Delayed Sensitization to Heat by Inhibitors of Polyamine-biosynthetic Enzymes

David J. M. Fuller and Eugene W. Gerner

Division of Radiation Oncology, Department of Radiology, University of Arizona Health Sciences Center, Tucson, Arizona 85724

ABSTRACT

Exposure of Chinese hamster ovary cells to the enzyme inhibitors methylglyoxal bis(guanylylhydrazone) and α-difluoromethylornithine (DFMO) results in increased sensitivity to hyperthermia. While methylglyoxal bis(guanylylhydrazone) demonstrates pronounced cytotoxicity at moderate extracellular concentrations, DFMO is tolerated well by this cell line at concentrations of up to 10 mM, as assayed by clonogenic survival after treatments at 37°C. An 8-hr preincubation at 37°C with either drug elicits increasing sensitivity to 43°C hyperthermia treatments with time after removal of the drug. In contrast to results obtained by heating in the presence of the drugs during the 8-hr exposure, DFMO acts as the more effective sensitizing agent for this delayed effect on progeny of DFMO-treated populations. This phenomenon seems to result from depletion of intracellular putrescine, because the effect can be at least partially recovered by providing the cells with an exogenous source of this diamine. The potential for in vivo heat sensitization by the non-toxic agent DFMO has yet to be investigated but may have intriguing clinical possibilities.

INTRODUCTION

Elevated temperatures (above 41°C) have been shown, both in vivo and in cell culture systems, to decrease cell survival as measured by clonogenic assay. It is well known that the cell-killing effects of drugs (8) and radiation (3, 4, 15) can be modulated by heat. These findings have obvious clinical relevance to cancer therapy, in which an array of mutually interactive modalities may prove to be less toxic to the patient than one or 2 modalities delivered at high levels. Work in our laboratory and others has included the investigation of the potential of heat-induced cell killing by the polyamines and their analogs (2, 7). Polyamines are organic polycations that occur at near-millimolar concentrations within mammalian cells, the precise function(s) of which remain(s) unclear, although some specific interactions have been described. Our data have shown sensitizing to 43°C in the order spermine > spermidine > putrescine at exogenous levels much lower than those seen intracellularly. This suggests a specific structurally related mechanism at the level of the cell membrane.

The diamine putrescine is derived from the decarboxylation of ornithine by the enzyme ODC. ODC can be rapidly and irreversibly inhibited by the drug DFMO. This is an enzyme-activated "suicide" inhibitor that rapidly depletes putrescine and spermidine in many systems (10). The compound is of particular interest since, unlike MGBG, it has minimal toxicity. It has been used successfully in vivo to treat parasitic protozoan diseases and has been under trial as a potential antitumor agent (1, 11).

Spermidine and, indirectly, spermine are synthesized from the diamine putrescine by the action in part of the enzyme S-adenosylmethionine decarboxylase (EC 4.1.1.50). MGBG is a competitive inhibitor of the enzyme and has been used as an antiproliferative drug in the treatment of acute myelocytic leukemia and more recently in some solid tumors (9). Although structurally related to spermidine, the compound has been found to interfere with the normal functioning of mitochondria, affecting both ultrastructure and mitochondrial DNA synthesis (5, 12, 13).

The purpose of this study was to determine whether these polyamine-depleting agents could be used to modulate the sensitivity of cells in culture to the cytotoxic effects of elevated temperatures.

MATERIALS AND METHODS

Cell Culture and Hyperthermic Treatment. CHO cells were maintained in monolayer at 37°C in McCoy's Medium 5A (Gibco Laboratories, Santa Clara, Calif.) supplemented with 10% fetal bovine serum (Gibco Laboratories). Hyperthermia treatments were carried out by sealing T-25 (Falcon Plastics, Oxnard, Calif.) flasks containing cells and 5 ml of medium with Parafilm (American Can Company, Greenwich, Conn.) and immersing them in a water bath at 43°C.

After heating, cells were trypsinized off the monolayer and counted (Coulter Counter; Coulter Electronics, Inc., Hialeah, Fla.). Dilutions of the cell suspensions were plated out and allowed to grow undisturbed. After 1 week, the cell colonies were stained and counted to estimate plating efficiency and percentage survival.

Drugs. MGBG was obtained from Aldrich Chemical Company (Milwaukee, Wis.). DFMO (Merrell-Dow Pharmaceutical, Inc., Cincinnati, Ohio) was generously donated by Merrell Research Center.

RESULTS

37°C Survival and Cell Proliferation. Chart 1 illustrates the effect of continuous exposure of CHO cells to DFMO and MGBG. Log-phase populations of cells were incubated at 37°C with 10 mM DFMO or 10 μM MGBG. Chart 1A shows the effect of continuous drug exposures of up to 24 hr on cell number increase. DFMO treatment does not cause a significant change from control cell numbers, whereas MGBG shows a blocking effect on cell division developing between 6 and 12 hr. This is associated with rapidly decreasing cell survival at this time, as seen in Chart 1B. Clonogenic survival assay indicates that DFMO-treated cells show no loss of colony-forming ability for treatment times up to 24 hr. MGBG, however, demonstrates considerable cytotoxicity, with survival values approaching 1% after 24 hr. For this reason, a treatment time of 8 hr was
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Chart 1. A, effect of drugs (DFMO, 10 mM; MGBG, 10 μM) on CHO cell proliferation. Asynchronously growing cultures (control; DFMO; MGBG) were continuously exposed to drugs and harvested by trypsinization at 6-hr intervals. B, effect of drugs (DFMO, 10 mM; MGBG, 10 μM) on cell viability as measured by clonogenic survival assay. Asynchronous cultures (control; DFMO; MGBG) were trypsinized at intervals after introduction of the drugs, and cells were plated out for survival assay.

chosen for subsequent studies as survival was still greater than 50% after this exposure duration.

Effect of Drugs on Heat-induced Cell Kill. In these experiments, survival values were corrected to show "heat-only" effects. Chart 2 shows the effect of 43°C on the survival of cells exposed to the drugs. MGBG sensitizes to heat primarily by decreasing the shoulder of the survival curve. At 90 min, almost a 2-log increase in cell kill is observed. DFMO elicits no significant sensitization.

Delayed Sensitization Effects. Chart 3A shows the increase in cell number with time after an 8-hr exposure to the drugs. DFMO-treated and control cells increase in number in a similar manner with a doubling time of approximately 14 hr, typical for this cell line. Cells exposed to MGBG increase in numbers after an initial lag period following drug removal.

Chart 3B shows the changes in sensitivity to 90 min at 43°C with time after the 8-hr exposure to either DFMO or MGBG. Control cells show survivals between 8 and 10% throughout the 48-hr period.

Drug treatment prior to heat, however, causes an increase in sensitivity becoming significant 24 hr after the end of the 8-hr drug exposures at 37°C. MGBG causes moderate sensitization with survival decreasing to around 1%. The DFMO-treated cells, however, display a high degree of delayed heat sensitivity with survivals shifted by more than 2 logs to 0.02% during the 48-hr interval between the end of drug exposure and the heat dose.

Protective Effect of Putrescine Added Exogenously. In these experiments, asynchronous cell populations were exposed to 10 mM DFMO for 8 hr. Selected cultures were simultaneously treated with exogenous putrescine at concentrations from 10^-6 to 10^-3 M. Following the protocol described for Charts 3A and 3B, drugs were washed from the monolayers with Puck's Saline A, and the cultures were allowed to grow in full medium for 36 hr, at which time all were subjected to 90 min at 43°C and assayed for survival. Data displayed in Chart 4 indicate a protective effect exerted by exogenous putrescine over the heat-sensitizing effect of DFMO. Although a return to control levels is not observed at the concentrations used, survival is raised from 0.1% (0 mM putrescine) to 2% (10^-3 M putrescine), an increase of 20-fold, expressed in a dose-dependent manner. Cultures were maintained in 10^-5 M amino-guanidine, an inhibitor of diamine oxidase, to prevent the formation of toxic by-products from the oxidation of putrescine.

DISCUSSION

The data presented here clearly show that both DFMO and MGBG are capable of eliciting a delayed and marked increase in sensitivity to heat in CHO cells. The phenomenon is intriguing in that this thermal sensitivity is displayed by cells that are the daughters of those treated with the drug.

MGBG, which is capable of sensitizing cells to elevated temperature-induced cytotoxicity when the 2 agents are used simultaneously (DFMO is ineffective for this sequence), shows
oratory has shown that the effect is dose dependent and essentially maximal at 10 mM.

Delayed potentiation by this drug surprising. Work in this lab coupled with the lack of immediate sensitizing to 43°C make the (Chart 1, 4). This ability to tolerate high levels (10 mM) of DFMO in the 24-hr period in which we have exposed CHO cells to it for it to exert its cytostatic effects, presumably through the depletion of some intracellular target. A discussion of these dual modalities is to be published in a short review (6).

The use of DFMO clinically as an antitumor drug has met with mixed results. Its major advantage is that it is minimally toxic, but cells must be exposed to the drug for long periods for it to exert its cytostatic effects, presumably through the depletion of intracellular polyamines. The drug, however, is toxic, but cells must be exposed to the drug for long periods to depletion of some intracellular target. A discussion of these dual modalities is to be published in a short review (6).

The results shown in Chart 4 suggest that, at least in part, putrescine can rescue cells from the effects of DFMO. Whether the potentiation is due to a depletion of putrescine or its next homolog, spermidine, in some critical intracellular target is unclear. DFMO stimulates S-adenosylmethionine decarboxylase 8-fold over control levels, and exogenously added putrescine may be rapidly incorporated into the polyamine pathway. This induction suggests that spermidine levels are depressed (10). In addition, despite the transient nature of the exposure of these cells to DFMO, ODC activity does not recover, despite cell proliferation thus limiting putrescine, the precursor to spermidine. Analyses of polyamine levels have confirmed that both putrescine and spermidine concentrations are reduced after exposure to DFMO. Longer treatments with exogenous putrescine can completely protect against the DFMO-induced delayed heat sensitization with considerable structural specificity. Of a range of diamines used, from 1,3-diaminopropane to 1,8-diaminooctane, only putrescine showed convincing ability to rescue cells from the effects of DFMO.

As for the nature of the target itself, it is tempting to speculate that it may reside in the cytoskeletal structure of the treated cells. Pohjanpelto et al. (14) have demonstrated that CHO cells selected as polyamine auxotrophs are dependent on exogenous putrescine for correct assembly of actin filaments and microtubules. Sunkara et al. (16) have shown that MGBG and a-methylornithine (an ODC inhibitor) will also interfere with the formation of actin bundles in cell cultures, a phenomenon that can be reversed by exogenous polyamines. It is important to emphasize the dual roles of the polyamines in the modulation of sensitivity to heat. In the past, data from our laboratory and others have indicated that extracellular polyamines can act to sensitize to heat when present at the time of heat shock (2, 3, 7). We have suggested that this may be a cell membrane phenomenon. In the present study, we have observed that treatment of cells with a polyamine biosynthesis inhibitor many hr prior to heating can evoke increased cell kill, possibly due to depletion of some intracellular target. A discussion of these dual modalities is to be published in a short review (6).

The use of DFMO clinically as an antitumor drug has met with mixed results. Its major advantage is that it is minimally toxic, but cells must be exposed to the drug for long periods for it to exert its cytostatic effects, presumably through the depletion of intracellular polyamines. The drug, however, is toxic, but cells must be exposed to the drug for long periods for it to exert its cytostatic effects, presumably through the depletion of intracellular polyamines. The drug, however, is cleared rapidly, and high chronic doses are required. We present here evidence that DFMO is capable of potentiating the effects of heat in CHO cells by acting on a target that is sensitive to short-term exposures. This protocol is readily achievable in vivo and offers intriguing possibilities for the clinical use of a nontoxic heat sensitizer.

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