversible. DNA MTase was not recovered from the insoluble fraction despite extraction with 0.5 M salt, detergent (1% Sarkosyl, 2% SDS), or boiling in the presence of SDS and a reducing agent. Furthermore, it is likely that the DNA-bound DNA MTase remains intact because the antibody used to detect DNA MTase was generated against an amino-terminal domain (amino acids 250–687), whereas binding to 5-azacytosine residues occurs at the active site in the carboxyl-terminal domain (amino acid 1226).

Although there is an overall concordance between the levels of DNA MTase and cytotoxicity, there appears to be a fundamental difference between ER-positive and ER-negative cells in their response to 5-aza-dC. The ER-positive cell line MCF-7 and the ER-negative cell lines Hs578t and MDA-MB-435 have similar levels of DNA MTase activity and cross-link formation, yet the MCF-7 cells are 10–25-fold less sensitive and do not undergo apoptosis in response to the drug. Our data show that 5-aza-dC-induced cytotoxicity in the Hs578t cells is in part caused by the reactivation of the growth suppressor gene, ER. However, we found no evidence for a significant effect of ER reactivation on the growth of MDA-MB-435 cells. Therefore, the increased sensitivity of the MDA-MB-435 cells relative to the MCF-7 cells may be the result of differences in downstream signaling events triggered by DNA MTase adduct formation.

For example, the response of ER-negative cells to 5-aza-dC involved activation of an apoptotic pathway, whereas we found little evidence of programmed cell death in the ER-positive cells. Another possibility is that the MCF-7 and MDA-MB-435 cells differ in their ability to repair DNA MTase-DNA adducts. The persistence of such complexes in some cell types may play a role in 5-aza-dC-induced mutations (21). Further study on the time course and reversibility of 5-aza-dC-mediated cross-link formation in the various cell lines may be useful in elucidating
any cell type-specific repair deficiencies. A final alternative explanation is that 5-aza-dC has effects on MDA-MB-435 cells which are unrelated to either reactivation of ER or formation of DNA MTase-DNA cross-links, which may include reactivation of other growth suppressor genes.

In summary, we have shown that sensitivity to 5-aza-dC is dictated primarily by intracellular DNA MTase levels and the formation of cross-links between DNA MTase and 5-azacytosine-substituted DNA. Therefore, 5-aza-dC, an agent that has undergone testing for the treatment of acute myelogenous leukemia and myelodysplastic syndromes, may also be efficacious in the treatment of ER-negative breast cancers and other tumors with high DNA MTase levels. Moreover, given the recent interest in gene-specific hypermethylation in carcinogenesis, we now provide evidence that demethylation and re-expression of aberrantly methylated growth suppressor genes may be a feasible alternative to cytotoxic agents in cancer therapy.

REFERENCES

1. El-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R. W. C., Falco, J. P., Hamilton, S. R., and Baylin, S. B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3470–3474
2. Issa, J.-P. J., Vertino, P. M., Wu, J., Sazawal, S., Celano, P., Nelkin, B. D., Hamilton, S. R., and Baylin, S. B. (1993) J. Natl. Cancer Inst. 85, 1235–1240
3. Belinsky, S. A., Nikula, K. J., Baylin, S. B., and Issa, J.-P. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4045–4050
4. Wu, J., Issa, J.-P. J., Herman, J., Bassett, D. E., Jr., Nelkin, B. D., and Baylin, S. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8891–8895
5. Laird, P. W., Jackson-Grusby, L., Fazeli, A., Dickinson, S. L., Edward Jung, W., Li, E., Weinberg, R. A., and Jaenisch, R. (1995) Cell 81, 197–205
6. Laird, P. W., and Jaenisch, R. (1994) Hum. Mol. Genet. 3, 1487–1495
7. Vertino, P. M., Yen, R. C., Gao, J., and Baylin, S. B. (1996) Mol. Cell. Biol. 16, 4555–4565
8. Ohtani-Fujita, N., Fujita, T., Azuki, A., Osifchin N. E., Robbins, D. D., and Sakai, T. (1993) Oncogene 8, 1063–1067
9. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J.-P., and Davidson, N. E. (1995) Cancer Res. 55, 4525–4530
10. Lapidus, R. G., Ferguson, A. T., Ottaviano, Y. L., Pearl, F. F., Smith, H. S., Westman, S. A., Baylin, S. B., Issa, J.-P., and Davidson, N. E. (1996) Clin. Cancer Res. 2, 805–810
11. Yoshida, K., Kuni, O., Chibana, A., Shimoyama, Y., Sugimura, T., and Hirohashi, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7416–7419
12. Lee, W. H., Morton, R. A., Epstein, J. I., Brooks, J. D., Campbell, P. A., Bova, G. S., Hsieh, W.-S., Isaac, W. B., and Nelson, W. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11733–11737
13. Ghabara, S., and Bhagwat, A. S. (1995) Biochem. J. 307, 87–92
14. Santi, D., Noturn, A., and Garrett, C. E. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6996–6997
15. Charache, S., Dover, G., Smith, K., Talbot, C. C., Moyer, M., and Boyer, S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4842–4846
16. Pinto, A., Attadine, V., Fasce, A., Ferrara, P., Spada, O. A., and Di Fiore, P. P. (1984) Blood 64, 922–929
17. Pinto, A., and Zagonel, V. (1993) Leukemia 7, 51–60
18. Alvarez, E., Elliott E., Houghton, A. N., and Kerbel, R. S. (1988) Cancer Res. 48, 277–278
19. Huang, Y. C., and Friedman, S. (1991) J. Biol. Chem. 266, 17424–17429
20. Jahlonen, E., Goitein, R., Marcus, M., and Cedar, H. (1985) Chromosoma 93, 152–156
21. Jackson-Grusby, L., Laird, P. W., Magge, S. N., Mueller, B. J., and Jaenisch, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4681–4685
22. Juttnermann, R., Liu, E., and Jaenisch, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11797–11801
23. Ottaviano, Y. L., Issa, J. P., Pearl, F. F., Smith, H. S., Baylin, S. B., and Davidson, N. E. (1994) Cancer Res. 54, 2552–2555
24. Ferguson, A. T., Lapidus, R. G., Baylin, S. B., and Davidson, N. E. (1995) Cancer Res. 55, 2279–2283
25. Garcia, M., Deroog, D., Freiss, G., and Rocheft, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11538–11542
26. Jiang, S. Y., and Jordan, V. C. (1992) J. Natl. Cancer Inst. 84, 580–591
27. Zajchowski, D. A., Sager, R., and Webster, L. (1993) Cancer Res. 53, 6004–6011
28. McCluskey, D. E., Casero, R. A., Woster, P. M., and Davidson, N. E. (1995) Cancer Res. 55, 3233–3236
29. Michalowski, L. A., and Jones, P. A. (1987) Mol. Cell. Biol. 7, 3076–3083
30. Ferguson, A. T., and Subramani, S. (1994) J. Virol. 68, 4274–4286
31. Subramanian, D., Kraut, E., Staubus, A., Young, D. C., and Muller, M. T. (1995) Cancer Res. 55, 2097–2103
32. Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R., and Sikorska, M. (1993) EMBO J. 12, 3679–3684
33. Vaux, D. L., and Strasser, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2239–2244
34. Gel, J. M. W., Robertson, J. F. R., Ellis, I. O., Willsher, P., McClelland, R. A., Hoyle, H. B., Kyme, S. R., Finlay, P., Blamey, R. W., and Nicholson, R. I. (1994) Int. J. Cancer 59, 619–628
35. Teixeira, C., Reed, J. C., and Pratt, M. A. C. (1995) Cancer Res. 55, 3902–3907
36. Mompnar, R. L. (1985) Pharmacol. Ther. 30, 237–299
37. Chabot, G., Bouchard, J., and Mompnar, R. L. (1983) Biochem. Pharmacol. 32, 1327–1328
38. Howell, A., DeFriend, D. J., Robertson, J. F., Blamey, R. W., Anderson, L., Anderson, E., Sutcliffe, F. A., and Walton, P. (1996) Br. J. Cancer 74, 300–308
39. Graham, J. D., Roman, S. D., McGowan, E., Sutherland, R. L., and Clarke, C. L. (1995) J. Biol. Chem. 270, 30693–30700
40. Christman, J. K., Schneiderman, N., and Acs, G. (1985) J. Biol. Chem. 260, 4059–4068
41. Klimasauskas, S., Kumar, S., Roberts, R. J., and Cheng, X. (1994) Cell 76, 357–369
42. Issa, J.-P. J., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., and Baylin, S. B. (1994) Nat. Genet. 7, 536–540
43. Issa, J.-P. J., Zehnbauer, B. A., Civan, C. I., Collector, M. I., Sharkis, S. J., Davidson, N. E., Kaufman, S. H., and Baylin, S. B. (1996) Cancer Res. 56, 973–977