Enhancement of Mitomycin C Cytotoxicity to Hypoxic Tumor Cells by Dicoumarol in Vivo and in Vitro

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ABSTRACT

Previous work by our laboratories demonstrated that dicoumarol can increase the enzymatic activation of mitomycin C (MC) to alkylating species by tumor cell sonicates under hypoxic conditions. To determine whether this increased generation of reactive metabolites would result in increased cytotoxicity, we examined the effect of this combination on the viability of EMT6 cells treated in vitro under hypoxic and oxygenated conditions. Dicoumarol increased the cytotoxicity of MC to these neoplastic cells under hypoxic conditions and decreased the toxicity of the antibiotic to aerobic cells. These findings suggested that dicoumarol might enhance the toxicity of MC to the hypoxic cells of solid tumors, without increasing the toxic side effects of the antibiotic to the host. Treatment of EMT6 tumor-bearing animals with both dicoumarol and MC significantly decreased the survival of the radioresistant hypoxic tumor cells from that obtained with MC alone. In contrast, the leukopenia produced by the antibiotic was not exacerbated by the addition of dicoumarol. These results suggest that a treatment regimen combining dicoumarol and MC might be a useful adjunct to radiation therapy for the eradication of the radioresistant hypoxic cells in solid tumors.

INTRODUCTION

Significant advances have been made toward the cure of rapidly growing cancers, while solid tumors with low growth fractions are often poorly responsive or nonresponsive to existing therapy. In these refractory tumors, hypoxic cell populations may well be a major factor limiting cure (8, 12). To develop an effective therapeutic strategy to attack hypoxic tumor cells, our laboratories have been studying agents designed to generate toxic species through reductive activation in hypoxic cells. We have investigated extensively the mechanism of activation of the bioreductive alkylating agent, MC, which is preferentially toxic to Sarcoma 180 and EMT6 mouse mammary tumor cells in the absence of oxygen (Footnote 4; Ref. 7). In the course of these studies, we observed that the anticoagulant dicoumarol increased the production of reactive species from MC in EMT6 cell sonicates under hypoxic conditions. If intact hypoxic cells treated with a combination of MC plus dicoumarol likewise produce increased amounts of alkylating species, and if this leads to increased cytotoxicity, then the combination of dicoumarol and MC should be more efficacious than MC alone in eliminating hypoxic cell populations in solid tumors. This paper describes experiments which demonstrate that the combination of dicoumarol and MC is superadditive in eradicating hypoxic tumor cells both in vivo and in vitro.

MATERIALS AND METHODS

MC was a gift from Bristol-Myers Laboratories (Syracuse, NY). All other materials were purchased from commercial sources: dicoumarol (Sigma Chemical Co., St. Louis, MO); gases (Presto Welding Service Centers, North Haven, CT); fetal bovine serum and Waymouth's medium (Grand Island Biological Co., Grand Island, NY); Zap-IsoT (Coulter Diagnostics, Inc., Hialeah, FL); and Cameo Quik Stain II (Cambridge Chemical Products, Inc., Ft. Lauderdale, FL).

BALB/c KBr mice approximately 3 months of age were used for in vivo studies. These animals were bred and maintained in a barrier system as described elsewhere (15). EMT6 cells (subline EMT6-Rw) were maintained by alternate passage in mice and in cell culture using techniques described previously (14, 17). Tumors were implanted by the i.d. injection of 2 × 106 cells into the flanks of mice (14, 17) and were allowed to develop for approximately 2 weeks, to a volume of about 100 cu mm, before use in experiments. Dicoumarol was administered i.p. (34 mg/kg) at 24 hr and 2 hr before sacrifice and was present continually in the drinking water (180 mg/liter) during the last 24 hr before sacrifice. MC (6 mg/kg) was injected i.p. 1 hr before sacrifice, since maximal MC cytotoxicity is expressed within the first hr after injection (16). MC and dicoumarol were dissolved in sterile 0.9% NaCl solution (saline) with stoichiometric amounts of NaOH added to dissolve the dicoumarol. Some animals were exposed to 1500 rads of whole-body X-irradiation, without anesthesia, exactly as described elsewhere (16). Radiation was given after drug treatment, since previous studies showed that the toxicity of MC to hypoxic tumor cells was independent of the order of administration (16). To assess tumor cell survival after treatment of mice in vivo, single cell suspensions were made from solid neoplasms, and the ability of the tumor cells to form colonies was determined as described previously (14, 16, 17). Surviving fractions were calculated as the ratios of the plating efficiencies of the cells from treated tumors and of cells from untreated control tumors assayed on the same day. The plating efficiencies of control tumors in these experiments averaged 33%.

The effects of the combination treatment on the hematopoietic system were assessed by serially sampling 10 μl of tail vein blood from individual animals and determining the total WBC content using a hemacytometer. The regimen of treatment with MC and with dicoumarol plus MC were the same as those described above for the studies with tumors, except that pyrogen-free saline was used to dissolve drugs. WBC counts for each group of 4 mice were expressed as the percentages of the initial, pretreatment level for that group. Differential counts of peripheral blood smears, stained with Wright-Giemsa stain, were performed using at least 100 leukocytes per animal.

Cultured cells were maintained at 37° in Waymouth's medium plus 15% fetal bovine serum in an atmosphere of 95% air and 5% CO2. For experiments with MC, exponentially growing cells in glass bottles were gassed for 3 hr with 95% air-5% CO2 or 95% N2-5% CO2. During the last hr, cells were exposed to 100 μM dicoumarol, to 1.5 μM MC, to dicoumarol and MC, or to the appropriate vehicle (0.5 M NaOH or 70%...
ethanol). Treated cells were suspended by trypsinization, counted, and assayed for viability by colony formation as described previously (14). Surviving fractions for drug-treated groups were calculated using the viabilities of the appropriate aerobic or hypoxic vehicle-treated controls, which averaged 68 and 52%, respectively.

To examine the effect of dicoumarol on cellular radiosensitivity, exponentially growing cultures were treated with 100 μM dicoumarol for 1 hr before and during irradiation with 250 kV X-rays. Aerobic experiments were performed using standard culture dishes (Costar). For irradiation of hypoxic cells, cultures in Permanox dishes (Lux), designed and fabricated to allow rapid removal of residual O2, were placed in steel vessels and gassed with 95% N2-5% CO2 for 1 hr before irradiation (15, 16, 17). Treated cells were assayed for colony-forming ability as described above.

RESULTS

The effect of dicoumarol on the cytotoxicity of MC to hypoxic neoplastic cells was assessed using EMT6 cells in vitro (Chart 1). MC was more cytotoxic to EMT6 cells in hypoxia than in the presence of oxygen, in agreement with previous studies (Footnote 4; Refs. 7 and 18). Under hypoxic conditions, the cytotoxicity of MC was increased by simultaneous treatment with dicoumarol; this increase was statistically significant. In contrast, under aerobic conditions, dicoumarol caused a slight, but significant, decrease in the toxicity of MC. Neither dicoumarol alone nor the vehicles altered the viability of the cells under the experimental conditions used.

The effect of dicoumarol on the efficacy of MC in vivo was assessed by examining the survival of cells from solid EMT6 tumors treated with MC, dicoumarol, or the combination of these 2 agents (Table 1). The effects of the drugs on hypoxic tumor cells were assessed by using 1500 rads of X-rays to selectively destroy the aerobic tumor cell population; it has been shown previously that this treatment will kill over 99.9% of the radiosensitive, aerobic tumor cells, leaving a surviving tumor cell population composed almost entirely of radioresistant hypoxic cells (12, 16, 17). Treatment of unirradiated tumor-bearing animals with the vehicle or dicoumarol had no effect on the viability of the tumor cells. Treatment with either MC alone or X-rays alone was cytotoxic and, as described in detail previously (16), the combination of X-rays and MC produced additive cytotoxicity. Dicoumarol added to the MC-radiation combination significantly increased the tumor cell kill over that produced by MC and X-irradiation in the absence of dicoumarol. This increased cytotoxicity must reflect the enhancement of MC toxicity in hypoxia by dicoumarol (Chart 1), rather than radiosensitization by dicoumarol, since dicoumarol did not act as a radiosensitizer for aerobic or hypoxic cells in vitro (Chart 2) and did not alter significantly the viability of tumor cells irradiated in vivo (Table 1).

Since hematological depression is the major dose-limiting toxicity of MC (2, 3), the effect of dicoumarol on MC-induced leukopenia was examined. Neither the repeated bleeding nor dicoumarol alone had any effect on peripheral blood indices (Chart 3). Both MC alone and MC plus dicoumarol caused marked decreases in the WBC counts to a nadir of 50% of the control values; the WBC count decreased faster in animals treated with MC plus dicoumarol than in those exposed to MC alone. The time course and the magnitude of the decrease after
DICOUMAROL-MC CYTOTOXICITY

Chart 3. Leukopenia produced by MC and dicoumarol. BALB/c mice were either untreated (○) or treated with dicoumarol (●), MC (●), or MC and dicoumarol (●), administered as described in "Materials and Methods" and Table 1. Points for MC and MC plus dicoumarol are the mean ± S.E. from 3 independent experiments. Points for untreated and dicoumarol-treated mice are the mean from 2 experiments. The mean initial WBC counts for the untreated, dicoumarol, MC, and MC plus dicoumarol groups were 1.60, 1.64, 1.85, and 1.58 × 10^7 WBC/ml of blood.

**DISCUSSION**

Investigations on the enzymatic bioactivation of MC demonstrated that addition of dicoumarol to hypoxic sonicates of EMT6 cells incubated with MC and an NADPH-regenerating system increased the rate of production of reactive metabolites, as measured by trapping with 4-(p-nitrobenzyl)pyridine. Dicoumarol is a potent inhibitor of DT-diaphorase, an enzyme which reduces quinones such as menadione, Adriamycin, and vitamin K (4, 9). In hepatocytes, menadione is reduced by DT-diaphorase to a nontoxic hydroquinone and by NADPH-cytochrome c reductase to a toxic semiquinone radical (1, 4, 6). Treatment of hepatocytes with dicoumarol markedly increased the cytotoxicity of menadione by (a) effectively increasing the concentration of the semiquinone radical and (b) blocking the competitive formation of the hydroquinone (1, 6). In contrast, dicoumarol has been reported to decrease the toxicity of the quinone, 3-bromomethylmenadione (19). Although the mechanism by which dicoumarol increases the generation of alkylating species from MC under hypoxic conditions is not established, it is reasonable to postulate that an increase in the production of reactive metabolites from MC should be reflected by an increase in the cytotoxicity of MC to hypoxic EMT6 cells in vivo and in vitro.

The toxicity of MC to hypoxic EMT6 cells in vitro was increased by simultaneous treatment with dicoumarol (Chart 1). This result is consistent with the hypothesis that DT-diaphorase metabolizes MC to a less toxic or nontoxic product and that the toxic MC species is produced in hypoxic cells by an enzyme such as NADPH-cytochrome c reductase and/or xanthine oxidase (Footnote 4; Refs. 10 and 13). Although the data for EMT6 cells in culture do not identify the mechanism of the increased cytotoxicity to hypoxic cells, they do suggest that treatment of animal tumors in vivo with MC plus dicoumarol should increase the kill of hypoxic tumor cells. In contrast, dicoumarol decreased the toxicity of MC to aerobic EMT6 cells. Since normal tissues are, for the most part, well vascularized and relatively well oxygenated, this finding suggests that simultaneous treatment with dicoumarol should not increase, and might possibly decrease, the toxic side effects of MC in vivo.

To assess these possibilities, animals bearing well-established i.d. implants of EMT6 tumors were treated with a combination of these agents (Table 1). Dicoumarol alone had no detectable effect on the viability of the tumor cells. The cytotoxic effects of MC in unirradiated tumors were similar with and without dicoumarol. This finding was predicted on the basis of the in vitro studies (Chart 1), since untreated EMT6 tumors contain approximately 80% aerobic cells (which at best would be protected only slightly from MC by dicoumarol) and about 20% hypoxic cells (which would be sensitized to MC by dicoumarol). Because aerobic cells are extremely sensitive to the cytotoxic effect of ionizing radiation, irradiation of the tumors with 1500 rads of X-rays decreases the survival of the aerobic cell population in these tumors to approximately 0.1%, while approximately 20% of the radioresistant hypoxic tumor cells survive. As a result, 95% of the tumor cells surviving irradiation with 1500 rads are hypoxic (12). In irradiated tumors, the combination of dicoumarol plus MC was extremely effective and killed 90% of the hypoxic tumor cell population that would have survived treatment with MC alone. This appears to reflect the effects of dicoumarol on the metabolism of MC, since dicoumarol does not alter the radiosensitivity of either hypoxic or aerobic EMT6 cells in vitro (Chart 2) or the survival of cells from tumors irradiated with 1500 rads in vivo (Table 1).

Most therapies used to eradicate neoplastic growths are toxic to the host as well as to the tumors. To ensure that dicoumarol did not increase the hematological toxicity of MC, the hematological status of the mice was examined after treatment with MC alone or in combination with dicoumarol; the amount of leukopenia produced by the combination was not significantly different from that produced by MC alone (Chart 3). These results agree with our in vitro findings, which predicted that dicoumarol would not enhance significantly the toxicity of MC to aerobic tissues.

These findings suggest that a combined regimen of X-irradiation, dicoumarol, and MC should be useful for treating localized solid tumors. Irradiation would eliminate oxygenated tumor cells, while the combination of MC and dicoumarol would attack the radiation-resistant, hypoxic tumor cells. The design of this anti-neoplastic regimen was derived from metabolic studies examining the mechanisms of the bioactivation of MC. Using this approach, it should be possible to develop an in vitro test to predict in advance whether a specific tumor might be treated more effectively with dicoumarol and MC, by using biopsied tissue to measure the enzymatic production of alkylating species from MC in the presence and absence of dicoumarol.
Coumarin anticoagulants have been tried previously in combination therapy as antineoplastic agents (5, 20). Bleeding episodes have been encountered with the prolonged dicoumarol treatments (11, 20) necessary to block coagulation by depleting reduced vitamin K (20). However, only a short treatment with dicoumarol is necessary to alter MC metabolism, since dicoumarol must be present only during the brief period when MC is present in the cells, as illustrated with the 1-hr exposure in Chart 1. In this study, a relatively short (24-hr) exposure to dicoumarol was found to increase the toxicity of MC to hypoxic tumor cells in vivo; it is probable that shorter pretreatments, with even less anticoagulant effect, would also suffice. It should be noted that attempts to avoid the remote possibility of anticoagulation by the administration of vitamin K might well be counterproductive, since we have found that the production of alkylating species from MC is blocked by menadione (vitamin K₃). ⁵

In conclusion, we have demonstrated that the combination of dicoumarol and MC is superadditive in decreasing the survival of hypoxic tumor cells and that this action occurs without increasing the degree of leukopenia produced by the antibiotic. The results were directly predictable from biochemical studies, which may provide a basis for an in vitro test to identify specific tumors that would benefit from this combination regimen. Our findings suggest that the combination of dicoumarol and MC might be unusually efficacious when combined with regimens using agents, such as radiation, which are most effective against aerobic tumor cells. Since many solid tumors appear to contain large numbers of hypoxic cells, which limit their curability by radiotherapy and chemotherapy regimens, the development of multiagent regimens with increased efficacy against hypoxic cells could be of great clinical significance.

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REFERENCES

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