In Vitro Thermochemotherapy of Human Colon Cancer Cells with cis-Dichlorodiammineplatinum(II) and Mitomycin C

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ABSTRACT

The thermosensitivity of human colon adenocarcinoma (LoVo) cells was investigated as a function of temperature and duration of heating in exponentially growing cultures. At 39–43°, time-dependent survival followed a simple exponential function. D0 values decreased progressively with a rise in temperature, from D0 at 40° = 38 hr to D0 at 42° = 17 hr to D0 at 43° = 1.5 hr, thus indicating relative thermoresistance of LoVo cells compared to Chinese hamster ovary cells. Dose-dependent 1-hr survival of LoVo cells treated with cis-dichlorodiammineplatinum(II) and mitomycin C was effectively modified when treatment was conducted under hyperthermic conditions. For both agents and cultures in exponential and stationary growth phases, hyperthermia abolished the initial shoulder portion and steepened the subsequent exponential part of the survival curves for dose-modifying factors at the 10% survival level of 1.5 to 2.0 at 41° and 2.6 to 2.8 at 42°. This significant enhancement of drug-induced cell kill by moderate hyperthermia suggests that thermochemotherapy with mitomycin C and cis-dichlorodiammineplatinum(II) should be tested clinically with both regional and total-body hyperthermia.

INTRODUCTION

Hyperthermia has been demonstrated to be lethal in a variety of experimental tumor cells (12–14, 20, 21, 25, 30), thus stimulating renewed interest in its clinical application, both alone and in conjunction with ionizing radiation and chemotherapy (for review, see Refs. 2, 8, 15 and 29). While regional hyperthermia has been shown to be effective and safe in the treatment of clinical and experimental animal tumors (9, 22, 23), total-body hyperthermia has generally been associated with considerable host toxicity and marginal-to-moderate antitumor efficacy (2, 5, 6, 18, 19, 26). In a previous clinical trial of whole-body heating, we reported moderate antitumor activity (primarily in malignant melanomas) with substantial host toxicity (3). Addition of melphalan did not improve the therapeutic efficacy of systemic thermotherapy. Similarly, combination of total-body hyperthermia with Adriamycin in a Phase II clinical trial at the National Cancer Institute has not revealed significant clinical responses suggestive of heat-drug synergism (7); in fact, there is evidence of augmented cardiomyopathy from thermochemotherapy with Adriamycin (17). In an extensive preclinical trial to elucidate potential synergism between systemic hyperthermia and a large variety of currently used chemotherapeutic agents, Rose et al. (27) did not identify any heat-drug pairs that significantly enhanced tumor shrinkage, survival, or cure rates in L1210-bearing mice beyond the potential of either treatment modality alone.

We therefore initiated in vitro investigations to search for heat-drug pairs that would exert synergistic, i.e., more than additive, cytotoxicity at temperatures that are safe for total-body hyperthermia, i.e., ≤42°. We now report on the lethal effects of hyperthermia alone and in combination with DDP3 and MC on cultured human colon cancer cells in exponential and stationary growth phases. While there was moderate cell kill from heat alone, temperatures of 41 and 42° significantly enhanced the lethal effects of both DDP and MC on cells in exponential and stationary phases of growth.

MATERIALS AND METHODS

Cell Lines. A human carcinoembryonic antigen-producing adenocarcinoma cell line (LoVo cells), the biological properties and growth kinetics characteristics of which have been reported (10, 11), was utilized. Monolayer cultures of LoVo cells were grown in Ham’s F-10 medium, supplemented with 20% fetal calf serum, glutamine, vitamins, and antibiotics. Cells seeded into 60-mm Petri dishes (5 × 10⁶ cells/dish) reached 8 days (11). For all experiments presented here, stock cultures and experimental culture dishes of LoVo cells were harvested by successive treatments with hyaluronidase (100 units/ml for 5 min at 37°) and trypsin (2.5% for 5 min at 37°); they were then counted with an electronic particle counter (Coulter Electronics, Inc., Hialeah, Fla.).

To determine the relative thermosensitivity of LoVo cells, parallel experiments were performed with CHO cells grown in McCoy’s Medium 5A (Iwakati and Grace modification), supplemented with 20% fetal calf serum, glutamine, and antibiotics, in a 5% CO₂ atmosphere. Stock cultures and experimental culture dishes were harvested with trypsin (0.25% for 5 min at 37°).

Drugs. MC was obtained as Mutamycin from Bristol Laboratories, Syracuse, N. Y. Five mg of drug were reconstituted in 10 ml of 0.9% NaCl solution. DDP, manufactured by Ben Venue Laboratories, Inc., Bedford, Ohio, was obtained from the Division of Cancer Treatment, National Cancer Institute, and dissolved in distilled water immediately before each experiment. Appropriate drug concentrations were made by dilution with fresh medium.

Lethal Effects. Suspension aliquots of 5 × 10⁶ cells were seeded into 60-mm Petri dishes. For experiments involving

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3 The abbreviations used are: DDP, cis-dichlorodiammineplatinum(II); MC, mitomycin C; CHO, Chinese hamster ovary; Dₜ, 95% threshold dose equal to the intercept with the abcissa of the exponential part of survival curve; Dₜ, mean lethal dose equal to the concentration required to reduce survival by 63% on the exponential part of survival curve.
cultures in exponential growth phase, the cells were incubated at 37° in a 5% CO2 atmosphere for 48 hr (LoVo cells) and 24 hr (CHO cells). The medium was then discarded, and cells were exposed to increasing concentrations of MC or DDP for 1 hr at temperatures ranging from 37 to 42°. Heat treatment was conducted by exposing the bottom surfaces of Petri dishes positioned on a tray in a specially constructed water bath, which allowed direct contact of the water to the bottom surfaces of the dishes. The bath was maintained within ±0.1° with a Lauda circulator and regulator (Messgeräte-Werk, Dr. R. Wobs-G K., Lauda, Germany). Thermocouple probes [Bailey Instruments, Inc., Saddlebrook, N. J.; accuracy, ±0.1° (S.D.)] were placed into several areas of the water bath and into Petri dishes without cells to assure even heat distribution on the water bath surface. With a specially designed Plexiglas chamber above the water surface, a 5% CO2 atmosphere was maintained at a temperature 0.1—0.3° lower than that of the water surface and the Petri dishes. The final treatment temperature in the Petri dishes was achieved within 1 to 3 min. After 1 hr of incubation, the drug-containing medium was discarded, and the cells were washed twice with Hanks’ balanced salt solution, harvested as a monodispersed suspension, and counted. For each experimental dose and time point, known aliquots were dispensed into triplicate sets of 60-mm Petri dishes so that 50 to 100 colonies would appear after incubation at 37° for 7 days (CHO cells) or 20 days (LoVo cells). The colonies were stained with 2% crystal violet in 95% ethanol. Viability was defined as the ability of single cells to give rise to a colony of more than 50 cells. In each experiment, the plating efficiency of at least 6 control cultures was assessed simultaneously, and all experiments were carried out at least twice. Control cultures consisted of cells treated in the same way as were the test cells but without receiving drugs and with or without exposure to heat. The survival functions for the test cultures were normalized with respect to the individual controls for each experiment. For combined heat-drug experiments, cell survival was normalized to survival of heat-treated cells in the absence of drug. In this series of experiments, the plating efficiencies under normothermic conditions were 30 to 55% for LoVo cells and 75 to 85% for CHO cells.

Experiments designed to delineate the time-dependent survival of LoVo and CHO cells at various temperatures were conducted in a fashion similar to those described above. The highest temperature used was 43°. At this temperature, <10% of the cells detached from the dish after treatment intervals of >24 hr. In additional control experiments, no significant differences in surviving fractions were demonstrated whether or not cells floating in supernatant medium were combined with monolayer cells before colony formation. A second control system was set up to determine the influence of harvesting procedures on the survival of heat-treated LoVo cells. For this purpose, adjusted cell numbers were seeded initially, so that there was no additional cell manipulation involved following heat treatment of exponentially growing cultures. These dishes were exposed for a given length of time to the experimental temperatures and returned to 37° for colony formation. No significant differences were observed between cultures exposed to routine harvesting methods with trypsin and hyaluronidase and those merely exposed to hyperthermia without further manipulation during the period between cell inoculation and survival analysis.

RESULTS

Chart 1 illustrates the time-dependent survival of exponentially growing LoVo cells exposed to increasing temperatures ranging from 39 to 43°. At all temperatures, hyperthermia reduced survival of LoVo cells in a simple exponential fashion.

Time-dependent survival declined gradually as temperature was increased from 39 to 42°; this was reflected in a decrease
in $D_0$ values from 38 hr at 40° to 17 hr at 42°. A sharp increase in cytotoxic activity ($D_0 = 1.5$ hr) was observed when the temperature was increased from 42 to 43°. Since most previous studies have been conducted in nonhuman cell lines, we contrasted the thermal sensitivity of LoVo cells to that of CHO cells in exponential growth phase (Chart 2). CHO cells displayed a threshold-exponential survival pattern with substantially steeper exponential portions ($D_0$ at 42° = 0.5 hr; $D_0$ at 43° = 0.3 hr) than those of LoVo cells.

Thermochemotherapy of LoVo cells with DDP produced similar 1-hr dose-dependent survival responses in both exponential ($D_0$ at 41° = 2.2 μg/ml, $D_0$ at 42° = 1.6 μg/ml; Chart 3) and stationary growth phase ($D_0$ at 41° = 2.0 μg/ml, $D_0$ at 42° = 1.15 μg/ml; not shown). The initial shoulder of normothermic treatment was abrogated and the subsequent exponential portions of survival curves were progressively deepened, resulting in dose-modifying factors at the 10% survival level of 1.9 to 2.0 at 41° and 2.6 at 42°.

Normothermic 1-hr exposure of LoVo cells to MC was characterized by a threshold-exponential cell kill function, which was similar for cultures in exponential and stationary phases of growth. Hyperthermic conditions (41 and 42°) abrogated the shoulder region and steepened the exponential cell kill with $D_0$ values of 0.8 μg/ml (exponential phase) and 0.6 μg/ml (stationary phase) at 41° and 0.4 μg/ml (exponential and stationary phase) at 42° (Chart 4). Dose-modifying factors at the 10% survival level were 1.5 at 41° and 2.6 at 42°.

**DISCUSSION**

Marmor (22) has indicated the relevance of studies concerning the critical temperatures for synergistic tumor and host toxicity from thermochemotherapy. Such investigations are performed in order to identify agents that deserve clinical investigation as components of systemic thermochemotherapy, where the maximum tolerated temperature is 42–43°. Recent *in vitro* investigations on normal human bone marrow stem cell survival have demonstrated the potential synergistic toxicity of *in vitro* thermochemotherapy with nitrosoureas (24). Once agents, the tumoricidal effects of which are significantly enhanced under hyperthermic conditions of ≥43°, have been identified *in vitro*, clinical synergism can be safely tested using local hyperthermia and systemic chemotherapy, before initiation of potentially toxic and ineffective systemic thermochemotherapy.

In the present study, we have addressed the question of whether systemically achievable hyperthermia of 41–42° can favorably alter the lethal efficacy of drugs active in a human colon cancer cell line (4). The human LoVo cells were considerably less thermosensitive than were CHO cells, a nonhuman mammalian culture. Consistent with reports on other cell systems (24), the thermal sensitivity of LoVo cells increased abruptly as the temperature was raised from 42 to 43°.

DDP and MC represent the 2 most effective agents among a large number of antitumor compounds investigated in this cell system to date (4). Simultaneous 1-hr exposure of LoVo cells to either chemotherapeutic agent and 41–42° hyperthermia resulted in significant modification of normothermic 1-hr dose-

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dependent survival curves. This was true for cells in both exponential and stationary growth phases, indicating that thermal enhancement of DDP- and MC-induced cell kill occurs regardless of tumor cell proliferative activity. This is of great importance for the treatment of clinical disease with generally low growth fraction and hence relatively high chemotherapy resistance (28). In particular, thermochemotherapy with DDP and MC abrogated the shoulder region and significantly steepened the exponential portion of the survival functions. Thus, the observed modification of chemotherapy dose-dependent survival by hyperthermia implies both an increase in the sensitivity of the critical target molecules to drug-induced lethal injury and especially a reduction in the ability of cells to absorb sublethal damage. The thermal enhancement of DDP-induced cell kill in LoVo cultures was slightly inferior to that noted by Hahn (15) in HA1 Chinese hamster cells.

Both DDP and MC have been reported to produce DNA cross-links, which may be the molecular injury associated with sublethal damage. The thermal enhancement of DDP-induced molecular damage under hyperthermic conditions is marked in the case of DDP treatment, suggesting an increased drug uptake, the more prolonged course of MC-induced cross-links would be consistent with inhibition of repair of MC-induced molecular damage under hyperthermic conditions.

We have since obtained clinical evidence of synergism between 43°C ultrasound hyperthermia and DDP in one patient with a head and neck squamous cell carcinoma that had been refractory to hyperthermia and DDP alone. Interestingly, there was a 75% tumor regression upon treatment with DDP (25 mg/m²) and 1-hr ultrasound hyperthermia at 43°C administered concurrently on each of 3 subsequent days. Alberts et al. (1) demonstrated differential in vivo toxicity of systemic thermochemotherapy with DDP in mice, measuring bone marrow stem cell survival of leukemic and normal colony-forming units in spleen.

Once clinical synergism between regionally applied moderate hyperthermia of ≤42°C and systemically administered MC or DDP chemotherapy has been demonstrated and substantiated, trials of total-body hyperthermia in conjunction with these agents appear to be justified.

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