Systemic Lidocaine Enhancement of Hyperthermia-induced Tumor Regression in Transplantable Murine Tumor Models

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ABSTRACT

Previously, we reported that local lidocaine infusion of a CA 755 mammary adenocarcinoma growing in C57BL × DBA/2 F1 mice, when combined with local heating for 1 hr in a 43.5°C water bath, significantly increased survival and inhibited tumor growth more than heating alone. Because of its clinical implications, systemic lidocaine was tested in the above model system and in a murine fibrosarcoma tumor model. An equivalent supraadditive, tumor-inhibitory effect of heat and lidocaine was obtained with both systemically and intratumor-administered lidocaine. The serum levels of lidocaine necessary to achieve tumor regression were within the therapeutic range for the control of arrhythmia in humans. Several treatment schedules, varying the mode of drug delivery, were evaluated. The effects of treatment on tumor growth characteristics were analyzed using an extension of the Cox survival model.

INTRODUCTION

Hyperthermia as a treatment modality for cancer is currently receiving renewed attention alone and in combination with a variety of chemotherapeutic agents (9, 12) and noncytotoxic drugs (19, 20). The potential use of nonchemotherapeutic drugs, e.g., membrane-active agents, which avoid the clinical problem of myelosuppression is supported by the results obtained in several preclinical studies (11, 19–22). The in vivo studies are of particular interest. The injection of LID directly into a transplantable mammary carcinoma growing in the hind leg of mice significantly increased the animals’ survival when combined with local heating for 1 hr in a 43.5°C water bath (20).

Since these observations have clinical implications, it was of interest to test whether systemic LID at nontoxic levels could also serve as a potentiator of hyperthermic killing. Two murine tumor models were used, one a murine fibrosarcoma and the other a mammary adenocarcinoma for which previous studies have demonstrated a synergistic interaction with local LID and hyperthermia (20).

Systemic LID proved to be equally as effective as i.t. injection of the drug for potentiation of cell killing by local hyperthermia. Blood levels of LID in combination with hyperthermia that achieve tumor cell killing in mice are within the nontoxic level for humans. Additional variables, such as temperature and time and site of LID administration, were evaluated with respect to tumor growth characteristics.

MATERIALS AND METHODS

Animal Models

M Murine Mammary Carcinoma. Young adult male C57BL × DBA/2 F1 (hereafter called BD2F1) mice weighing approximately 30 g and grafted with mammary adenocarcinoma strain CA755 were used. The average number of malignant cells required to produce tumors in 50% of inoculation sites for this mammary carcinoma is 18 (10); this suggests that this tumor model must be weakly antigenic at best. For transplantation, tumors were removed from donor mice, and a crude suspension was prepared with the aid of a Snell cytosieve and then injected s.c. in the hind leg below the knee as described previously (20). When the tumors measured approximately 4 mm in mean diameter, the mice were randomly assigned to treatment groups as designated below. Tumor sizes were determined 3 times a week as described below. A complete response was defined as disappearance of all measurable tumor with a response duration of at least 12 days. By the 12th day posttreatment, animals that had not achieved a complete remission were generally premorbid, had significant tumor necrosis, and therefore were sacrificed. Those animals demonstrating tumor responses were all sacrificed at Day 29. Our previous experience (20) showed that complete remissions lasting for 29 days correlated with survivals of greater than 250 days.

Mice were anesthetized by i.p. injection of 14 mg of chloral hydrate. They were placed on special carriers with the tumor-bearing leg drawn through an opening for immersion in a Tecam constant-temperature bath with a TU Tempunit circulating heater and a Yellow Springs Instrument telethermometer thermistor probe to monitor water temperature. The legs of the mice were gently held in place by masking tape over the upper portion of the limb during heating. The tumor-bearing legs were exposed to bath temperatures of 43.5 ± 0.1°C (S.D.) for 1 hr unless otherwise noted. The water surface was insulated by floating plastic spheres 2 cm in diameter both as an aid in maintaining constant bath temperature and to further insulate the remainder of the animals’ bodies from heating. The air temperatures above the water bath in the vicinity of the mice never exceeded 37°C. The difference between the core temperature of the tumor and the water bath was determined using a pair of copper-constantin thermocouples and a microvolt meter. Because of the damage done by the insertion of the thermocouple, none of the animals used for temperature measurements were included in the tumor growth analyses. The temperature difference between the water bath and the tumor core was less than 0.15°C.

Lidocaine-HCl (Elkins-Sinn, Inc.) was infused by injection into 3 areas of the tumor in a total volume of 0.05 ml (i.t.) within 5 min prior to heat treatment (unless otherwise specified), or injections were administered s.c. in non-tumor-bearing regions of the animal and/or i.p. Nine treatment regimens were used. Comparison treatments include no treatment and heat only (43.5°C for 1 hr). LID (2 mg s.c.) was given in the nontumorous leg or in the tumorous leg distant from the tumor site. LID (2 mg i.t.) was injected as in previously reported studies (20). Injections i.p. (1 mg) were given 60 min prior to heating in one of 4 regimens: (a) no second injection; (b) second i.p. injection 1 min before heating; (c) second injection given s.c. 1 min prior to heating; and (d) same as c except heating for 30 min only. No LID-only groups were included in this study; previous studies (20) have shown no difference between LID only and no treatment. In this study, we did not combine i.p. LID with 42.5°C heating in the mammary...
carcinoma studies reported here, since previous work demonstrated no synergism at this temperature with i.t. LID (20).

**Murine Fibrosarcoma.** Female C57BL/6 mice weighing approximately 30 g were grafted with a methylicholanthrene-induced fibrosarcoma were also used. Gross tumor was diced under aseptic conditions and treated with 0.25% trypsin for 45 min, cells were rinsed and diluted using Roswell Park Memorial Institute medium (KC Biological, Lenexa, Kans.). A volume of 0.3 ml containing 10^6 cells was injected into the hind foot pad of etherized mice. On Day 15 after injection (mean tumor diameter, 3.0 mm), the mice were randomly assigned to 7 treatment groups of 12 mice each: Group 1, no treatment; Group 2, anesthetized only; for Groups 3 to 7, anesthetized plus Group 3, heat only at 42.5°; Group 4, heat only at 43.5°; Group 5, heat (42.5°) plus 1.5 mg LID i.p. given 1 hr prior to heating; Group 6, heat (43.5°) plus 1.5 mg LID i.p. given 1 hr prior to heating; Group 7, no heat, anesthetized, followed immediately by 1.5 mg LID i.p. Mice were anesthetized by i.p. injection of 7 mg chloral hydrate (3 animals died from anesthesia). They were placed on carriers with the tumor-bearing foot drawn through an opening for immersion in a constant-temperature bath for 60 min as described above. Tumor size was determined daily as described below.

The footpad was chosen as the injection site for the following reasons: (a) previous experience with this murine fibrosarcoma within our laboratory group was at this injection site; (b) the change in tumor volume at this site was a simple exponential function for a sufficient length of time for determination of the pre- and post-tumor growth rate; (c) it was found that the footpad could be heated efficiently and conveniently in a water bath; and (d) heating at this site does not result in the elevation of core temperature as measured by rectal probe.

**Tumor Size and Growth Determinations**

The major and minor axis lengths of the tumor were measured, and the product of the major axis and the square of the minor axis was used as a volume index. Semilogarithmic plots of the volume index versus time were prepared for each tumor. The volume index is converted to an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments. The time-to-fixed-volume, 1000 cu mm for an ellipsoidal volume by multiplying by \( \pi r^2 / 6 \). The regrowth straight line was extended back to the original volume and regrowth delay estimated as the difference between this time point and the time of treatment. This estimate of delay is a minimal value as an increase in doubling time is found with some treatments.

**Analysis**

Often in in vivo experiments, some animals show local tumor control, and/or some animals are removed from the experiment for reasons such as histological studies. Animals such as these are referred to as censored from the study. Commonly used descriptive statistics and models are inadequate in analyzing the results from experiments containing censored animals, because they frequently do not use the data from the censored animals. Denekamp (6), Begg (1), and others at the Ninth Gray Conference have discussed this problem in some detail (7). The Cox survival model (3) as modified by Breslow (2) provides a solution (8) to the problem by including censored animals in the analysis. While the Cox model was initially proposed for analysis of time-to-death data, the assumptions underlying the model may be used with any time-to-defined-event data (17).

The modified Cox survival model is especially useful because it allows one to use categorical, ordinal, and continuous covariates. This model permits the representation of treatments and injections in terms of dummy variables that take on yes (1) or no (0) values. For instance, 2 dummy variables can describe 3 treatments, e.g., no heating (0,0), 43.5° for 30 min (1,0), and 43.5° for 60 min (0,1). Combinations of 4 such 0 or 1 variables uniquely describe 9 experimental treatments.

The initial tumor size, estimated as the product of the major axis length and the square of the minor axis length, was treated as a covariate for all preliminary analyses. In the case of the adenocarcinoma, this factor varied little and was found not to be significant. Therefore, in the final analyses, initial size was included only for the fibrosarcoma.

The computer program (COVAR) used was based on an algorithm developed by Crowley and Hu (4) and programmed by R. Spry of the Biostatistics Section of the Wisconsin Clinical Cancer Center (5). The program estimates the coefficients of the model for all factors specified and computes a relevant statistic for testing the significance of each factor. The program permits the preparation of tabular listings of the proportion of mice having a fixed response at a specified time as well as the production of graphical presentations of the model results. Validity of this model, when dealing with phenomenon involving the time elapsed to a fixed response, can be partially tested by examination for linearity on a semilog plot of the percentage of animals showing the property being studied against time. Both regrowth delay-time and the time-to-fixed-volume show such linearity on a semilog plot. An example of such a plot for regrowth delay was published in the monograph for a meeting on hyperthermia (14).

**LID Determinations**

Serum LID levels were determined using a standard clinical gas chromatographic method. LID concentrations are determined by comparing the chromatographic peak heights to known standards of the same relative retention time. The assay is linear up to 10 µg/ml. Higher levels must be diluted. The detection level for this assay is 0.2 µg/ml. Recovery is 90 to 95%. The only known interfering substance is pentobarbital. In our laboratory, the standard deviations on 3-µg/ml and 8-µg/ml controls are 0.20 and 0.47 µg/ml, respectively. For this assay, a cardiac therapeutic range is 2 to 6 µg/ml.

For the assay, a Hewlett Packard 402 gas chromatograph equipped with a 6-ft-long, 2-mm (inside diameter) glass column packed with 1% OV-17 on Gas Chrom Q 100/120 mesh was used; oven temperature was 215°.

Samples (1 ml of plasma) were prepared by adding 0.5 ml of solution containing carbocaine as an internal standard plus 1.0 ml of borate buffer. Each sample was extracted into chloroform at a basic pH and evaporated using air while vortexing. Samples were dissolved in ethyl acetate and injected into the gas chromatograph.

LID levels were determined on the pooled blood of 4 animals for each time point. Blood was obtained by decapitation. The time elapsed to collect the blood from 4 animals was ~1.75 min.

**RESULTS**

**Murine Mammary Carcinoma.** Repeating treatments reported earlier (20), with 2 groups of animals, we confirmed that 43.5° heating for 1 hr showed some retardation of tumor growth with no complete responses (0 of 6) and that similar heating with the addition of i.t. administration of LID resulted in a 33% complete response rate (2 of 6). A similar complete response rate was observed if LID (followed 1 min later by heating for 60 min) was administered s.c. in the nontumorous leg (2 of 5) or the tumorous leg distant from the tumor site (2 of 8). Administration of LID i.p. followed 60 min later by heating resulted in a complete response rate of 33% (3 of 9). If a second injection of LID was given (either s.c. or i.p.) 60 min after an i.p. injection and then the animals were heated for 1 hr, comparable complete response rates are obtained, i.e., s.c. (6 of 15). If 2 such injections of LID were given, with heating for 30 min instead of 1 hr, no response was seen (0 of 6).

In all groups in which LID was combined with 43.5° heating
for 1 hr, a pronounced shift in the tumor volume-time curve is found. This shift, measured as the tumor regrowth delay time and the time to fixed volume, has been analyzed using the Cox model. Because LID i.p. 60 min before heating results in a very low value of systemic LID (see below) at the time of the second injection and heating, the animals are grouped for analysis in terms of their injection at the time of heating.

Median regrowth delay in the experiments reported here for no treatment (0 days), heating for 1 hr at 43.5° (2.9 days), and heating for 1 hr at 43.5° with i.t. LID (11.4 days) was essentially the same as in earlier unpublished studies: 0, 2.9, and 8.0 days. Survival data for these earlier studies have been published (20).

The observed regrowth delays were essentially the same within all the i.p. and s.c. LID administration groups; therefore, the data have been pooled. The median regrowth delay was 14.3 days following heat (43.5° for 1 hr) combined with i.p. LID and 14.2 days for s.c. LID.

Median days to 1000 cu mm in the experiments reported here were 9.4 days (no treatment), 12.0 days (43.5°, 1 hr), 20.5 days (43.5°, 1 hr, i.t. LID), 22.7 days (43.5°, 1 hr, s.c. LID), and 23.2 days (43.5°, 1 hr, i.p. LID). In the previous study referenced above, the median days to fixed value were, as in the case of regrowth delay, essentially the same as the values found in the present study.

The percentage of animals with tumors that show no regrowth 30 days posttreatment (~25%) is very similar to the percentage of 250-day survival of animals given i.t. injections of LID and heated to 43.5° for 1 hr [based on long-term follow-up of animals reported previously (20)].

The BD2F mice treated at the same time as the mice noted in the above experiments were utilized to determine the serum LID levels. As expected, an i.p. injection of LID (2 mg) resulted in the rapid attainment of serum levels of this drug, i.e., 5.8 μg/ml at 15 min, in 30-g mice. Levels at 60 min were <1.5 μg/ml. Interestingly, similar levels were seen if the same dose was given s.c. as illustrated in Chart 1. If animals were given 2 mg of LID i.p. and then 60 min later were given chloral hydrate anesthesia and a second s.c. dose (2 mg LID) and heating, the LID levels produced were comparable to a single s.c. injection (2 mg) with immediate heating (see Chart 1).

The serum levels of LID dropped significantly 30 min after the first i.p. injection in unanesthetized mice (Chart 1, left) which explains the similarity of the 2 regimens. The difference in the LID levels at 30 min comparing the right and left sides of Chart 1 correlates with the presence and absence of chloral hydrate anesthesia. Unanesthetized animals consistently have low levels after 30 min, while animals that receive chloral hydrate have significant levels until 60 min after administration of LID. This difference in LID levels may be due in part by the dropping of the core temperature (~6°) of mice immediately after chloral hydrate administration. Heating mice as described returns core temperatures to base-line values of ~37° within 10 min. Although detailed studies of LID absorption distribution and metabolism are possible, they are beyond the scope and interest of these studies.

**Fibrosarcoma.** The raw data (connected by straight-line segments) for 3 treatment regimens are shown in Chart 2 to illustrate the range of tumor growth characteristics which we observed.

In Chart 2 are presented the tumor volume versus time posttreatment for: no treatment (left); 43.5°, 1 hr (middle); and 43.5°, 1 hr, plus 2 mg LID (right). Comparison of these 3 panels illustrates the extent of the increase in delay of regrowth when LID is combined with hyperthermia in the fibrosarcoma model. In this group, 2 animals were sacrificed at Day 15 for histology and ultrastructure analysis. In contrast to the adenocarcinoma in which this treatment yields 25% long-term (>250 days) control, no such complete control was observed in this study for the fibrosarcoma.

Chart 3 presents the results of the Cox analysis of the fibrosarcoma model data. There is a significantly greater than additive effect of LID with 43.5° heating for 1 hr (p < 0.05). The effect of LID shown at 42.5° is in the same direction but is not significant at p = 0.05. Table 1 presents the median values for days to 500 cu mm and the regrowth delay in days for each of the treatment regimens used on the fibrosarcoma model. For each treatment, in addition to a median value, a normal deviate statistic (Z) is listed along with a p value for the hypothesis that any increase in time to effect is, in truth, equal to zero.

The addition of LID does not increase either the time to fixed volume or the regrowth delay. Heat alone at 42.5° for 1 hr significantly increases time to 500 cu mm but shows only a suggestion of effect on regrowth delay (p = 0.055). Both parameters are significantly increased by 43.5° heating for 1 hr. The interaction of heat plus LID was assessed directly. In effect, the hypothesis tested is whether or not there is supraadditivitly of heat and LID effects. For both measures of tumor response, 43.5° for 1 hr plus LID showed a significant interaction (supraadditivity). For 42.5°, 1 hr, plus LID, the supraadditive increase in time to fixed volume is significant but the regrowth delay is not.

**DISCUSSION**

The observations made with the mouse adenocarcinoma model confirm our previous work (20) which demonstrated potentiation of hyperthermic tumor killing with local anesthetic. The present report extends our previous understanding by demonstrating that systemic LID, when combined with local hyperthermia, is as effective as is i.t. injection of LID with heating for the local control of tumor. We reported that tumor control in this model system confers survival advantage (20). Interestingly, administration of LID (i.p.) either just prior to heating or 1 hr before heating resulted in the same supraadditive killing of tumor cells. We have observed this same lack of time dependence on
the administration of LID (i.e., just before heating versus 1 hr before heating) in i.t. studies of this model. If, however, tetracaine is used i.t. as an anesthetic agent, responses are seen only if administration is just prior to heating. Although the rapidity of achieving a steady blood level after s.c. LID was unexpected, it was consistent with the observed tumor responses; i.e., there were no significant differences between different routes of LID administration. A review of the literature demonstrates that s.c. administration of LID leads to similar levels in humans (13).

While survival is considered by many to be the ultimate measure of therapeutic success, investigational evaluation of a new therapy often relies on measures of tumor response. For the adenocarcinoma, the interruption of tumor growth is evidenced by a median delay after heat alone of 2.9 days, while the interaction of LID with 43.5° heating for 1 hr increases the median delay to 11.4 (t.i.), 11.4 (s.c.), and 14.3 (i.p.) days. For the fibrosarcoma, 43.5° alone results in a median delay of 7.0 days which is further increased by addition of LID to 12.0 days.

Subsequent to these studies, a series of animals bearing the mammary carcinoma were treated with 43.5° heating for 1 hr with 2 mg LID i.p. Those animals which remained disease free for up to 3 months were sacrificed and found by light and electron microscopy to be free of residual cancer.

The results obtained with the fibrosarcoma model confirm the supraadditive effectiveness of systemic LID in combination with hyperthermia discussed above by also demonstrating local tumor control in a mesenchymal tumor known to be both immunogenic and spontaneously metastasizing. Studies of systemic hyperthermia (42°) using a pig model revealed no normal tissue toxicity with the same serum levels of LID used in the current studies (15). Further, in vivo studies combining whole-body hyperthermia with LID (41–42°) in a murine leukemia (AKR) model (18) confirm in vitro studies of AKR leukemia (41.8°) (16) which demonstrate an enhancement by LID of tumor kill. Such results have encouraged our use of systemic hyperthermia and anesthetic agents clinically.

As discussed previously (19), the above findings are consistent with the hypothesis that the physical state of the cell membrane is a major factor in the death of cells exposed to hyperthermia. Further research to elucidate the fundamental molecular basis of LID-heat interaction is essential and ongoing.

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*4 M. B. Yatvin, unpublished data.

*5 A. Clark, H. I. Robins, W. H. Dennis, and M. B. Yatvin, manuscript in preparation.
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