The Role of Hyperthermia in Regional Alkylating Agent Chemotherapy

Omar I. Abdel-Wahab,[1] Elizabeth Grubbs,[1] Benjamin L. Viglianti,[3] Tsung-Yen Cheng,[1] Tomio Ueno,[1] SaeHee Ko,[1] Zahid Rabbani,[2] Simon Curtis,[1] Scott K. Pruitt,[1] Mark W. Dewhirst,[2,3] Doug S. Tyler[1]

Departments of [1]Surgery and [2]Radiation Oncology, Duke University School of Medicine, Durham, North Carolina, and [3]Department of Biomedical Engineering, Duke University, Durham, North Carolina

ABSTRACT

The role of hyperthermia during regional alkylating agent chemotherapy is controversial. The aim of this study was to determine the exact contribution of hyperthermia to tumor response during isolated limb infusion with L-phenylalanine mustard. Rats bearing rodent fibrosarcoma on the hindlimb underwent isolated limb infusion with saline, saline plus heat, L-phenylalanine mustard, L-phenylalanine mustard under conditions of normothermia, or L-phenylalanine mustard plus hyperthermia. Heat was administered locally using an in-line hot water circulation loop. Treatment with L-phenylalanine mustard at a concentration of 15 or 50 μg/mL was ineffective at producing tumor growth delay ($P = 0.24$ and 0.41, respectively). Furthermore, thermal enhancement of L-phenylalanine mustard activity was not seen at 15 μg/mL. However, administration of high-dose L-phenylalanine mustard, 50 μg/mL, with increasing amounts of heat yielded increasing tumor growth delay, increased regressions, and decreased proliferative index. Although L-phenylalanine mustard infusion under normothermia yielded a tumor growth delay of 7.1 days, combination L-phenylalanine mustard + hyperthermia treatment produced tumor growth delay of 27.0 days ($P < 0.01$; with two of five animals showing a complete response). Four hours after isolated limb infusion, 50.9% of cells in tumor treated with L-phenylalanine mustard + hyperthermia experienced apoptosis, whereas only 18.1, 16, and 4.4% of cells underwent apoptosis after treatment with L-phenylalanine mustard, saline + hyperthermia, or saline. The mean concentration of L-phenylalanine mustard within tumor relative to perfusate following isolated limb infusion was found to be similar among all groups at 0.023, 0.025, and 0.032 in animals undergoing isolated limb infusion with L-phenylalanine mustard, L-phenylalanine mustard + normothermia, and L-phenylalanine mustard + hyperthermia, respectively. These data indicate a synergistic cytotoxic effect of L-phenylalanine mustard + hyperthermia in isolated limb infusion, which is not attributable to enhanced tumor drug uptake.

INTRODUCTION

Isolated limb perfusion is a treatment for regionally advanced melanomas of the extremities, as well as unresectable sarcomas of the extremities, which has been in use since the late 1950s (1). Isolated limb perfusion is a surgical procedure in which high-dose chemotherapeutic agents are delivered regionally to an extremity in hopes of eliminating in-transit metastases while limiting systemic exposure and toxicity. Currently there are three main indications for isolated limb perfusion: (1) therapeutic isolated limb perfusion for locoregionally advanced melanoma, which presents as locally recurrent melanoma, satellitosis (the presence of small foci of tumor adjacent to a larger primary tumor), or in-transit metastases; (2) therapeutic isolated limb perfusion for locally advanced, unresectable sarcomas of the extremities; and (3) prophylactic isolated limb perfusion as an adjunct to surgical excision of high-risk primary or recurrent melanoma (2). Isolated limb perfusion has the theoretical benefit over surgical resection in that it allows one to treat an entire area at risk of recurrence, and it differs from traditional systemic chemotherapy in that doses 10 times higher than the allowed systemic dose can be used.

Isolated limb perfusion is performed by dissecting out and cannulating the main artery and vein of an extremity (3). The arterial and venous cannulas are then connected to a pump oxygenator. To further isolate the extremity, a tourniquet is wrapped around the proximal portion of the extremity. With the tourniquet in place, the pump oxygenator circulates blood to the extremity while maintaining normal tissue oxygenation and pH (4). A chemotherapy agent is then perfused through the isolated limb for 1 hour. After the hour, the extremity is washed out, the cannulas removed, and the vessels repaired (3).

Recently isolated limb infusion has appeared as a simplified and less invasive alternative to isolated limb perfusion (5). Isolated limb infusion differs from isolated limb perfusion in that arterial and venous catheters are inserted percutaneously, without surgery, and a bypass circuit is not involved (6). Although hyperthermia is used, the chemotherapy is given for a shorter period of time to an extremity that is both hypoxic and acidotic. Response rates to regional chemotherapy using the

Received 1/16/04; revised 3/26/04; accepted 4/6/04.
Grant support: NIH Grant K08 CA74241 (D. Tyler), a grant from the Department of Surgery, Duke University Medical Center (D. Tyler), Department of Defense Breast Cancer Research Program DAMD17-03-1-0348 (B. Viglianti), and NIH/NCI CA42745 Grant (M. Dewhirst).
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Doug S. Tyler, Box 3118 Medical Center, Duke University Medical Center, Durham, NC 27710. E-mail: tyler002@acpub.duke.edu.

technique of isolated limb infusion are comparable with those seen with isolated limb perfusion (7).

Although many cytotoxic drugs have been studied in isolated limb infusion and isolated limb perfusion, melphalan (L-phenylalanine mustard) has emerged as the most widely used drug. In the late 1960s, Cavaliere *et al.* (8) first added hyperthermia to regional perfusion with L-phenylalanine mustard. This was done because of emerging evidence of a synergistic cytotoxic interaction between L-phenylalanine mustard and hyperthermia from *in vitro* studies (9–11). Since then, hyperthermia has been widely performed in conjunction with isolated limb perfusion (usually by heating the perfusate to 39°C to 41°C and by applying external warming blankets to prevent heat loss).

Despite the frequent use of hyperthermia during isolated limb perfusion, clinical studies examining the use of hyperthermia in regional perfusion have not clearly demonstrated a benefit in perfusion with the addition of hyperthermia (12, 13). Furthermore, all studies examining the use of hyperthermia in isolated limb perfusion have been retrospective studies. Moreover, only one study has contained a controlled normothermia arm (12). This is important because it has long been believed that when isolated limb perfusion is performed without the addition of heat, the ischemic limb and tumor experience a relative hypothermia, which is a condition believed to limit the effectiveness of chemotherapy (12). Thus, all studies that have shown a benefit in hyperthermic isolated limb perfusion *versus* isolated limb perfusion without the addition of heat have failed to prove whether it is the addition of hyperthermia or the prevention of hypothermia, which is the cause for enhanced therapeutic benefit in hyperthermic isolated limb perfusion. Indeed, a retrospective study by Klasse *et al.* (12) comparing 218 patients who underwent hyperthermic isolated limb perfusion, 39°C to 40°C, to 166 patients who underwent isolated limb perfusion under normothermic heating conditions, 37°C to 38°C, failed to show a benefit in disease-free or overall survival in those patients undergoing hyperthermic perfusion.

To fully define the role of hyperthermia in regional alkylating agent chemotherapy, we devised a novel study using the controlled application of known amounts of localized heat in a rodent fibrosarcoma model of isolated limb infusion. We, as well as others, have shown that isolated limb infusion and isolated limb perfusion are feasible in rodent models and yield results similar to those seen clinically (14, 15).

On the basis of prior *in vitro* (16–19) and animal studies (20–22) on combined use of hyperthermia and alkylating agent chemotherapy, our hypothesis was that the addition of hyperthermia would enhance the cytotoxic activity of L-phenylalanine mustard over isolated limb infusion performed without the addition of hyperthermia. We sought evidence for this by comparing tumor growth delay, number of tumor regressions, tumor proliferative index, and tumor apoptotic index following isolated limb infusion with L-phenylalanine mustard, L-phenylalanine mustard + normothermia, and L-phenylalanine mustard + hyperthermia. We believed that although infusion under normothermic conditions may provide some benefit, the greatest therapeutic benefit would be seen with isolated limb infusion under conditions of hyperthermia. Furthermore, our initial hypothesis was that hyperthermia may contribute to increased tumor cell death by increasing tumor tissue uptake of drug as has been shown by some prior studies (17, 23).

MATERIALS AND METHODS

Animals. Seven to 9-week-old female Fischer 344 rats were purchased from Harlan (Harlan Sprague Dawley, Indianapolis, IN), housed in a temperature controlled room with a 12-hour light-dark cycle, and food and water were provided *ad libitum*. Weight on day 0 of treatment was 167.75 ± 2.21 g (mean weight ± SD). Rats were monitored for general well-being, weight, tumor volume, and functional ability of leg after survival surgery. A grand total of 68 rats was used in this study. All animal experimental protocols were approved by The Duke Institutional Animal Care and Use Committee.

Tumors. Rodent fibrosarcoma tumor was obtained from Dr. Mark Dewhirst of Duke University. Stock tumors growing in the hindlimb of Fischer rats were excised and cut into small pieces. Viable 1.5-mm^3 tumor pieces were transplanted s.c. into the right hindlimb of the rats and allowed to grow to 15 mm in diameter, which took 1.5 to 2 weeks. A rodent fibrosarcoma tumor model was chosen as this tumor is conveniently transplantable, has been previously extensively studied in *in vivo* hyperthermia research experiments, and is similar to tumor types, which are clinically treated with regional chemotherapy (22, 24, 25).

Drug for Therapy Studies. Melphalan (MW 305; Sigma Chemicals, St. Louis, MO) was administered in the perfusate for limb infusion at a concentration of 15 or 50 μg/mL in 0.9% sodium chloride. In clinical use of isolated limb infusion, L-phenylalanine mustard dosage is chosen based on a patient's estimated limb volume (6, 7). The two L-phenylalanine mustard dosages used in this study were chosen because they represent two possible dosages within the range of L-phenylalanine mustard dosages used in the clinical setting.

Therapeutic Studies. After transplantation of fibrosarcoma tumor, groups of five to seven randomly selected rats were treated with saline, saline under hyperthermic tumor conditions, L-phenylalanine mustard, L-phenylalanine mustard under controlled normothermic conditions, or L-phenylalanine mustard under hyperthermic conditions once their tumors reached 15 mm in diameter. Two concentrations of L-phenylalanine mustard (15 and 50 μg/mL) were used in the limb infusion perfusate in the survival studies. Tumor growth was then compared between groups to determine the contribution of heating to normothermic and hyperthermic conditions on tumor response.

Isolated Limb Infusion Technique. Rats were anesthetized with 10 mg i.p. ketamine and 2 mg i.p xylazine before undergoing isolated limb infusion. The animals then received a low-dose inhaled isoflurane anesthesia, 1 to 3%, for the duration of the procedure.

Through an inguinal incision, the right femoral artery and vein were isolated using an operating microscope for the dissection. The side branches of the femoral artery and vein were ligated with 6-0 silk ties. A tourniquet was placed loosely around the proximal thigh, excluding the femoral vessels. The proximal femoral artery and vein were then ligated with 6-0 silk ties. The femoral artery was cannulated through a small arteriotomy with a 24-gauge catheter, and the arterial catheter was

attached to a peristaltic pump (Master Flex model 7524-00; Cole-Parmer Instrument Co., Vernon Hills, IL). The femoral vein was then cannulated. The venous cannula drained by gravity into a reservoir. Once both cannulas were in place, the pump and tubing were primed with saline, the thigh tourniquet was tightened, and infusion was initiated at a flow rate of 1.5 mL/minute. The perfusate consisted of 0.9% sodium chloride or L-phenylalanine mustard at the desired concentration. A 15-minute infusion was performed, followed by a 2-minute washout infusion with 0.9% sodium chloride. This particular length of infusion time was chosen because it approximates the length of infusion time performed clinically in isolated limb infusion and provides a similar thermal dosage to that seen clinically (6). After the wash, the arterial and venous cannulas were removed, and the femoral artery and vein were ligated distally with 6-0 silk ties. In the survival experiments that involved *in vivo* tumor measurements, the wound was sutured with a 4-0 Vicryl suture.

Heating Method and Determination of Temperature Distribution. Rats were anesthetized with pentobarbital (50 mg/kg i.p. Nembutal) and a 16 gauge catheter was placed through the center of the tumor. The core temperature of the rats was maintained with a rectal temperature-regulated heating pad. Heated water (50.1°C, 1.8 ml/second) was passed through the catheter to heat the surrounding tissue. The resulting surface temperature of the catheter was 6°C cooler than the temperature of the heated water. Temperature profiles were measured with a k-type 33.5-gauge hypodermic thermocouple (Omega Probes, Stamford, CT) in four rats with tumors of 17 to 19 mm in diameter after tumors reached steady-state temperature (15 to 20 minutes). The measurements were located on three orthogonal planes to the heating catheter, one in the central plane and the other two 2 mm from the center. Each plane had four measurement tracks taken 2 and 4 mm from the centerline of the tumor defined by the catheter. The thermocouple was inserted 13 mm from the surface of the tumor and retracted every 1 mm, yielding 4×13 measurements per plane. The tracks were taken in random order, and the measurements within each plane were averaged and converted to a radial distance from the catheter. This yielded 26 radial measurements from 2 to 9 mm for each plane. The results for each were then fitted to the radial one-dimensional steady-state heat transfer equation using Gauss-Newton nonlinear regression implemented on MATLAB (The MathWorks, Inc., Natick, MA; Fig. 1).

Hyperthermia and Controlled Normothermia Treatment. Before surgery for isolated limb infusion, all animals had their tumor catheterized with a 16 gauge needle such that the catheter penetrated the approximate tumor center, traversing the tumor to reemerge through the other side. For those animals whose tumor was heated to normothermic or hyperthermic conditions, both ends of the catheter were attached to tubing with heated water pumping through it. Heating was initiated at the onset of surgery (defined as the time of first incision) and discontinued with the conclusion of the surgery (defined as the conclusion of infusion). On the basis of the temperature profile created by the heating apparatus, a temperature of 43°C was chosen for the heating surface of the catheter in the hyperthermia treatments. This was chosen to heat the majority of the surface area of the tumor to temperatures between 38°C and 43°C, which is comparable with temperatures applied clinically

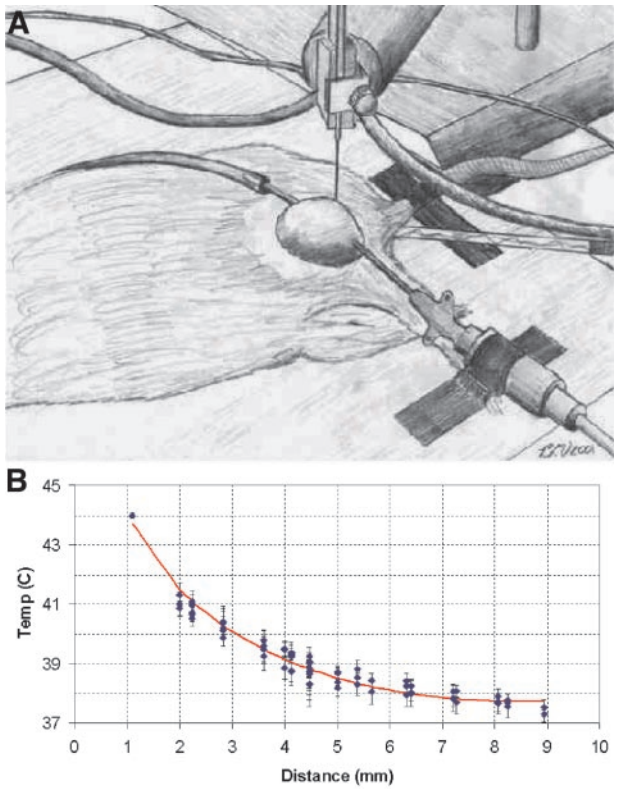

Fig. 1 *A*, illustration depicting method of localized heating using an in-line heated water circulation loop. Tumors were heated by placing a 16 gauge catheter across the approximate center of the tumor. Both ends of the catheter were then connected to tubing containing heated water pumping through it. The temperature in the tumor tissue was monitored using hypodermic thermocouples as illustrated. This unique method of heating was chosen because it allowed for a rise in temperature directly at the tumor, which was easily controlled and consistently reproducible from experiment to experiment (illustration by Benjamin Viglianti). *B*, a radial temperature profile created from multiple temperature measurements in fibrosarcoma tumors by the heating apparatus. On the basis of this temperature profile, the goal temperature for the tumor center in hyperthermic and controlled normothermic treatments was chosen.

in hyperthermic isolated limb infusion and hyperthermic isolated limb perfusion (2, 26). For the treatments involving heating the tumor to maintain normothermic conditions, a temperature of 37.5°C was used for the heating surface of the catheter. This was done to ensure that the tumor temperature would remain above 35°C without being heated to hyperthermic conditions. Also, this temperature range was chosen as it resembles that used clinically in isolated limb infusion and isolated limb perfusion under normothermic conditions (2, 12). Temperature was monitored in all animals by thermocouples located in the tumor at 1, 3, and 8 mm from the approximate tumor center, as well as from muscle on the opposite unaffected leg. Temperature readings were recorded every 2 minutes throughout the surgery. After completing all survival surgeries in which heat was applied, a hyperthermia dosage was calculated for each animal in terms of the conventional hyperthermic dosage, the cumulative number of equivalent minutes at 43°C (CEM43°C). This was done as described in previous reports (27, 28) using the

following equation: $CEM43°C = tR^{(43 - T)}$, where t represents the time interval (in minutes) and T represents the temperature within that time interval. R is the number of minutes needed to compensate for a one-degree temperature change above or below the 43°C breakpoint ($r = 0.45$ if $T \geq 43°C$ and $r = 0.25°C$ if $T < 43$; refs. 27, 28). CEM43°C was calculated for each temperature measurement from each of the three positions on the tumor examined. A total CEM43°C for each animal was then calculated, and an average total CEM43°C was determined for each treatment group. Average total CEM43°C was compared between all treatment groups where hyperthermia was applied using Wilcoxon rank-sum test to ensure that a similar thermal dosage was used in all groups.

Tumor Measurement. Tumors were measured every other day with Vernier calipers (Scientific Products, McGraw, IL), and any ulcer was measured in the same fashion. Tumor volume was calculated according to the formula (length) $\times$ $(\text{width})^2/2$, and the volume of any ulcer was subtracted from the total tumor volume. Percent change in tumor volume was calculated, and animals were followed until a tripling of tumor volume or 60 days occurred.

Assessment of Tumor Response. Time (in days) taken to reach a tripling of tumor volume was calculated from tumor volume measurements using linear regression. The average tumor tripling time was then calculated. Response to treatment was assessed by growth delay and regressions. Growth delay (in days) was defined as the difference in tumor tripling time in treated *versus* control animals. Regressions were defined as the number of tumors whose volume decreased over two consecutive measurements. The thermal enhancement ratio was defined as the ratio of the growth delay of the group treated with L-phenylalanine mustard plus normothermia or hyperthermia to that of the group treated with L-phenylalanine mustard alone. Statistical analysis was performed using the Wilcoxon rank order test for growth delay and the Fisher's exact test for tumor regressions.

Melphalan Analysis. Tumor-bearing animals underwent nonsurvival isolated limb infusion with L-phenylalanine mustard, 50 μg/mL, L-phenylalanine mustard, 50 μg/mL, under controlled normothermic tumor conditions, and L-phenylalanine mustard, 50 μg/mL, under hyperthermic tumor conditions (four animals per group). Inflow perfusate from the limb infusion perfusate chamber was collected every 5 minutes and placed immediately on dry ice. Within 10 minutes after completion of isolated limb infusion, fibrosarcoma tumors, as well as muscle from the operative limb, were harvested and snap-frozen. L-Phenylalanine mustard concentrations in perfusate, tumor, and muscle were quantitated using a modification of the published high-performance liquid chromatography procedures (29). Separation was performed on a Nova-Pak C_{18} reversed-phase column (Waters Associates, Milford, MA) by using isocratic elution with a mobile phase of methanol and water containing a 0.01 mol/L low UV Pic B8 (octane sulfonic acid; Water Associates) at ambient temperatures. The eluent was monitored by using a fluorescence detector (Shimazdzu, Kyoto, Japan) with excitation and emission wavelengths of 260 and 350 nm, respectively.

Frozen samples of perfusate were thawed at room temperature and injected onto the column. Tissue samples were homogenized in ethanol and the homogenate was extracted thrice, concentrated under nitrogen, resuspended in ethanol, and injected onto the column. L-Phenylalanine mustard stock solutions were prepared in methanol containing 0.1 N hydrochloric acid. Standards were prepared by spiking plasma and then treating them in the same way as the samples. The unknown L-phenylalanine mustard concentrations were determined from linear regression analysis of the standards, using integrated peaks.

Immunohistochemical Staining for Proliferation Marker Ki-67. Fibrosarcoma tumors were taken from animals undergoing isolated limb infusion with saline, saline + hyperthermia, L-phenylalanine mustard, 50 μg/mL, L-phenylalanine mustard, 50 μg/mL, under normothermic tumor conditions, and L-phenylalanine mustard, 50 μg/mL, under hyperthermic conditions (two animals per group) 24 hours after isolated limb infusion. Immunohistochemical staining was then performed on 0.5-μm slices of cryosectioned tumor. Sections were washed in PBS to remove frozen mounting media. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS for 10 minutes, followed by a 6-minute wash in PBS. Sections were then incubated with primary anti-Ki-67 antibody (clone B56, diluted 1:20; BD Biosciences, San Diego, CA) for 1 hour at room temperature, followed by a 6-minute wash in PBS. Detection was continued by incubating with secondary biotinylated antibody (1:200; Jackson Immunoresearch, West Grove, PA) and avidin-biotin peroxidase complexes (1:200; Jackson Immunoresearch) for 30 minutes (30, 31). Reaction products were revealed with 3,3′-diaminobenzidine as the chromogen, and sections were counterstained with Harris' hematoxylin.

When evaluating the results of the immunostaining, the tumor areas with the highest density of positive nuclear staining were chosen. Five high-powered fields (10×40) were chosen from each slide. The proliferative index was then determined by dividing the total number of positive cells by total number cells in all five fields. All samples were scored independently by two observers. When the interobserver difference differed by >5%, the sample was rescored by the two investigators together.

Histopathologic Study of Apoptosis. Fibrosarcoma tumors were taken from animals 4 and 24 hours after isolated limb infusion with saline, saline + hyperthermia, L-phenylalanine mustard, 50 μg/mL, and L-phenylalanine mustard, 50 μg/mL, plus hyperthermia. At the time of sacrifice, tumors were fixed in 10% buffered formalin (pH 6.9 to 7.1). Apoptosis was detected on 0.5-μm slices of tissue by labeling the exposed of ends of DNA generated during apoptosis using the Klenow FragEL kit (Oncogene, San Diego, CA; ref. 32). Briefly, sections were first deparaffinized by immersion in xylene followed by rehydration in ethanol. Specimens were then permeabilized by incubation with 20 μg/mL proteinase K 1:100 in 10 mmol/L Tris (pH 8). Endogenous peroxide activity was blocked with 3% H_2O_2 in methanol for 5 minutes. DNA labeling was then performed by allowing specimens to equilibrate in Klenow Equilibration Buffer for 30 minutes followed by 1.5 hours of incubation with Klenow-labeling reaction mixture (33). The labeling reaction was then terminated, and labeled ends of DNA fragments were revealed with 3,3′-diaminobenzidine. Sections were then counterstained with methyl green. The percentage of cells undergoing apoptosis was then quantified as described above for proliferative index.

RESULTS

Tumor Temperature Distribution during Isolated Limb Infusion. Figs. 2 and 3 show representative graphs of temperature *versus* time during rodent limb infusion surgery. As evident from Fig. 2, temperature from sites on both extremities decreased during surgery. However, with placement of the tourniquet around the operative leg and initiation of infusion with room temperature perfusate, tissue temperatures in the affected leg dropped to hypothermic conditions. Temperature in the operative extremity in this representative animal fell to 27°C to 25.5°C during the 15-minute infusion. This temperature was hypothermic relative to core rodent body temperature as well as to the opposite leg. A similar pattern was seen repeatedly during limb infusion with saline or L-phenylalanine mustard without the addition of heat. As shown in Table 1 and Fig. 4, average temperature from six survival animals during isolated limb infusion with saline ranged from 29.0°C to 29.3°C. Similarly, temperature during isolated limb infusion with L-phenylalanine mustard ranged from 27.5°C to 28.1°C.

Having demonstrated that the rodent limb experienced hypothermia during isolated limb infusion without the addition of heat, isolated limb infusion under hyperthermic, as well as controlled normothermic conditions, were performed. Fig. 3 shows temperature distribution *versus* time during isolated limb infusion under controlled normothermic conditions (Fig. 3*A*) and hyperthermic conditions (Fig. 3*B*). Because tumors were heated using an in-line hot water circulation loop, the temperature of the water used to heat the tissues is displayed. Importantly, Fig. 4 and Table 1 show that animals undergoing infusion with saline + hyperthermia, L-phenylalanine mustard, 15 μg/mL, plus hyperthermia, and L-phenylalanine mustard, 50 μg/ml, plus hyperthermia had their tumors heated to comparable temperatures for similar amounts of time. This yielded similar hyperthermia dosages as evidenced by average total CEM43°C values, which were not statistically significant between hyperthermia treatment groups (Table 1). Because heating was applied locally at the tumor site, temperature in the tissues of the leg beyond the tumor's circumference were not influenced by the heating device. As evident in Fig. 3, *A* and *B*, although tumor temperature was elevated, temperature in the gastrocnemius muscle of the leg where heat was applied was still hypothermic relative to the opposite nonisolated leg.

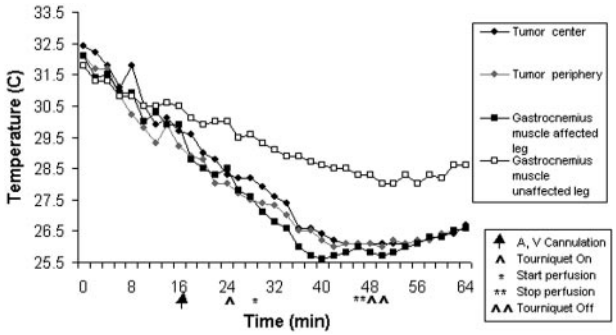

Fig. 2 Representative graph of temperature distribution *versus* time during isolated limb infusion with L-phenylalanine mustard, 50 μg/mL, without the addition of heat. The temperature in both legs decreased throughout the surgery. However, temperature dropped to a greater extent in the operative leg. The rate with which temperature dropped increased greatly with placement of tourniquet and initiation of perfusion with room temperature perfusate. By the end of the 15-minute perfusion, the temperature of the affected limb in this animal was 25.5°C.

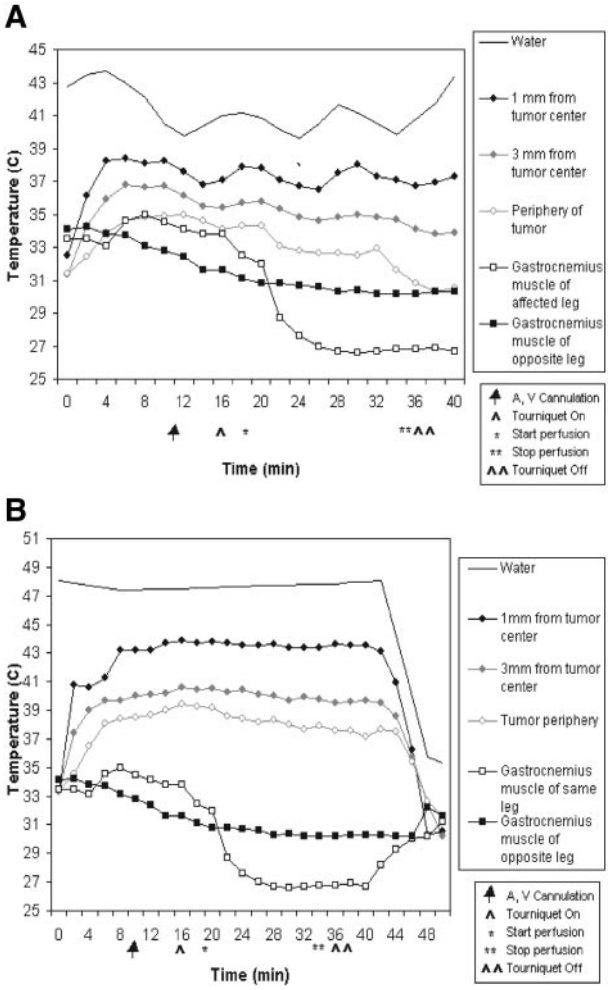

Fig. 3 Representative graphs of temperature distribution *versus* time during isolated limb infusion in animals undergoing isolated limb infusion with L-phenylalanine mustard, 50 μg/mL, under controlled normothermic conditions (*A*) and hyperthermic conditions (*B*). Temperature of the water in the in-line hot water circulation loop used to heat tumors is shown. Because heat was applied locally at the tumor site, temperature in the tissues of the leg beyond the circumference of the tumor was unaffected by heating.

Therapeutic Studies. The addition of hyperthermia alone produced a statistically significant growth delay of 4.4 days ($P < 0.05$) but did not produce any regressions in tumor volume. Although a 15-minute infusion with L-phenylalanine mustard, 15 and 50 μg/mL, without the addition of heat was ineffective at producing growth delay, the addition of hyperthermia significantly improved tumor response at both concentrations of L-phenylalanine mustard. At the lower dose of L-phenylalanine mustard studied, the addition of heat produced growth delays of 2.1 days, which was very similar to that of the

Table 1 Average tumor temperature and thermal dosimetry during isolated limb infusion surgery

			Treatment group*			
Location of temperature reading	Saline ($n = 6$)	LPAM ($n = 10$)†	LPAM (50 μg/mL) + controlled NT ($n = 5$)	Saline + HT ($n = 5$)	LPAM (15 μg/mL) + HT ($n = 7$)	LPAM (50 μg/mL) + HT ($n = 6$)
1 mm from tumor center	29.1 (1.0)	27.5 (0.9)	37.3 (0.2)	42.0 (1.2)	42.7 (0.1)	42.5 (0.3)
3 mm from tumor center	29.3 (1.0)	27.7 (1.0)	36.1 (0.9)	39.5 (0.7)	40.7 (0.7)	39.3 (1)
Tumor periphery (8 mm from tumor center)	29.0 (1.0)	28.1 (1.6)	34.1 (1.5)	37.8 (0.6)	37.8 (0.6)	36.8 (1.4)
Duration of heating‡			37.3 (1.5)	37.5 (1)	36.0 (1.35)	38.5 (1)
Average total CEM43°C†				60.8	74.1 (0.07)	74.5 (0.12)

* Average tumor temperature (with SD in parentheses) is displayed.

† Average total CEM43°C in minutes for each treatment group in which HT was applied. *P* values (in parentheses) comparing the CEM43°C of LPAM + HT groups to saline + HT group is shown.

‡ Average number of minutes tumor was heated (with SD in parentheses) is displayed.

Abbreviations: LPAM, L-phenylalanine mustard; NT, normothermia; HT, hyperthermia; CEM43°C, cumulative number of equivalent minutes at 43°C.

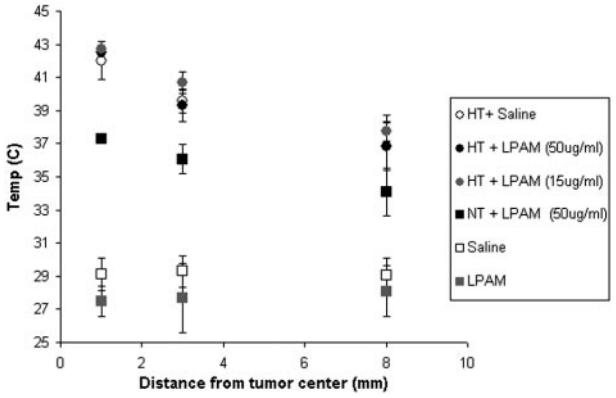

Fig. 4 Temperature distribution *versus* distance from tumor center in animals undergoing isolated limb infusion. Each point represents the average tumor temperature at a location on the tumor with *vertical bars* representing SD in average temperature between animals. These temperature recordings are from the survival animals, which were followed after treatment to determine the effect of heat on tumor response in isolated limb infusion. As evident from this graph, (1) all tumors treated with hyperthermia (HT) were heated to comparable temperatures, and (2) the temperature of tumors, which were not heated, fell into hypothermic range. Data from this figure is shown in quantitative form in Table 1. NT, normothermia; LPAM, L-phenylalanine mustard.

growth delay produced by L-phenylalanine mustard alone (Table 2). However, because the SD in tumor tripling time in the L-phenylalanine mustard, 15 μg/mL, plus hyperthermia group was so small, the growth delay produced by L-phenylalanine mustard + hyperthermia was unexpectedly found to be statistically significant ($P < 0.05$), whereas the growth delay produced by L-phenylalanine mustard alone was not significant ($P > 0.05$). At a higher dose of L-phenylalanine mustard, 50 μg/mL, the effect of heat on L-phenylalanine mustard infusion was much more evident. As shown in Fig. 5, at the higher dose of L-phenylalanine mustard, increasing amounts of heat yielded increased growth delay. Two animals of the five treated with L-phenylalanine mustard, 50 μg/mL, plus hyperthermia experienced complete responses (defined as no gross evidence of tumor by 60 days after treatment). With the addition of heat to normothermic conditions, growth delay increased from 1 to 7.1 days ($P < 0.05$). With further heating of the tumor to hyperthermic conditions, growth delay increased to 27 days ($P < 0.01$; Fig. 6). In addition to increased growth delay, the addition of heat also yielded statistically significant regressions in tumor volume, as well as increased thermal enhancement ratios (Table 3).

Although the use of heat produced no noticeable differences in toxicity to animals in most cases, one of the six animals in the high-dose L-phenylalanine mustard + hyperthermia group experienced an amputation of the affected limb 1 week after surgery. This animal was euthanized according to animal protocol, and its data were not used in this study.

Effect of Hyperthermia on Tumor Melphalan Levels. To determine whether the improved tumor response seen under hyperthermia conditions was related to alterations in tumor uptake of drug, we examined tumor melphalan concentrations following isolated limb infusion. Hyperthermia did not produce a statistically significant change in tumor L-phenylalanine mustard concentrations, tumor-muscle L-phenylalanine mustard con-

Table 2 Effect of isolated limb infusion ± localized mild hyperthermia in rat fibrosarcoma

Treatment group	Growth delay (d)*	Regressions	Amputations
Saline		0/6	0/6
Saline + HT	4.4 (0.01)	0/5	0/5
Melphalan (15 μg/mL)	2.3 (0.24)	0/5	0/5
Melphalan (15 μg/mL) + HT	2.1 (0.02)	0/7	0/7

NOTE. Tumor-tripling time: number of days required for tumor to reach a volume three times volume on day of treatment. Number in parentheses, SE.

* Number in parentheses is *P* value relative to saline obtained using Wilcoxon rank-sum test. Although growth delay of melphalan (15 μg/mL) and melphalan (15 μg/mL) + HT are similar, the large SE in growth delay of melphalan (15 μg/mL) resulted in statistically insignificant growth delay.

Abbreviation: HT, hyperthermia.

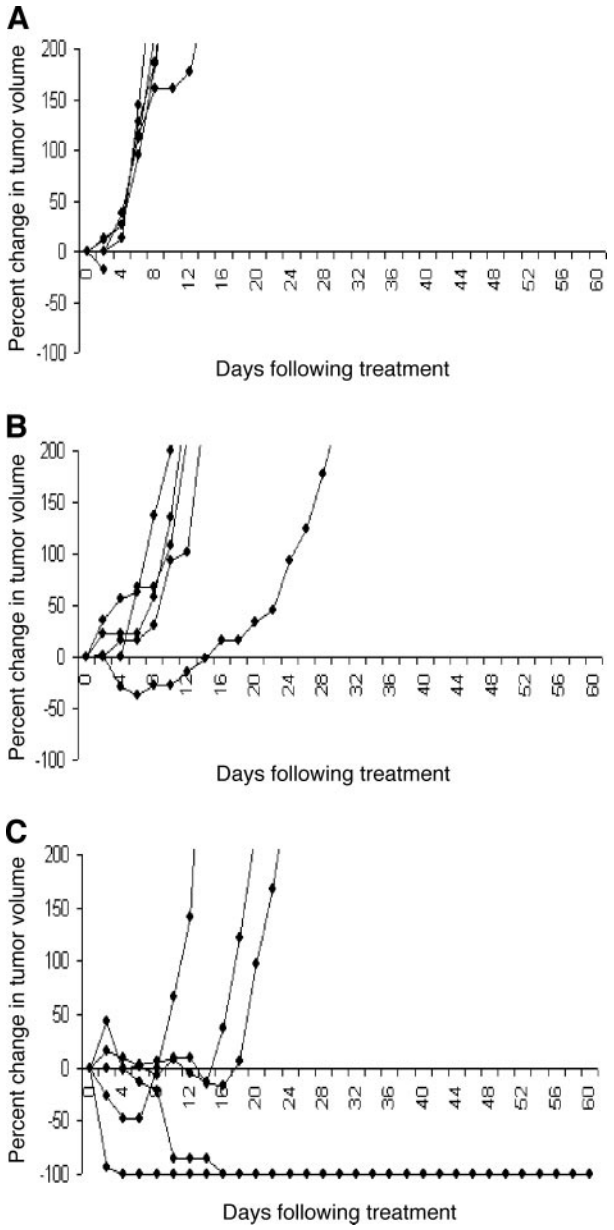

Fig. 5 Percent change in tumor volume *versus* days after isolated limb infusion with L-phenylalanine mustard, 50 μg/mL (*A*), L-phenylalanine mustard, 50 μg/mL, under controlled normothermic conditions (*B*), and L-phenylalanine mustard, 50 μg/mL, under hyperthermic conditions (*C*). Each line represents the tumor growth of an individual animal. Animals were followed until tumors tripled in size or 60 days. Growth delay was significant in melphalan + normothermia group ($P < 0.05$) and highly significant in the L-phenylalanine mustard + hyperthermia group ($P < 0.01$).

centrations, or tumor-inflow perfusate concentrations from samples taken within 30 minutes of isolated limb infusion (Fig. 7).

Tumor Cell Proliferation after Isolated Limb Infusion. To examine the effect of various treatment regimens on cellular proliferation we examined the Ki-67 proliferation index 24 hours after isolated limb infusion. The proliferative index was found to be highest in saline treated animals, followed by L-phenylalanine mustard, 50 μg/mL, alone and saline + hyperthermia groups (Fig. 8). Although the average proliferative index of animals in the L-phenylalanine mustard + hyperthermia group was not statistically significant relative to saline control ($P = 0.1$ using Wilcoxon rank-sum test), there was a general trend toward decreased proliferative index with addition of hyperthermia and L-phenylalanine mustard. Animals treated with L-phenylalanine mustard, 50 μg/mL, plus normothermia and L-phenylalanine mustard + hyperthermia had the lowest proliferative index with 14.9 ± 3.6 and 9.0 ± 4.3% (average index ± SE) of cells being positive for Ki-67, respectively.

Apoptotis after Isolated Limb Infusion. The percentage of cells in fibrosarcoma tumor undergoing apoptosis was determined 4 and 24 hours after isolated limb infusion. Although the amount of apoptosis in animals treated with saline-isolated limb infusion was minimal, treatment with saline + hyperthermia or L-phenylalanine mustard alone increased the percentage of apoptosis to 16.1 and 18.1%, respectively (Fig. 9). Combination L-phenylalanine mustard + hyperthermia treatment resulted in a much larger extent of apoptosis with 50.9% of cells containing apoptotic nuclei. Interestingly, by 24 hours after isolated limb infusion, the extent of apoptosis had decreased considerably in the combination L-phenylalanine mustard + hyperthermia-treated tumors, and this level of apoptosis was comparable with animals treated with L-phenylalanine mustard alone.

DISCUSSION

The current studies demonstrate enhancement of L-phenylalanine mustard activity against rodent fibrosarcoma tumor during limb infusion with concomitant administration of localized hyperthermia at the tumor site. We found that in limb infusion performed without the addition of heat, temperatures within the operative leg consistently fell to hypothermic conditions. However, using a unique method of heat application, we were able to heat tumors to a normothermic or hyperthermic temperature range in a controlled and reproducible manner. The ability to administer doses of hyperthermia to the tumor site in this way is essential because variations in tumor temperature as small as

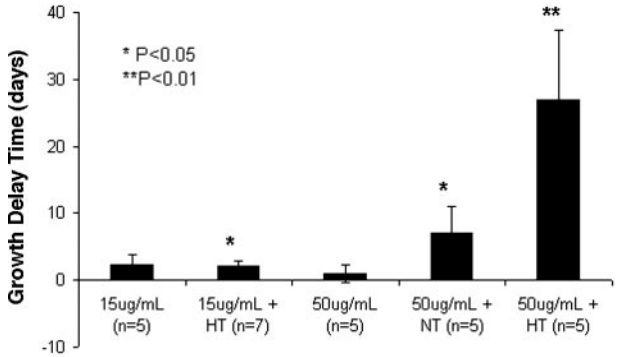

Fig. 6 Growth delay time of rodent fibrosarcoma after isolated limb infusion with melphalan +/− the addition of localized heat. The *vertical bars* indicate SE in average tumor tripling time. HT, hyperthermia; NT, normothermia.

Table 3 Effect of isolated limb infusion with and without the addition of localized mild hyperthermia in rat fibrosarcoma

Treatment group	Tumor-tripling time (days)*	Growth delay (days)†	Thermal enhancement ratio‡	Regressions§	Local control¶	Amputations
Saline	7.9 (0.5)			0/6	0/6	0/6
Saline + HT	12.3 (0.99)	4.4 (0.01)		0/5 (1)	0/5	0/5
Melphalan (50 μg/mL)	8.9 (1.3)	1 (0.41)		0/5 (1)	0/5	0/5
Melphalan (50 μg/mL) + controlled normothermia	15.0 (3.9)	7.1 (0.01)	7.1	1/5 (0.45)	0/5	0/5
Melphalan (50 μg/mL) + HT	34.9 (10.4)	27 (0.008)	27.0	4/5 (0.015)	2/5	1/6

* Mean number of days taken for individual tumors to reach a volume of three times their untreated volume on day 1. Number in parentheses, SE.

† The difference between the mean time required for tumors in treated and saline groups to reach a volume three times their untreated volume on day of treatment. Number in parentheses, *P* of growth delay compared with saline obtained using Wilcoxon rank-sum test.

‡ Thermal enhancement ratio = growth delay of melphalan + HT group/growth delay of melphalan alone group.

§ Regressions defined as number of animals that experienced a decrease in tumor volume over two consecutive measurements. Number in parentheses is *P* of number regressions compared with saline obtained using Fisher's exact test.

¶ Local control defined as no tumor present after 60 days of treatment.

Abbreviation: HT, hyperthermia.

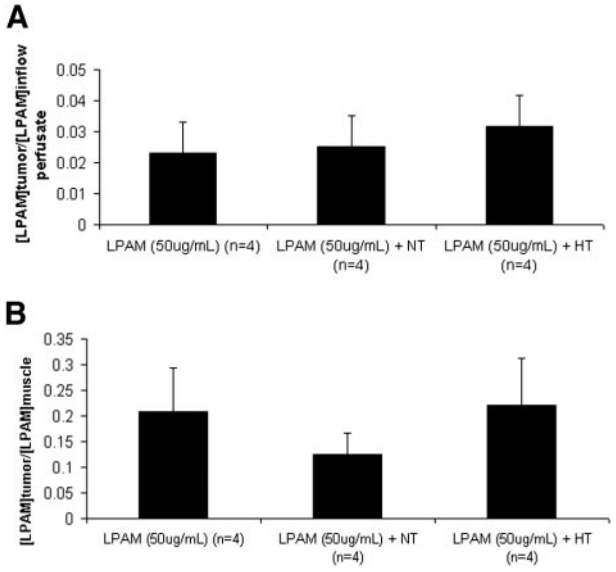

Fig. 7 Mean melphalan tumor-inflow perfusate concentrations (*A*) and tumor-muscle concentration (*B*) from samples taken within 30 minutes after isolated limb infusion. *Vertical bars* represent SE in ratio between animals. Localized hyperthermia (HT) did not appear to significantly alter total tumor drug concentrations. *n* represents number of animals studied. NT, normothermia; LPAM, L-phenylalanine mustard.

2°C maintained for ≥30 minutes can result in vastly different fractions of tumor cell killing (27, 34).

As stated earlier, no prospective randomized studies have been performed to support the use of hyperthermia in isolated limb perfusion, and some retrospective studies have questioned the benefit in hyperthermic perfusions over perfusion with heating to normothermic conditions. Our results provide evidence of increased tumor growth delay, increased number of regressions in tumor volume, and decreased tumor proliferative index as temperatures were increased from hypothermic, 27.5°C to 28.7°C, to normothermic, 34.1°C to 37.3°C, to hyperthermic, 36.8°C to 42.5°C, during L-phenylalanine mustard-isolated limb infusion. This result strongly parallels *in vitro* studies by Hahn (16), which classified alkylating agents into a category of drugs that yield a continuous increase in cytotoxicity with increasing temperature. In addition, quantitative histologic analysis for apoptosis following isolated limb infusion revealed that combination L-phenylalanine mustard + hyperthermia yielded a significantly higher percentage of apoptotic cells than treatment with L-phenylalanine mustard or hyperthermia alone. However, we found that the improved effect of L-phenylalanine mustard with addition of heat cannot be explained by increased uptake of drug to tumor tissue.

Thermal enhancement of L-phenylalanine mustard activity was striking at the elevated dose of L-phenylalanine mustard studied but modest at low concentrations of L-phenylalanine mustard studied. Our results suggest that the interaction between hyperthermia and L-phenylalanine mustard at the higher dose studied was synergistic. This conclusion was implied because the growth delay produced by combination hyperthermia and L-phenylalanine mustard therapy (27.0 days) was unequivocally greater than the addi-

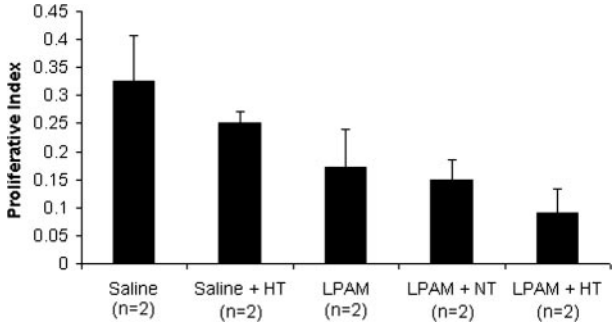

Fig. 8 Ki-67-proliferative index of rodent fibrosarcoma tumor 24 hours after isolated limb infusion with saline, saline + hyperthermia (HT), L-phenylalanine mustard (LPAM), 50 μg/mL, LPAM + normothermia (NT), or LPAM + HT. Proliferative index was determined by dividing total number of positive cells by total number of cells in five high-powered fields (10 × 40). Shown is the average proliferative index with *vertical bars* representing SE in index between tumors (*n* = number of tumors examined). Although the average proliferative index relative to saline did not reach statistical significance for any group, there was a trend toward decreased proliferative index 24 hours after isolated limb infusion with the addition of heating plus LPAM.

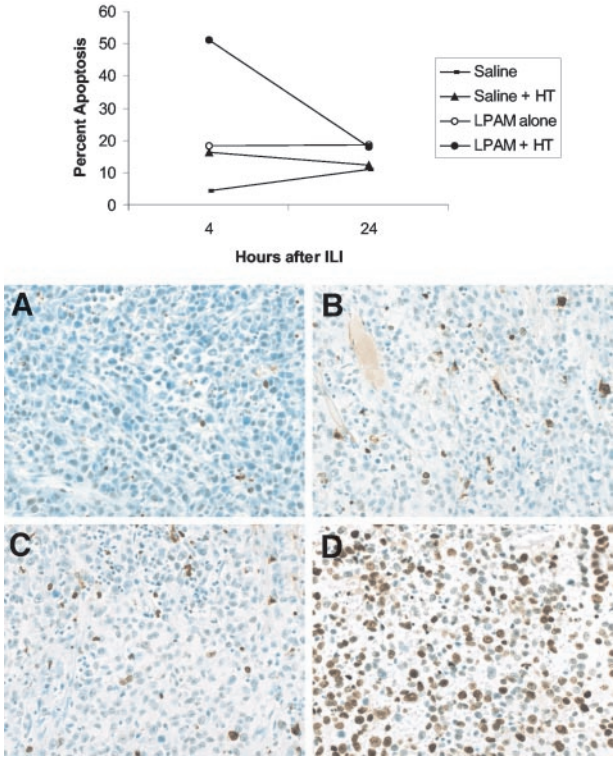

Fig. 9 Graph (*top*) depicting percentage of apoptotic nuclei 4 and 24 hours after isolated limb infusion (ILI). The graph demonstrates that tumor treated with L-phenylalanine mustard (LPAM) + hyperthermia (HT) experienced a significantly greater amount of apoptosis than the other treatments 4 hours after ILI. However, the level of apoptosis fell to control levels by 24 hours after treatment. Below the graph are photographs depicting fibrosarcoma tumor tissue stained for apoptosis 4 hours after treatment with saline (*A*), saline + HT (*B*), LPAM (*C*), and LPAM + HT (*D*) (10 × 40). Detection of apoptotic nuclei (*brown*) was performed by immunohistological labeling of exposed ends of fragmented DNA created during apoptosis. Apoptotic cells (*stained brown*) are much more numerous in the tumor treated with LPAM + HT (*A*) than in the other tissues.

tive growth delay of hyperthermia alone (4.4 days) and L-phenylalanine mustard alone (1 day). In the model of limb infusion used in the current study, the concentration of L-phenylalanine mustard achieved in the tumor tissue was usually <10% of the L-phenylalanine mustard concentration administered in the perfusate (Fig. 7). This inefficient delivery of drug to the tumor tissue may explain why thermal enhancement of melphalan activity was only evident at elevated concentrations of L-phenylalanine mustard. Administration of L-phenylalanine mustard at a concentration of 15 μg/mL may have resulted in such a low concentration of drug in the tumor that any interaction with elevated temperatures could not have been evident. This is consistent with animal studies using systemic administration of the alkylating agent cyclophosphamide, which revealed that thermochemotherapy was only successful when plasma and tissue levels of drug were sufficiently elevated (22).

In addition to greater growth delay and increased number of tumor regressions, combination hyperthermia and L-phenylalanine mustard treatment yielded a trend toward decreased tumor proliferation as shown by Ki-67 expression 24 hours after isolated limb infusion. Ki-67 is an antigen expressed in all phases of the cycle beyond G_0 and is a commonly used measure of cell proliferation (35). Plaat *et al.* (36) have found that the posttreatment proliferative index in soft tissue sarcomas from patients undergoing hyperthermic isolated limb perfusion with L-phenylalanine mustard correlates with overall survival after treatment. In contrast, posttreatment mitotic and apoptotic activity was not clearly associated with survival. In the present study, combination hyperthermia + L-phenylalanine mustard yielded the smallest Ki-67-proliferative index 24 hours after isolated limb infusion. This result additionally underscores the therapeutic benefit in addition of hyperthermia to melphalan limb perfusion.

Multiple hypotheses have been proposed to explain the mechanism of interaction between hyperthermia and alkylating agents. Several authors have proposed that hyperthermia increases drug delivery to the tumor site as well as increases tumor cell uptake of drug. Clark *et al.* (17) studied a human melanoma cell culture model of isolated limb perfusion and found that application of mild hyperthermia, 41.5°C, for 1 hour resulted in increased cellular accumulation of L-phenylalanine mustard. However, increased tumor accumulation of drug with addition of heat has not been a consistent finding in *in vivo* studies. For example, Honess *et al.* (37) reported that after 45 minutes of systemic heating to 41°C in mice, tumor-plasma L-phenylalanine mustard ratios in the RIF-1 murine tumor were higher in unheated animals. Similarly, Laskowitz *et al.* (38) found no difference in L-phenylalanine mustard uptake in rhabdomyosarcoma xenograft after 70 minutes of regionalized heating to 42°C. Likewise, in the current study, there was no significant difference in tumor L-phenylalanine mustard concentrations, tumor-perfusate ratio of L-phenylalanine mustard concentration, or tumor-muscle ratio of L-phenylalanine mustard concentration. Thus, thermal enhancement of L-phenylalanine mustard cytotoxicity could not be explained by increased uptake of drug.

One explanation for the discrepancy in findings between *in vitro* and *in vivo* studies is that in *in vitro* systems, tumor cell accumulation of drug is not dependent on blood flow, whereas in *in vivo* systems, blood flow is a major determinant of tissue drug delivery. Although it is commonly believed that hyperthermia increases blood flow, controversy exists as to the actual response of blood flow to hyperthermia, and the response is often heterogeneous and unpredictable (23). Also, in all of the *in vivo* studies discussed above, L-phenylalanine mustard concentrations were measured in the homogenized bulk of tumor. None of these studies examined whether hyperthermia increased intracellular:extracellular concentration of drug. In contrast, the *in vitro* study by Clark *et al.* (17) found that intracellular concentrations of L-phenylalanine mustard were consistently higher than medium concentrations of drug and that hyperthermia increased the cell:medium ratio of L-phenylalanine mustard. Although we have shown that total tumor concentration of L-phenylalanine mustard did not increase with the addition of heat, we cannot conclude that thermal enhancement of L-phenylalanine mustard activity was not due to increased intracellular accumulation of the drug.

Even if hyperthermia does not increase tumor intracellular concentration of L-phenylalanine mustard, there is evidence that

hyperthermia increases the rate by which L-phenylalanine mustard creates interstrand cross-links in DNA. Urano and Ling (18) observed an increase in the rate constant by which L-phenylalanine mustard creates adducts with DNA with increases in temperature in mouse fibrosarcoma II cells. Moreover, there is evidence from *in vitro* studies of platinum-alkylating agents that the cellular ability to repair DNA adducts may be impaired after hyperthermia treatment (19). Increased DNA cross-link formation and decreased ability to repair adducts are possible mechanisms by which hyperthermia and L-phenylalanine mustard interact in hyperthermic isolated limb infusion. Future work examining the effect of varying thermal dosage on DNA cross-link formation after isolated limb infusion would help to additionally elucidate the mechanism of alkylating agent potentiation by hyperthermia.

In this study, hyperthermia and L-phenylalanine mustard were found to have a synergistic effect on tumor growth delay after hyperthermic isolated limb infusion. To determine whether the tumor response witnessed *in vivo* was due to induction of apoptotic cell death, the percentage of cells undergoing apoptosis was examined after isolated limb infusion. Alkylating agents have been shown to induce apoptosis at low concentrations while inducing necrosis at higher concentrations (39). Similarly, the mode of cell death induced by hyperthermia is thought to depend on the dose of hyperthermia administered (40). A suggested explanation for this phenomenon is that at low levels of damage to the cell, the cell maintains the ability to initiate programmed cell death. In contrast, when the insult to the cell is severe, signaling necessary to initiate apoptosis is impaired and the cell dies by necrosis instead. At the same time, the sensitivity of cells to hyperthermia- and chemotherapy-induced apoptosis varies widely between cell types. For instance, heating WW1 Burkitt lymphoma cells to 43°C for 30 minutes induces apoptosis in >95% of cells, whereas the same heat regimen induces apoptosis in <5% of MM96L melanoma cells (41). This wide variability in apoptotic response is found even among different cell lines of the same tumor type. Sekiguchi *et al.* (42) discovered that two different cell lines of ovarian carcinoma exhibited different modes of cell death when exposed to the same toxic dose of cisplatin. Such findings have led to the belief that the mode of cell death is dependent upon genotype of the cell, which, in turn, determines the function of proteins necessary for apoptosis induction such as p53 (43, 44). In the present study, we found that at 6 hours after isolated limb infusion, there was significantly greater percentage of apoptotic nuclei in the combination L-phenylalanine mustard + hyperthermia treatment group than any other group. However, by 24 hours after treatment, the level of apoptosis approached that of control treatment groups. This observation is consistent with the findings of Sakaguchi *et al.* (24), who determined that the maximum level of hyperthermia-induced apoptosis in rats bearing rodent fibrosarcoma occurred 6 hours after treatment. By 24 hours after hyperthermia treatment, apoptosis levels had reached that of the nonheated control tumor. Furthermore, it was seen that the rodent fibrosarcoma tumor was relatively resistant to heat-induced apoptosis compared with the Ward colon carcinoma tissue, which was similarly treated. Thus, the predominant mode of cell death appears to depend on the severity of treatment, the tumor cell type, and the posttreatment time at which the tumor is studied. Because the amount of apoptotic death has been shown to vary based on tumor genotype, examination of apoptosis after isolated limb infusion is limited in this study by the use of a single tumor model. Investigation of hyperthermic potentiation of alkylating agents on other tumor types (such as melanoma) is currently being carried out using a similar model to that used in this study. Furthermore, the observation that different thermal dosages may affect the mode of cell death underscores the need for future studies on molecular mechanisms by which thermochemotherapy leads to induction of apoptosis (44).

The fact that the extent of apoptosis varied based on posttreatment time may be explained by the well-characterized pathology of apoptotic cell death. When cells die by apoptosis, they die singly and are rapidly eliminated by neighboring healthy parenchymal cells and macrophages (20). Thus, if tissue is examined for apoptosis shortly after the application of cytotoxic damage, cells undergoing apoptosis will be more likely to be present in the tissue and detectable by apoptosis detection methods. However, if tissue is examined for apoptosis at a time distant from cytotoxic damage, the extent of apoptosis may appear diminished as many of the apoptotic cells will have been removed from the tissue (45).

Although combined use of hyperthermia and L-phenylalanine mustard provided the greatest induction of apoptosis and therapeutic benefit, there was some evidence of adverse effects of combined thermochemotherapy in this study. This was suggested by the occurrence of an amputation in one animal after isolated limb infusion with L-phenylalanine mustard, 50 μg/mL, and hyperthermia. Although isolated limb infusion is thought to be associated with less morbidity than isolated limb perfusion, the lack of limb oxygenation in isolated limb infusion may be associated with untoward effects (7). However, the fact that the only animal to experience limb toxicity in this study underwent isolated limb infusion with L-phenylalanine mustard + hyperthermia suggests that the combined use of hyperthermia and L-phenylalanine mustard was the cause of the amputation rather than the isolated limb infusion procedure. Nevertheless, the present study supports the administration of heat during isolated limb infusion and isolated limb perfusion with L-phenylalanine mustard. Although the greatest thermal enhancement of L-phenylalanine mustard activity was seen under hyperthermia tumor conditions, 36.8°C to 42.5°C, our results provide evidence of increased thermal enhancement of L-phenylalanine mustard activity with heating to maintain normothermic conditions, 34.1°C to -37.3°C, as well. These findings suggest that the application of any amount of heat to prevent hypothermic conditions will yield greater therapeutic benefit in regional chemotherapy administration as compared with using no heat at all and will improve the therapeutic index for melphalan in the management of extremity malignancies.

REFERENCES

1. Creech O, Krementz E, Ryan R, Winbald J. Chemotherapy of cancer: regional perfusion utilizing an extracorporeal circuit. Ann Surg 1958; 148:616.

2. Hohenberger P, Kettelhack C. Clinical management and current research in isolated limb perfusion for sarcoma and melanoma. Oncology 1998;55(2):89–102.

3. Kroon BB. Regional isolation perfusion in melanoma of the limbs; accomplishments, unsolved problems, future. Eur J Surg Oncol 1988; 14(2):101–10.

4. van de Merwe SA, van den Berg AP, Kroon BB, van den Berge AW, Klaase JM, van der Zee J. Modification of human tumour and normal

tissue pH during hyperthermic and normothermic antiblastic regional isolation perfusion for malignant melanoma: a pilot study. Int J Hyperthermia 1993;9(2):205–17.

5. Mian R, Henderson MA, Speakman D, Finkelde D, Ainslie J, McKenzie A. Isolated limb infusion for melanoma: a simple alternative to isolated limb perfusion. Can J Surg 2001;44(3):189–92.

6. Lindner P, Doubrovsky A, Kam PC, Thompson JF. Prognostic factors after isolated limb infusion with cytotoxic agents for melanoma. Ann Surg Oncol 2002;9(2):127–36.

7. Thompson JF, Kam PC, Waugh RC, Harman CR. Isolated limb infusion with cytotoxic agents: a simple alternative to isolated limb perfusion. Semin Surg Oncol 1998;14(3):238–47.

8. Cavaliere R, Ciocatto EC, Giovanella BC, et al. Selective heat sensitivity of cancer cells. Biochemical and clinical studies. Cancer (Phila.) 1967;20(9):1351–81.

9. Woodhall B, Pickrill KL, Georgiade NG, Mahaley MS, Dukes HT. Effect of hyperthermia upon cancer chemotherapy-application to external cancer of head and face structures. Ann Surg 1960;151:750–9.

10. Crile G. Heat as an adjunct to the treatment of cancer: experimental studies. Cleve Clin Quart 1961;28:75–89.

11. Shingleton WW, Bryan FA, O'Quinn WL, Krueger LC. Selective heating and cooling of tissue in cancer chemotherapy. Ann Surg 1962; 156:408–16.

12. Klaase JM, Kroon BB, Eggermont AM, et al. A retrospective comparative study evaluating the results of mild hyperthermic versus controlled normothermic perfusion for recurrent melanoma of the extremities. Eur J Cancer 1995;31A(1):58–63.

13. Di Filippo F, Anza M, Rossi CR, et al. The application of hyperthermia in regional chemotherapy. Semin Surg Oncol, 1998;14(3): 215–23.

14. Kelley ST, Menon C, Buerk DG, Bauer TW, Fraker DL. Acidosis plus melphalan induces nitric oxide-mediated tumor regression in an isolated limb perfusion human melanoma xenograft model. Surgery (St. Louis) 2002;132(2):252–8.

15. Grubbs E, Friedman H, Colvin M, et al. In-transit melanoma: The role of alkylating agent resistance in regional therapy. J Am Coll Surg. In press 2004.

16. Hahn G. Hyperthermia and cancer. New York: Plenum Press; 1982.

17. Clark J, Grabs AJ, Parsons PG, Smithers BM, Addison RS, Roberts MS. Melphalan uptake, hyperthermic synergism and drug resistance in a human cell culture model for the isolated limb perfusion of melanoma. Melanoma Res 1994;4(6):365–70.

18. Urano M, Ling CC. Thermal enhancement of melphalan and oxaliplatin cytotoxicity in vitro. Int J Hyperthermia 2002;18(4):307–15.

19. Hettinga JV, Lemstra W, Meijer C, et al. Mechanism of hyperthermic potentiation of cisplatin action in cisplatin-sensitive and -resistant tumour cells. Br J Cancer 1997;75(12):1735–43.

20. Toyota N, Strebel FR, Stephens LC, et al. Therapeutic efficacy and apoptosis and necrosis kinetics of doxorubicin compared with cisplatin, combined with whole-body hyperthermia in a rat mammary adenocarcinoma. Int J Cancer 1998;76(4):499–505.

21. Takemoto M, Kuroda M, Urano M, et al. The effect of various chemotherapeutic agents given with mild hyperthermia on different types of tumours. Int J Hyperthermia 2003;19(2):193–203.

22. Urano M, Kuroda M, Nishimura Y. For the clinical application of thermochemotherapy given at mild temperatures. Int J Hyperthermia 1999;15(2):79–107.

23. Song CW. Effect of local hyperthermia on blood flow and microenvironment: a review. Cancer Res 1984;44(10 Suppl):4721s–30s.

24. Sakaguchi Y, Stephens LC, Makino M, et al. Apoptosis in tumors and normal tissues induced by whole body hyperthermia in rats. Cancer Res 1995;55(22):5459–64.

25. Wondergem J, Bulger RE, Strebel FR, et al. Effect of cis-diamminedichloroplatinum(II) combined with whole body hyperthermia on renal injury. Cancer Res 1988;48(2):440–6.

26. van der Zee J, Kroon BB, Nieweg OE, van de Merwe SA, Kampinga HH. Rationale for different approaches to combined melphalan and hyperthermia in regional isolated perfusion. Eur J Cancer 1997; 33(10):1546–50.

27. Dewhirst MW, Viglianti BL, Lora-Michiels M, Hanson M, Hoopes PJ. Basic principles of thermal dosimetry and thermal thresholds for tissue damage from hyperthermia. Int J Hyperthermia 2003;19(3): 267–94.

28. Thrall DE, Rosner GL, Azuma C, et al. Using units of CEM 43°C T90, local hyperthermia thermal dose can be delivered as prescribed. Int J Hyperthermia 2000;16(5):415–28.

29. Adair CG, Burns DT, Crockard AD, Desai ZR, Harriott M. Modified extraction and chromatography for the measurement of plasma melphalan by ion-pair high-performance liquid chromatography. J Chromatogr 1984;336(2):429–33.

30. Huuhtanen RL, Blomqvist CP, Wiklund TA, et al. Comparison of the Ki-67 score and S-phase fraction as prognostic variables in soft-tissue sarcoma. Br J Cancer 1999;79(5–6):945–51.

31. Elias JM. Cell proliferation indexes: a biomarker in solid tumors. Biotech Histochem 1997;72(2):78–85.

32. Gu Y, Jow GM, Moulton BC, et al. Apoptosis in decidual tissue regression and reorganization. Endocrinology 1994;135(3):1272–9.

33. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119(3):493–501.

34. Oleson JR, Samulski TV, Leopold KA, et al. Sensitivity of hyperthermia trial outcomes to temperature and time: implications for thermal goals of treatment. Int J Radiat Oncol Biol Phys 1993;25(2):289–97.

35. Gerdes J, Li L, Schlueter C, et al. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 1991;138(4):867–73.

36. Plaat BE, Molenaar WM, Mastik MF, et al. Hyperthermic isolated limb perfusion with tumor necrosis factor alpha and melphalan in patients with locally advanced soft tissue sarcomas: treatment response and clinical outcome related to changes in proliferation and apoptosis. Clin Cancer Res 1999;5(7):1650–7.

37. Honess DJ, Donaldson J, Workman P, Bleehen NM. The effect of systemic hyperthermia on melphalan pharmacokinetics in mice. Br J Cancer 1985;51(1):77–84.

38. Laskowitz DT, Elion GB, Dewhirst MW, et al. Hyperthermia-induced enhancement of melphalan activity against a melphalan-resistant human rhabdomyosarcoma xenograft. Radiat Res 1992;129(2): 218–23.

39. Fernandes RS, Cotter TG. Apoptosis or necrosis: intracellular levels of glutathione influence mode of cell death. Biochem Pharmacol 1994; 48(4):675–81.

40. Hildebrandt B, Wust P, Ahlers O, et al. The cellular and molecular basis of hyperthermia. Crit Rev Oncol Hematol 2002;43:33–56.

41. Harmon BV, Takano YS, Winterford CM, Gobe GC. The role of apoptosis in the response of cells and tumours to mild hyperthermia. Int J Radiat Biol 1991;59(2):489–501.

42. Sekiguchi I, Suzuki M, Tamada T, Shinomiya N, Tsuru S, Murata M. Effects of cisplatin on cell cycle kinetics, morphological change, and cleavage pattern of DNA in two human ovarian carcinoma cell lines. Oncology 1996;53(1):19–26.

43. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. Cell 2002;108(2):153–64.

44. Ohnishi K, Ohnishi T. Heat-induced p53-dependent signal transduction and its role in hyperthermic cancer therapy. Int J Hyperthermia 2001;17(5):415–27.

45. Stephens LC, Hunter NR, Ang KK, Milas L, Meyn RE. Development of apoptosis in irradiated murine tumors as a function of time and dose. Radiat Res 1993;135(1):75–80.