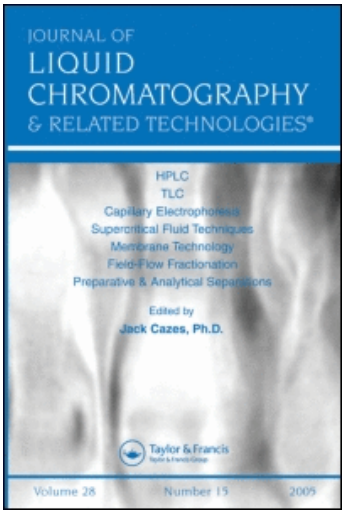

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DETECTION OF DRUG-INDUCED DNA HYPERMETHYLATION IN HUMAN TUMOR CELLS EXPOSED TO CANCER CHEMOTHERAPY AGENTS

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ABSTRACT

A method is described for the quantitative analysis of drug-induced DNA hypermethylation in human tumor cells exposed in vivo or in vitro to DNA synthesis-inhibiting levels of cancer chemotherapy agents. The method relies upon isocratic separation of formic acid hydrolysates of DNA using an Aminex A-9 column converted to the cation form. When combined with pre-incorporation of uridine or deoxycytidine labeled specifically in the 6-position, the method is sensitive in the picomolar range for 5-methylcytosine, and is specific for the detection of cytosines methylated during the period of drug-induced DNA synthesis inhibition. The method can be used to quantitate DNA hypermethylation occurring in patient bone marrow or peripheral blood specimens in vivo during chemotherapy.

INTRODUCTION

DNA methylation is an enzymatically mediated process in which specific cytosines within newly polymerized DNA are methylated on

the 5-carbon position. Most DNA methylation in vertebrates occurs within the dinucleotide CpG and most CpGs in vertebrate DNA are methylated. Although de novo methylation of unmethylated double-stranded DNA is possible, most methylation reactions appear to be of the "maintenance" type, in which modification in the newly synthesized strand occurs across from a methylated cytosine within the parental strand. In this way, patterns of methylation established during embryogenesis can be maintained. There is a lag between DNA synthesis and DNA methylation, and the transient presence of hemimethylated DNA within and behind replication forks (methylation as yet only on the parental strand), is thought to have biological importance (1). Furthermore, considerable evidence suggests an important role for DNA methylation as a controlling mechanism in gene expression (2). Thus, in general, methylated genes are not transcribed whereas unmethylated genes are transcribed.

We have previously shown that exposure to DNA synthesis-inhibiting levels of cancer chemotherapy agents produces a profound increase in levels of DNA 5-methylcytosine (3,4). Presumably, under conditions in which DNA synthesis is inhibited, methylase enzymes "catch up" to replication forks and prevent the formation of transiently unmethylated daughter strand DNA. Continued inhibition of DNA synthesis results in hypermethylation within stalled replication forks, and such hypermethylation is capable of reaching profound levels (5). Because of the potential importance of drug-induced DNA hypermethylation occurring during

exposure to DNA synthesis-inhibiting drugs, including cancer chemotherapy agents, a sensitive method for detecting hypermethylated DNA was developed. This method is described.

MATERIALS

All drugs were obtained from the SIGMA Chemical company (St. Louis, MO), and were used without further purification. Reagent grade solvents were obtained from Aldrich (Milwaukee, WI). Aminex A-9 was obtained from BioRad laboratories (Richmond, CA). Fetal Calf serum was obtained from the Lineberger Cancer Center (Chapel Hill, NC), and was heat inactivated at 60 degrees C for 30 minutes. All tissue culture media and supplements were obtained from GIBCO (Grand Island, NY).

METHODS

Column Preparation

The Aminex A-9 resin (Bio-Rad) was converted to the cation form and slurried in 0.2 M ammonium acetate, pH 5.5, and the 250 X 4 mm stainless steel column packed and checked using this same buffer. Guard columns (30 X 4 mm) were packed using this same resin and buffer, and inserted into a standard cartridge holder.

Sample Preparation

CCRF/CEM human acute lymphoblastic leukemia cells (ATTC, Rockville, MD) were grown in suspension in T-75 tissue culture flasks at a concentration of 0.25 million per ml in RPMI media supplemented with 10% fetal calf serum. Incubations were perform-

ed in a water-jacketed CO2 incubator (5.0%) maintained at 37 degrees C in a humidified atmosphere (93%). Twenty-four hours after inoculation, media was changed and cisplatinum was added at the indicated concentrations. Immediately following addition of drug, 10 uCi/ml 6-[3H]-deoxycytidine or 6-[3H]-uridine (Moravek Biochemicals, Brea, CA) was added. Cultures were harvested 24 hours later. For estimation of DNA synthesis rates, parallel cultures were exposed to 0.5 uCi/ml [3H]-thymidine in place of labeled uridine or deoxycytidine for 24 hours under identical treatment conditions. Nanomoles newly incorporated thymidine (hot) per mol total thymidine (hot plus cold as quantitated from a standard curve generated for thymidine absorption at 254 nm) were then calculated for each treatment point.

Patient samples, consisting of either peripheral blood or bone marrow, were collected into heparinized evacuated tubes at specific times before, during and after treatment and the red cell fraction was allowed to settle at unit gravity for 20 minutes. The upper serum fraction containing most of the tumor specimen and remaining white cell fraction was carefully removed and 1.0 ml aliquots were placed into 30 mm tissue culture dishes containing 10 uCi/ml 6-[3H]-deoxycytidine. This technique permitted drug concentrations within patient serum to be retained during the period of 6-[3H]-deoxycytidine labeling and formation of hypermethylated DNA. Samples were subsequently prepared for analysis in a manner analagous to that for CCRF/CEM cells.

DNA Hydrolysis

DNA was prepared essentially according to the method of Jones and Taylor (6). Hydrolysis was carried out in 60 mm culture tubes with PFTE seals at 180 degrees centigrade for 40 minutes in 88% formic acid. Formic acid was removed under a purified nitrogen stream following temperature reduction of hydrolyzed samples to -20 degrees C to minimize pressurization. Hydrolysates (100 microgram samples) were taken up in 100 microliters of 0.1 M HCl and injected directly into the loading port of a Waters or Bio-Rad HPLC system.

HPLC Conditions

The column was operated under the following conditions: Flow rate, 0.7 ml/minute; temperature, 60 degrees C maintained with a Bas LC-22A temperature controller; eluant, 0.2 M ammonium acetate, pH 5.5, purged; operating pressure, 1500 psi. An ISCO model 2150 Peak separator was used to select cytosine and 5-methylcytosine UV absorption peaks (254 nm for cytosine and 280 nm for 5-methylcytosine, using a Waters model 440 absorption detector or Bio-Rad model 2100 UV/VIS monitor) and fractions were collected at 30 second intervals commencing with the cytosine signal using a Haake-Buchler LC-100 fraction collector. Liquiscint (National Diagnostics), 5.0 ml, was added and samples were quantitated using a Packard TriCarb model 2200CA liquid scintillation counter optimized for tritium. Results were calculated as the ratio of the radiolabeled fraction (dpm) coeluting with 5-methylcytosine to the fraction coeluting with total cytosines (5-methylcytosine plus

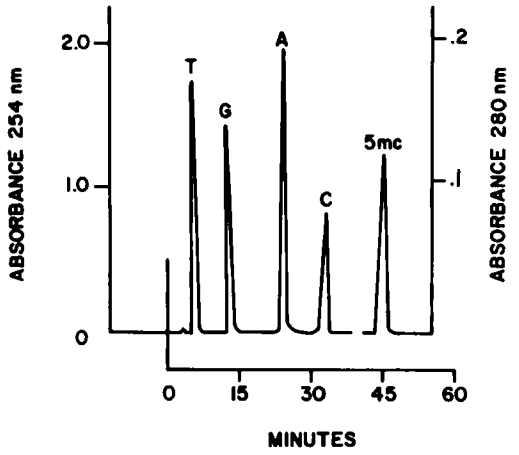

FIGURE 1. Aminex A-9 Separation of Hydrolyzed DNA Bases. Formic acid-hydrolyzed DNA (100 ug in 100 ul of 0.1 M HCl) was injected using a Waters U6K system. Elution characteristics are described within the text.

cytosine). Resolution of hydrolyzed DNA bases is depicted in Figure 1.

RESULTS

Exposure of CCRF-CEM acute lymphoblastic leukemia cells grown in tissue culture to cytotoxic concentrations of cisplatinum induced a significant hypermethylation of DNA as shown in Table 1. This effect was dose dependent. The extent of drug-induced DNA hypermethylation at increasing drug dosage also correlated strongly with cytotoxic response (data not shown). A bone marrow specimen obtained from a patient with acute myeloblastic leukemia obtained 24 hours after treatment with high dose cytosine arabinoside (araC) and cultured in the patient's own serum for an addi-

TABLE 1

Cisplatinum-induced DNA Hypermethylation of CCRF/CEM Human Lymphoblastic Leukemia Cells

Concentration of cisplatinum (uM)	5mC/5mC+C (% of Control)	% of Control DNA Synthesis
0.0	3.73 ±0.19 (100)	100 ±5
1.0	3.92 ±0.23 (105)	74 ±8
10.0	5.95 ±0.61 (152)	11 ±9
100.0	12.33 ±2.15 (330)	0.7±2

CCRF/CEM cells were exposed to the indicated concentrations of cisplatinum for 24 hours, in the presence of 6-[3H]-deoxycytidine (10 uCi/ml), and DNA prepared for analysis as described within the text. For analysis of DNA synthesis, parallel cultures were exposed to 0.5 uCi/ml [3H]-thymidine for 24 hours. Results represent three experiments performed in triplicate, and are presented ± standard error of the mean.

TABLE 2

Cytosine Arabinoside-induced DNA Hypermethylation Occurring In In Bone Marrow Aspirates of a Patient With Acute Myeloblastic Leukemia

Treatment status	5mC/5mC+C (% of Control)	% Pretreatment DNA Synthesis
Pretreatment	3.3 ±0.4 (100)	100 ±8
Following HDAC	6.9 ±1.7 (209)	9 ±4

Bone marrow aspirates were obtained 12 hours after initiation of high dose cytosine arabinoside (HDAC; 3grams/square meter) therapy. Patient was a 21 year old male with a diagnosis of acute myeloblastic leukemia. Bone marrow aspirate was determined by cytology to be >97% tumor blasts. Leukemic cells were incubated in patient's own serum in the presence of 10 uCi/ml 6-[3H]-deoxycytidine for 24 hours, then processed as for CCRF/CEM cells. Results represent the average of three samples processed individually, ± the range.

tional 24 hours showed a similar DNA hypermethylation response, as indicated in Table 2. Although parallel experiments to quantitate DNA synthesis inhibition using [3H]-thymidine were not possible in this patient, previous work has shown that incorporation of 6-[3H]-deoxycytidine into DNA cytosine provides an estimation of DNA synthesis which agrees closely with that obtained using thymidine incorporation. Using this parameter, araC-induced DNA hypermethylation occurring in the in vivo/in vitro experiment reported here also correlated closely with degree of DNA synthesis inhibition.

DISCUSSION

Drug-induced DNA hypermethylation may have profound biological consequences. Since methylated genes are usually transcriptionally inactivated, drug-induced DNA hypermethylation may be capable of silencing active genes. During cancer chemotherapy, such an event could initiate the development of a drug-resistant cell population. For example, in order for the chemotherapeutic agent araC to be cytotoxic to leukemia cells, it must be activated by deoxycytidine kinase to its triphosphate form, araCTP. Since araC is itself capable of inducing DNA hypermethylation, occasional drug-induced hypermethylation-inactivation of the deoxycytidine kinase gene within a minor fraction of a tumor cell population would create clonal populations refractory to further araC therapy. Similar situations can be envisioned for other agents requiring activation to cytotoxic forms, e.g., thioguanine

(HPRT gene inactivation), 3′-azido-2′-dideoxycytidine (AZT; thymidine kinase gene inactivation), etc. Furthermore, this mechanism of drug resistance suggests that hypermethylation induced by one drug may also produce resistance to other drugs by a similar gene inactivation mechanism.

The technique described here has been optimized for the detection of hypermethylated DNA in both established cell lines and in fresh human leukemia specimens. Even under conditions in which DNA synthesis is inhibited by greater than 99%, 5-methylcytosine levels can be quantitated accurately. This procedure may permit the determination of the biological effects of drug-induced DNA hypermethylation, particularly with respect to such important clinical problems as the development of drug resistance during cancer chemotherapy.

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