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Increased Tumor Control Rates in Murine Fibrosarcoma by Combined Therapy with L-Alanosine and Radiation

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L-Alanosine, an analog of L-aspartic acid, was investigated as one of a series of chemical compounds that may have inhibitory effects on the repair of potentially lethal damage caused by radiation using an *in vivo* murine fibrosarcoma (Meth-A tumor) in BALB/cBy male mice. The combined treatment of single administration of L-alanosine (600 mg/kg) and single dose of X-irradiation (20 Gy) on Meth-A tumors produced 62% tumor control, while the radiation alone resulted in less than 5% tumor control. The potentiating effect by L-alanosine was higher when the drug was administered 8 h prior to X-irradiation. The dose modification factor of the drug is estimated to be 1.4 for Meth-A tumor. The increased tumor control rates with combined alanosine and radiation were highly dependent upon the time and sequence of the combined treatment. The reason for reduced efficacy at treatment times of less than 8 h prior to X-irradiations appears to be related in part to the modulation of the body temperature by L-alanosine when combined with Ketamine, an anesthetic agent.

Key words: L-Alanosine — Radiation — Murine fibrosarcoma

In the treatment of human cancer by ionizing radiation, there are several factors responsible for intrinsic radioresistance of the tumor. Of these, the efficiency of repair of potentially lethal damage (PLD) caused by radiation is one of the most important factors.[1,2] While the molecular mechanisms of PLD repair are not fully understood, their implications in clinical radiotherapy are well appreciated.

There are two classes of drugs that have been reported to inhibit repair of PLD. They are inhibitors of DNA repair synthesis such as β-arabinofuranosyladenine (β-Ara-A),[3-6] fludarabine phosphate,[7] and other purine nucleoside analogs such as 3′-deoxy-adenosine[6,8] and energy depleters (e.g. lonidamine[9,10]). L-Alanosine, an antibiotic produced by *Streptomyces alanosinicus*,[11,12] which may belong to the latter class, has been demonstrated to have antitumor activity in some animal systems,[12,13] and is undergoing phase I-II clinical trials for antitumor activity in humans.[13] The compound was investigated for its radiosensitizing effects on a murine tumor model based on the premise that PLD repair inhibition by L-alanosine would potentiate radiation damage.

MATERIALS AND METHODS

Tumor system Isogenic BALB/cBy male mice, 6–8 weeks old (Jackson Lab., ME), were inoculated with single cell suspensions of methylcholanthrene-induced fibrosarcoma (Meth-A tumor) cells which were obtained from Dr. T. Boyse, Sloan-Kettering Institute, and have been maintained in ascites form. For tumor transfer, cells were collected from peritoneal ascites and suspended in minimal essential medium for inoculation. Viability of the cells was determined by trypan blue dye exclusion and was regularly greater than 99%. Cell numbers were counted with a Coulter Zm counter followed by appropriate cell dilution. Then 10^6 cells were inoculated intramuscularly in the distal thigh region. At 7 to 10 days after the inoculation, animals whose tumor volumes were 600 ± 100 mm^3 were used for these studies.

Tumor growth delay and tumor control assay The tumor volume analysis was carried out by measuring maximal diameters of the tumor-bearing thighs. Tumor volumes were calculated by the experimental formula

$$V = d^3 - 36d$$

in which d is the average diameter (mm) of the tumor-bearing thigh. The accuracy of this method was confirmed by water displacement studies.[14] All tumor volume data points were plotted as the log of the average tumor volume per group vs. days post treatment. Dose-dependent growth delays were defined as the time required for mean tumor volumes to regrow to the respective initial treatment volume. Local tumor control was defined as a non-palpable tumor at 60 days post-treatment.

X-Irradiation Irradiation was performed on anesthetized mice (Ketamine-HCl, 100 mg/kg). An AG. E. Maxitron operated at 300 kVp and 20 mA with 2 mm Cu filtration at a dose rate of 100 cGy/min and a target-skin distance of 50 cm was used. Animals were secured in a circular

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lucite jig with 4 mm lead shielding through which the tumor-bearing legs projected for X-irradiation.

Drugs L-Alanosine was supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The drug was made up fresh in saline prior to each experiment. The drug was administered with 0.2 ml inocula into the peritoneal cavity of mice at selected time intervals according to the experimental protocol.

Body temperature measurements Because purine nucleoside analogs have been reported to decrease the body temperature,[15] temperature measurements were recorded (Doric Tendicator) using rectal thermocouple probes (Bailey Instrument Inc.) at various times after drug administration.

RESULTS

The toxicity of L-alanosine to BALB/cBy mice Previous studies have indicated the LD_{10} dose of L-alanosine in BDF_1 mice to be 1600 mg/kg.[13] The LD_{10} in BALB/cBy male mice was considerably lower (700 mg/kg), especially in combination with anesthetic (ketamine-HCl 100 mg/kg). As a result, all the following experiments were performed at the dosage of 600 mg/kg or less.

The sequence and timing of radiation and L-alanosine Figure 1 shows tumor volume curves as a function of days after treatment with L-alanosine 600 mg/kg alone, 20 Gy of radiation alone, and the combination of radiation and L-alanosine administered one hour before X-irradiation. The volume of the tumor treated with L-alanosine alone corresponded to 2.5 days of growth delay, decreasing from 600 to 400 mm^3 in 24 h, but increasing immediately thereafter. Growth delays of the tumor treated with radiation alone and with the combination of radiation and L-alanosine were 16 and 25 days, respectively. From comparisons of these values, it is confirmed that the effect of the combination is greater than a simple additive effect.

Prior to determining the dose-modifying factor of L-alanosine, it was important to isolate which specific treatment sequence and timing of L-alanosine administration with respect to X-irradiation would result in optimal

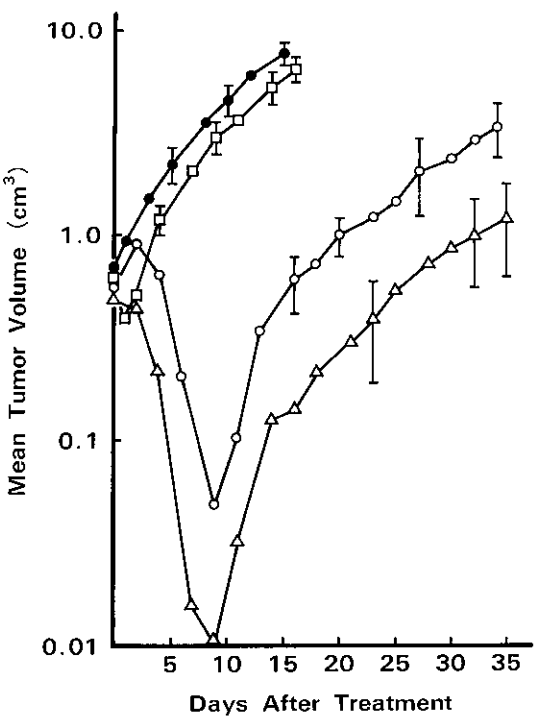

Fig. 1. Growth delays of Meth-A tumor treated with a single dose of radiation and/or L-alanosine. ●, control, untreated tumor; □, L-alanosine 600 mg/kg alone; ○, radiation 20 Gy alone; △, L-alanosine 600 mg/kg plus radiation. L-Alanosine was injected intraperitoneally into mice at one hour before 20 Gy of irradiation. Each data point represents the average obtained from 8–10 animals. Bars, SD.

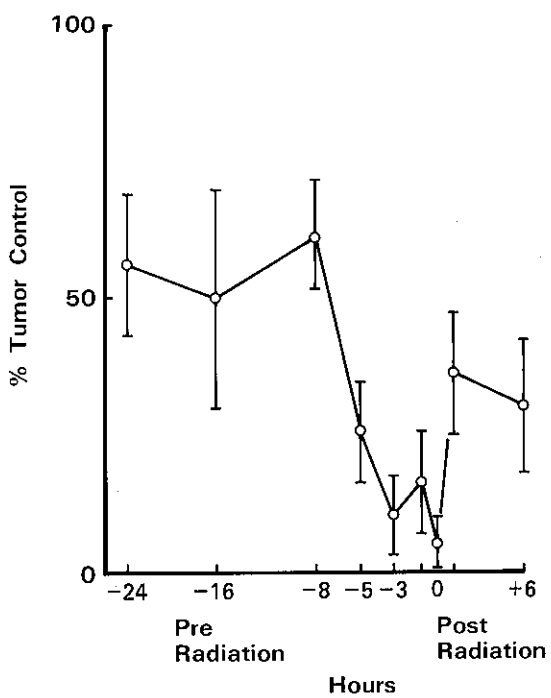

Fig. 2. The local tumor control rate of Meth-A fibrosarcoma as a function of time between irradiation and L-alanosine injection. L-Alanosine, 600 mg/kg was administered intraperitoneally at different time points in combination with a single dose of irradiation, 20 Gy. Tumor control was defined as a non-palpable tumor at 60 days post-treatment. Each data point was obtained from the results of 8–30 mice. The bar represents the SEM.

tumor control. Figure 2 depicts the tumor control rate as a function of time between radiation (20 Gy) and L-alanosine (600 mg/kg) administration. Increased radiosensitization was demonstrated at 8, 16 and 24 h before X-irradiation. Although there was no statistically significant difference at these treatment times, the maximum effect (62% tumor control) was observed at 8 h before irradiation. Drug administration at 5, 3, 1 h and immediately before and 1 h after X-irradiation produced less sensitizing effects when compared to the 8-h treatment group. The administration of L-alanosine 6 h after X-irradiation also resulted in 30% tumor control compared to 0% control rate at 20 Gy alone.

Similarly, the tumor control rate as a function of L-alanosine dosage administered 8 hours prior to 20 Gy X-irradiation substantiates the dose dependency of the radiosensitizing effects observed (Fig. 3).

Determination of dose modification factor (DMF) The results of the preceding studies (Figs. 2 and 3) demonstrate that optimal treatment effects on the Meth-A fibrosarcoma could be obtained when the drug was administered 8 h before radiation at the dose of 600 mg/kg. When the radiation dose that produced local tumor control of 50% (TCD_{50}) was compared for Meth-A-bearing mice receiving radiation and concomitant L-alanosine (600 mg/kg) or single dose radiation alone, a DMF of 1.4 was obtained for isoeffects (Fig. 4).

There was no disproportionately enhanced difference in skin reaction between the radiation alone group and the combined treatment group.

Lowering body temperature with L-alanosine and Ketamine-HCl L-Alanosine alone at a dose of 600 mg/kg was not found to alter the body temperature. Ketamine-HCl alone at a dose of 100 mg/kg resulted in a drop in temperature of approximately 2°C at the nadir 30 min after injection, and normal temperature was recovered after 5 h (Fig. 5). When L-alanosine and Ketamine-HCl were administered simultaneously, however, the fall in rectal temperature increased dramatically (6–7°C). These effects were observed even when L-alanosine was administered within 3 h prior to Ketamine-HCl injection,

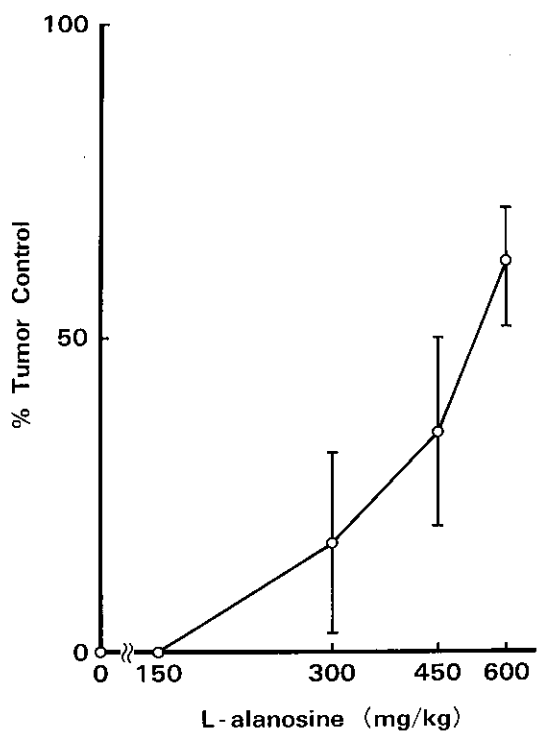

Fig. 3. The percent tumor control of Meth-A fibrosarcoma as a function of dose of L-alanosine administered 8 h before X-irradiation (20 Gy). Each data point was obtained from the results of 10–30 animals. The bar represents the SEM.

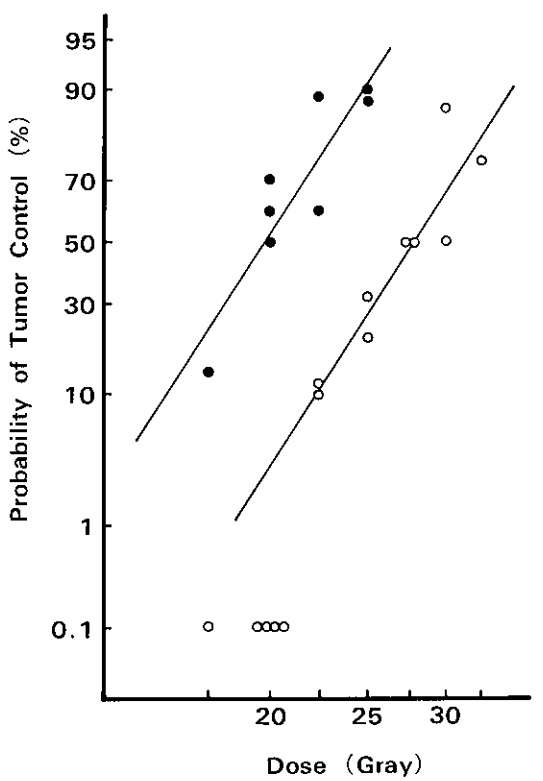

Fig. 4. The percent tumor control of Meth-A fibrosarcoma as a function of dose of X-irradiation. A single dose of 600 mg/kg L-alanosine was given 8 h before various doses of X-irradiation. Each data point was obtained from the results of 9–10 animals. The abscissa is in proportion to 2^n Gy. The lines were drawn by the minimum square method. TCD_{50} of radiation alone (○) was 28.0 Gy; TCD_{50} of the combined treatment (●) was 20.0 Gy. The dose modification factor is 1.40 (28.0/20.0).

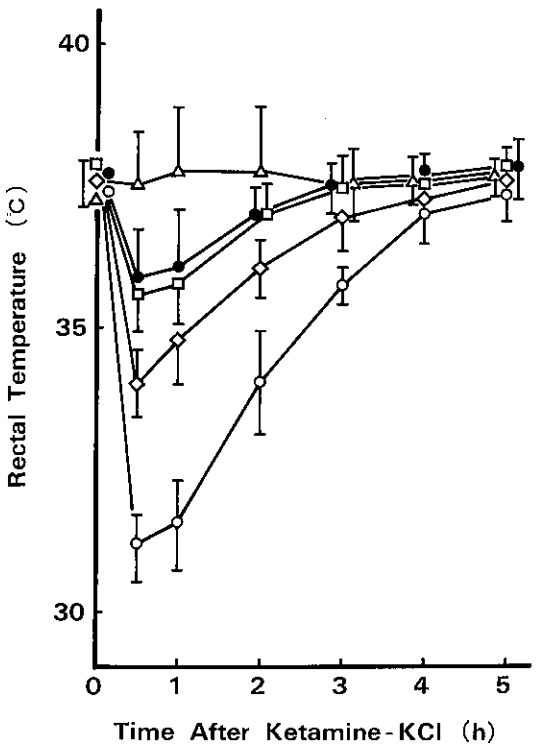

Fig. 5. Mouse body temperature as a function of time after Ketamine-HCl administration: ●, Ketamine-HCl 100 mg/kg alone; ○, L-alanosine plus Ketamine-HCl simultaneous injection; ◇, L-alanosine 3 h pre-injection plus Ketamine-HCl; □, L-alanosine 8 h pre-injection plus Ketamine-HCl; △, L-alanosine 600 mg/kg alone and in this case alone the abscissa represents time after L-alanosine administration. Each data point represents the average temperature obtained from 5–10 mice. The bar represents SD.

although the absolute temperature reduction was not as great as with simultaneous administration.

The administration of L-alanosine at 8 h prior to anesthesia gave a temperature response comparable to that with Ketamine-HCl alone, while L-alanosine one hour after Ketamine-HCl injection resulted in only a marginal reduction in rectal temperature.

DISCUSSION

The data presented demonstrate that the tumor control rates of Meth-A fibrosarcoma were significantly increased when the radiation was combined with L-alanosine. The effect was greater than a simple additive effect, and was directly dependent on drug concentration and the sequence of drug administration prior to radiation.

Several biological and biochemical factors may be involved in influencing the interaction of L-alanosine and radiation. It was observed that L-alanosine (600 mg/kg) alone reduced the tumor volume from 600 to 400 mm^3 at 24 h after treatment, unlike other inhibitors of PLD repair we have studied.[7,9,10] Although this effect was transient (Fig. 1), the reduction of tumor size was observed even at 8 h, although the volume difference was not as significant as the 24-h volume reduction. This decrease in tumor volume may not be an absolute indication of tumor cell reduction as the dynamics of cell loss and blood vessel reduction within tumor cannot be rapidly changed in one day. The decrease in tumor volume would, however, result in an increase of blood supply per unit volume of tumor tissue. This could result in the better oxygenation of any radioresistant hypoxic foci within the tumor.

The time dependence associated with L-alanosine radiosensitization appears to require some biochemical interaction or metabolic processing. In this respect, there is supportive evidence that DNA synthesis of P388 cells when measured by thymidine incorporation is totally inhibited between 2 and 24 h after L-alanosine administration.[16] This may contribute to the increased effects observed when the drug was administered 8 through 24 h pre-irradiation. Additionally, the lowering of body temperature observed by concomitant L-alanosine and Ketamine-HCl was dependent on the time interval between the drug administrations. When L-alanosine was administered 8 h prior to Ketamine-HCl anesthesia, there was no additional lowering in temperature compared to Ketamine-HCl alone. The lowering of body temperature has both radioprotective and/or radiosensitizing effects associated with it.[17-20] Lowering the tumor temperature without changing the blood supply would support the radiosensitizing effects by virtue of decreasing oxygen consumption of aerobic cells in the tumor tissue. This would result in greater oxygen distribution to hypoxic cells. However, lowering of the whole body temperature is always accompanied with a lowering of the basal metabolic rate, with subsequent reduction of the whole body blood circulation rate. In the case of L-alanosine, the biochemical activation process appears to be time dependent for radiosensitization. Consequently, the lowering of body temperature should result in a decreased drug metabolism, ultimately modifying the radiation interaction. This may be the primary reason why administration of L-alanosine immediately before irradiation was less effective than administration at intervals equal to or greater than 8 h before irradiation.

Finally, the sensitization effects seen at 1 and 6 h after irradiation may be explained by L-alanosine inhibition of PLD repair. The PLD repair is predominantly completed within 6 h after irradiation *in vitro*.[1,2,8] The time course

of PLD repair in solid tumors, however, appears more variable, ranging to 24 h after irradiation.[2]

Several PLD repair inhibitors have been reported as potential radiosensitizers. 3′-Deoxyadenosine, a purine nucleoside analog in the same class as L-alanosine, was found to enhance radiation effects, but it was too toxic for clinical use.[6, 8] β-Ara-A, an inhibitor of DNA synthesis, was shown to be a potent inhibitor of PLD repair in cell culture,[3, 4, 6] but did not show any radiation potentiation effect on murine tumors.[8] Biochemically, β-Ara-A was found to be rapidly inactivated by adenosine deaminase present in the serum and other tissues. Fludarabine phosphate, an analog of β-Ara-A, which is not inactivated by adenosine deaminase, has been reported to have pronounced radiation enhancement effects on a murine tumor.[7] Since L-alanosine has several unique features as a radiosensitizer as described above, verifying its biochemical mechanisms of radiosensitization may lead to new insights into radiation biology and therapy.

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