Effect of Step-Down Heating on the Cytotoxicity of Adriamycin, Bleomycin, and cis-Diaminedichloroplatinum

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ABSTRACT

The cytotoxicity of a number of anticancer drugs can be potentiated by mild hyperthermia. Since the cytotoxicity of low-hyperthermic temperatures in the range of 39–43°C can be potentiated by using a step-down heating (SDH) sequence (short times at ≥45°C followed by longer times at lower temperatures), we investigated the combined effects of SDH (specifically 45°C, 10 min, followed by time at 41.5°C) and three chemotherapeutic drugs known to be more toxic at elevated temperatures.

In agreement with previous observations with Chinese hamster cells, we found that cultured RIF tumor cells showed an increased rate of cell killing at 41.5°C when the low-temperature heat treatment was preceded by an acute heat shock of 10 min, 45°C. Similarly, RIF cells showed enhanced cytotoxicity of Adriamycin, bleomycin, and cisplatin at 41.5°C over that at 37°C, as reported previously for hamster cells. When SDH and drug exposures were combined, the rate of cell killing was greater than that observed with the drugs at a constant 41.5°C. SDH alone reduced the D0 on the 41.5°C survival curve from approximately 500 min to 100 min after 10 min at 45°C. Adriamycin cytotoxicity (0.3 μg/ml) at 41.5°C was characterized by a D0 of approximately 160 min for treatment times of 1 to 4 hr; with SDH, this was reduced to 52 min. Similarly, bleomycin (5 μg/ml) and cisplatin (0.5 μg/ml) showed a decrease in D0 by factors of 1.4 (68 to 48 min) and 2 (25.2 to 13.5 min), respectively, with the SDH sequence when compared with drug toxicity at 41.5°C alone. However, the sensitization to cisplatin was the same when 10 min at 45°C was followed by drug exposure at 37°C, or at 41.5°C. The results indicate that Adriamycin and bleomycin, but not cisplatin, cytotoxicity was increased by SDH over that achievable with 41.5°C alone. Possible clinical implications are discussed briefly.

INTRODUCTION

Previous study, primarily that by Hahn (4), has demonstrated that the cytotoxicity of some anticancer drugs can be significantly enhanced by mildly elevated temperatures. These observations led to several clinical trials of whole-body hyperthermia (6, 8) combined with chemotherapeutic agents in cancer patients. Unfortunately, this combination appears to be only modestly successful (6).

The combination of local-regional hyperthermia with anticancer drugs is not limited to only those temperatures that can be tolerated systemically (7). The use of locally high temperatures presents dosimetry problems, however, since large tissue masses are difficult to heat uniformly, and nonuniform localized heating will result in a variable biological response. SDH (3) may be one way of avoiding some of the problems associated with intense high-temperature hyperthermia. Furthermore, SDH may be expected to potentiate cytotoxicity of some drug-hyperthermia treatment combinations (5). In this paper, we report our results with the combination of SDH and 3 anticancer drugs (Adriamycin, bleomycin, and cisplatin) on the killing of RIF tumor cells in vitro. The drugs were chosen because each has been shown to interact with both hyperthermia (4) and X-radiation (1–3) in a supraadditive manner, and the concentrations were chosen for relatively low (37°C) toxicity, i.e., such that the longest drug exposures would not reduce cell survival below 1%.

MATERIALS AND METHODS

Cell Culture. RIF tumor cells (9) were used in these experiments. These cells were grown in vitro in a minimal essential medium supplemented with 10% (v/v) fetal calf serum, penicillin (11 units/ml), and streptomycin (100 μg/ml) (Grand Island Biological Co., Grand Island, NY). Cells were maintained in logarithmic growth phase at 37°C in a 5% CO2–95% air atmosphere; the population doubling time was approximately 12 hr. Cell number was determined with an electronic particle counter (Model ZBI; Coulter Electronics, Inc., Hialeah, FL). RIF cells were passed every 3.5 days in vitro and returned to grow in C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) following the protocol of Tweney and others (9).

Cell Viability Measurements. Cell viability was measured by colony formation in vitro. Briefly, variable numbers of cells were plated into 25-cm² flasks (Corning Plastics, Corning, NY) 16 hr prior to the treatments outlined below. After treatments, cells were washed twice, overlaid with 5 ml of fresh medium, and incubated at 37°C for 8 to 10 days for colony formation. Colonies were scored when they contained more than 50 cells. The plating efficiencies of untreated cells were between 30 and 35%. Surviving fractions as plotted represent the average (± S.E.) of 4 to 6 flasks/experiment and 2 replicate experiments.

Drug Treatments. Exponentially growing cells were treated with different concentrations of cis-diaminedichloroplatinum(II) (cisplatin; Bristol Laboratories, Inc., Syracuse, NY), bleomycin (Bristol Laboratories), or Adriamycin (Farmitalia Co., Milan, Italy) for up to 4 hr at either 37 or 41.5°C, or they were treated at 45.0°C for 10 min followed by various times at 41.5°C or 37°C up to 4 hr. Stock solutions of the drugs in water were prepared immediately before use. Appropriate dilutions were added to fresh growth medium to produce the desired drug concentrations. After treatment, the medium was removed and the cultures were washed twice with 3 ml of warm Puck’s Saline A (Grand Island Biological Co.), refed with 5 ml of fresh medium, and incubated at 37°C for 8 to 10 days for colony formation. In each experiment, only one drug concentration was used (0.5 μg cisplatin/ml, 5.0 μg bleomycin/ml, 0.3 μg Adriamycin/ml), and the drug was added before any hyperthermia treatments.

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3 The abbreviation used is: SDH, step-down heating.

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**RESULTS**

Hyperthermia at 41.5° for up to 6 hr is relatively nontoxic to RIF cells *in vitro*, with cell survival remaining above 30%. However, 45° hyperthermia for 10 min immediately prior to 41.5° hyperthermia reduced cell survival to approximately 2% after subtracting cell killing by the initial 45° heat shock (Chart 1). The approximated $D_0$ values for the 2 survival curves are 515 and 102 min at 41.5°, respectively. The reduction of the $D_0$ by a factor of 5 is similar to that reported for Chinese hamster ovary cells in response to the same temperature combination (5), even though Chinese hamster ovary cells are more heat sensitive.

The results of combinations of hyperthermia at 41.5° and 45° with exposure to cisplatin during variable times are shown in Chart 2. The rate of cell killing at 37° was characterized by a $D_0$ of about 90 min, which was decreased to a $D_0$ of 25 min at 41.5°. A similar survival response was attained after 10 min at 45°, followed by a drug exposure at either 37° or 41.5°. This result implies that cell killing by cisplatin and SDH was subadditive; i.e., that heat sensitization at 45° to cisplatin was effectively saturated, yielding a subsequent time-dependent exponential loss of cell survival. For this drug, the use of SDH probably would offer no cytotoxin advantage over constant-temperature hyperthermia other than that due to the 45°, 10-min treatment alone, unless a tissue-specific variation in the drug-heat interaction were demonstrated.

Adriamycin combined with hyperthermia at the same 2 temperatures resulted in the survival curves demonstrated in Chart 3. Cell killing at 37° was marked by a biphasic response; in agreement with data in the literature (4), most cell killing occurred during the first hr. Prolonged exposure without addition of fresh Adriamycin produced continued cell killing with a $D_0$ of approximately 150 min. When cells were exposed to Adriamycin at 41.5°, cell killing was enhanced primarily during the initial phase (first hr). With additional time, the exposure to Adriamycin reduced cell survival rate both at 37 and 41.5°. The combination of SDH and Adriamycin, however, resulted in an increased rate of cell killing during the entire interval of 0 to 4 hr, characterized by a $D_0$ of 52 min, 41.5° ($D_0 = 158$ min, 41.5° without prior 45° hyperthermia). The additional sensitization by SDH was not associated solely with 45° heat conditioning as with cisplatin (see above), as 10 min, 45° immediately prior to a 37° Adriamycin

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**Chart 1**. Cell survival curves for asynchronous RIF tumor cells *in vitro* at 41.5° or at 41.5° immediately following a heat shock of 10 min, 45° (SDH). The cells were plated approximately 16 hr before the beginning of the experiment and heated when the cells were reattached to the plastic and had resumed exponential growth. The $D_0$ for the 41.5° curve, based on heating times between 1.5 and 6 hr, is 515 min; for the SDH curve, the $D_0$ is 102 min, 41.5° using heating times of 0 to 6 hr. Bars, S.E.

**Chart 2**. Survival response of RIF cells to cisplatin (0.5 μg/ml) exposures at 37°, 41.5°, or with SDH. The drug was added at zero time (•, ○) or just prior to 45° hyperthermia (■, □). After the indicated interval, the drug was removed by rinsing twice with warm 0.9% NaCl solution, and the medium was replaced. Calculated $D_0$ were 13.5 min, 41.5° (■, □) and 25.2 min, 41.5° (○). The datum labeled CisP, 20 sec, 37° is a control that involved the addition of the drug at room temperature, followed by immediate removal and rinsing. This control reflects the cell killing from rapid binding of the drug to cells and to plastic and the toxicity of residual drug bound to plastic and/or released from damaged cells. The survival curves have been corrected for this effect. Bars, S.E.
exposure did not sensitize cells to Adriamycin (Chart 3). In fact, it is notable that prior treatment at 45° for 10 min markedly protected surviving cells against Adriamycin cytotoxicity. This finding is in agreement with data published by Hahn (4) and suggests that clinical administration of Adriamycin should not follow heat exposure.

Bleomycin, like Adriamycin, killed most cells during the initial first hr of exposure (Ref. 4; Chart 4). However, in contrast to Adriamycin, cell killing at 41.5° in the presence of bleomycin was enhanced also after the first hr of drug exposure, yielding an exponential loss of survival with a $D_0$ of 68 min, at 41.5°. Prior 45° hyperthermia (SDH) further reduced the $D_0$ to 48 min, 41.5°. Hyperthermia at 45°, followed by bleomycin exposure at 37°, however, also showed a low degree of sensitization; normalizing the survival curve labeled 45° × 10 min + Bleo at 37° to the origin shows that the survival plateau is about a factor of 2 lower than the 37° control curve.

DISCUSSION

The observations made in this study suggest that the combination of SDH and Adriamycin or bleomycin can be used to enhance the rate of cell killing over that achieved with low-temperature hyperthermia alone. However, the data provide no direct information on possible mechanisms underlying the observed phenomena. Nevertheless, these observations can be used on an empirical basis for the formulation and testing of specific hypotheses on the modification of drug damage under SDH conditions. For example, one could design specific experiments to investigate the role of DNA repair in the contrasting patterns of SDH-drug interaction with cisplatin versus bleomycin. However, with their present limitations, these data cannot be used alone for proposing SDH-drug combinations that might show selective clinical antitumor activity; separate studies need to be conducted with SDH-drug combinations with normal versus tumor tissues to assess their potential for enhancing therapeutic gains. The data do suggest, however, that cisplatin is the least likely of the 3 drugs to show increased therapeutic gain in combination with SDH, since this temperature manipulation failed to enhance the rate of cell killing by cisplatin over that achieved with constant-temperature 41.5° hyperthermia.

These studies were conducted in order to examine possible strategies for using chemotherapeutic drugs at temperatures inducible with local-regional heating. Most laboratory investigations have studied the cytotoxicity of anticancer drugs at temperatures appropriate for use with whole-body hyperthermia, where the highest peak temperature used has been 42.4° (6). We have shown that, for bleomycin and Adriamycin, an increased rate of cell killing is observed when these drugs are combined with a higher temperature (45°) even when heat exposure is
short (10 min). These results suggest that, in spite of the relative lack of success of whole-body hyperthermia combined with systemic chemotherapy (6, 8), improved tumoricidal results might be expected when selected chemotherapeutic agents are used with temperatures achievable local-regionally. In addition, anticancer drugs could be combined with both local-regional hyperthermia and systemic hyperthermia. This approach might allow maximal heat- and drug-induced cell killing to occur at sites of bulky tumor where local-regional temperatures can be higher than those systemically tolerable, while circulating drug at lower, whole-body hyperthermic temperatures might effectively treat micrometastatic disease. In vivo confirmation of these in vitro results is underway.

REFERENCES

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