Hyperthermic Enhancement of Cell Killing by Mitomycin C in Mitomycin C-resistant Chinese Hamster Ovary Cells

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ABSTRACT

The interaction of hyperthermia and mitomycin C (MMC) was studied in wild-type Chinese hamster ovary cells and in Chinese hamster ovary cells selected for resistance to MMC. Hyperthermic potentiation of MMC activity was maximal if heating was done simultaneously, or up to 3 h following MMC exposure. Heat enhanced the effect of MMC in both drug-sensitive and -resistant cells. Dose enhancement ratios increased from 1.3–2.0 at 42°C to 2.6–3.8 at 43.5°C and were similar for all cell lines. Cellular uptake of MMC was determined by high pressure liquid chromatography. MMC uptake was similar in the drug-sensitive and -resistant cell lines at 37°C. Hyperthermia (43.5°C) increased cellular uptake of MMC by 78 and 27% in MMC-sensitive and -resistant cell lines. MMC-resistant cells were more sensitive to heat alone at 42, 43, and 44°C. The results suggest that tumor cells that have developed resistance to MMC might be treated effectively with MMC combined with heat to augment the response to MMC. Resistance to MMC may also render tumor cells more sensitive to hyperthermia.

INTRODUCTION

Hyperthermia potentiates the cytotoxicity of several chemotherapeutic agents including doxorubicin (1, 2), cisplatin (2, 3), BCNU (2, 4), and MMC (5, 6). Several investigators have reported clinical trials combining hyperthermia with chemotherapeutic agents (7, 8).

While in vitro and in vivo studies show that heat potentiates the action of various drugs, most previous investigations have been performed with cells that are sensitive to the drug alone. In the clinical setting, however, patients treated with thermochemotherapy are likely to have tumors that have already demonstrated resistance to chemotherapeutic agents. Drug resistance may alter the response of cells to thermochemotherapy. For example, hyperthermia does not augment the action of DX against cells selected for resistance to DX (9) suggesting that thermochemotherapy with DX may be no better than hyperthermia alone for DX-resistant tumors. The same may be true for other chemotherapeutic agents.

MMC is an antibiotic with activity against a variety of human tumors (10). Several investigators have shown that hyperthermia acts synergistically in vitro with MMC against MMC-sensitive cells (5, 6). Because human tumors frequently develop resistance to MMC, we have studied whether or not hyperthermia augments drug action against MMC-resistant cells.

MATERIALS AND METHODS

Cells and Culture Conditions. All studies were done with Chinese hamster fibroblasts, HA-1 cells, and their MMC-resistant variants, HA-M3 and HA-M4. The cells were maintained in Eagle's minimal essential medium (Gibco, Santa Clara, CA) supplemented with 15% fetal calf serum and gentamycin (25 μg/ml). Cultures were kept at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO2 and routinely checked for Mycoplasma contamination. Exponentially growing cultures, used for all experiments, were prepared by plating 2.0 × 10⁶ cells in 60-mm Petri dishes on day 0. Experiments were performed on day 2 when the cell density reached 0.8–1.2 × 10⁶ cells/cm².

MMC-resistant cell lines, designated HA-M3 and HA-M4, were developed by growing cells in progressively higher concentrations of MMC. The HA-M3 line was grown in a final concentration of 0.04 μg/ml MMC prior to being returned to drug-free medium. HA-M4 cells were grown in a final concentration of 0.06 μg/ml MMC prior to being returned to drug-free medium. Both drug-resistant lines were passed in drug-free medium for at least 1 month before being used in experiments. They were maintained from that time on in drug-free medium and showed no loss of drug resistance during the 12 months within which experiments were performed. Plating efficiencies for all lines were 85–95%, with doubling times of 15, 17, and 17 h for HA-1, HA-M3, and HA-M4, respectively.

Drug and Heat Treatment. An MMC stock solution was prepared by diluting freshly mixed MMC (Bristol-Myers, Syracuse, NY; kindly supplied by Dr. James H. Keller) to a concentration of 50 μg/ml in sterile water. Multiple 2-ml aliquots were separately frozen in vials at −20°C and protected from light. The same stock of drug was used for all experiments. Immediately prior to each experiment, a vial of MMC was thawed to room temperature and diluted to the appropriate concentration with full medium. For MMC exposure, normal medium was removed and replaced with drug-containing medium. Control experiments were performed to determine that up to 2 × 10⁴ MMC-killed cells did not appreciably alter the plating efficiency of untreated cells. Immediately after MMC treatment, the medium was withdrawn, the cells were washed once with phosphate-buffered saline, trypsinized, counted on a Coulter Counter, and plated at appropriate concentrations to yield 50–200 colonies/dish. The dishes were incubated at 37°C for 8–10 days, at which time surviving colonies were stained with crystal violet, and those with 50 or more cells were counted with a dissecting microscope.

Heating of cell monolayers was done in water baths in specially designed incubators. The pH was maintained at 7.2–7.4 by a regulated flow of 95% air and 5% CO2. The temperature of the water bath was controlled to within ±0.1°C. Medium with or without MMC was placed over the cells immediately prior to heating. Cells were irradiated with a 250 kVp X-ray machine (Westinghouse) at 250 kV, 15 mA, and a dose rate of 300 rads/min.

Cellular Uptake and Efflux of MMC. Cellular uptake and efflux of MMC were measured by high pressure liquid chromatography, with some modification of the technique of Den Hartigh et al. (11). Eight to nine million exponentially growing cells were exposed to MMC in 100-mm Petri dishes. Following drug exposure, the cells were rinsed in the dish 6 times with cold phosphate-buffered saline (the amount of MMC left in the dish remained constant after 5 washes). Following rinsing, the cells were lysed with distilled water, scraped into a conical glass centrifuge tube, and extracted with a 1:1 (w/v) mixture of 2-isopropanol and chloroform. The cell lysate was pipetted 6 times and centrifuged at 1500 rpm for 15 min. The lower, organic phase was collected and dried at 40°C under a stream of nitrogen. The sediment was resuspended in 100 μl of methanol, and a 30-μl sample was injected onto a Bondapak C18 reverse-phase column [30 cm x 3.9 mm inside diameter; particle size, 10 μm (Waters Associates, Milford, MA)]. Mitomycin C was monitored by absorption at 364 nm (Hewlett Packard 1040). MMC was seen as a distinct peak at 8.5–9.0 min elution time. Absorbance was linearly related to drug quantity from 6–60 ng/injec-

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3 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosoure; MMC, mitomycin C; DX, doxorubicin; DER, dose-enhancement ratio.
tion volume. Total MMC was adjusted for cell number and expressed as ng MMC/10^6 cells.

RESULTS

The effect of time sequencing on the interaction of heat with MMC in HA-1 cells is shown in Fig. 1. The interactive effect is maximal when MMC and heat are given simultaneously or if the heat treatment is given up to 4 h following drug exposure. All experiments were subsequently performed with simultaneous heat and drug exposure for 1 h. SE bars in Fig. 1 and all subsequent figures represent the composite of at least 3 replicate, separate experiments.

The response of the parent line, HA-1, and the 2 resistant variants to exposure to MMC for 1 h at 37°C are shown in Fig. 2. At the 50% survival level, the HA-M3 and HA-M4 cell lines were 3.0- and 2.6-fold, respectively, more resistant to MMC than the parent line. While HA-M4 had been grown in a concentration of 0.06 µg/ml prior to being returned to normal medium, it showed no more drug resistance than did HA-M3, which was grown in a concentration of MMC of 0.04 µg/ml. The degree of drug resistance seen here was the maximum that could be achieved with the method of adaptation used. Above a concentration of 0.06 µg/ml, cell growth slowed markedly and the cells could not be maintained in culture.

The cytotoxicity of MMC at 37, 42, 43, and 43.5°C is shown in Fig. 3. The survival curves in the lower panels have been corrected for heat killing. Enchancement of drug cytotoxicity after correction for heat killing represents synergism (12). Both drug-resistant cell lines showed potentiation of the effect of MMC by hyperthermia.

DERs were calculated as the MMC concentration needed to produce a 20% survival level in the presence of heat (corrected for heat killing) divided by the concentration needed to give a 20% survival in the absence of heat (Fig. 4). There was a marked increase in the DER, from 1.3-2.0 at 42°C to 2.6-3.8 at 43°C. While the survival levels of the drug-resistant cell lines are higher at any given drug concentration and temperature, the DERs are similar to those of the drug-sensitive parent line. The

![Fig. 2. Response of MMC-sensitive (HA-1) and -resistant (HA-M3 and HA-M4) cell lines to MMC at 37°C for 1 h. Bars; SE; U, μ.](image)

DERs were similar whether calculated at the 10, 20, or 50% survival levels.

The heat response of the cell lines at 42, 43, and 44°C is shown in Fig. 5. Both drug-resistant cell lines showed some increased heat sensitivity at all temperatures tested; HA-M4 was more heat sensitive than HA-M3. At 1.5 and 4 h exposure to 44 and 43°C, respectively, survival of HA-M4 cells was significantly lower than that for HA-M3 cells (P < 0.05, 2-tailed t test).

Uptake of MMC at 37 and 43.5°C for HA-1 cells and HA-M3 cells is shown in Fig. 6. Cells were exposed to 8.0 µg/ml MMC for 5-60 min. MMC uptake was similar between the 2 cell lines. Survival of HA-1 cells after 1 h exposure to 8.0 µg/ml MMC at 37 and 43.5°C was 3.6 × 10^-4 and 2.1 × 10^-3, respectively. For HA-M3, survival at 37 and 43.5°C was 9.4 × 10^-2 and 2.5 × 10^-3, respectively. By integrating the areas under the curves, MMC uptake in HA-M3 cells was 26% greater than in HA-1 cells after 1 h exposure at 37°C. Elevated temperature was found to increase MMC uptake by 78 and 27% for HA-1 cells and HA-M3 cells, respectively. Binding of MMC to dishes that contained no cells was less than 5% of the amount in cell-containing dishes. Increasing the exposure temperature did not increase drug binding to empty Petri dishes. Efflux of MMC was similar between the 2 cell lines (Fig. 7).

DISCUSSION

Mitomycin C is an antibiotic which acquires cytotoxic properties after reduction to an alkylating form inside cells (13). It is preferentially activated by hypoxic cells (14) and may therefore show some degree of selectivity for solid tumors. Previous investigators have shown that the action of MMC against drug-sensitive cells is augmented by hyperthermia (5, 6). We have shown here that the same phenomenon occurs in cells selected for resistance to MMC. While MMC-resistant cells were still relatively resistant at elevated temperatures, hyperthermia en-
Fig. 3. Response of cells to MMC exposure for 1 h at 37, 42, 43, and 43.5°C. Top, combined effect of MMC and heat at the temperatures shown. Cell survival has been corrected for cytotoxicity by heat alone (bottom). Bars, SE; U, µ.

Fig. 4. DERs at 42, 43, and 43.5°C. Calculations were made after correction for heat killing. Bars, SE.

Enhanced drug action to the same extent in drug-resistant and -sensitive cells. The DERs of 1.4–4 were calculated at drug concentrations that are clinically achievable in serum (15). The DERs for both the MMC-sensitive and -resistant cells increased markedly above 42°C, temperatures which are attainable with localized rather than whole-body hyperthermia.

While uptake of MMC at 37°C was greater in the resistant cell line (HA-M3) as compared to HA-1 cells, the former had a 14% greater cell volume, accounting at least in part for the increased MMC accumulation. MMC efflux was similar in our drug-sensitive and -resistant cells. By extracting the cells in an organic phase, we only measured the unbound or cytoplasmic MMC. MMC that has covalently bound to DNA, RNA, or protein will be left either in the aqueous phase, or at the interface between the aqueous and inorganic phase of the extraction volume and will not be measured. Orstavik (16) used 3H-labeled MMC to show that cytoplasmic (unbound) MMC accounts for 60% of the total cellular drug. Because changes in cellular permeability should directly affect the unbound, or
cytoplasmic drug concentration, it appears that resistance of our cells to MMC is due to something other than a change in cellular permeability to the drug. The differences in drug sensitivity may be due to changes in the rate of activation of the drug (17), changes in the repair kinetics of DNA damage, or to other as yet undefined factors.

The mechanism of hyperthermic potentiation of chemotherapeutic agents is probably multifaceted. It may involve changes in drug accumulation (1), drug activation (5), alterations in DNA repair (18), or increased rates of intracellular alkylation. There is limited information on the role of various postulated mechanisms. We found an increase in cellular accumulation of MMC at elevated temperature, suggesting that alterations in cellular permeability may play a role in hyperthermic potentiation of MMC. Teicher et al. (5) have shown that there is increased activation of MMC at elevated temperature, which may also account in part for increased cytotoxicity at elevated temperature. The fact that heating cells after MMC has been removed from the medium still potentiates MMC cytotoxicity (Fig. 2), however, argues against the importance of permeability or activation changes at elevated temperatures; rather, it points to the importance of alterations in damage-repair processes.

MMC-resistant cells demonstrated an increased sensitivity to heat alone. Drug resistance is not necessarily associated with increased heat sensitivity. Previous investigators have shown that doxorubicin (9) and BCNU (4) resistance does not alter the response of cells to hyperthermia alone. Collateral sensitivity is an unusual phenomenon (19). Cross-resistance between chemotherapeutic agents is much more common. We do not know why MMC resistance would confer an increased sensitivity to heat. Considerable differences in heat sensitivity between cell lines are common (20) but are as yet unexplained. Cell surface protein changes have been associated with MMC resistance (21). Membrane effects have also been implicated in
the mechanism of heat killing (22). It is possible that membrane alterations are a common factor in MMC resistance and heat sensitivity.

Because the vast majority of solid tumors rapidly develop resistance to the available chemotherapeutic agents, it will be important in thermochemotherapy trials to use drugs whose action against drug-resistant cells is potentiated by heat. Da Silva et al. (4) recently showed that BCNU-resistant cells are more responsive to BCNU in the presence of heat. BCNU and MMC, therefore, may both be good drugs to use in combination with hyperthermia against solid tumors that have failed to respond to conventional chemotherapy regimens. In contrast, Wallner and Li (9) have shown that DX-resistant cells do not respond any better to DX in the presence of heat, suggesting that DX-resistant tumors would not respond better to DX plus heat than they would to heat alone.

The choice of drugs, heat and drug sequencing, and temperatures achievable may all affect the outcome of further attempts to combine chemotherapeutic agents with hyperthermia. In vitro studies such as those described here should provide direction for further in vivo work and clinical applications and may alert clinicians to potential pitfalls of thermochemotherapy.

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