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ORIGINAL ARTICLE

Faheez Mohamed · O. Anthony Stuart · Olivier Glehen
Muneyasu Urano · Paul H. Sugarbaker

Optimizing the factors which modify thermal enhancement of melphalan in a spontaneous murine tumor

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Abstract *Background*: Hyperthermia enhances the cytotoxicity of some chemotherapeutic agents. Both clinical and laboratory studies suggest melphalan may be an important drug when hyperthermia is added to chemotherapy treatments. Factors that may modify the thermal enhancement of melphalan were studied to optimize its clinical use with hyperthermia. *Methods*: The tumor studied was an early-generation isotransplant of a spontaneous C3Hf/Sed mouse fibrosarcoma, Fsa-II. All studies were performed under supervision of the Animal Care and Use Committee. Hyperthermia was administered by immersing the tumor-bearing foot into a constant temperature water bath. Four factors were studied: duration of hyperthermia, sequencing of hyperthermia and melphalan, intensity of hyperthermia, and tumor size. To study duration of hyperthermia tumors were treated at 41.5°C for 30 or 90 min immediately after intraperitoneal administration of melphalan. For sequencing of hyperthermia and melphalan, animals received hyperthermia treatment of tumors for 30 min at 41.5°C immediately after drug administration, both immediately and 3 h after administration of drug or only at 3 h after administration of drug. Intensity of hyperthermia was studied using heat treatment of tumors for 30 min at 41.5 or 43.5°C immediately following drug administration. Effect of tumor size was studied by delaying experiments until three times the tumor volume (113 mm^3) was observed. Treatment of tumors was for 30 min at 41.5°C immediately following drug administration. Tumor response was studied by the mean tumor growth time. *Results*: Hyperthermia in the absence of melphalan had a small but significant effect on tumor growth time at 43.5°C but not at 41.5°C. Hyperthermia at 41.5°C immediately after melphalan administration doubled mean tumor growth time at 30 min and caused a threefold increase at 90 min ($P = 0.0002$) when compared to tumors treated with melphalan alone at room temperature. Application of hyperthermia for one-half hour immediately following drug administration was the most effective in delaying tumor growth. No significant difference in mean tumor growth time was observed with an increase in temperature from 41.5 to 43.5°C. For large tumors heat alone and melphalan alone caused a moderate increase in tumor growth delay. These effects in large tumors were greatly increased by a combination of chemotherapy and hyperthermia. *Conclusions*: From our data it would appear that the administration of intraperitoneal melphalan immediately prior to 90 min of heat at 41.5°C may optimize anti-neoplastic activity. These data may be useful in formulating clinical protocols in which melphalan and heat are combined.

Keywords Hyperthermia · Intraperitoneal chemotherapy · Thermal enhancement · Animal tumors

F. Mohamed · O. A. Stuart · O. Glehen · P. H. Sugarbaker (✉)
The Washington Cancer Institute,
Washington Hospital Center, 106 Irving Street,
NW, # 3900N, Washington, DC 20010, USA
E-mail: Paul.Sugarbaker@medstar.net
Tel.: +1-202-8773908
Fax: +1-202-8778602

M. Urano
Memorial Sloan-Kettering Cancer Center,
1275 York Avenue, New York, NY 10021, USA

Introduction

It has been well established in cultured mammalian cells and in animal tumors that hyperthermia can enhance cytotoxicity of some chemotherapeutic agents [1, 2]. Clinical efforts to combine these two treatment modalities have been less fruitful than might be expected from laboratory data. Many factors remain poorly understood, including the optimum duration of hyperthermia treatment, the appropriate scheduling of drug and heat, proper heat intensity, and size of tumor for treatment. Unsolved clinical problems include heterogenous temperature distribution in tumors, normal tissue damage,

and technology to heat tumors deep within the patient. Also, selection of the most effective agents to use at elevated temperatures requires further investigation. The drugs of choice at physiological temperatures may not be the drugs of choice at elevated temperatures.

The duration of hyperthermia treatment may play a role in modifying chemotherapy response and is dependent upon plasma half-life of a drug, and retention of drug in tumor tissue [1]. A prolonged heat treatment alone may retard tumor growth, but this effect can be augmented if coupled to a drug that is retained within tumor tissue. Although many studies have shown the potential for an increased response to chemotherapy with the simultaneous use of hyperthermia and drug, this may not be the most effective timing for all agents [3, 4]. Delayed application of heat following chemotherapy administration may maximize thermal enhancement for certain drugs [5]. There is also evidence that progressive temperature elevation may result in a progressive increase in the cytotoxicity of some chemotherapeutic agents [6]. The optimum temperature for hyperthermic enhancement of melphalan has not been defined in vivo. Tumor size is also important. The fraction of hypoxic cells and thus cellular acidity increases with tumor volume leading to increased thermal sensitivity [7].

In a recent study of the thermal enhancement of new chemotherapeutic agents at moderate hyperthermia (defined in this manuscript as 41.5°C), the most promising agent was found to be melphalan [8]. Melphalan is a well-known anti-neoplastic alkylating agent that has been in use to treat cancer patients for over 50 years [9]. It has markedly increased pharmacological activity with heat, both in vitro and in vivo [1, 6]. Recent animal studies in three different tumors demonstrated melphalan to be the most effective agent at elevated temperatures [2].

The purpose of this work was to identify the optimum duration, heat-drug sequencing, temperature intensity, and tumor size for thermochemotherapy using melphalan. Thermochemotherapy with melphalan is being considered for use in heated intraoperative intraperitoneal chemotherapy protocols for patients with carcinomatosis, sarcomatosis, and peritoneal mesothelioma.

Materials and methods

Mice and tumor

One hundred and fifty C3Hf/Sed mice weighing between 17 and 24 g were obtained from a single breeding colony (Charles River Laboratories, Inc., Wilmington, MA). At least ten mice were used in each group. Animals were kept two per cage in our animal facility and were allowed free access to food and water. These experiments were conducted with approval from our Animal Care and Use Committee.

Tumors were early generation isotransplants of fibrosarcoma, FSa-II, which arose spontaneously in a C3Hf/Sed mouse. The single cell suspensions were prepared by trypsinization with 0.25% trypsin (Gibco, Grand Island, NY), and the number of cells was counted on a hemocytometer. Tumor cells were harvested, and a uniform tumor inoculum was aliqouted and frozen at −80°C. This uniform tumor inoculum was used for all experiments. Ten microliters of the single cell suspensions (approximately 10^6 cells) were inoculated subcutaneously through a 22S gauge Hamilton microliter syringe (Hamilton Co., Reno, NV, USA) into the dorsum of the mouse right hind foot. Treatment was administered when tumors reached 34 or 113 mm^3. Animals were sacrificed when tumors reached 700 mm^3.

Melphalan (Sigma-Aldrich Co., St. Louis, MO) was given at a dose of 16 mg/kg as used by Urano et al. [1]. The drug was given as a single intraperitoneal injection at a constant volume of 0.02 ml/g body weight. Melphalan was solubilized in 2% hydrochloric acid and appropriately diluted. The drug was administered within a few minutes of its dilution in normal saline solution.

A uniform heat treatment was achieved by immersing the tumor-bearing leg into a constant temperature water bath as described by Urano and colleagues [7]. Room temperature was between 22 and 24°C. The following factors were studied: duration of hyperthermia, sequencing of hyperthermia and drug, temperature, and tumor size. No toxicities or deaths from treatments were observed.

Duration of hyperthermia at 41.5°C

Animals were assigned to hyperthermia treatment of tumors for 30 or 90 min immediately after drug administration. These durations were chosen as they are achievable in a clinical setting.

Sequencing of hyperthermia and drug

All hyperthermia treatment of tumors was for 30 min at 41.5°C. Animals were assigned to one of three groups: hyperthermia given immediately following administration of drug; hyperthermia given both immediately and 3 h after administration of drug; and hyperthermia given only at 3 h after administration of drug.

Intensity of hyperthermia

Animals were assigned to receive heat treatment of tumors for 30 min at 41.5 or 43.5°C. The body temperatures and the tumor temperatures were not measured. The mice were not cooled while the right hind foot was heated. The temperature within the tumor or within the right hind foot was not measured.

Tumor size

Two tumor sizes were studied. Heat treatment of tumors for 30 min at 41.5°C was administered when tumors reached 34 mm^3 (4 mm diameter) or 113 mm^3 (6 mm diameter).

Controls

Control groups received no chemotherapy at room temperature, melphalan at room temperature, heat alone at 41.5°C for 30 or 90 min or heat alone for 30 min at 41.5°C or 43.5°C. Controls for 113 mm^3 tumors included no chemotherapy at room temperature, melphalan alone at room temperature or heat alone at 41.5°C for 30 min. The control animals did not receive intraperitoneal saline.

Evaluation of results

Tumor response was studied by the tumor growth time assay as previously described [10]. Three diameters of each tumor, a, b, and c, were measured at least three times a week and the formula πabc/6 was used to determine the tumor volume. The growth curve was drawn for each tumor and the tumor growth time was determined. For each factor studied the mean tumor growth time was calculated. This was defined as the mean time required for tumors to reach 700 mm^3 from the treatment day. The longer the mean tumor growth time, the more effective the treatment. Histologic studies of the tumors were not performed.

Statistical analysis

Statistical analysis was performed using the tumor growth time. Data were analyzed with the Mann–Whitney test (two-tail) using Prism for Windows, version 3.0 (Graph Pad Software Inc., San Diego, CA). For all statistical procedures values for $P<0.05$ were considered significant.

Results

Duration of hyperthermia

For the control groups receiving hyperthermia alone, no significant difference in tumor growth time was demonstrated at 41.5°C based on duration of hyperthermia (Fig. 1). The mean tumor growth time of untreated 34 mm^3 tumors (± SD) at room temperature was 12.1±1.1 days. The mean tumor growth time following hyperthermia alone for 30 min was 12.2±0.9 days, and was 14.2±2.9 days following 90 min of hyperthermia alone. The tumor growth at 43.5°C for 30 min was 15.3±1.5 days. This was a significant difference ($P=0.008$).

The group treated with melphalan at room temperature had a mean tumor growth time of 13.0±1.4 days (Fig. 2). The mean tumor growth time of animals treated with melphalan doubled to 27.6±2.5 at 30 min of hyperthermia ($P<0.001$) and tripled to 38.1±2.9 ($P<0.0001$) days at 90 min at 41.5°C. There was a significant increase in tumor growth time with increasing duration of hyperthermia treatment ($P=0.0002$).

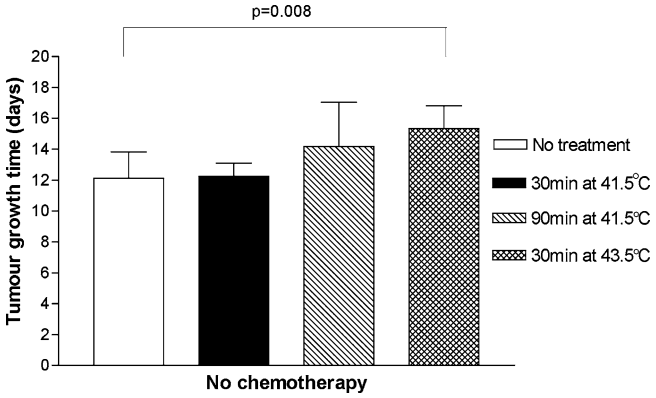

Fig. 1 Mean tumor growth time with no chemotherapy at 41.5°C for 30 or 90 min and at 43.5°C for 30 min. *Error bars* represent standard deviation

Sequencing of hyperthermia and drug

Hyperthermia treatment immediately following administration of melphalan was effective at delaying tumor growth (Fig. 3). Tumor growth time increased from 13.0±1.4 to 27.6±2.5 days with the addition of 30 min of heat. A second hyperthermia treatment 3 h following this did not appear to result in an additional effect ($P=0.7789$). A single hyperthermia treatment 3 h after drug administration gave results similar to those of drug alone with mean tumor growth time of 14.4±1.5 days compared to 13.0±1.4 days ($P=0.07$) for melphalan alone

Temperature

The mean tumor growth time increased significantly following a 30-min hyperthermia treatment with melphalan at 41.5°C ($P<0.0001$) and at 43.5°C ($P<0.0001$). In the presence of melphalan an increase in temperature from 41.5 to 43.5°C did not significantly increase the tumor growth time ($P=0.1304$) (Fig. 4).

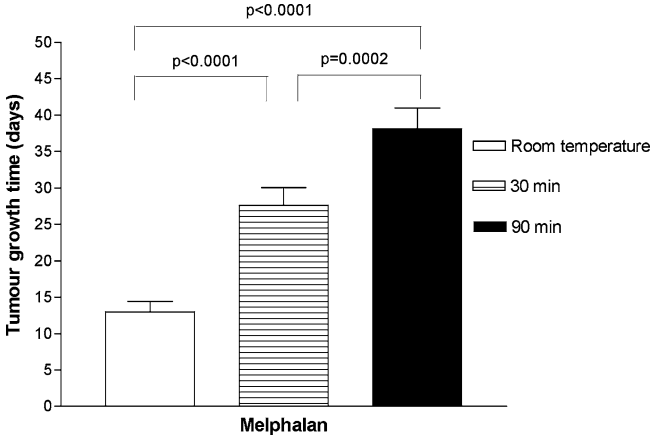

Fig. 2 Mean tumor growth time with melphalan at 41.5°C for 30 or 90 min

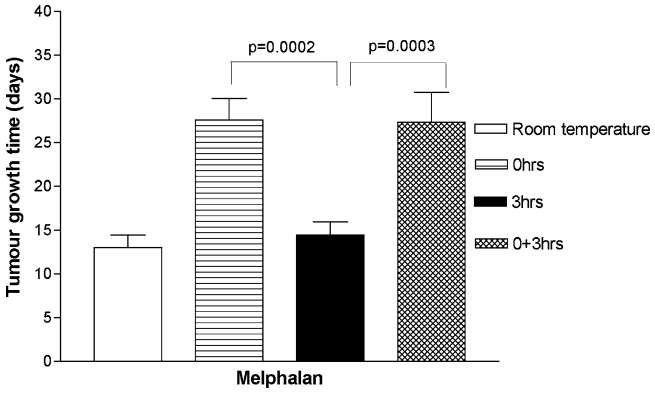

Fig. 3 Mean tumor growth time following hyperthermia at 41.5°C for 30 min, immediately after, 3 h after, and immediately and 3 h after melphalan administration

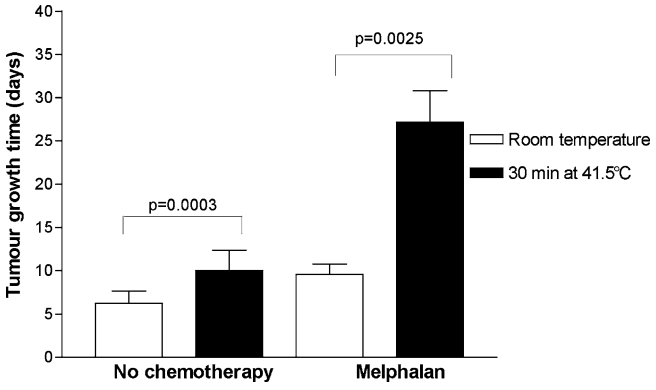

Fig. 5 Mean tumor growth time for 113 mm^3 tumors at room temperature and with melphalan at 41.5°C for 30 min

Tumor size: 113 mm^3 tumors

Hyperthermia alone at 41.5°C for 30 min significantly increased tumor growth time when compared to untreated tumors at room temperature (Fig. 5). The mean tumor growth time at room temperature was 6.2±1.4 days and at 41.5°C was 10.0±2.4 days ($P=0.0003$). Melphalan at 41.5°C for 30 min significantly delayed tumor growth compared to melphalan at room temperature. When treated at room temperature or 41.5°C, the groups receiving melphalan had mean tumor growth times of 9.6±1.2 and 27.2±3.7 days ($P=0.0025$).

Discussion

Hyperthermia has been shown to be a potentiator of the cytotoxic effects of cancer chemotherapy [1, 3]. These findings have renewed interest in thermochemotherapy as a treatment modality, and have led to promising results from trials of hyperthermic chemoperfusion for extremity sarcoma and melanoma [11, 12]. In recent studies, heated intraoperative intraperitoneal chemotherapy combined with cytoreductive surgery has proven effective in the treatment of peritoneal carcinomatosis [13]. However, the optimal drugs and the factors which determine or modify their activity with hyperthermia remain unclear.

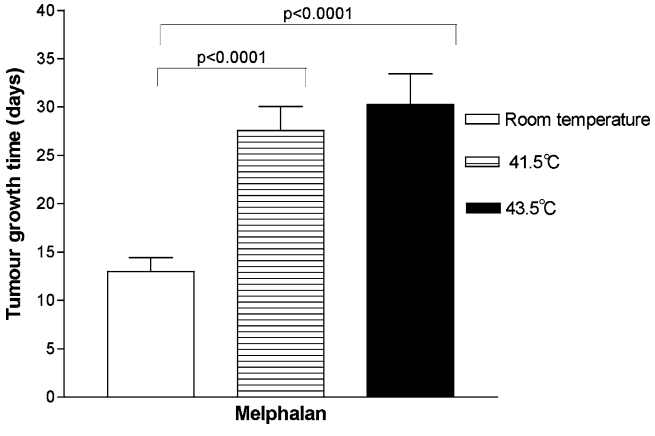

Fig. 4 Mean tumor growth time with melphalan at 41.5 or 43.5°C for 30 min

Recent studies performed with melphalan have suggested that it may be the drug of choice at elevated temperatures [2, 8]. Melphalan exerts its anti-neoplastic effect through the formation of interstrand DNA cross-links. It is thought that the formation of these DNA cross-links is facilitated at elevated temperatures leading to enhanced cell kill [6]. As shown in previous studies, a large dose of melphalan resulted in the greatest augmentation of cytotoxic effects [6]. Consequently, a dose of 16 mg/kg was used in these experiments.

In our study prolonged heating of the tumor with melphalan further decreased the rate of tumor progression. Heating melphalan at 41.5°C for 30 min or 90 min increased mean tumor growth time 100 and 200%, respectively, compared to melphalan at room temperature. The importance of heating time may depend on the pharmacokinetics of the chemotherapy used. Agents such as cyclophosphamide require activation in the liver before uptake by the target tissue. The plasma half-life of cyclophosphamide is only approximately 20 min in mice after an intraperitoneal administration [14]. This means hyperthermia must be applied concomitant with drug administration; prolonged heating may provide little or no advantage. Other agents, such as cisplatin, which require no in vivo activation may have a short plasma half-life. Thirty minutes of heat may produce optimum enhancement following intraperitoneal administration [15]. However, in addition to plasma half-life, retention of drug in tumor tissue may also be important. Even after elimination from plasma, residual drug in tumor tissue may be augmented by heat for a longer period of heat. A study of the effect of 45 min of systemic heating at 41°C on melphalan pharmacokinetics in C3H mice reported a plasma elimination half-life of 24–44 min following a 7.5 mg/kg intraperitoneal administration of drug. The half-life of melphalan was

longer in heated than unheated animals. Our data showed that an increase in the duration of hyperthermia at 41.5°C from 30 to 90 min resulted in a significant delay in tumor growth when applied immediately after intraperitoneal administration of melphalan. This may result from both slower plasma elimination and prolonged tumor tissue retention of drug causing a long-term acceleration of DNA cross-linking at 41.5°C. Some data have suggested that prolonged heating may reduce blood flow (due to sludging) in tumor tissue resulting in no further incorporation of drug [16]. From our data using melphalan in this mouse footpad assay does not indicate sludging of blood with up to 90 min of heating by immersion.

Heat drug sequencing has been shown to be important in a number of studies with different drugs. Thermal enhancement of gemcitabine with hyperthermia was only found by administering the drug 20 or 24 h before heat treament [17]. Similarly Robins and colleagues showed the importance of appropriate time scheduling to obtain optimal interactions of TNF-alpha and melphalan given in combination with 41.8°C hyperthermia in vitro [18, 19]. Our data show that simultaneous administration of melphalan and heat appears the most effective in enhancing performance. Applying hyperthermia 3 h following administration of drug was similar to treatment with melphalan at room temperature. Our knowledge of melphalan pharmacokinetics would suggest that both plasma and tumor concentrations following a single intraperitoneal administration would be minimal at this time. Also, application of an additional 30 min of heat at 3 h following 30 min of simultaneous heat and drug produced no additional effect.

There is evidence that the sensitivity of cells (and tissues) to hyperthermia is transiently but markedly reduced following an initial heat treatment [19, 20, 21]. This resistance, which has been termed "thermotolerance," is expressed in cells which survive one episode of hyperthermia. The development of thermotolerance appears to be related to heat-shock proteins, hsp 70, in particular [22, 23]. The time required for the development of maximum thermotolerance varies with the magnitude of initial heat treatment, from a few hours following a very mild heat treatment to 10–15 h following severe initial treatments. Thermotolerance protects cells against repeated hyperthermia not only with respect to the maintenance of their reproductive ability but also with respect to general cell metabolism [24]. In our tumor model with melphalan, an absence of effect seen with an additional heat treatment at 3 h may be due to acquired thermotolerance. More knowledge of the decay of this thermotolerance, which can be slow and variable, is needed before fractionated treatments of hyperthermia and melphalan are undertaken.

Our data comparing hyperthermia alone at 41.5 and 43.5°C showed that hyperthermia alone for 30 min at 43.5°C resulted in a significant delay in tumor growth time which was not observed at 41.5°C. This confirms previous studies showing that hyperthermia alone for 30 min at a temperature below 42.5°C did not prolong tumor growth time, but at temperatures of 43.5 and 44.5°C a significant delay in tumor growth occurred [25, 26]. When combined with melphalan administered immediately prior to heat no significant difference was observed in tumor growth time between the 41.5 and 43.5°C group. Above 43°C there is evidence that hyperthermic inactivation of mammalian cells in vitro results from denaturation of chromosomal protein which is irreversible, and not selective for malignant cells. At 43.5°C the predominant anti-neoplastic effect may be heat damage rather than enhancement of chemotherapeutic agent.

Another relevant observation is that tumor microcirculation may collapse at temperatures above 43°C [27]. This phenomenon would reduce melphalan uptake into tumor tissue and result in no additional tumor control at 43.5°C. For clinical application of these experiments, temperatures below 42°C for whole body and regional hyperthermia may be maximally effective, well tolerated and easier to achieve.

The tumor sizes used in our study were 34 mm^3 (4 mm diameter) and 113 mm^3 (6 mm diameter). Our data showed that melphalan at 41.5°C for 30 min was found to be as effective in delaying tumor growth for both sizes of tumor. In contrast hyperthermia alone at 41.5°C for 30 min was shown to significantly delay tumor growth of the 113 mm^3 tumors, but not the 34 mm^3 tumors. These observations support those of Urano and colleagues [7] who found that the thermal sensitivity of tumor cells in vivo can be substantially modified by tumor size at the time of treatment. Animal tumors contain aerobic and hypoxic tumor cells. Aerobic cells exist near blood vessels and receive a sufficient supply of oxygen and nutrients for metabolism, while hypoxic cells are more distant from blood vessels receiving less oxygen [28]. In hypoxic foci the pH may be low because of accumulation of lactic acid, the product of anaerobic metabolism [29]. The decrease of extracellular pH has been shown in cultured mammalian cells to be associated with increasing thermal sensitivity [30, 31]. The fraction of acidic cells, their acidity and therefore thermal sensitivity may increase with tumor size over 34 mm^3. When melphalan was administered with hyperthermia at 41.5°C to 113 mm^3 tumors, the mean tumor growth time was similar to that observed for smaller tumors. This suggests that for the larger tumor size thermochemotherapy was as effective if not more so in controlling tumor growth.

The impressive results obtained in combining melphalan with hyperthermia have prompted the initiation of a clinical protocol combining heated intraoperative intraperitoneal melphalan with cytoreductive surgery for peritoneal carcinomatosis. To date 12 patients have been treated. From our data it would appear that the administration of intraperitoneal melphalan simultaneously with 90 min of heat at 41.5°C may optimize its anti-neoplastic activity for residual tumor nodules.

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