Interaction of Hyperthermia and Radiation in Murine Cells: Hypoxia and Acidosis in Vitro, Tumor Subpopulations in Vivo

Terence S. Herman, Beverly A. Teicher, Sylvia A. Holden, and Laura S. Collins

ABSTRACT

To better understand the effect of the level of oxygenation and pH on the heat-radiation interaction, these factors were modeled in vitro using FSAIIIC cells in monolayer and correlated with the response of Hoechst 33342 dye-defined FSAIIIC tumor subpopulations treated in vivo. Exposure to both 42°C and 43°C for 1 h in culture prior to graded single fractions of radiation resulted in a striking decrease in the radiation oxygen enhancement ratio which was pH as well as temperature dependent. The oxygen enhancement ratio at 37°C and pH 7.40 (or pH 6.45) was 2.9, but decreased to 1.4 at 42°C at normal pH, 1.2 at low pH, and 1.0 at 43°C at both pH values tested. This decrease in the oxygen enhancement ratio resulted from a far more marked decrease in Dv values for the radiation survival curves of hypoxic cells compared to normally oxygenated cells at elevated temperatures. In addition, the shoulder region of the radiation survival curves was significantly decreased with increasing temperatures and the magnitude of the decrease was greatest in hypoxic cells at low pH. In vivo treatment followed by immediate tumor excision showed that bright cells (presumably oxygenated cells at normal pH) were ~2-fold more sensitive to 10 Gy of radiation than were dim cells (presumably hypoxic cells at low pH) but that dim cells were 2.5-fold more sensitive to 43°C for 30 min hyperthermia. The combination of hyperthermia followed by radiation proved to be 1.8-fold more toxic to dim than to bright cells. Both hyperthermia alone and hyperthermia plus radiation, in contrast to radiation alone, were significantly more cytotoxic when tumors were left in situ for 24 h prior to excision as compared with immediate excision. These results indicate that hyperthermia markedly sensitizes hypoxic cells at low pH to the cytotoxic effects of radiation, as well as effectively killing cells in this tumor subpopulation.

INTRODUCTION

Many solid tumors have been shown to contain cellular subpopulations which, because they are distant from the tumor vasculature, exist at low levels of oxygenation (1-4), pH (1, 5, 6), and proliferation status (7, 8). These clonogenic malignant cells are likely to be a particularly difficult therapeutic problem because anticancer agents may penetrate these poorly vascularized regions in inadequate concentrations and hypoxic cells are relatively resistant to the cytotoxic effects of ionizing radiation (9) and some chemotherapeutic drugs (10-13).

The cytotoxicity of hyperthermia, however, is oxygen independent (14) and is actually increased in cells that are nutrient deficient and/or at low pH (14-19). In vivo, therefore, hypoxic cells which are relatively resistant to other anticancer therapies are likely to be more sensitive to hyperthermia. In addition, it is possible that these poorly vascularized environments will be less able to dissipate heat so that higher temperatures will also be achieved in these tumor regions (20-22).

Several clinical studies (23-27) have now shown that the addition of local hyperthermia to radiation therapy can substantially improve local tumor control. Investigators in this field, however, have argued whether the major beneficial effects of the addition of hyperthermia to radiation in the clinic are predominantly the result of radiosensitization or independent heat killing of hypoxic radioresistant cells. We have examined the effects of hypoxia and pH on the heat-radiation interaction modeled in vitro in an effort to further study this question. In addition, we have used the Hoechst 33342 dye method, which we have adapted for use in the FSAIIIC tumor system (28), to define the survival of putative oxic and hypoxic cells after in vivo hyperthermia, radiation, and combination treatments.

MATERIALS AND METHODS

In Vitro Survival Studies. FSAIIIC murine fibrosarcoma cells (29, 30) grow as monolayers in αMEM (1) (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum (Sterile Systems, Logan, UT). For experiments, FSAIIIC cells were grown in plastic culture flasks and used when in exponential growth.

Production of Hypoxia. To produce hypoxia, the plastic flasks, containing exponentially growing monolayers in complete medium plus serum, were fitted with sterile rubber septums and exposed to a continuously flowing 95% N2/5% CO2 humidified atmosphere for 4 h at 37°C as previously reported (13, 31). 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 septum without disturbing the hypoxia. To confirm the quality of the hypoxia produced, the oxygen content of 3 representative flasks at 37, 42, and 43°C was monitored during the 4-5 h at 37°C and 1 h at elevated temperature and during the radiation delivery using a microneedle Clark electrode (model 747; Diamond General Corp., Ann Arbor, MI). Very careful attention was given to temperature monitoring during both the calibration and experimental measurements with the oxygen electrode. The oxygen tension in the media varied between 0 and 3 mm Hg, with a mean of 0.5 during the test periods. No consistent pattern of variability was observed and the PO2 measurements were not higher at 42 or 43°C.

pH Alterations. The pH of the medium was adjusted using a sodium bicarbonate (NaHCO3)/5% CO2 buffer system (32). 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 (pH 7.40) was replaced with media without NaHCO3, and flasks were purged with either 95% air/5% CO2 for normally oxygenated conditions or gassed with 95% N2/5% CO2 for 4 h at 37°C for hypoxic experiments as stated above. Thus, only acute changes in pH were studied. After completion of the treatment, the monolayers were washed with 0.9% phosphate-buffered saline, suspended by trypsinization, and plated in normal pH complete media for colony formation. The pH of control flasks with media and cells was monitored at various stages in the experimental process. The pH values recorded did not vary by more than 0.03 pH unit from the desired 6.45 or 7.40.

Heat Treatments. Exponentially growing cells were exposed to temperatures of 37°C, 42°C, or 43°C for 1 h. Heating was accomplished in a Plexiglas water tank with a continuous inflow and outflow system controlled by a water temperature controller (Braun Thermomix 1460; Braun Instruments, Bethlehem, PA) (33). Cells undergoing heating in sealed plastic flasks (Falcon Plastics) containing 5 ml of complete...
medium. Water temperature could be maintained at ±0.10°C (SD). Temperatures in the water bath were measured using multiple mercury thermometers calibrated against thermometers verified for accuracy by the National Bureau of Standards.

Radiation Treatments. Normally oxygenated or hypoxic conditions were maintained throughout the radiation treatment. Irradiation was carried out using a 137Cs irradiation unit (Gammacell 40; Atomic Energy of Canada, Ltd.) at a dose rate of approximately 1.05 Gy/min at 25°C. X-ray doses of 2.5, 5, 10, or 15 Gy were used.

Cell Viability Measurements. Cell viability was measured by the ability of single cells to form colonies in vitro, as described previously (13, 31). Following treatment, suspensions of known cell numbers were plated in plastic Petri 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 experiment was repeated 3-5 times and each data point per experiment represents the results of 3 different dilutions of cells plated in triplicate.

Tumor. The FSA1C fibrosarcoma (29) adapted for growth in culture (FSA1C) (30) was carried in male C3H/FeJ mice (The Jackson Laboratory, 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 male C3H/FeJ mice 8-10 weeks of age.

Tumor Subpopulation Studies: Tumor Growth and Hoechst 33342 Labeling. When the tumor volumes were approximately 50-100 mm³ (about 1 week after tumor cell implantation), in those groups receiving hyperthermia, heat was delivered locally as a single dose to the tumor-bearing limb by immersion in a specially designed water bath at 44°C which allowed the centers of tumors to reach 43 ± 0.2°C as measured by a digital readout thermistor (Sensortech, Inc., Clifton, NJ) placed into the center of the tumor in selected control animals as described previously (34). In those groups receiving radiation, X-rays were delivered locally to the tumor-bearing limb at a dose of 10 Gy. No anesthetic was used.

Hoechst 33342 (2 mg/kg; Aldrich Chemical Co., Milwaukee, WI) dissolved in phosphate-buffered saline was administered by tail vein injection (0.25 ml) to tumor-bearing mice either 20 min after treatment or 24 h after treatment (28). Tumor cell suspensions were prepared by excising the tumor 20 min after i.v. administration of the dye (35-38) under sterile conditions and single cell suspensions of tumor cells were prepared for the colony-forming assay (39). To remove contaminating erythrocytes, 0.17% NH₄Cl was added to the tumor cell pellets for 3 min at room temperature just after filtering through gauze. The cells were washed once with αMEM supplemented with 10% fetal bovine saline, filtered through a syringe fitted with a 40-μm nylon mesh filter to remove cell clumps, counted, and centrifuged at 200 x g. Cells were then resuspended at a concentration of 2 × 10⁶ cells/ml in αMEM supplemented with 10% fetal bovine serum. No significant difference in the numbers of cells recovered as compared with untreated tumors was found in any of the experimental groups and no trends relative to specific types of treatments or to the interval between treatment and excision were observed. Since the numbers of recovered cells per treatment group typically varied by 15%, only cell losses of greater than 20% would have been detectable.

Flow Cytometry and Sorting. The fluorescence of the cells from tumors was analyzed and the cells were sorted using the Coulter Epics V (Hialeah, FL) instrument. Hoechst 33342 intensity was measured using an argon ion laser with excitation at 350-360 nm (40 mW power) with emission monitored through a 457 nm long pass and 530 nm short pass filter. The fluorescence distributions were divided into ten fractions based on Hoechst 33342 intensity. Two sorted fractions of cells were collected, one which contained the brightest 10% of cells and the other containing the dimmest 20% of cells. No significant difference in the fluorescent patterns of the sorted cellular populations at 20 min or 24 h after treatments as compared with cells from untreated tumors was observed. The cells were washed once with αMEM containing 10% fetal bovine serum and plated for colony formation. After 1 week, colonies were stained with crystal violet and colonies of >50 cells were counted manually. The plating efficiency for the unsorted population was 15.5 ± 2.7%. For the 10% brightest cells, the plating efficiency was 9.2 ± 1.6% and for the 20% dimmest cells the plating efficiency was 5.5 ± 1.4%. The survival results are expressed as the surviving fraction ± SE of the treated bright and dim fractions compared to the bright and dim untreated controls, respectively.

Calculated Values. The following calculated values were used to describe the effect of environmental alterations on the radiation survival curve: (a) the D₀ is defined as the dose in Gy which reduces the number of surviving cells to 37% on the exponential portion of the radiation survival curve (40); (b) the radiation OER is the D₀ under hypoxic conditions + D₀ under oxic conditions; (c) the TEF is the D₀ at 37°C + D₀ at elevated temperatures; and (d) the quasithreshold dose (D₀) is defined as the dose in Gy at which the exponential portion of the radiation survival curve, extrapolated backwards, cuts the dose axis drawn through a surviving fraction of unity for radiation alone or through the survival after exposure to hyperthermia alone when radiation and hyperthermia are combined.

RESULTS

The thermosensitivity of cells in culture under normally oxygenated and hypoxic conditions has been investigated previously in a variety of cell lines (41-47). The effect of acid pH on the cytotoxicity of hyperthermia has also been studied by several laboratories in a variety of cell lines (14-19). It is likely that in tumors the environment of some cell subpopulations is characterized by both hypoxia and relatively acidic pH. These conditions have been modeled in the experiments presented in Fig. 1. FSA1C cells in exponential growth were made hypoxic by circulating an atmosphere of 95% N₂/5% CO₂ over the cells for 4 h prior to and during radiation treatment, and they were made acutely acidic by replacing normally buffered media with media containing NaHCO₃ 4 h prior to and during treatment (see "Materials and Methods"). For killing by hyperthermia alone, we observed that exposure to 42°C or 43°C for 1 h caused essentially the same loss of viability in both normally oxygenated and hypoxic cells at normal pH (SF, 0.79 ± 0.04

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and 0.69 ± 0.05 at 42°C and 0.47 ± 0.03 and 0.42 ± 0.04 at 43°C in normally oxygenated and hypoxic cells, respectively. Exposure to 42°C and 43°C at pH 6.45 caused slightly greater cytotoxicity (SF, 0.72 ± 0.07 in normally oxygenated and 0.50 ± 0.10 in hypoxic cells at 42°C and 0.40 ± 0.05 inoxic and 0.35 ± 0.05 in hypoxic cells at 43°C). The greater cytotoxicity of temperatures in the 42–43°C range to hypoxic cells at low pH compared to normally oxygenated cells at low pH has been reported previously (14).

The OER observed at 37°C and pH 7.40 was 2.9 ± 0.2. When the cells were exposed to 42°C at normal pH for 1 h prior to irradiation, the OER was reduced to 1.4 ± 0.1 due to a greater increase in the radiosensitivity of the hypoxic versus normally oxygenated cells at this temperature. In this case, the TEF in the normally oxygenated cells was only 1.4 but TEF was 2.40 in the hypoxic cells.

When FSaIIC cells were exposed to 43°C at normal pH for 1 h prior to radiation treatment, there was no difference between the slopes of the survival curves for the normally oxygenated and hypoxic cells, resulting in an OER of 1.0, and equal killing of cells under both conditions was observed. The TEF in the normally oxygenated cells was found to be 1.20 after the 43°C 1-h exposure and 3.30 in the hypoxic cells. Therefore, the combination of treatment at 43°C for 1 h followed by radiation treatment at normal pH resulted in considerably more sensitization of hypoxic versus oxic cells to radiation.

When media at normal pH were replaced with media at pH 6.45 for 4 h prior to and during radiation treatment, the results presented in the lower panels of Fig. 1 were obtained. Hypoxic FSaIIC cells which were exposed to pH 6.45 at 37°C were slightly (but not significantly) more sensitive to the cytotoxic action of radiation, resulting in an OER for these cells of 2.7 ± 0.2. The radiation survival curve of normally oxygenated cells was unaffected by acidic pH.

When cells under both normally oxygenated and hypoxic conditions at pH 6.45 were exposed to 42°C for 1 h prior to radiation, the OER for the hypoxic cells was reduced to 1.2 ± 0.1 because of a large increase in the radiation sensitivity of the hypoxic cells and very little change in the radiosensitivity of the corresponding normally oxygenated cells. The TEF in the oxygenated cells was only 1.02; however, the TEF in the hypoxic cells was 2.20 compared to the corresponding cells at 37°C and pH 6.45 and was 2.40 compared to hypoxic cells at 37°C and normal pH.

When the hyperthermic temperature was increased to 43°C at pH 6.45 for 1 h prior to radiation treatment, the OER was 1.0, indicating that the slopes of the survival curves for the normally oxygenated and hypoxic cells were the same. The TEF in the normally oxygenated cells was 1.07 compared to normally oxygenated cells at 37°C. The TEF in the hypoxic cells at pH 6.45 following exposure to 43°C for 1 h prior to radiation was 2.82 compared to hypoxic cells at 37°C and pH 6.45 and it was 3.10 compared to hypoxic cells at 37°C and pH 7.40.

In addition to slope changes in the survival curves of the FSaIIC cells, the addition of hyperthermia also resulted in a decrease in the shoulder regions of the radiation survival curves which was temperature, level of oxygenation, and pH dependent. The D, for normally oxygenated FSaIIC cells at 37°C and normal pH was 3.3 Gy, and for hypoxic cells under the same conditions the D, was 3.7 Gy. At normal pH and at increasing hyperthermic temperatures, the D, for normally oxygenated FSaIIC cells decreased to 2.9 Gy following a 1-h exposure to 42°C and to 2.4 Gy after exposure to 43°C. In hypoxic cells at normal pH, treatment with hyperthermia prior to radiation decreased the D, to 2.5 Gy with pretreatment at 42°C for 1 h and to 1.9 Gy with pretreatment at 43°C for 1 h. Thus, the shoulder region of the survival curves was decreased relatively more in the hypoxic than in the oxic cells after exposure to moderate hyperthermic temperatures at normal pH.

Maintaining the cells for 4 h at pH 6.45 prior to and during radiation treatment at 37°C resulted in a slightly, but not significantly, lower D, in both normally oxygenated and hypoxic cells compared to cells under the same conditions at pH 7.40. For normally oxygenated cells at pH 6.45 the D, was 3.2 Gy and for hypoxic cells at pH 6.45 the D, was 3.6 Gy. The acidity of the environment had no effect on the alteration in D, produced by treatment at 42°C for 1 h in either normally oxygenated or hypoxic FSaIIC cells compared with the same treatments at normal pH. The D, for normally oxygenated cells following exposure to 42°C and pH 6.45 was 2.9 Gy and that for hypoxic cells after 42°C for 1 h at pH 6.45 was 2.5 Gy. Following exposure to 43°C for 1 h at pH 6.45, however, a significant further effect in the shoulder region of the radiation survival curve was observed. In this case, for the normally oxygenated cells the D, was reduced to 1.9 Gy and for hypoxic cells the D, was 0.9 Gy. Overall, therefore, the reduction of the shoulder of the radiation survival curve caused by hyperthermia in hypoxic cells was greater than that for normally oxygenated cells under acidic conditions, as it was under normal pH conditions, and this difference was accentuated by exposure to 43°C versus 42°C.

We have used the diffusion of Hoechst 33342 dye and flow cytometry to examine the effects of in vivo heat, radiation, and heat plus radiation treatments on bright (presumably oxygenated cells at normal pH) and dim (presumably hypoxic cells at acidic pH) tumor cell subpopulations (Fig. 2). Tumor cell survival following excision was measured both immediately (20 min) after treatment and at 24 h following treatment. The 24-h interval should have allowed for repair and other secondary effects to occur. For treatment in vivo, the hyperthermia exposure was 43°C for 30 min and the radiation treatment was 10 Gy locally to the tumor-bearing limb. Initially (at 20 min), hyperthermia produced approximately a 1.5-fold greater cell kill in the dim cells compared to the bright cells (SF, 0.40 ± 0.06 versus 0.57 ± 0.05). Twenty-four h later there was an increase in tumor cell kill in both the bright and the dim subpopulations. The increase in cell kill over 24 h was 1.7-fold in the bright cells and 2.9-fold in the dim cells; therefore, at 24 h posttreatment, hyperthermia was 2.5-fold more toxic to the dim tumor cell subpopulation than to the bright tumor cell subpopulation (SF, 0.14 ± 0.04 versus 0.35 ± 0.05). With radiation therapy, on the other hand, at 20 min after exposure to X-rays, the survival of the dim cells was 1.6-fold greater than that of the bright cells (SF, 0.12 ± 0.02 versus 0.075 ± 0.015). By 24 h, however, there was evidence of potentially lethal damage repair in both the bright and dim cells. Thus, at 24 h in the bright cells, there was a 1.6-fold increase in cell survival; and in the dim cells, there was a 1.5-fold recovery in cell survival (SF, 0.11 ± 0.02 versus 0.22 ± 0.05). Therefore, there was no significant difference in the potentially lethal damage repair observed in the two tumor subpopulations. Overall, then, at 24 h posttreatment, there was a 1.9-fold sparing of dim cells by 10 Gy of radiation compared to the kill of bright cells. By comparison, using similar methodology in the KHT sarcoma, Siemann and Keng (48) found a 2.2–2.3-fold sparing of the dim cell subpopulation compared to the brightly fluorescent subpopulation after treatment of the tumors in vivo with 10 Gy. In addition, Chaplin et al. (49) in
Gy of X-rays increased the kill of the bright tumor cells by 2.9-fold. The addition of hyperthermia treatment to 10 Gy between bright and dim cell populations, the magnitude of which appeared to reflect both inhibition of the repair of potentially lethal heat damage. Alternatively, local hyperthermia has been shown to promote cellular migration of immune effector cells into tumors (57) and 24 h may provide enough time for effector cells to reach tumors. The interaction of hyperthermia and radiation under environmental conditions likely to be present in tumor subpopulations (47). In vitro we found that oxic and hypoxic cells at normal pH were equally sensitive to exposure to 42°C or 43°C for 1 h but that at pH 6.45 oxic and hypoxic cells were slightly more sensitive to the heat exposure. Several investigators (14) have reported previously that both normally oxygenated and hypoxic cells are sensitized to hyperthermia under acutely acidotic conditions. Some investigators (50, 51) have also reported that hypoxic cells at low pH are more sensitive to heating than are oxic cells. Although, in general, longer periods under hypoxic conditions were necessary to unmask this effect in these previous studies (50, 51) than the 4 h we utilized, this interaction may be cell line dependent.

The method for inducing hypoxia which we used in vitro seemed successful since an OER of 2.9 at 37°C was observed. At 42°C or 43°C the reduced solubility of oxygen and the decreased respiration of cells at these temperatures could have contributed to some reoxygenation of the cell cultures. However, measurements with a Clark-type electrode yielded a mean oxygen tension of 0.5 mm Hg (range, 0–3), which was not temperature dependent. The 3-mm Hg readings were momentary, while the 0–1-mm Hg readings were sustained, indicating that the 3-mm Hg recordings probably represented instrument fluctuation.

The in vitro experiments were designed to study the effect on tumor cell survival of heat and radiation, both immediately after treatment and at 24 h after treatment, so that essentially full expression of damage and repair could be evaluated. It is interesting to note (and quite fortunate) that we found no significant difference in the fluorescence patterns of the Hoescht 33342 dye at either 20 min or 24 h after exposure to 43°C for 30 min as compared with that seen in untreated tumors, despite the work of others (52) which has demonstrated an effect of exposure to 45.5°C on uptake of this dye into cells (53, 54). Perhaps the milder treatment (43°C) for 30 min as well as the 20-min and 24-h time periods between treatments and injection of dye dampened these effects.

Directly after exposure to 43°C for 30 min, bright (presumably oxygenated cells at normal pH) exhibited measurable cytotoxicity (SF, 0.57 ± 0.05), while dim (presumably hypoxic cells at low pH) exhibited slightly more lethal damage (SF, 0.40 ± 0.06). By 24 h, however, the cytotoxicity of this treatment was measurably greater in both cellular subpopulations but more so in the dim cells (SF, 0.35 ± 0.05 versus 0.14 ± 0.04). Kang et al. (55) and Marmor et al. (56) also found that cells from tumors left in situ expressed more hyperthermic cytotoxicity than cells excised directly following treatment. Kang et al. (55) attributed this finding to a persistent decrease in perfusion in heated tumors which presumably further compromised the environments of some tumor cells and prevented repair of potentially lethal heat damage. Alternatively, local hyperthermia has been shown to promote cellular migration of immune effector cells into tumors (57) and 24 h may provide enough time for some expression of the cytotoxic actions of these cells. In our studies, the increased expression of cytotoxicity at 24 h versus 20 min was greater in the dim population, indicating...
that environmental conditions did play a role in this observation.

The lethal effect of exposure to 10 Gy of radiation in oxygenated cells at 37°C in vitro at normal pH (SF, 0.05 ± 0.01) was slightly greater than in the oxygenated cells isolated as the bright fraction from tumors at 20 min (SF, 0.08 ± 0.03). Conversely, the cytotoxicity of 10 Gy was less in hypoxic cells at low pH in vitro (SF, 0.31 ± 0.05) than in hypoxic cells isolated as the dim fraction from tumors at 20 min (SF, 0.31 ± 0.08). These results indicate (as would be expected) that the bright and dim populations are not homogeneously oxygenated or hypoxic cells as can be modeled in vitro, so that the effect of the level of oxygenation on radiation cytotoxicity was diluted in vivo as compared with the ideal experimental conditions in vitro. The Hoechst 33342 method can provide only partially pure oxic versus hypoxic fractions. The fact that clearly observable differences in survival were reproducibly evident, however, indicated to us that the effect of heat as an adjuvant to radiation on the killing of dye-selected subpopulations was valid.

In vitro the effect of hyperthermia on the radiation survival curve was temperature, oxygen, and pH dependent. In the FSaIIC tumor cells the effect of exposure to 42°C or 43°C on the radiation OER was striking because the TEFs were far more markedly increased in the hypoxic versus the oxic cells at both normal and acidic pH. Even at 42°C the OER was reduced from 2.9 to 1.4 at pH 7.40 and to 1.2 at pH 6.45. At 43°C the OER was 1.0 at both pH values tested. A controversy has existed in the literature regarding this point for more than a decade. Both Robinson and Wizenberg (41) and Kim et al. (42, 43) reported that the effect of hyperthermia on radiation toxicity was far more marked in hypoxic than in oxic cells, while Power and Harris (44) could not substantiate this difference and attributed the previous reports to acid pH and/or nutrient deficiency caused because hypoxia was created by metabolic depletion. In our studies, however, the pH was carefully controlled and fresh medium was used during treatment; while pH 6.45 did slightly increase the hyperthermic effect on the OER at 42°C (but not at 43°C), this was clearly a minor effect.

In addition to slope (D0) changes, hyperthermia also affected the shoulder regions of the radiation survival curves as measured by Dq values. The effect of hyperthermia on the Dq was also temperature, oxygen, and pH dependent. The FSaIIC cells exhibited a fairly broad shoulder on the radiation survival curves at 37°C under normally oxygenated conditions which was even broader in hypoxic cells. At hyperthermic temperatures the Dq values in both normally oxygenated and hypoxic cells were significantly decreased but relatively more so in the hypoxic than the normally oxygenated cells, and this effect was greater after exposure to 43°C than after treatment at 42°C. At acidic pH the decrease in the Dq caused by prior exposure to hyperthermic temperatures was unchanged following treatment at 42°C but was further significantly reduced after exposure to 43°C. The magnitude of the effects was large so that the Dq of normally oxygenated cells at normal pH decreased from 3.3 Gy at 37°C to 2.4 Gy after heating at 43°C for 1 h, and for hypoxic cells at low pH it decreased from 3.6 Gy at 37°C to only 0.9 Gy after 1 h at 43°C. Similar trends caused by hyperthermia on the shoulder regions of radiation survival curves have been reported previously by Holahan et al. (17, 18). Since standard radiation fractions used in the clinic average 180–300 cGy, these changes in the Dq values are likely to be of even more therapeutic significance than D0 changes.

The in vivo results for the combination of hyperthermia and radiation are somewhat at variance with the in vitro results. The magnitude of the cell kill achieved by exposure to 43°C for 1 h plus 10 Gy radiation in both the normally oxygenated cells at normal pH and the hypoxic cells at low pH in vitro was significantly greater than in both the bright and dim cells in vivo. This was especially true when tumors were excised shortly after exposure to heat and radiation rather than excised at 24 h when greater hyperthermic killing as well as likely hyperthermia-induced inhibition of potentially lethal radiation damage repair contributed to significantly increased cell killing. In addition, the in vitro data indicate that the cell kill achieved by exposure to 43°C for 1 h then 10 Gy of radiation should be greater in hypoxic cells at low pH than in oxygenated cells at normal pH. In vivo, however, the cytotoxicity of 43°C for 30 min followed by 10 Gy was slightly greater in the bright (oxygenated) than in the dim (hypoxic) cells when excision occurred shortly after treatment (at 20 min). By 24 h, however, the cytotoxicity measured in the hypoxic cells was clearly greater, as predicted by the in vitro results. The reasons for these disparities are not clear, although the difference in the heating duration (1 h in vitro versus 30 min in vivo) must have contributed partially to these differences. In previous investigations using a median tissue culture dose assay (58, 59) or an [3H]iododeoxyuridine incorporation viability assay (60, 61) hypoxic tumor cells were found to be either selectively killed or selectively radiosensitized by hyperthermic treatment.

In any case, the pattern of cytotoxicity observed both in vitro and in vivo may have significant clinical implications. Exposure to 43°C for 30 min (a clinically achievable local hyperthermia treatment) more than overcame the relative resistance of dim (hypoxic) cells to 10 Gy of radiation when measured at 24 h as predicted by the in vitro results. Hyperthermia has been shown to induce an initial increase in tumor blood flow in rodents (53, 54). An increase in blood flow which resulted in transient reoxygenation of hypoxic cells at the time of irradiation, followed by a reversion to a diminished perfusion state and sorting of these cells in the dim subpopulation, would have led to an increase in the observed heat-radiation cytotoxicity within the dim cell population.

The cytotoxic effect of exposure to 43°C for 30 min alone was quite significant, especially in the dim cells where nearly a 1-log kill (SF, 0.14 ± 0.04 versus 0.35 ± 0.05 in bright cells) was observed at 24 h. The in vitro curves showed that hyperthermia decreased the D0, especially under acidic conditions, so that exposure to radiation fractions in the clinically relevant 250–500-cGy range resulted in about a 25% increase in cell kill of oxygenated cells at normal pH and about a 50% increase in the cell kill of hypoxic cells at low pH. It has long been argued whether the main clinical effect of hyperthermia in the heat-radiation combination is as a radiosensitizer or as an independent killer of hypoxic cells. While obviously either or both effects may predominate, depending on the temperatures achieved and the radiation dose delivered, the present results suggest that for standard radiation fractions and clinically achievable temperatures, hyperthermic radiosensitization of hypoxic cells is probably quite cytotoxically significant. Along with direct thermal killing, then, this effect very likely plays a critical role in the improved clinical results observed when this combination is used.

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