Drug-induced DNA Hypermethylation and Drug Resistance in Human Tumors

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ABSTRACT

Drug-induced DNA hypermethylation was observed to constitute one component of the response of human tumor cells to toxic concentrations of commonly used cancer chemotherapy agents. In both human lung adenocarcinoma cells (HTB-54) and human rhabdomyosarcoma cells (CC1-136), pulse exposures to the topoisomerase II inhibitors etoposide and nalidixic acid; to the antibiotic doxorubicin; to the microtubule inhibitors vincristine, vinblastine, and colchicine; to the DNA cross-linking agent cisplatinum; to hydroxyurea; and to the antimetabolites 1β-β-arabinofuranosylcytosine, 5-fluorouracil, 5-fluorodeoxyuridine, and methotrexate were associated with profound drug-induced DNA hypermethylation. Exposure of human T-lymphocytes (MOLT-4) to toxic pulse doses of 3'-azidodeoxythymidine was associated with similar drug-induced DNA hypermethylation. In every case, drug-induced DNA hypermethylation was observed only when the degree of DNA synthesis inhibition caused by the drug exceeded 90% and when drug levels or duration of exposure was sufficient to kill 90-100% of exposed cells. Drug-induced DNA hypermethylation was shown not to represent a tissue culture phenomenon, since it occurred in vivo during high-dose 1β-β-arabinofuranosylcytosine and hydroxyurea treatments in two leukemic patients. Drug-induced alterations in DNA methylation were frequently biphasic, with hypomethylation occurring at drug concentrations which produced mild DNA synthesis inhibition and which killed less than 50% of exposed cells. Exposure to the alkylating agents 1,3-bis(2-chloroethyl)-1-nitrosourea and cyclophosphamide and to the antimetabolites 5-azacytidine and 6-thioguanine was associated with DNA hypomethylation at all studied concentrations in HTB-54 cells. Drug-induced DNA hypermethylation could be blocked by preexposure to hypomethylating agents administered at nontoxic to mildly toxic concentrations. Drug-induced DNA hypermethylation may be capable of creating drug-resistant phenotypes by inactivating genes the products of which are required for drug cytotoxicity. Perhaps paradoxically, drug-induced DNA hypermethylation may also produce a second class of drug-resistant tumor cells, characterized by overexpression of particular gene products, by potentiating the process of gene amplification.

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MATERIALS AND METHODS

Drugs and Chemicals. Drugs tested included etoposide, nalidixic acid, doxorubicin, vincristine, vinblastine, colchicine, cyclophosphamide, BCNU, cisplatinum, ara-C, TG, 5-FUra, FdUrd, 5-aza-dC, methotrexate, hydroxyurea, and AZT. Drugs were obtained from the Aldrich Chemical Company (Milwaukee, WI) or from the oncology pharmacy of Pratt County Memorial Hospital (etoposide, BCNU). AZT was obtained from the Burroughs-Wellcome Company (Research Triangle Park, NC). Drugs were tested for purity by HPLC or thin layer chromatography and not used if less than 99%. Aqueous preparations (0.9% NaCl solution) of all drugs were used, except for TG, which was dissolved at high concentration (10 mg/ml) in dimethyl sulfoxide and subsequently diluted in 0.9% NaCl solution. All drugs were prepared immediately prior to use and filter sterilized.

Cell Lines and Clinical Specimens. Two of the human cell lines used for these studies, HTB-54 lung epidermoid carcinoma and CCI-136 rhabdomyosarcoma, were obtained from Professor Robert Bolande, Department of Pathology, East Carolina University. Cell lines were grown in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum (Lineberger Cancer Research Center Tissue Culture Facility). Certain experiments were performed using HTB-54 cells adapted for growth in PC-1 serum-free media (Ventrex, Portland, ME). MOLT-4 cells were obtained from Professor Judy Thomas, Department of Surgery, School of Medicine, East Carolina University, and were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum. Clinical specimens were obtained from a 21-year-old male patient (K. E.) having a diagnosis of acute myelomonocytic leukemia (class IV) (bone marrow, >97% leukemic blasts) in relapse and a 60-year-old female patient (F. R.) having a diagnosis of acute myelocytic leukemia (bone marrow, >97% leukemic blasts). Clinical specimens were kindly provided by Drs. C. Tate Holbrook (patient K. E.) and Spencer Raab (patient F. R.) of the divisions of pediatric and adult Hematology/Oncology, Department of Medicine, East Carolina University, respectively. Incubations were at 37°C in a humidified atmosphere containing 5% CO2.

Assay of DNA Hypermethylation. HTB-54 or CCI-136 cells were plated at a density of 0.3 × 106/60-mm Falcon Primaria tissue culture dish and exposed to drugs at the indicated concentrations 24 h later. MOLT-4 cells were grown in suspension culture to a concentration of 0.2 × 105/ml. Drug exposure occurred for 24 or 48 h, as indicated, in the presence of [6-3H]deoxycytidine or [6-3H]thymidine (10 μCi/ml). Patient specimens consisted of bone marrow aspirates or RBC-depleted fractions of peripheral blood obtained before and after in vivo drug exposure. The serum fraction containing leukemic blasts from a 21-year-old male patient diagnosed with acute myelomonocytic leukemia was kindly provided by Dr. C. Tate Holbrook.

DNA Synthesis. The ability of drug-exposed and control HTB-54, CCI-136, or patient cells to incorporate [3H]thymidine, [3H]thymidine, or [3H]deoxycytidine into nuclear DNA was assayed by HPLC analysis of free bases obtained from formic acid DNA hydrolysates. Eclusion conditions and column characteristics were as reported above for measurement of DNA methylation levels.

RESULTS

Drug-induced DNA Hypermethylation in Human Lung Epidermoid Carcinoma Cells (HTB-54). In vitro exposure of the HTB-54 human lung epidermoid carcinoma cell line to a variety of clinically useful cancer chemotherapy agents induced significant hypermethylation within DNA of exposed cells (Fig. 1). Such drug-induced DNA hypermethylation was frequently preceded by hypomethylation at low to moderately cytotoxic drug concentrations. For example, HTB-54 cells responded in a biphasic manner to exposure to the topoisomerase II inhibitors etoposide, nalidixate, and doxorubicin (Fig. 1A), or to the microtubule inhibitors vincristine, vinblastine, and colchicine (Fig. 1B).

The alkylating agents cyclophosphamide and BCNU induced DNA hypomethylation in HTB-54 cells at all tested concentrations (Fig. 1C). This was in sharp contrast to cisplatinum, which induced profound levels of DNA hypermethylation.

Of the tested antimetabolites, ara-C, 5-FUra, FdUrd, and methotrexate induced significant DNA hypermethylation (Fig. 1D). TG produced DNA hypomethylation in HTB-54 cells to a significant degree and in a dose-dependent fashion.

Dependence of Drug-induced DNA Hypermethylation upon DNA Synthesis Inhibition. The simplest explanation to account for drug-induced DNA hypermethylation is that methylases overtake drug-inhibited replication forks and, due to their longer residence times over newly replicated DNA, modify a greater percentage of DNA cytosines than would normally be the case. If this model is correct, then DNA hypermethylation should correlate closely with the degree of DNA synthesis inhibition induced by various drugs. Fig. 2 illustrates that such a close correlation between DNA synthesis inhibition and drug-induced DNA hypermethylation does indeed exist. In fact, hypermethylation appeared to occur primarily when DNA synthesis inhibition exceeded approximately 90%. This level of DNA synthesis inhibition appeared also to be associated with a threshold for cytotoxicity, with maximum hypermethylation occurring under conditions producing minimum viability.

Drug-induced DNA hypermethylation resulting from DNA synthesis inhibition was not an effect limited to HTB-54 human lung epidermoid carcinoma cells. As illustrated in Fig. 3, CCI-136 human rhabdomyosarcoma cells also showed extensive hypermethylation in response to DNA synthesis-inhibiting and cytotoxic doses of various chemotherapy agents. Fig. 4 indicates that the reverse transcriptase inhibitor AZT produced DNA hypermethylation in MOLT-4 human T-lymphocytes in a dose-dependent manner which correlated directly with degree of DNA synthesis inhibition.

Drug-induced DNA Hypermethylation at Physiologically Relevant Drug Concentrations. One argument that might be made against the physiological relevance of drug-induced DNA hypermethylation is that its induction to the highest observable levels required drug concentrations that are not practical in a clinical setting. Figs. 2A and 3A, insets, indicate that longer term exposure to lower levels of drugs produced DNA hypermethylation comparable in extent to short term exposure to higher levels of drugs. For both hydroxyurea and cisplatinum, 48-h exposure to levels of drug within the micromolar range resulted in significant DNA hypermethylation.

In experiments designed to assay the potential for drugs to induce DNA hypermethylation in vivo, pretreatment samples were obtained from patients about to undergo chemotherapy and these were compared to posttreatment samples with respect to their 5-mCyt content. The results of Fig. 5 were obtained from bone marrow specimens containing a greater than 97% pure population of leukemic blasts from a 21-year-old male patient diagnosed with acute myelomonocytic leukemia in relapse. Significant DNA hypermethylation was observed to have occurred within 12 h following a 3-h infusion of HDAC (3 g/
DRUG-INDUCED DNA HYPERMETHYLATION

Fig. 1. Drug-induced alterations of 5-mCyt content in drug-exposed human tumor cells. HTB-54 human lung epidermoid carcinoma cells were maintained in log phase as monolayers and exposed for 24 h to the indicated concentration of drugs, plus 10 μCi/ml [3H]uridine or [3H]deoxycytidine. Results are reported as the ratio of (cpm in 5-mCyt)/(cpm in cytosine + 5-mCyt). Points, mean for three experiments performed in triplicate; bars, SEM.

m²). At 72 h beyond HDAC infusion, viable leukemic blasts (as judged by their ability to incorporate radiolabeled deoxycytidine and thymidine into polymeric DNA) were observed to have a 5-mCyt content approximately double that of pretreatment blasts. Significant DNA hypermethylation was retained for 30 days after HDAC infusion, as surviving blasts repopulated the marrow. Extensive DNA hypermethylation then followed combination chemotherapy consisting of hydroxyurea (1200 mg/m² p.o. at t = 0), etoposide (100 mg/m² i.v. over 45 min at t = 1 h), and ara-C (500 mg/m² i.v. over 15 min at T = 2 h). Content of 5-mCyt in bone marrow aspirates obtained 26½ hr after cessation of therapy was approximately 3.5 fold higher than in pretreatment samples. This patient proved refractory to therapy and subsequently died. Fig. 6 illustrates data obtained from peripheral blood (97% pure malignant population) from an adult patient with acute lymphocytic leukemia before and after treatment with hydroxyurea (1.5 g/m²). By 7 h posttreatment, a greater than 5-fold increase in 5-mCyt content of leukemic blast DNA was evident.

Blockade of Drug-induced DNA Hypermethylation. Fig. 7 shows that, by the prior addition of 5-aza-dC to cultures of HTB-54 cells about to undergo exposure to various concentrations of cisplatinum, it is possible to titrate the induction of DNA hypermethylation back toward normal values. At most concentrations of cisplatinum, 10⁻⁷ M 5-aza-dC was found to be sufficient to block cisplatinum-induced DNA hypermethylation. Similarly, 5-aza-dC within this concentration range was found to be sufficient to block ara-C-induced DNA hypermethylation (Fig. 8). Of interest is the fact that concentrations of 5-aza-dC sufficient to block drug-induced DNA hypermethylation are within the noncytotoxic range in our assay system.

Nalidixic acid is a topoisomerase II inhibitor that we had shown previously to inhibit DNA methylation in hamster fibrosarcoma cells (14). When HTB-54 cells were pretreated with nalidixic acid prior to exposure to various concentrations of cisplatinum, cisplatinum-induced DNA hypermethylation was blocked over a wide concentration range (Fig. 9). This effect occurred even at concentrations of nalidixic acid shown in parallel experiments to induce DNA hypermethylation. No evidence was obtained for modification of survival in cells pretreated with hypomethylating agents, beyond that attributable to the additive effects of the drugs used alone.

DISCUSSION

We have shown that one consequence of exposure of human tumor cells to cytotoxic chemotherapy agents in vitro and in vivo is drug-induced DNA hypermethylation. A wide variety of agents possessing diverse mechanisms of action were shown to produce this effect in a dose-dependent manner. Levels of drug-
induced DNA hypermethylation were observed to correlate directly with the degree of DNA synthesis inhibition and to the degree of cytotoxicity induced. In this respect, drug-induced DNA hypermethylation at position 5 of cytosine appeared to resemble the toxicity-associated alkylation of guanine reported by Shank (27). In both human lung adenocarcinoma cells (HTB-54) and human rhabdomyosarcoma cells (CCI-136), pulse exposures to the topoisomerase II inhibitors etoposide and naldixic acid; the antitumor antibiotic doxorubicin; the microtubule inhibitors vincristine, vinblastine, and colchicine; the ribonucleotide reductase inhibitor hydroxyurea; the DNA cross-linking agent cisplatinum; and the antimetabolites ara-C, 5-FUra, FdUrd, and methotrexate were associated with significant drug-induced DNA hypermethylation. Pulse exposures of MOLT-4 lymphocytes to cytotoxic concentrations of AZT produced similar levels of drug-induced DNA hypermethylation.
The topoisomerase II inhibitors etoposide and nalidixic acid and the antitumor antibiotic doxorubicin inhibited DNA methylation at concentrations eliciting low to moderate levels of cytotoxicity, however. One possible explanation for this effect is that topoisomerase II activity and DNA methylase activity may be linked within the complex structure of the nuclear matrix. Thus, one function of topoisomerase II might be to constrain DNA within a conformation that makes it a good substrate for DNA methylase. Topoisomerase II inhibitors may down-regulate this function, resulting in the release of DNA.
Drug-induced DNA hypermethylation following in vivo exposure to hydroxyurea. The RBC-depleted fraction of peripheral blood obtained at the indicated times before and after in vivo drug exposure were rapidly exposed to 25 μCi/ml [6-3H]deoxycytidine (16 Ci/mmol; 1.0 mCi/ml). Columns, mean for three to four specimens obtained at each treatment point; bars, SEM.

Fig. 7. Blockade of cisplatinum-induced DNA hypermethylation. HTB-54 cells were pretreated for 15 min with the indicated concentrations of 5-aza-dC prior to exposure to cisplatinum and [6-3H]uridine. Points, mean for three experiments performed in triplicate; bars, SEM.

Fig. 8. Blockade of ara-C-induced DNA hypermethylation. HTB-54 cells were pretreated for 15 min with the indicated concentrations of 5-aza-dC prior to exposure to ara-C and [6-3H]deoxycytidine. Points, mean for three experiments performed in triplicate; bars, SEM.

from that conformation which can be efficiently methylated. Exposure to the alkylating agents BCNU and cyclophosphamide produced hypomethylation at all drug concentrations tested, a result which was predicted based upon the previous observations of Cox (28) and Wilson and Jones (29) that compounds structurally related to these drugs inhibited methylyase activity. The hypomethylation produced by TG in HTB-54 cells was unexpected but can possibly be explained in the following way. TG is incorporated into DNA and would be expected to replace CpG dinucleotides (the preferred substrate for vertebrate DNA methylases) with CpTG isomeric dinucleotides. HTB-54 cell DNA methylase may be incapable of modifying CpTG dinucleotides contained within its recognition sequence(s).

The extreme level of DNA-hypermethylation induced by cisplatinum exposure is noteworthy. This drug produces good response rates in human ovarian, testicular, bladder, and head and neck cancer, yet the emergence of cisplatinum resistance is nearly an inevitable consequence of therapy (30). One possible explanation for the opposite effects upon DNA methylation induced by BCNU and cyclophosphamide as compared to cisplatinum may be that the former agents may directly inactivate methylase enzyme, while cisplatinum may be selective for producing lesions at the level of DNA.

Possible Mechanisms of Induction of Drug-resistant Phenotypes Resulting from Drug-induced DNA Hypermethylation

Gene Inactivation. There exist diverse mechanisms by which tumor cells can escape cytotoxic chemotherapy (31–33). One of these is by the reduction in expression or activity of gene products required for the metabolic activation of the applied drug. Examples of this mechanism include inactivation of the dCK gene locus during the development of resistance to ara-C. In the absence of dCK enzyme expression, ara-C cannot be metabolized to its cytotoxic form, ara-C triphosphate. As we have seen, ara-C exposure induces substantial increases in the level of DNA methylation. In those hypomethylated but viable tumor cells in which the dCK gene is methylated by this mechanism, occasional methylation-dependent inactivation of the dCK gene loci and subsequent resistance to ara-C may occur.

A variety of other genes might also be inactivated by drug-induced DNA hypermethylation with the result being the induction of drug-resistant tumor cell clones. For example, metabolic activation by cytochrome P-450 is required for such agents as cyclophosphamide to exert tumor cell cytotoxicity. Drug-induced DNA hypermethylation-inactivation of the relevant P-450 locus during combination chemotherapy would provide a mechanism for cyclophosphamide-resistant tumor cell clones to become established. Similarly, drug-induced DNA
Drug-induced DNA Hypermethylation

Levels of drug-induced DNA hypermethylation similar to those associated with \textit{in vitro} drug exposures were observed to occur \textit{in vivo}, during patient chemotherapy with HDAC or hydroxyurea. This finding indicates that drug-induced DNA hypermethylation is not simply a tissue culture phenomenon but may have clinical relevance as well. Since hypermethylation could be blocked by preexposure to methylation-blocking agents, it may be possible to design chemotherapeutic protocols in which drug combinations are selected based upon their ability to maintain a normal or near normal tumor cell DNA methylation content. Thus, doses of drugs such as 5-aza-dC, BCNU, etoposide, TG, etc., may be selected not on the basis of their ability to elicit tumor cytotoxicity but rather for their ability to block drug-induced DNA hypermethylation. 5-aza-dC preceeding cisplatinum therapy, for instance, would have the effect of preventing cisplatinum-related DNA hypermethylation. If DNA hypermethylation is a contributing factor in the development of drug-resistant phenotypes, the appropriate selection of drug combinations to minimize this effect could improve both rate of induction and the length of duration of patient response to chemotherapy. Since DNA methylation-blocking activity occurs at nontoxic concentrations of 5-aza-dC and topoisomerase II inhibitors, the use of these compounds in this way would represent a departure from the recent trend toward high-dose drug regimens. It would also represent a novel form of biochemical modulation, designed to reduce the occurrence of a drug-mediated DNA lesion having the potential for initiating drug resistance.

Drug-induced DNA hypermethylation could itself constitute a component of drug-induced cytotoxicity. To the extent that this is true, attempts to block drug-induced DNA hypermethylation could result in reduced efficacy of drug therapy. As such, drug-induced DNA hypermethylation may constitute both a toxic response to drug exposure and a mechanism by which maximum heterogeneity can be achieved within a tumor cell population.

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