ABSTRACT

Mitomycin C, a bioreductive alkylating agent with clinical utility against several human tumors, was found to be selectively toxic at a relatively low concentration (1.5 μM) to EMT6 tumor cells made chronically hypoxic by preincubation in 95% N₂-5% CO₂ for 4 hr prior to drug exposure. This selective cytotoxicity correlated well with the preferential activation and metabolism of mitomycin C by sonicated cell preparations. The bioactivation of mitomycin C to an alkylating agent by EMT6 and Sarcoma 180 cell sonicates required hypoxic conditions and a reduced nicotinamide adenine dinucleotide phosphate-generating system. Furthermore, the formation of reactive drug metabolites and the disappearance of mitomycin C from the reaction mixture were inhibited by carbon monoxide. The presence of potassium cyanide in the incubation mixture did not affect either the rate of overall metabolism or the rate of formation of reactive metabolites. A high rate of disappearance of mitomycin C from the medium of intact cultures of EMT6 cells was found only in those cultures which were made chronically hypoxic. These data suggest that bioreductive alkylating agents like mitomycin C have the potential to attack selectively the chemotherapeutically resistant hypoxic cell component of solid tumors. Thus, agents capable of bioreductive alkylation should be useful adjuncts to existing therapeutic regimens which are effective against well-oxygenated cells.

INTRODUCTION

The bioreductive alkylating agents are a class of drugs which require reductive biotransformation in order to exhibit alkylation activity. Lin et al. (10, 11) postulated that hypoxic tumor cells exist in environments which are conducive to the reductive processes necessary to activate bioreductive alkylating agents. As a result, hypoxic tumor cells would be expected to activate preferentially and therefore be selectively susceptible to drugs of this class. Because hypoxic tumor cells are relatively resistant to radiotherapy and chemotherapy (9), agents directed specifically against the hypoxic cell component of solid tumors would be extremely useful adjuncts to current cancer therapy.

One clinically used agent which can function as a bioreductive alkylating agent is mitomycin C (Chart 1). It has been shown previously that mitomycin C is activated to an alkylating species by reduction of the quinone moiety and subsequent loss of the methoxy group (6, 7, 14, 20). Spontaneous rearrangement of the reduced molecule leads to the production of a highly reactive quinone methide intermediate (see Chart 1) which can alkylate DNA, RNA, protein, and other cellular molecules. Schwartz (19) has demonstrated that the liver has an enzymatic system capable of metabolizing mitomycin C under anaerobic conditions. The enzyme system which carries out the reduction is located in both the microsomal and nuclear fractions (8). However, to exhibit selective cytotoxicity towards hypoxic tumor cells, bioreductive alkylating agents such as mitomycin C must be activated by cancer cells in situ.

The data presented in this report demonstrate that mitomycin C can be activated to an alkylating species under hypoxic conditions in vitro by cells from 2 experimental mouse tumor lines, Sarcoma 180 and EMT6. In addition, the cytotoxic effects of mitomycin C were investigated using cells maintained under different states of oxygenation. At low drug concentrations, mitomycin C was found to be selectively toxic to hypoxic tumor cells in vitro.

MATERIALS AND METHODS

Drugs. Mitomycin was the gift of Dr. Maxwell Gordon of the Bristol-Myers Co. (Syracuse, N. Y.). NADP⁺, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were obtained from standard chemical sources unless otherwise specified.

Tumor Cells. Experiments using EMT6 mouse mammary tumor cells were performed in vitro. The techniques used for propagating the cells and measuring cell survival by colony formation have been described previously (16, 17). To obtain sufficient quantities of cells for in vitro metabolism studies, the EMT6 cells also were grown as spheroids. Approximately 3 × 10⁶ cells were added to Optilux plastic Petri dishes containing 20 ml of fresh Waymouth's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with fetal bovine serum and antibiotics as specified previously (16). The medium was changed twice a week for 3 weeks; the spheroids were then harvested and washed with 0.9% NaCl solution before the cells were prepared for use in metabolism studies.

Sarcoma 180 cells were collected as described previously (1) from ascites fluid in the peritoneal cavities of female CD-1 mice (20 to 30 g) 7 days after implantation of 1 × 10⁶ cells. The cells were washed 3 times with 0.9% NaCl solution to remove RBC and were then prepared for use in metabolism studies.

Metabolism Studies. EMT6 or Sarcoma 180 cells were suspended in 1 volume of distilled water and incubated for 10 min on ice to facilitate disruption of the cells. After addition of 1.8% NaCl solution to achieve isotonicity, the cell suspensions were sonicated 3 times for 10 sec each using a Branson
Mitomycin Activation by Hypoxic Tumor Cells

sonicator at a setting of 25. The sonicated cell preparations were diluted with 0.1 M Tris-HCl buffer (pH 7.4) and used in metabolism and alkylation assays.

Incubation mixtures for metabolism experiments with sonicated cell preparations contained 0.72 μmol NADP⁺, 5 μmol MgCl₂, 5 μmol glucose 6-phosphate, 1.25 units glucose-6-phosphate dehydrogenase and 2.0 mg cell sonicate protein. The total volume of the reaction mixture was 1.0 ml. Hypoxic conditions were achieved by stoppering flasks and pregassing reaction mixtures for 10 min with prepurified nitrogen which contained less than 10 ppm oxygen (Matheson Gas, Rutherford, N. J.). As noted, all reactions were performed under a continuous flow of nitrogen. The incubation flasks were then warmed for 2 min to generate NADPH, and the reaction was initiated by the addition of mitomycin C (0.30 μM final concentration) dissolved in acetone. The NADPH-regenerating system continuously provides sufficient quantities of NADPH for the reaction investigated in these experiments. Because the reaction catalyzed by glucose-6-phosphate dehydrogenase does not require O₂, this method of producing NADPH is insensitive to the presence or absence of O₂. Furthermore, the absorbances of samples containing only the NADPH-regenerating system were similar under aerobic and hypoxic (N₂) atmospheres, suggesting that similar quantities of the cofactor were produced under these conditions. After 20 min, the incubations were stopped by adding 1 ml of saturated Ba(OH)₂ solution and 1 ml of 15% ZnSO₄ to precipitate protein. Mitomycin C concentrations in the supernatant were then determined as described below.

Reactive metabolites of mitomycin C were estimated by a modification of the method described by Wheeler et al. (23). Incubation mixtures used to estimate the relative amounts of biotransformation products with alkylation capabilities were the same as those described for the metabolism experiments, except that 10 μl of a 10% acetone solution of 4-(p-nitrobenzyl)pyridine (Aldrich Chemical Co., Milwaukee, Wis.) were added to the samples prior to incubation. After incubation, each reaction was terminated by adding 2 ml of acetone and 1 ml of 1 M NaOH, and the sample was extracted immediately with 4 ml of ethyl acetate. The organic and aqueous phases were separated by centrifugation for 2 min at 1000 × g, and the absorbance of the organic layer at 540 nm was determined. The incubations and subsequent extractions for estimating the alkylation metabolites were carried out under subdued light.

Metabolism studies were performed using exponentially growing monolayers of EMT6 cells, obtained by planting 5 × 10⁶ cells in 25-sq cm Corning tissue culture flasks containing 5 ml of supplemented Waymouth's medium and incubating the flasks for 3 days in a humidified atmosphere of 95% air-5% CO₂. The medium was changed just before the experiment. Mitomycin C was added to cells maintained under different states of oxygenation to produce a concentration of 0.03 μM, and the cells were incubated with the drug for 1 hr. At the end of the incubation period, the medium was removed and the protein was precipitated by the addition of 1 ml of saturated Ba(OH)₂ and 1 ml of 15% ZnSO₄. Mitomycin C concentrations in the supernatant were determined as described below. Cells were harvested by trypsinization and counted. The total number of cells per flask was calculated, and the rates of metabolism were expressed as nmol of mitomycin C consumed per hr per 10⁶ cells.

Cytotoxicity Studies. Cytotoxicity studies were performed using exponentially growing monolayer cultures of EMT6 cells, handled as described above. The medium was changed just before the experiment. Cultures were incubated under different states of oxygenation and mitomycin C was added to the culture medium to produce a concentration of 1.5 μM. After a 1-hr incubation, the cells were harvested by trypsinization, and cell survival was assayed by colony formation in vitro as described previously (16, 17).

Hypoxic Conditions. Cells were made hypoxic by sealing 25-sq cm cell culture flasks with thick rubber sleeves. The flasks were then fitted with 20-gauge needles for inflow and outflow of gases. The flasks were gassed continuously with a humidified mixture of 95% N₂-5% CO₂ (Matheson Gas, Rutherford, N. J.). With this technique, drug could be added directly to cultures without breaking the hypoxia, by injecting a small volume of drug through the sleeve. Normally aerated cultures were incubated in a humidified atmosphere of 95% air-5% CO₂. Acutely hypoxic conditions were defined as those in which cultures were gassed with 95% N₂-5% CO₂ for 1 hr, beginning immediately after the addition of drug to normally aerated cultures. Chronically hypoxic cells were preincubated for 4 hr with a humidified atmosphere of 95% N₂-5% CO₂ prior to the addition of drug.

Analyses. Protein was determined by the method of Lowry et al. (12). Mitomycin C concentrations were estimated by determining the absorbance of the quinone form of the drug at 363 nm, and the measured absorbance was then related to that obtained using known amounts of the drug. Any contribution from NADPH to the absorbance of mitomycin C at 363 nm was eliminated by determining drug-related absorbances against appropriate samples containing only the NADPH-regenerating system. The limit of sensitivity for the method was 0.1 μg/ml.
RESULTS

Mitomycin C was activated by sonicated cell preparations obtained from either Sarcoma 180 or EMT6 tumor cells as shown in Charts 2 and 3, respectively. The results are expressed as a percentage of the nitrogen control incubations. The rates of disappearance of mitomycin C from these control incubations were 1.00 ± 0.08 (S.E.) and 1.40 ± 0.22 nmol/min/mg protein for the sonicated cell preparations obtained from Sarcoma 180 and EMT6 cells, respectively. Furthermore, the rates of formation of alkylating species were similar for Sarcoma 180 cell preparations (0.92 ± 0.10 ∆AA540/min/mg) and for EMT6 cell sonicates (1.13 ± 0.07 ∆AA540/min/mg). The disappearance of mitomycin C and the formation of alkylating metabolites, as measured with the trapping reagent 4-(p-nitrobenzyl)pyridine, were dependent upon both time and protein concentration being linear for 30 min under the conditions used at protein concentrations up to 5 mg/ml (data not shown).

As shown in Charts 2 and 3, the metabolism of mitomycin C, as determined by its disappearance from the incubation mixture, required an NADPH-generating system, was inhibited strongly by oxygen, was not affected by 0.5 mM potassium cyanide (Chart 3), and was inhibited by carbon monoxide (Chart 3). The effect of carbon monoxide on mitomycin C metabolism could be reversed partially with high concentrations (1 mM) of drug (data not shown). Those manipulations which inhibited the rate of disappearance of mitomycin C also depressed the rate of formation of metabolites capable of alkylation. These results suggest that the generation of reactive metabolites by sonicated tumor cell preparations was related to, or was the direct consequence of, the metabolism of the antibiotic.

To examine the relationship between the utilization of mitomycin C and the cytotoxic activity of the drug, the disappearance of mitomycin C from the medium and the cytotoxicity of the antibiotic were determined using EMT6 cell cultures incubated under different states of oxygenation. The spectrophotometric assay for mitomycin C was relatively insensitive; this necessitated studying the metabolism of mitomycin C at concentrations of 0.03 mM in the medium. Only under conditions of chronic hypoxia was there a high rate of drug disappearance from the culture medium (Chart 4). In contrast, under conditions Chart 4. Metabolism of mitomycin C by intact EMT6 cells in culture. Cells were incubated with drug at a concentration of 0.03 mM under different states of oxygenation as described in the text. Results are expressed as the mean ± S.E. for 3 experiments.

Chart 5. Survival of EMT6 cells exposed to mitomycin C in vitro at a concentration of 1.5 μM under different states of oxygenation as described in the text. Results are expressed as the mean ± S.E. for 6 determinations. The cloning efficiency of untreated cells exposed to air-5% CO2 was 61%.
in which significant oxygen concentrations existed during at least part of the incubation period of the cells with mitomycin C (i.e., normal aeration and acute hypoxia), relatively little drug disappeared from the medium.

Chart 5 shows the fraction of EMT6 tumor cells that survived exposure for 1 hr to 1.5 \( \mu M \) mitomycin C under various states of oxygenation. Results are expressed as a percentage of untreated control cultures, which had a cloning efficiency of 61%. Untreated hypoxic cell cultures (both acutely and chronically hypoxic cultures) were included in each experiment. In the absence of mitomycin C, the cloning efficiencies of the hypoxic cells were equivalent to those of the untreated normally aerated cells. As can be seen in Chart 5, the surviving fractions of cells exposed to mitomycin C under normal aeration or acute hypoxia were similar. However, cells exposed to mitomycin C under conditions of chronic hypoxia showed a significantly lower surviving fraction that did normally aerated or acutely hypoxic cells.

**DISCUSSION**

The hypoxic cells of solid tumors are known to limit the curability of experimental animal tumors by large doses of radiation (9). Hypoxic cells may also be relatively resistant to conventional chemotherapy for several reasons. Because hypoxic tumor cells exist at relatively great distances from blood vessels, drugs with physicochemical properties not conducive to diffusion through tissue may not reach the hypoxic regions of the tumor and, therefore, may be ineffective in attacking hypoxic cells. In addition, agents which are rapidly metabolized and cleared from the body may not reach hypoxic cells in sufficient concentrations to produce biological effects. Furthermore, many hypoxic tumor cells are probably quiescent or are cycling with prolonged, abnormal cell cycle times. As a result, these cells may not be susceptible to those anticancer drugs which are active only against proliferating cells. Chemical agents directed specifically against hypoxic tumor cells would therefore be valuable adjuncts to both existing chemotherapy and radiotherapy.

Bioreductive alkylating agents are envisioned to be a series of drugs directed against hypoxic tumor cells. These agents were hypothesized (10, 11) to be activated selectively by hypoxic tumor cells because these cells exist in environments which are conducive to reductive processes. The results presented in this paper using the prototype bioreductive alkylating agent, mitomycin C, strongly support this conceptual basis for a selective chemotherapeutic attack on hypoxic cells. Sonicated preparations from EMT6 and Sarcoma 180 cells were able to metabolize mitomycin C, and the biotransformation of the drug by the cell preparations appeared to result in the production of a metabolite(s) with alkylating properties, as is evidenced by the formation of an alkylated derivative of \( 4-(p\)-nitrobenzyl)pyridine. The enzyme system present in these malignant cells has properties similar to that responsible for mitomycin C metabolism and activation in the liver (8, 9, 19). The tumor cell enzyme(s) which activates mitomycin C requires pyridine nucleotide reducing equivalents and is sensitive to oxygen. The lack of effect by potassium cyanide on the metabolism of the drug suggests that mitochondrial processes were not involved in the activation of mitomycin C by neoplastic cells. The inhibition of mitomycin C metabolism by carbon monoxide, however, supports the concept that a cytochrome P-450 enzyme system may be involved. Further work with other inhibitors will be required to elucidate conclusively the enzymatic machinery responsible for the biotransformation of mitomycin C.

It had been hypothesized by Lin et al. (10, 11) that, if mitomycin C is activated preferentially under hypoxic conditions, the drug should be selectively toxic to hypoxic tumor cells. The data presented in this report show that cells made hypoxic in culture are much more sensitive to the cytotoxic actions of mitomycin C than are cells maintained under normal aeration. Unpublished work in our laboratory has demonstrated that the selective toxicity of mitomycin C towards hypoxic tumor cells is exhibited over a concentration range of \( 10^{-9} \) to \( 10^{-5} \) M. Previous experiments have shown that EMT6 cell cultures made hypoxic by the technique used in these experiments have radiation dose-response curves similar to those of EMT6 cells rendered severely hypoxic in vitro by more rigorous techniques (18). Cultures gassed for 1 hr (acutely hypoxic cells) become radiobiologically hypoxic only during the end of their incubation period. Neither the gassing procedure per se nor the moderate hypoxia that developed during the early part of these incubations altered the metabolism and cytotoxicity of mitomycin C from that seen in the aerobic cultures. Severe hypoxia throughout the 1-hr incubation with the drug, however, resulted in increased metabolism and cytotoxicity. The similarity of the radiation dose-response curves for hypoxic EMT6 cells in culture and in solid tumors (18) suggests that the chronically hypoxic cells employed in the system used in these studies represent an acceptable model for the radiation-resistant, hypoxic cells of solid tumors.

The data obtained by examining the disappearance of the drug from the culture medium of intact cells is consistent with a markedly greater rate of metabolism of mitomycin C by cells maintained under conditions of chronic hypoxia. For methodological reasons, these data were obtained at a much higher drug level than that used in the cell survival studies. Although the generation of metabolites with alkylating potential could not be measured directly in the experiments with intact cells, the results are consistent with the findings from the sonicated cell preparations and indicate that an increase in the rate of drug disappearance from the culture medium occurred only under conditions of chronic hypoxia and that oxygen strongly inhibited the reaction. Because drug uptake into cells maintained under normal aeration or hypoxia could not be determined, it is possible that differences in intracellular steady-state levels of drug under these 2 conditions may contribute to the overall rate of drug disappearance from the medium. Integration of all of these results suggests that the enhanced cytotoxic activity of mitomycin C under hypoxic conditions is the consequence of increased metabolic activation of this drug to an alkylating species.

Although mitomycin C is selectively toxic to hypoxic cells at low concentrations, this agent is capable of killing well-oxygenated cells at higher levels of drug. Bachur et al. (2, 3) have reported that mitomycin C can undergo 1-electron reduction and subsequent reoxidation by molecular oxygen to generate superoxide radicals. Superoxide anions, in turn, could lead to other oxygen-containing species, such as hydrogen peroxide and the hydroxyl radical (4), and could result in significant cytotoxicity if the cells could not effectively detoxify these.
reactive forms (15). This cyclic reduction and oxidation of mitomycin C by well-oxygenated cells may be responsible for the toxicity of the drug to aerated cells in tumors and normal tissues. Because normal tissues are well oxygenated and unlikely to activate the drug by bioreductive mechanisms, it is possible that the clinical use of mitomycin C could be improved by the use of more appropriate treatment regimens which would take advantage of the selectivity of this agent at low concentrations for hypoxic cells.

Agents other than mitomycin C are known to be activated to cytotoxic metabolites under hypoxic conditions. Studies by several workers have shown that CHO and Ehrlich ascites cells made hypoxic in vitro can reduce misonidazole to the corresponding amine and generate hydroxylamine and/or a nitro-radical anion as cytotoxic species (5, 13, 22). Although originally envisioned solely as agents which would sensitize hypoxic cells to radiation, recent experimental results have suggested a possible role for the nitroimidazoles as chemotherapeutic agents directed against hypoxic tumor cells to be used in combination with agents designed to attack well-oxygenated neoplastic cells (21, 24). Such approaches would appear to be somewhat limited by the relatively large doses of the nitroimidazoles required to produce cytotoxicity. The results obtained in the present study, as well as those obtained with the nitroheterocyclic radiosensitizing compounds, suggest that it is indeed possible to attack selectively hypoxic tumor cells, and they encourage the development of new bioreductive alkylating agents with increased cytotoxicity to hypoxic cells.

REFERENCES

Preferential Activation of Mitomycin C to Cytotoxic Metabolites by Hypoxic Tumor Cells

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