Combined Modality Therapy with Bleomycin, Hyperthermia, and Radiation

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ABSTRACT

In an attempt to develop better combination therapies for use with local radiation, the interaction between bleomycin and hyperthermia ± radiation was studied in the FSAIIIC tumor system. In cells exposed in vitro to bleomycin at 37°C and at pH 7.40, the drug was substantially more toxic toward normally oxygenated than hypoxic cells. At hyperthermic temperatures (42° or 43°C), however, the differential killing between the normally oxygenated and hypoxic cells disappeared and bleomycin became significantly more toxic. Exposure to bleomycin at pH 6.45 did not substantially alter the cytotoxicity of the drug at 42° or 43°C. In tumor growth delay experiments, combining bleomycin, hyperthermia, and radiation induced long delays, and the more successful sequences were bleomycin—radiation—hyperthermia or bleomycin—hyperthermia—radiation. If radiation was given prior to drug and hyperthermia, however, the sequence was significantly less effective. In tumor excision experiments performed 24 h after treatment, increasing doses of bleomycin produced a shallow, log-linear increase in tumor cell kill at 37°C, but bleomycin followed by hyperthermia (43°C, 30 min) led to about 1 log more cell killing. Administration of bleomycin just prior to treatment with a single dose of radiation was cytotoxicity additive. In this assay the most effective tridational treatment sequence was bleomycin—hyperthermia—radiation.

In tumor subpopulations defined by Hoechst 33342 dye staining, bleomycin at 37°C was about two-fold more toxic toward the bright (presumably well-oxygenated) cells than toward the dim (presumably hypoxic) cell subpopulation. The addition of hyperthermia following bleomycin produced nearly a log more tumor cell killing in both the bright and dim tumor cells. The combination of bleomycin followed by hyperthermia and then radiation was at least additive in the bright cells and caused a large cell kill, but, in comparison, there was marked sparing of the dim cells. These results indicate that treatment with bleomycin and hyperthermia in conjunction with radiation can add substantially to tumor cell killing. This combination is significantly less effective in the hypoxic thanoxic tumor regions, however, in spite of in vitro data which demonstrate that the cytotoxicity of bleomycin at hyperthermic temperatures is not oxygen-dependent.

INTRODUCTION

The advantage of combination therapy for the treatment of disseminated malignant diseases such as leukemia is well recognized (1). Combined modality therapy to achieve control of locally advanced malignancies, however, has been utilized less frequently. Since radiation therapy is usually the most appropriate modality for the treatment of nonresectable local tumors (2), it seems reasonable to develop therapeutic strategies which will augment the antitumor effects of this treatment.

The use of radiation sensitizing drugs (3), chemotherapeutic agents (4), and hyperthermia (5) in conjunction with radiation therapy have each shown promise in the clinic. The rationale for combining hyperthermia and a chemotherapeutic agent with radiation for the treatment of local tumors is twofold: firstly, some chemotherapeutic agents (6-8) and hyperthermia (9-11) can effectively attack radioresistant subpopulations of the tumor such as hypoxic cells at low pH, and secondly, some chemotherapeutic drugs are cytotoxically interactive with both radiation and hyperthermia (12).

The primary effect of bleomycin appears to result from the fragmentation of DNA (13, 14), through formation of a DNA-bleomycin-ferrous ion-dioxygen complex which reduces molecular free oxygen to yield a highly reactive species, producing both single and double strand breaks in DNA (15-18). Bleomycin is significantly more toxic to normally aerated than hypoxic cells in culture (8, 19-21). The cytotoxicity of bleomycin is also markedly increased at hyperthermic temperatures (22-25), and bleomycin has been shown to potentiate the cytotoxic effects of radiation (26-31). In addition, the cell kill achieved by bleomycin at hyperthermic temperatures has been shown to increase at acidic pH, and so the combination of bleomycin and heat may be effective against cells in poorly perfused areas of tumors. Bleomycin is also only minimally bone marrow suppressive (32, 33) and could be scheduled repeatedly with hyperthermia and radiation during a conventionally fractionated course of radiation therapy. We have, therefore, examined the interaction of bleomycin plus hyperthermia with radiation in an effort to explore this combination for possible use in the clinic.

MATERIALS AND METHODS

Drugs. Bleomycin (Blenoxane) was a gift from Bristol Laboratories (Syracuse, NY).

In Vitro Survival Studies. FSAIIIC murine fibrosarcoma cells grow as monolayers in αMEM (Grand Island Biological Co., Grand Island, NY) supplemented with 10% FBS (Sterile Systems, Logan, UT). For experiments, FSAIIIC cells were grown in plastic culture flasks and used when in exponential growth (21).

Heat Treatments. Exponentially growing cells were exposed to temperatures of 37, 42, or 43°C for 1 h. Heating was accomplished in a PleXiglas water tank with a continuous in-flow and out-flow system controlled by a water temperature controller (Braun Thermomix 1460; B. Braun Instruments, Bethlehem, PA) (34, 35). Cells underwent heating in sealed plastic flasks (Falcon Plastics, Oxnard, CA) containing 5 ml of complete medium. Water temperature could be maintained at ± 0.10°C (SD). Temperature in the water bath was measured using multiple mercury thermometers calibrated against thermometers verified for accuracy by the National Bureau of Standards.

Production of Hypoxia. To produce hypoxia, the plastic flasks, containing exponentially growing monolayers in complete medium serum, were fitted with sterile rubber septa and exposed to a continuously flowing 95% N2/5% CO2 humidified atmosphere for 4 h at 37°C as previously reported (8). Parallel flasks were maintained in 95% air/5% CO2 at the end of 4 h, the drug or vehicle was added to the flasks by injection through the rubber septa without disturbing the hypoxia.

pH Alterations. The pH of the medium was adjusted using a sodium bicarbonate (NaHCO3)/5% CO2 buffer system (35, 36). For medium with serum, the lowest pH that could be achieved with 5% CO2 without NaHCO3 was 6.43 ± 0.01. The reduced solubility of CO2 at 40-45°C increased the actual pH to 6.45 as measured by a bioprobe combination pH electrode (Orion Research, Cambridge, MA).

For altered pH experiments, the original phosphate buffered medium

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2 To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.
Drug Treatments. Experimentally growing cells were exposed to varying concentrations of bleomycin in 25 cm² flasks for 1h at 37, 42, or 43°C. Non-drug-treated controls were handled identically. Addition of the drug solution did not significantly alter the pH of the culture. After treatment, the medium was removed and the cultures were washed twice with PBS at 37°C and trypsinized (0.01% for 2–3 min). Following this procedure, known numbers of cells were plated into plastic culture dishes for colony growth as described below.

Cell Viability Measurements. Cell viability was measured by the ability of single cells to form colonies in vitro, as described previously (8). Following treatment, suspensions of known cell numbers were plated in plastic culture dishes and allowed to grow in a 37°C incubator under standard culture conditions for 8–10 days. After this time interval, macroscopic colonies were stained with crystal violet in methanol containing 3.7% formaldehyde and were counted manually. Each data point per experiment represents the results of three different dilutions of cells plated in duplicate and each experiment was repeated 3–5 times.

Tumor. The FSaII fibrosarcoma (37) adapted for growth in culture (FSaIIIC) (38) was carried in C3H/He male mice (The Jackson Laboratories, Bar Harbor, ME). For the experiments, 2 × 10⁶ tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of C3H/He male mice 8 to 10 weeks of age.

Tumor Growth Delay Experiments. When the tumors were approximately 50 mm³ in volume (about 1 week post tumor cell implantation), treatment was initiated. In those groups receiving the drug, bleomycin (15 mg/kg) in 0.9% PBS (0.2 ml) was injected as a single dose i.p. on the first day of treatment. In those groups receiving hyperthermia, heat was delivered as a single dose on Day 1 of the treatment locally to the tumor-bearing limb for 30 min by immersion in a specially designed Plexiglas water bath at 44°C which allowed the centers of tumors to reach 43 ± 0.2°C as measured by a digital read-out thermistor (Sensor-tech Inc., Clifton, NJ) placed into the center of the tumor in selected control animals as previously described (38). In those groups receiving radiation, X-rays were delivered locally to the tumor-bearing limb at a dose of 3 Gy daily for 5 days. No anesthetic was used. The progress of each tumor was measured three times per week until it reached a volume of 500 mm³. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm³ compared to the untreated controls. Each treatment group had seven animals and the experiment was repeated three times. Days of tumor growth delay are the mean ± standard error for the treatment group compared to the control.

Tumor Excision Assay. When the tumors were approximately 50 mm³ in volume (about 1 week after tumor cell implantation) the animals were injected i.p. with various doses of bleomycin (0, 10, 20, or 30 mg/kg) alone or immediately followed by hyperthermia (43°C, 30 min), as described above, to the tumor-bearing limb. Other unanesthetized treatment groups received various doses of radiation (5, 10, or 15 Gy) whole body alone or in combinations with i.p. bleomycin (10 mg/kg) and/or hyperthermia (43°C × 30 min) in various treatment schedules. Three treatment groups received tricornality combinations: (a) radiation (5, 10, or 15 Gy) followed by bleomycin (10 mg/kg) then hyperthermia (43°C, 30 min); (b) bleomycin (10 mg/kg) followed by radiation (5, 10, or 15 Gy) then hyperthermia (43°C, 30 min); or (c) bleomycin (10 mg/kg) followed by hyperthermia (43°C, 30 min) then radiation (5, 10, or 15 Gy). Mice were sacrificed and soaked in 95% ethanol 24 h after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei with two scalpels. Four tumors were pooled to make each treatment group.

RESULTS

As has been shown previously in EMT6 cells (8), in Ehrlich ascites tumor cells (19) and in the human sarcoma cell line MES-SA (20), we found that bleomycin was more toxic toward normally oxygenated FSaIIIC cells in culture than toward hypoxic FSaIIIC cells at 37°C (Fig. 1). The survival curves of
FSaIIIC cells exposed to bleomycin for 1 h at 37°C and pH 7.40 under both normally oxygenated and hypoxic conditions appeared biphasic (Fig. 1A). The drug was more toxic toward the oxygenated cells over the entire concentration range, reaching a maximum differential of about 10-fold at 150 and 200 μM of bleomycin. At 42°C and pH 7.40, there was increased kill of both normally oxygenated and hypoxic FSaIIIC cells by bleomycin (Fig. 1B). Surprisingly, at this hyperthermic temperature, the differential kill between the normally oxygenated and hypoxic cells disappeared. The survival curves at 42°C were still primarily biphasic; however, there was a steeper slope in the final phase of the curves than was seen at 37°C. In the normally oxygenated cells there was about 5-fold additional cell kill at 42°C following exposure to 200 μM of bleomycin than at 37°C. In the hypoxic cells there was about 2 logs additional cell kill at 42°C by 200 μM bleomycin compared to the same drug concentration at 37°C. At 43°C and pH 7.40 the biphasic nature of the survival curves for both normally oxygenated and hypoxic cells was maintained (Fig. 1C), but the slope of the terminal portion of the survival curves at 43°C was steeper than at either 42° or 37°C. As at 42°C, there was no significant difference in the killing of normally oxygenated or hypoxic cells at 43°C, and there was a 7- to 10-fold increase in the level of cell kill at 200 μM bleomycin at 43°C compared to 42°C. Therefore, at 43°C and normal pH, exposure to the highest bleomycin concentration tested for 1 h led to an additional 2 logs of cell kill in normally oxygenated cells and an additional 3 logs of cell kill in hypoxic cells compared to treatment at 37°C.

When the same series of studies were carried out at acidic pH, the results in the lower three panels of Fig. 1 were obtained. At pH 6.45 and 37°C, the survival curves were less biphasic (i.e., more nearly exponential) under either normally oxygenated or hypoxic conditions (Fig. 1D). Although bleomycin was minimally more cytotoxic to normally oxygenated than hypoxic cells at pH 6.45, the differential was much less than at pH 7.40 and 37°C. The cell killing by 200 μM bleomycin in normally oxygenated cells at 37°C and pH 6.45 was about 4-fold greater than at pH 7.40, and the killing of hypoxic cells by the same drug concentration at 37°C and pH 6.45 was about 10-fold greater than at pH 7.40.

As was seen at pH 7.40, at 42°C and pH 6.45 the protective effect of hypoxia against bleomycin cytotoxicity disappeared. With 200 μM of bleomycin at 42°C and pH 6.45 there was about a two-fold additional kill of normally oxygenated cells and about 10-fold increased kill of hypoxic cells compared to the same drug concentration at 37°C and pH 6.45. There was no difference between the normally oxygenated and hypoxic cell killing seen at 42°C at either pH 7.40 or pH 6.45 with 200 μM bleomycin. Similarly, at 43°C and pH 6.45 there was about 1 log additional cell kill of both normally oxygenated and hypoxic cells by 200 μM bleomycin compared to the cell kill with the same drug concentration at 42°C and pH 6.45, but the addition of the acidic pH environment of bleomycin treatment at 43°C also did not affect the overall level of killing of either normally oxygenated or hypoxic cells compared to treatment at 43°C and normal pH. Therefore, both acid pH and hyperthermia had similar effects on the killing of FSaIIIC cells following exposure to bleomycin in that the levels of cytotoxicity were significantly increased in oxic cells and more increased in hypoxic cells. A combination of low pH and hyperthermia, however, did not further increase the concentration-dependent lethality of 1 h exposure to bleomycin over that achieved at hyperthermic temperatures at normal pH.

Growth delay of the FSaIIIC fibrosarcoma tumor produced by combinations of bleomycin, hyperthermia, and radiation are shown in Table 1. A 30-min treatment at 43°C produced a tumor growth delay of about 1.4 days. Bleomycin at 10 mg/kg administered i.p. in a single dose was not very effective in the FSaIIIC fibrosarcoma and produced a tumor growth delay of about 1.3 days. Bleomycin (10 mg/kg) followed by hyperthermia (43°C, 30 min) caused a tumor growth delay of about 5.7 days (probably more than additive). Radiation was administered as five fractions of 3 Gy once per day for 5 days to simulate a weekly fractionation scheme similar to that used in the clinic. This radiation schedule produced a tumor growth delay of 6.2 days in the FSaIIIC fibrosarcoma. Bleomycin (10 mg/kg) given i.p. just before radiation on Day 1 of the 5-day schedule increased the growth delay achieved to about 8.0 days, and appeared probably additive with the radiation schedule. Similarly, hyperthermia given just before radiation produced a growth delay of about 8.4 days which was also probably additive.

| Treatment group | Tumor growth delay, days*
<table>
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<tr>
<td>43°C, 30 min</td>
<td>1.4 ± 0.7</td>
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<tr>
<td>Bleomycin (10 mg/kg)</td>
<td>1.3 ± 0.5</td>
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<td>X-rays (5 × 3 Gy)</td>
<td>6.2 ± 1.5</td>
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<td>Bleo → heat</td>
<td>5.7 ± 1.1</td>
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<tr>
<td>Bleo → X-rays</td>
<td>8.0 ± 1.7</td>
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<tr>
<td>Heat → X-rays</td>
<td>8.4 ± 2.2</td>
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<tr>
<td>X-rays → bleo → heat</td>
<td>12.0 ± 2.3</td>
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<tr>
<td>Bleo → X-rays → heat</td>
<td>16.0 ± 3.4</td>
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<td>Bleo → heat → X-rays</td>
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* Tumor growth delay in the difference in the number of days for the treated tumors to reach 500 mm³ compared to untreated control tumors. The data presented are the means of 14 animals ± SEM.

Heat was delivered as a single dose on Day 1 of treatment locally to the tumor-bearing limb by emersion in a water bath at 44°C which allowed the tumors to reach 43°C.

Bleomycin was injected in normal saline as a single dose i.p. on Day 1 of treatment.

X-rays were delivered locally to the tumor-bearing limb at a dose of 3 Gy daily for 5 days. No anesthetic was used.
Three sequences of trimodality therapy given on Day 1 of the 5-day radiation schedule were tested. When radiation (3 Gy) was given first followed by bleomycin (10 mg/kg) then hyperthermia (43°C, 30 min) on treatment Day 1, the tumor growth delay was about 12 days. Administering the bleomycin first, followed by radiation then hyperthermia produced a tumor growth delay of about 16 days. If bleomycin was given first followed by hyperthermia and then radiation, the tumor growth delay was 14.5 days. There was no significant difference between the tumor growth delay produced by the treatment sequences bleomycin—radiation—hyperthermia and bleomycin—hyperthermia—radiation; however, the sequence in which radiation was given prior to the drug and hyperthermia was significantly less effective.

Tumor cell survival from FSaIIIC fibrosarcoma tumors was assayed from animals receiving a range of bleomycin doses with and without hyperthermia given immediately following the drug (Fig. 2). Bleomycin in increasing doses produces a relatively shallow, log-linear increase in tumor cell kill. Hyperthermia itself killed 30–40% of the FSaIIIC tumor cells treated in vivo. The combination of bleomycin followed by hyperthermia led to about 1 log more tumor cell kill over that produced by the drug alone. The slope of the tumor cell survival curve for the drug plus hyperthermia is essentially parallel to that of the drug alone, indicating killing of an additional tumor subpopulation by hyperthermia plus bleomycin rather than a true dose modifying effect by hyperthermia on bleomycin cytotoxicity.

Administration of bleomycin (10 mg/kg) just prior to treatment with a single dose of radiation led to a small increase in tumor cell kill compared to radiation alone, indicating a primarily additive effect of the two treatments (Fig. 3). Similarly, when the radiation treatment was preceded by hyperthermia (43°C, 30 min), there was also a small increase in the killing of tumor cells which appear to represent an additive effect of these two treatments.

The same three sequences of the bleomycin trimodality treatment as tested in the tumor growth delay experiments but using various single fraction doses of radiation were examined in the tumor excision assay (Fig. 3). There was a significant difference in tumor cell survival depending on the sequence of treatment which was used. The least effective treatment sequence was again radiation followed by bleomycin administration and then hyperthermia; however, even in this case, at the radiation dose of 5 Gy there was an additional 1 log of tumor cell kill with the addition of bleomycin and hyperthermia. Some of the effect of the drug plus hyperthermia was lost at the highest dose of radiation (15 Gy), since only ~0.7 log additional killing was observed, which may indicate an overlap in the tumor cell populations which are killed by radiation alone at this high dose and by the trimodality treatment. Administering the bleomycin first, followed by radiation then hyperthermia produced 1.5- to 2.5-fold greater tumor cell kill than did radiation—bleomycin—hyperthermia. The most effective treatment sequence in this assay, however, was bleomycin then hyperthermia followed by radiation. This sequence was 8- to 9-fold better than the least effective trimodality sequence, and, at the lowest radiation dose of 5 Gy, produced 2 logs greater cell kill than radiation alone. For this sequence, much of the cytotoxic effect
was maintained throughout the radiation dosage range, so even when 15 Gy of X-rays was used there was still a 33-fold increase in tumor cell kill compared to radiation alone.

In an effort to discern the effectiveness of these various treatments on environmentally determined tumor subpopulations, tumors were treated as in the above tumor cell excision assays and then sorted into subpopulations by the Hoechst dye method (Fig. 4). The 10% brightest cells are felt to represent a population near the tumor vasculature (oxic) and the 20% dimmest to represent a cellular population distal from the tumor vasculature (hypoxic) (43). Hyperthermia (43°, 30 min) was slightly more cytotoxic toward the dim cell population (~60% cell kill) than toward the bright cells (~50% cell kill). The single dose of radiation (10 Gy) was twofold more toxic toward the bright cell subpopulation (~90% cell kill) than toward the dim cells (~80% cell kill). Bleomycin (10 mg/kg) alone was also about twofold more toxic toward the bright cell subpopulation (~40% cell kill) than toward the dim cell subpopulation (~20% cell kill). The addition of hyperthermia just after treatment with bleomycin produced a marked increase (7- to 8-fold) in cell kill of both the bright and dim subpopulations, so that the relative twofold sparing of the dim cells was maintained. In the bright cell subpopulation the combination of bleomycin and radiation was essentially additive; however, in the dim cell population the tumor cell killing by bleomycin and radiation was about twofold more effective than predicted by simple additivity.

Only the most effective trimodality sequence was studied with this method. The combination of bleomycin followed by hyperthermia and then radiation produced about a 2.8-log cell kill in the bright cells, and about 1.6 logs of cell kill in the dim cells. Overall, therefore, this combined treatment approach led to about a 1.2-log sparing of the dim cell subpopulation compared to the bright cell subpopulation. Whereas for the bright cells the surviving fraction for the trimodality treatment was very close to the product of the surviving fractions for the bimodality treatments, cell kill in the dim population was about 5-fold less than predicted by this simple calculation of additivity.

DISCUSSION

It has been generally accepted that the antitumor activity of bleomycin can be attributed to its ability to mediate DNA degradation by a mechanism which is metal ion and oxygen dependent. It has previously been shown that bleomycin is more cytotoxic toward normally oxygenated and hyperoxygenated cells in culture than toward hypoxic cells (8, 19–21) and that bleomycin is more cytotoxic toward normally oxygenated cells in culture at hyperthermic temperatures (22–25). We have examined the cytotoxic effect of bleomycin on FSaIIIC cells in culture under normally oxygenated and hypoxic conditions at normal and acidic pH and at normal and hyperthermic temperatures. When bleomycin exposure occurred at 42° or 43°C and at normal pH, we observed a steepening of the slope of the terminal portion of the survival curve, as was seen by Urano et al. (25). More interestingly, however, the greater cytotoxicity of bleomycin in normally oxygenated versus hypoxic cells disappeared at 42° and 43°C. There is evidence that the bleomycin-iron complex can cleave DNA by an oxygen-independent mechanism and that the product of that reaction is different from the product of the oxygen-dependent mechanism (15, 44–47). It may be that at hyperthermic temperatures the oxygen-independent mechanism by which bleomycin degrades DNA becomes a more significant component of drug action and that this damage, which may be repaired at normal temperature (48–50), may be fixed by the heat treatment (24).

Acidic pH has been reported to increase the cytotoxicity of bleomycin at hyperthermic temperatures (6, 25, 32). In the FSaIIIC cells used in these studies, however, while treatment at pH 6.45 at 37°C did significantly increase the concentration-dependent cytotoxicity of bleomycin, this was not the case at 42° or 43°C. The mechanisms responsible for the increased cytotoxicity of bleomycin at hyperthermic temperatures appear to involve increased drug retention in cells, increased DNA damage and decreased DNA repair (24), but mechanisms responsible for the effect of acidic pH on bleomycin-induced lethality have yet to be defined. The fact that acidic pH did not further sensitize cells to bleomycin at 42° or 43°C, however, suggests that low pH and mildly hyperthermic temperatures may share some of the same sensitizing mechanisms.

In tumor growth delay experiments we modeled a trimodality schedule of bleomycin, hyperthermia, and radiation which was similar to what could be used in the clinic on a weekly basis. Bleomycin and hyperthermia were given on treatment Day 1 and fractionated radiation was given on treatment Days 1 through 5. Bleomycin is most effective in animal tumors if given in multiple doses, and it has previously been shown that bleomycin plus hyperthermia is most effective in animal tumors when administered on a fractionated schedule with hyperthermia given immediately after the drug or simultaneously with the drug (51–54).

In the FSaIIIC growth delay experiments, neither bleomycin nor 43°C, 30-min hyperthermia was very effective treatment alone (about 1.4 and 1.3 days delay, respectively). The combination, however, produced about a 5.7-day delay which was probably more than additive. The growth delay produced by the trimodality treatment was clearly related to the treatment schedule. When bleomycin was given after radiation, the resultant growth delay probably reflected additivity of the bleomycin and hyperthermia components to the radiation component. However, when bleomycin was given prior to radiation, and hyperthermia was given either prior to or after radiation, supra-additive growth delays were produced (~14.5 and 16.0 days, respectively). These results suggest that there is an important cytotoxic interaction between the bleomycin-hyperthermia combination and radiation since the effects of either bleomycin or hyperthermia when added to X-rays singly were only additively cytotoxic.

The various single and double modality treatments were also assessed for cytotoxicity following tumor excision. At a relatively low dose of bleomycin (10 mg/kg), the addition of 43°C, 30-min hyperthermia dramatically increased the cell kill achieved. Further increasing the bleomycin dose did not alter the magnitude (~1 log) of this interaction. The parallel character of the tumor cell survival curves at 37° and 43°C with larger doses of bleomycin suggests that the addition of hyperthermia to the drug treatment was not dose modifying, although at all drug doses tested hyperthermia plus drug was clearly more than additively cytotoxic, confirming the growth delay results. Clinically, the fact that the magnitude of the hyperthermia-bleomycin interaction was maximum at the lowest dose of bleomycin tested is probably more significant than the mechanistic implication of dose modification. This is because bleomycin’s pulmonary and other normal tissue toxicities cause it to be given at doses which correspond to the low dose region of the survival curve.

The excision assay data for the bimodality combinations
including X-rays also confirmed the growth delay results. Both bleomycin plus X-rays and heat plus X-rays were essentially additive in terms of the cytotoxicity produced across a dose range of single radiation fractions. Again, there was a tendency for a greater cytotoxic interaction for either heat or bleomycin with X-rays at the lowest dose (5 Gy) as opposed to higher dose fractions. This finding also has positive clinical implications since the fraction size used in human beings is nearly always 6 Gy or less.

The excision assay data for the different trimodality sequences tested, once again, closely followed the growth delay data, although in these experiments the sequence bleomycin→heat→X-rays produce superior cytotoxicity. Also as in the growth delay experiments, administering radiation prior to bleomycin and heat was least cytotoxic, presumably because this sequence did not take maximum advantage of the radiosensitizing properties of bleomycin plus hyperthermia treatment since the drug was not present in the tumor during radiation. This result is identical to that we have previously reported for trimodality treatment using cisplatin, also investigated in the FSaIIIC tumor system (55). Administering the antitumor drug just before local hyperthermia may take advantage of the initial increase in tumor blood flow which occurs (56, 57) to deliver more drug to the tumor. In addition, an increase in the distribution of the drug in the tumor may also occur (58). Thus, when radiation immediately follows the drug plus heat, the tumor level of drug is probably high and the close scheduling of hyperthermia and radiation optimizes the cytotoxic interaction between these two modalities as well (since simultaneous treatment is impractical) (55).

The Hoechst dye experiments were conducted in order to examine the cytotoxicities of these treatments in tumor cell populations near versus distant from the vasculature (40–43). The relative resistance of hypoxic cells to radiation has been invoked as a cause of radiation treatment failure (3). In addition, chemotherapeutic agents may penetrate these underperfused areas poorly and some drugs, such as bleomycin, are less effective killers of hypoxic cells at normal temperatures (8). On the other hand, hyperthermia kills cells at low pH better than at normal pH and hypoxia is not protective against heat-induced lethality (59). Treatment combinations which include hyperthermia, therefore, may have significantly increased cytotoxicity in the hypoxic tumor cell subpopulation. Our data showed that most of these assumptions, primarily based on in vitro data, were correct. Hyperthermia alone was slightly more cytotoxic in the dim cells, while radiation was about twofold less toxic to the dim cells. The combination of these two treatments was additively cytotoxic and actually achieved slightly more killing in the dim tumor cells. However, 43°C, 30-min hyperthermia given just before 10 Gy of radiation was only able to achieve about 1.3 logs of killing.

Bleomycin at 37°C killed about 20% fewer dim than bright cells, due probably to the protection against bleomycin afforded by hypoxia (8, 19–21), and perhaps to poorer drug penetration. When bleomycin was given just prior to heat, however, the combination was essentially additively cytotoxic in both subpopulations, but there remained approximately 10% less cell kill in the dim cells. Bleomycin given just before radiation also proved an additively cytotoxic combination but achieved approximately 30% less kill in the hypoxic tumor cells. Finally, when the sequence bleomycin→hyperthermia→radiation was tested (which was the optimum schedule in the whole tumor excision assay), a substantial increase in killing of the bright cells was achieved (~2.8 logs killed) and the three modalities appeared at least additively cytotoxic. In the dim cells, however, the bleomycin trimodality therapy was much less effective, killing only about 1.6 logs and appeared less than additive. The reason why bleomycin, hyperthermia, and radiation are far less effective in the dim cell population is not clear. It may be that while the cytotoxicity of bleomycin is not oxygen dependent at 43°C, the interaction between the bleomycin plus hyperthermia combination and radiation requires oxygen to be maximally cytotoxic.

These results suggest that bleomycin plus hyperthermia can add substantially to the tumor cell kill, tumor growth delay and potentially curative capability of fractionated radiation therapy. Unfortunately, this trimodality combination is less effective in poorly perfused areas of the tumor. The bleomycin trimodality combination might still be curative, however, if significant reoxygenation of the tumor (60) occurs during fractionated treatment. Alternatively, there may be more appropriate anticancer agents to use with heat and radiation instead of or in addition to bleomycin.

REFERENCES


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