Modulation of the Cytotoxicity of Mitomycin C to EMT6 Mouse Mammary Tumor Cells by Dicoumarol in Vitro

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ABSTRACT

Aerobic and hypoxic cultures of EMT6 mouse mammary tumor cells were used to study the effects of dicoumarol (DIC) on the cytotoxicity of mitomycin C (MC). DIC protected aerobic cells from MC toxicity, but sensitized hypoxic cells to the cytotoxic actions of this antibiotic. Survival curves for cells treated with 1.5 μM MC ± 100 μM DIC for different periods of time under aerobic or hypoxic conditions showed that DIC acted as a dose-modifying agent, that is, an agent which changed the slopes, but not the shapes, of the MC survival curves. Experiments that examined the effects of the DIC concentration on the modulation of MC cytotoxicity revealed significant perturbations in MC toxicity with a DIC concentration of 100 μM and increased sensitization/protection with increasing levels of DIC. DIC altered the toxicity of MC only when it was present during exposure of the cells to MC. Treatment with DIC before or after (but not during) MC did not alter the amount of cytotoxicity. Addition of DIC to cell cultures seconds before the addition of MC was as effective as addition of DIC 30 min to 2 h before MC. Taken together, these findings suggest that DIC reversibly inhibits one or more enzymes involved in the activation and inactivation of MC, and that this modulation of the enzymatic processing of MC alters the cytotoxicity of the drug.

INTRODUCTION

MC has been used for many years in the therapy of cancer, both as a single agent and as part of combination chemotherapy regimens (1, 2). Recently, our groups have explored the possibility of using MC in combination with radiotherapy in regimens designed to capitalize on the differences in the toxicities of the two modalities (3–9). Radiation is far more effective in killing aerobic cells than in killing hypoxic cells (10, 11), while MC is more toxic to cells under hypoxic conditions than to aerobic cells (12, 13). As solid tumors include both hypoxic and aerobic cell populations (11), we hypothesized that regimens combining these two agents might be particularly efficacious in the treatment of solid malignancies. Such regimens were tested in laboratory animals with encouraging results (5, 6, 13–16), and a prospective clinical trial testing MC as an adjunct to radiotherapy in carcinoma of the head and neck (7) showed a significant improvement in local control with the combined modality regimen over that achieved with radiotherapy alone.

Previous studies in our laboratories showed that DIC, an inhibitor of DT-diaphorase (17), could be used to modulate the toxicity of MC to EMT6 tumor cells (18, 19). In vitro, DIC increases the toxicity of MC to hypoxic cells, but decreases the toxicity of the drug to aerobic cells. We suggested that DIC might be used to improve the therapeutic ratios attainable with MC in those situations in which the toxicity of MC to hypoxic tumor cells was critical in determining its antineoplastic efficacy, as, for example, in regimens combining MC with radiation (19). Initial studies with mice suggested that this approach had merit and indicated the value of further studies of the interactions of MC and DIC (19).

In the experiments described in this paper, aerobic and hypoxic cultures of EMT6 mouse mammary tumor cells were used to examine in more detail the effects of DIC on the cytotoxicity of MC. These data will be useful in developing optimized regimens of administration of DIC in vivo and will also provide some insights into the mechanism by which this agent modulates the toxicity of MC.

MATERIALS AND METHODS

All experiments were performed using EMT6 mouse mammary tumor cells (subline EMT6-Rw). The origin and characteristics of these cells and the techniques used to propagate and handle them are described in detail elsewhere (14, 20). All experiments were performed using exponentially growing monolayer cultures, propagated in Waymouth's medium supplemented with 15% fetal bovine serum and antibiotics; these were produced by plating 1 to 2 × 10⁵ cells, harvested from exponentially growing stock cultures, into glass milk dilution bottles and allowing the cultures to grow for 3 to 4 days at 37°C in a humidified atmosphere of 95% air/5% CO₂. Because of availability problems the source of the medium (Gibco, Grand Island, NY, or KC Biological, Kansas City, KS) and serum (Gibco, Grand Island, NY; KC Biological, Kansas City, KS, or Flow Labs, McLean, VA) varied during the course of these studies. This variation and batch-to-batch variations in MC lots had some effect on the absolute toxicity of MC (e.g., Fig. 1 versus Fig. 2) but did not alter the DIC sensitization/protection phenomenon described in this paper. To produce hypoxia in the cultures, the medium overlaying the cultures was removed, and the cells were recovered with 5 ml of fresh Waymouth's medium. The flasks were then stoppered with thick rubber sleeves and gassed with a humidified mixture of 95% N₂/5% CO₂ (<10 ppm O₂) as detailed previously (12).

This technique produces radiobiological hypoxia in the cultures after about 1 h of gassing. In the experiments reported here, cultures were gassed for 2 h before treatment with drugs, except as noted in the figure legends. Cultures were treated by injecting the drugs through the septum, without interrupting the flow of gas.

Cell viability was assessed using a colony formation assay described in detail elsewhere (20). In this assay, treated monolayers were rinsed and trypsinized, the suspended cells were counted and diluted, and varying numbers of cells were plated into 60-mm tissue culture dishes containing Waymouth's medium. The cultures were incubated for 2 wk at 37°C to allow viable cells to grow into macroscopic colonies; only colonies containing more than 40 cells were counted. Surviving fractions for treated cultures were calculated using the plating efficiencies of untreated control cultures assayed on the same day; these control plating efficiencies were generally ~60 to 70%. The total numbers of cells suspended from treated and control cultures were compared to detect any rapid cytolysis occurring as a result of treatment; none was observed in these studies.

MC was provided through the generosity of the Bristol Myers Company, Wallingford, CT. MC was dissolved in ethanol and was protected from light. DIC, purchased from sigma Chemical Company, St. Louis, MO, was dissolved in 0.5 N NaOH.

RESULTS

Studies reported previously (18, 19) showed that 100 μM DIC added to the medium overlying EMT6 cell cultures a few...
MODULATION OF MC TOXICITY BY DICOUMAROL

Fig. 1. Effects of 100 μM DIC on the survival curve of cells treated with 1.5 μM MC for various periods of time under aerobic or hypoxic conditions. Aerobic treatments, closed symbols; hypoxic treatments, open symbols. MC alone (O, •); DIC + MC (O, □); vehicles only (V, △); DIC only (A, ▲). Points, geometric mean of survivals determined from 3 to 8 independent experiments; bars, SE shown only for the 2-h points, to simplify the figure; SEs for other points are smaller.

Fig. 2. Effect of the concentration of DIC. Aerobic cultures (•) were treated with 1.5 μM MC for 2 h; DIC was added just before the addition of MC. Hypoxic cells (○) were treated with 1.5 μM MC for 1 h; DIC was added just before MC. The different durations of MC treatment in air and hypoxia were used to obtain similar cytotoxicities. Cultures treated with the vehicles or DIC plus aerobic (△) or hypoxic (□) incubations (without MC) were included to assess the effects of the experimental manipulations. Points, geometric mean of survivals determined in 4 independent experiments; bars, SE shown where larger than the points.

seconds before the addition of 1.5 μM MC for 1 h increased the toxicity of the antibiotic to hypoxic cells, but decreased the toxicity of MC to aerobic cells. The experiments shown in Fig. 1 examined the effects of a similar treatment with DIC on the survival curves for cultures treated with 1.5 μM MC for different periods of time. In these studies, 100 μM DIC was added to the medium overlying aerobic or hypoxic cultures a few seconds before addition of MC; this protocol resulted in essentially simultaneous exposure to DIC and MC. As shown in Fig. 1, simultaneous treatment with DIC altered the MC survival curves for both aerobic and hypoxic cells, protecting the aerobic cells from MC toxicity and sensitizing the hypoxic cells to the toxicity of the antibiotic. Under both aerobic and hypoxic conditions, DIC appeared to act as a dose-modifying agent [that is, an agent which alters the slope of the curve without modifying the basic shape of the survival curve (21)].

The importance of DIC concentrations was assessed by comparing cell survivals in cultures treated with 1.5 μM MC alone or in combination with DIC at different levels (Fig. 2). DIC was added to the culture medium seconds before the addition of MC. Aerobic cultures were treated with MC for 2 h, hypoxic cultures for 1 h; because of the greater toxicity of MC in hypoxia, these MC treatments produced similar cytotoxicities. The survivals of cultures exposed to DIC began to separate from those of cultures treated with MC alone at ~100 μM DIC. The sensitivity of hypoxic cultures increased progressively with increasing DIC concentration. The sensitivity of aerobic cultures decreased initially, and then plateaued at DIC concentrations of 300 to 1000 μM; at these levels the surviving fractions obtained with MC plus DIC were 20-fold higher than those obtained with MC alone and only slightly lower than the survivals obtained with DIC alone.

Fig. 3 and Table 1 show the results of several experiments assessing whether pretreatment with DIC increases its efficacy in modulating MC toxicity and assessing the effect of varying the sequence and timing of the DIC and MC treatments. As in the experiments shown on Fig. 2, aerobic cells were treated with 1.5 μM MC for 2 h; hypoxic cells were treated with 1.5 μM MC for 1 h to obtain roughly similar cytotoxicities with MC alone under these different conditions. Because of the findings shown on Fig. 2, a 300 μM concentration of DIC was used in these studies.

DIC increased the survival of aerobic, MC-treated cells when administered simultaneously with MC (Fig. 3; Table 1); pretreatment with DIC for 30 min to 2 h before addition of MC did not increase the protection over that obtained with only simultaneous exposures (Fig. 3). When cultures were treated with DIC before or after (but not during) the MC treatment, cell survivals were similar to those of cultures treated with MC alone (Table 1). Treatment with the experimental manipulations and vehicles, without drugs, produced no significant toxicities (Fig. 3; Table 1). DIC alone was not cytotoxic under the conditions used in these experiments (Fig. 1 to 3).

DIC and Table 1 also show the results of similar studies with hypoxic cultures. Treatment with DIC a few seconds before addition of MC sensitized hypoxic cells to the toxic action of MC. Pretreatment with DIC for 30 min to 2 h before MC did not alter the sensitization from that obtained with simultaneous exposures. Sequential 1-h treatments with MC and DIC, in either order, separated by a 1-h incubation to reestablish severe hypoxia, produced toxicities similar to those obtained with MC alone (Table 1). Control cells, manipulated in the same manner as drug-treated cells with hypoxic incubations, washing, and vehicles had viabilities similar to untreated cells (Fig. 3; Table 1).

In summary, hypoxic cells were sensitized to the toxic effects of MC by DIC, while aerobic cells were protected from MC. Both effects required the presence of DIC at the time of exposure to the antibiotic, and neither effect was augmented by pretreatment of the cultures with DIC.
DISCUSSION

The experiments described in this paper were undertaken to examine further the effects of DIC on the toxicity of MC to tumor cells and also to provide information which might assist in elucidating the mechanism by which DIC modulates the toxicity of MC.

The results shown in Fig. 1 reveal that DIC given simultaneously with MC acts as a dose-modifying agent, increasing the slope of the survival curve for cells treated with the antibiotic in hypoxia and decreasing the slope of the survival curve for cells treated in air. In general, dose-modifying agents modify the amount of damage produced by the cytotoxic agent, the fixation of this damage, or the repair of this damage. This can occur through direct interactions between the two agents, through interactions between the dose-modifying agent and the lesions produced by the toxic agent, or through other, less direct, mechanisms. As EMT6 cells do not appear to repair sublethal or potentially lethal lesions induced by MC (14, 22, 23), modulation of repair processes by DIC seems unlikely to cause the observed changes in MC toxicity. The fact that DIC has opposite effects in air and hypoxia suggests that this agent is in some way modulating the enzymatic activation and inactivation of MC by the cells. Other potential mechanisms of DIC action (e.g., alteration of the entry or efflux of MC by DIC, DIC interacting with activated MC, DIC reacting with radicals produced by MC, etc.) would be expected to have similar effects in air and hypoxia. Studies of the uptake and efflux of radiolabeled porfiromycin in EMT6 cells under aerobic and hypoxic conditions showed that DIC did not alter the rates or extents of uptake of that radiolabeled drug (24), suggesting that changes in intracellular drug processing, rather than changes in drug uptake, are responsible for the DIC-induced changes in the cytotoxicity of MC and porfiromycin.

The concentrations of DIC necessary to modulate the cytotoxicity of MC in air and hypoxia are similar, are in the range reported for inhibition of enzymes by DIC (17), and are sufficient to abolish measurable DT-diaphorase activity in EMT6 homogenates (18). The concentrations of DIC required to modulate MC cytotoxicity are far in excess of the MC concentrations (100 µM DIC versus 1.5 µM MC); this probably reflects the fact that DIC competes with NAD(P)H for the NAD(P)H binding site on the reductases (17), rather than competing for the binding site occupied by MC. If DIC is acting as an inhibitor of an enzyme(s) involved in the metabolism of MC, the different effects produced by DIC in air and hypoxia imply that the enzyme(s) inhibited by DIC has opposite effects in air and hypoxia. Protection from MC damage in air by DIC implies that DIC inhibits a process which activates MC to a toxic species; in contrast, sensitization to MC by DIC in hypoxia implies that DIC inhibits a process which protects against MC damage (e.g., a detoxification process). If a single enzyme is responsible for both effects, this enzyme must have opposite effects in air and hypoxia; if two enzymes are inhibited by DIC, one must activate MC to a toxic product in air, and one must detoxify MC in hypoxia.

DIC must be present simultaneously with MC to modulate the cytotoxicity of the antibiotic. The fact that treatment with DIC before, but not during, MC treatment does not alter the toxicity of the antibiotic indicates that DIC neither induces new enzymes nor irreversibly inhibits or activates existing enzymes which process MC. The finding that exposure to DIC after MC treatment is without effect suggests that DIC does not act by influencing slow damage fixation or repair processes in MC-treated cells. The fact that addition of DIC seconds before MC is as effective as addition up to 2 h before the antibiotic indicates that DIC acts rapidly, and again suggests modulation of the activity of an existing enzyme, rather than induction of new enzymes or changes in enzyme levels.

The development of protocols using DIC to modulate the efficacy of MC in vivo is facilitated by a consideration of these results. As DIC must be present only during MC treatment to modulate MC metabolism, clinical regimens should be designed to obtain maximal DIC levels in tumor cells at the time of MC.
treatment. Prolonged DIC treatments, which may result in anticoagulation because of a depletion of the reduced form of vitamin K, are unnecessary for augmenting the antineoplastic efficacy of MC.

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