Interaction of Ultrasonic Hyperthermia with Two Alkylating Agents in a Murine Bladder Tumor

Frank W. Longo, Philip Tomashefsky, Bernard D. Rivin, and Myron Tannenbaum

Charles R. Lachman Ultrasound Laboratory in the Department of Urology [F. W. L., B. D. R.] and the Department of Pathology [P. T., M. T.], College of Physicians and Surgeons, Columbia University, New York, New York 10032

ABSTRACT

Fischer rats bearing s.c. implants of TCT-4909 bladder tumor were treated either with ultrasonic hyperthermia (US) (44.2°, 20 min) or either of two alkylating agents, thiopeta or Cytoxan (CTX) or in combinations of chemotherapeutic agent and heat at specific time intervals. Applying US 20 hr before either agent (thiopeta, 2 mg/kg i.p.; or CTX, 50 mg/kg i.p.) had a less than additive effect upon tumor growth. CTX, administered 20 hr before US, resulted in a significantly increased tumor volume-doubling time compared to CTX only. This was not true for thiopeta. With both agents, a synergistic effect was obtained when US and the agent were applied within 1 hr of each other, but the maximum was observed when the US had been applied 30 min before injection of agent. The intratumor temperature had decayed to normal at the time of injection. Radiolabeled alkylating agents injected at different times after US showed decreased uptake of label up to 20 hr after heating. Tritiated thymidine uptake was also reduced over the same period. Nuclear morphometry indicated increased nuclear condensation in parallel with the reduced uptakes described above. The data suggest that the synergism was not due to increased uptake of agent into heated tissue nor to the direct activation of alkylating activity by heat. It was demonstrated that heat had a rapid and marked inhibitory action upon DNA synthesis. This could have augmented the delayed but prolonged DNA inhibition caused by the alkylating agents to produce a synergistic effect. The apparent prolongation of the growth-inhibitory effect of CTX or thiopeta by heat may be due to the thermal inhibition of the enzymes responsible for the recovery of DNA after alkylation. The precise mechanism for the synergy may vary with the agent, the dose, the equilibrium temperature and its dwell time, and the interval between modalities. The influence of each of these parameters will require further investigation.

INTRODUCTION

The primary limitation upon the use of antineoplastic alkylating agents in vivo is the effect these agents have upon the host. The primary sites of toxicity are the bone marrow, the gastrointestinal tract, and the gonads (7). Myelosuppression is the usual dose-limiting toxicity of both thiopeta and CTX, resulting in a leukopenia and thrombocytopenia which can be fatal. Factors which enhance the tumoricidal effect without increased systemic toxicity might enable the clinician to use chemotherapeutic agents at relatively nontoxic doses without loss of efficacy.

There is sufficient experimental evidence to indicate that heat therapy alone or in combination with either radiation or chemotherapy causes a regression of malignant tumors (5, 16, 21). Both in vitro and in vivo experiments have demonstrated synergism between hyperthermia and many chemotherapeutic agents (2, 9, 10, 21, 34). The rationale for this synergism and the mechanism of cell damage remain obscure. Experimental data have suggested that at elevated temperatures, there is an increase in the uptake of some chemical agents and, thus, enhancement of their cytotoxicity (1, 25). Johnson and Pavelec (13) determined the rate of inactivation of Chinese hamster fibroblasts by thiopeta as a function of the ambient temperature. When their rate constants were plotted on Arrhenius coordinates, a sharp increase in the rate of inactivation was evident when temperatures exceeded 44°. In this experiment, heat was applied while the chemical factor was present. Other investigators (11) have not found this to be a requirement. Hahn (9) and Meyn et al. (19) have proposed that the synergistic effect of heat is due either to increased membrane permeability to chemical agents or to a thermodynamically induced increase in specific activity of the agent.

In several earlier publications, we demonstrated a method for the production of local hyperthermia within a tumor through the absorption of ultrasonic energy (16, 26, 30). Although nonthermal effects (e.g., particle acceleration and cavitation) of US have been demonstrated, these either did not cause direct structural damage or were not significant at the intensity levels used in our experiments (14, 15).

We have developed a delivery system which can be accurately positioned and is controlled by a preset intratumoral temperature. The absorbed energy results in an elevated temperature distribution limited to the target area which can be maintained at any desired level for any length of time. We were interested in determining whether such a system could act as local enhancement to parenteral chemotherapy with a concomitant therapeutic gain (24).

Since we have already had experience with the therapy on a s.c. implanted murine tumor model using ultrasonically generated heat (16), we investigated the interaction of this heat with 2 alkylating agents, cyclophosphamide (CTX) and N,N',N'-triethylenethiophosphoramide (thiopeta). These agents were chosen because they are considered to be cell cycle-phase independent, in general, although there is some evidence that CTX shows a greater kill in the S phase (7, 31). Sequence and time studies would therefore not be further complicated by problems of synchronization or phasing. Thiopeta is currently one of the options available clinically for the intravesical treatment of recurrent low-stage bladder tumors (28). Preliminary experiments in our laboratory had shown that our model tumor was sensitive to CTX. It has been reported that while actual coincidence of heat and chemical agent was not a requisite, the timing of the 2 modalities was critical (1, 33). Therefore, we investigated the application of each factor in different time sequences. Further,
we conducted uptake experiments to determine whether heat increased the permeation of tumor tissue by the alkylating agents.

**MATERIALS AND METHODS**

**Tumor.** Female Fischer 344 rats weighing between 100 and 150 g were given implantations s.c. with the transitional cell tumor TCT-4909 in the right flank. Two hundred rats were used in these experiments. The tumor was originally obtained from Dr. W. H. Kaplan, University of Washington Medical School, Seattle, and has been characterized (32). In our laboratories, the tumor has been transplanted every 2 to 3 weeks through some 75 generations. The tumors were implanted by placing a 2- to 3-cm piece of freshly obtained tumor into a s.c. pocket. Experiments were started when the implant was 10 to 14 days old, at which time the tumors were well established.

**Ultrasonic Hyperthermia.** The s.c. implanted tumors were treated by an ultrasonic transducer applicator which delivered relatively unfocused energy confined to the area of the tumor system. That portion of ultrasonic energy which was absorbed within the tumor gave rise to an elevated spatial temperature distribution which was reproducible. The magnitude of the energy transmitted and that level at which the temperature was maintained in equilibrium were below the values that we had determined to be significantly cytotoxic in previous experiments (16).

The transducer assembly contained a 2.25-MHz piezoelectric ceramic disc which transmitted an acoustic wave to the tumor through a coupling disc which transmitted an acoustic wave to the tumor through a coupling mismatch at the interfaces. The tumor area was further treated with a commercial dehydrating agent (Nair). Ten minutes before sonication, the rats were anesthetized with pentobarbitol sodium solution (Fort Dodge Laboratories, Fort Dodge, Ind.) at a dose of 32 mg/kg i.p. On a few occasions, this had to be supplemented with ether. The anesthetized animals were positioned and restrained with their non-tumor-bearing sides in contact with a Curophane membrane interfacing a sound-absorbing water bath. The animal's body temperature and the temperature at the base of the tumor were monitored by thermocouple probes (Bailey Instruments, Saddle Brook, N. J.). The thermal probes were 0.009 inch in diameter and were implanted into the tumor using a retractable 20-gauge needle. For each experiment, the transient power was preset to a maximum average value of 17.3 watts (3.4 watts/sq cm) and automatically reduced, in the steady equilibrium state, to a value ranging between 0.8 watt/sq cm and 1.7 watts/sq cm.

The rate of heating was controlled by the temperature near the base of the tumor. This rose from body temperature to a preset level of 44.2 ± 0.2° (S.E.) and was held fixed at this temperature for 20 min by a closed-loop feedback delivery system (24). The initiation of 44° was used as the reference point for timing pre- or postheating chemotherapy.

Calibration of the transducer was performed using instruments developed in our laboratory (24). These instruments had been compared previously with standards at the Bureau of Radiological Health, Washington, D. C.

**Chemotherapy.** The agents were made up freshly in distilled water just prior to use. Thiotepa (Lederle Laboratories, Pearl River, N. Y.) was given at a dose of 2 mg/kg in a concentration of 3 mg/ml aqueous CTX (Merck, Sharp & Dohme, Rahway, N. J.) was given at a dose of 50 mg/kg in a concentration of 20 mg/kg i.p. The chemotherapeutic agents were administered either alone or in conjunction with US according to the 4 different schedules shown in Table 1A.

**Tracer Studies.** [ring-4-14C]Cyclophosphamide, 40 to 60 mCi/mmol, was purchased from New England Nuclear (Boston, Mass.). This was injected i.p. at the rate of 1 μCi/100 g according to the schedule in Table 1B, and included nonsensitized controls. The agent was received in benzene:ethanol. This was further diluted with benzene and distributed into small tubes. The benzene was evaporated under a stream of N2 and the tubes stored in the refrigerator. NaCl solution (0.9%) was added as needed, so that each injection was freshly prepared. [U-14C]Thiotepa was obtained in powder form from Lederle Laboratories and contained 2 mCi/mmol (10.7 μCi/mg).

At each injection period, the same sequence was observed. At 1 hr after injection of labeled compound, the rats were deeply anesthetized with pentobarbitol (45 mg/kg). Blood was obtained by heart puncture into heparinized glassware. An aliquot was placed in a counting vial, and the blood was solubilized with Protosol (New England Nuclear):ethanol (1:3). One-half ml of 30% H2O2, to decolorize, and then 10 ml of Bioflour (New England Nuclear) were added. The blood was counted in a Beckman LS-200 using appropriate standards and blanks. At sacrifice, the tumor was removed and obvious necrotic tissue was cleaned away. The tissue was stored 1 to 2 weeks in the freezer until analysis. At analysis, the tumor was homogenized in water (9 volumes), and aliquots were taken for counting. One-half of 1 ml of homogenate was solubilized with 1 ml of Protosol. This was then treated as above, but the final solution in Bioflour was neutralized with 0.5 ml of 0.5 M HCl. Efficiency of the 14C count in blood and tissues was about 80%. Counts were reported as dpm/g of tissue or dpm/ml of whole blood.

[methyl-3H]Thymidine (New England Nuclear) (20 mCi/mmol) was given i.p. at 5 μCi/100 g. Blood was handled as described above. The homogenized tumor was precipitated with 2 volumes of 1.0 M perchloric acid and extracted and quantified according to the method of Burton (4). Other aliquots were placed in scintillation vials with 10 ml of Bioflour for determination of counts. These were reported as dpm/mg of DNA corrected for blood levels.

**Morphometry.** Routine hematoxylin-and-eosin-stained sections were examined with a Zeiss MOP-3 analyzer. Under low-power light microscopy, representative fields were chosen, and the nuclei of the tissue sections were measured at ×2500. Nuclear surface areas were expressed in sq μm and represented in histograms at 2-sq μm intervals. Sections were obtained from rats killed either 45 min, 90 min, 20 hr, or 44 hr after sonication. These correspond to the sacrifice times in the uptake studies.

**Calculation.** Tumor volumes were computed from the 3 largest mutually perpendicular diameters. These were measured every 3 to 4 days. The average rate of decrease in tumor volume over a specified period (Days 3 to 14) was computed by regression analysis. The rates obtained were compared by the appropriate F test as described by Snedecor and Cochran (26). The rate of tumor growth expressed, as the time required to reach twice the initial volume, was determined for each animal, and the mean ± S.E. of each group was derived. These were compared by Student's t test. In some groups, individual tumors were apparently totally destroyed and did not regrow during the study period, which was
RESULTS

Control tumors grew at their expected linear rates during the first 3 weeks of the experiments when plotted on a logarithmic scale (Chart 1). Tumor volumes had an average doubling time of 2.71 ± 0.36 days. When a suboptimal level of US was used alone, there was a small effect upon the growth rate which increased the tumor volume-doubling times to 6.28 ± 1.43 days. This delay was significant at \( p = 0.025 \) (Table 2). Thiotepa alone increased the doubling time to 20.8 ± 1.55 days. This was highly significant \( (p < 0.001) \). While the combinations of thiotepa and US given 20 hr apart in either direction had increased doubling times, these increases were not significantly different from the effect of thiotepa alone (Table 2). However, injecting thiotepa either 15 min before or 30 min after heating resulted in doubling times which were greater than the sum of the effect of each modality applied separately (Table 2). We defined an ER as the inhibition due to the chemical agent plus US divided by the effects of each modality applied separately (Table 2). An analysis of the data showed that the immediate effect of the thiotepa:US combination was to increase the rate at which the tumor volumes diminished after treatment and, thus, it reached a deeper nadir than did the single agents (Chart 1A). This rate of decline was significantly different from that of thiotepa alone \( (F \) test, \( p < 0.005) \). The combined action of CTX and US was to delay the start of regrowth of tumor by 11 days as compared to CTX alone (Chart 1B).

For purposes of simplification and because the 2 groups were similar, the data from the rats given injections 15 min before and those given injections 30 min after the onset of 44° were combined in Chart 1 but are listed separately in Table 2. It should also be noted that for purposes of determination of slopes, the period between Days 3 and 14 was used, since the first 2 to 3 days usually showed an increase in volume regardless of treatment and subsequent direction of volume change (Chart 1).

The incorporation of \( ^{3}H \) thymidine into the TCT-4909 tumor was inhibited by US. This was observed in animals given injections 15 min before sonication and sacrificed 60 min later. When labeled thymidine was injected 30 min postsonication, there was a significantly greater inhibition which lasted for at least 20 hr. At 44 hr, the uptake of label was no longer significantly different from that of controls (Chart 2). The inhibition of thymidine uptake by heat has also been observed in vitro (18).

An analysis of the data showed that the immediate effect of the thiotepa:US combination was to increase the rate at which the tumor volumes diminished after treatment and, thus, it reached a deeper nadir than did the single agents (Chart 1A). This rate of decline was significantly different from that of thiotepa alone \( (F \) test, \( p < 0.005) \). The combined action of CTX and US was to delay the start of regrowth of tumor by 11 days as compared to CTX alone (Chart 1B).
In order to determine whether there were morphological criteria which correlated with the observed biological and biochemical effects, representative nuclear areas were measured. The morphometric data are presented in Fig. 3. The surface areas of untreated nuclei had a normal distribution and a limited range. However, within 45 min after the start of heating, there was a shift in average nuclear area and a greater variation of sizes (Chart 3). Ninety min after heating, the range of normal-appearing nuclei had shifted so far to the lower end of the scale that there was little overlap with the control. The distribution of these nuclei was bimodal. Coinciding with this new distribution was the appearance of a population of anucleolar nuclei at the low end of the scale. At 20 hr, nuclear sizes were in the normal range. The nuclear areas measured at 44 hr were slightly larger than were controls, but anucleolar nuclei population was again seen.

Mitotic indices were initially 0.8% but dropped to 0 at the 45- and 90-min points. They began to return at 20 hr (0.5%) and exceeded the control at 44 hr (1.3%).

**DISCUSSION**

Our data confirm that systemic chemotherapy utilizing either of 2 antineoplastic alkylating agents and localized induced US are synergistic when applied in appropriate time sequences. The reason for this synergism remains obscure. Using other models, several authors have suggested that this synergism is due to the increased entry of chemotherapeutic agent into tissue because of increased blood flow or membrane conformation changes after heating. For instance, Rochlin et al. (25) have demonstrated that the isolated perfused dog hind limb binds more thiotepa at 39° than it does at 24°. In mice, Alberts et al. (1) showed that another alkylating agent, cis-platinol, was taken up by the P388 tumor to a greater extent at 42.3° than at 37°. The present experiments do not confirm this on a weight basis. However, the equilibrium temperatures utilized in our experiments were higher than those cited. Conner et al. (6) have pointed out that cytological damage proceeds at a rapid pace at temperatures above 43°. Interestingly, Tacker and Anderson (29) found that ultrasonically heated bladder tumors took up free methotrexate better at 37° than at 42°.

It has been reported that heat does not increase tumor blood flow compared to that of normal tissue (27). In addition, the inhibitory effect of US upon vascular dynamics is considered to be greater than is the same degree of nonultrasonic heating (12). In our studies, we have consistently demonstrated significant intravascular stasis. These factors could have contributed to the decreased uptake that we had observed. Earlier, we had shown that US damages cellular membranes (17). This has also been seen by other workers (20). The presence of both morphologically damaged and intact membranes in the same specimen suggests that there were intermediate stages, not visible, in which permeability was altered. While our studies did not show increased entry of chemical agents into the cell as measured at one point in time, however, there was demonstrable radiolabeled agent present at all times studied.

Meyn et al. (19) incubated Chinese hamster ovary cells in the presence of cis-platinol and found more DNA cross-linking at 43° than at 37°. This result would require heating in the presence of agent. In our experiments, the greatest degree of synergism was seen when heating was begun 30 min before and was ended 10 min before the injection of alkylator. During this 10-min interval, the intratumoral temperature returned to its presonication level. CTX requires activation in the host liver, and this activation level. CTX requires activation in the host liver, and this activation does not peak within 30 min (3). Therefore, in our best regimen, the times of heating and of application of chemotherapeutic agent were distinctly separated. This suggests that there was no direct thermodynamic effect upon a biochemical reaction. Meyn et al. (19) also point out that the rate of cell "kill" increased with temperature faster than did the rate of cross-linking, presuming another unknown phenomenon involved. Johnson and Pavlec (13) found that below 44°, the alkylating effect of thiotepa predominated but, above that temperature, thermal denaturation of protein predominated.
ACKNOWLEDGMENTS

REFERENCES

13. Johnson, H. A., and Page, M. T.将达到thiopeta or CTX. In 1966, it was suggested by Popovic and Masironi (23) that a temperature differential between the tumor and the host leads to increased metabolic demand in the tumor, with inadequate ability to meet this demand. In addition to the direct cytotoxic action of a chemotherapeutic agent, the tumor is starved or, at least, rendered relatively ischemic. The resulting hypoxia could cause lethal changes in intracellular pH (22).
Interaction of Ultrasonic Hyperthermia with Two Alkylating Agents in a Murine Bladder Tumor

Frank W. Longo, Philip Tomashesky, Bermard D. Rivin, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/43/7/3231

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.