Sensitivity of Human Cells to Mild Hyperthermia

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ABSTRACT

The cytotoxic effects of short duration, high temperature, and long duration, low temperature hyperthermia were determined in human cells growing in culture. The human tumor cell lines A549 (lung carcinoma), WiDr (colon carcinoma), and U87MG (glioblastoma) were used. In addition, a normal human lung fibroblast cell type 18Lu was used. Sensitivity to direct cell killing was measured at 41, 43, and 45°C. Heat induced perturbations of cell cycle and proliferation were also analyzed. The results obtained on sensitivity of the above human cell lines at 43 and 45°C are similar to those of the previous work of others in that the human cell lines were observed to be relatively resistant to thermal killing at 43 or 45°C, when compared to heat sensitive rodent cell lines. The comparison is important because most prior hyperthermia research has been performed with rodent cells and clinical protocols have been designed with the use of rodent data. In contrast to the 43°C response, most of the human cells we tested were killed by 41°C heating to an extent greater than that observed for rodent cells. The heat sensitivities of the four different human cell lines varied widely. This appeared to be due to differences in both intrinsic heat sensitivity and tolerance development. During 41°C heating, human cells did not proliferate and cell cycle perturbations developed but did not correlate with sensitivity to killing. Our heat sensitivity measurements point out the shortcomings of using data derived from rodent systems to predict clinical outcome of hyperthermia therapy.

INTRODUCTION

Enthusiasm for the use of long duration, mild temperature hyperthermia in conjunction with low dose rate irradiation in the treatment of certain malignant diseases has recently developed. Results from in vitro experiments have demonstrated that heating rodent cells at 40 or 41°C during low dose rate irradiation can greatly enhance cell killing (1-5). These previous observations predict greatly enhanced tumor cell killing from the addition of long duration, mild hyperthermia to 41°C during low dose rate irradiation can greatly enhance cell killing of certain malignant diseases has recently developed. Results from in vitro experiments to predict clinical outcome of hyperthermia treatment require an understanding of the role of exposure conditions such as temperature, length of heating, and sequencing of heat and radiation. Predicting optimal parameters from experimental results requires the utilization of pertinent exposure conditions and appropriate biological models. Most basic research in the hyperthermia field has been performed on rodents or rodent cells (6). Although some aspects of the response of cells to heat are ubiquitous, other characteristics are undoubtedly species and cell type specific. Cellular sensitivity to hyperthermic killing is one characteristic that varies widely in different cell types. The clinical importance of this last aspect has been demonstrated by several investigators who have reported that cell lines of human origin are generally more heat resistant than rodent cells (7-9). Although little is known about the effectiveness of heat in directly killing human tumor cells in situ, the poor outcome of clinical trials using hyperthermia as a single modality (10) may be a reflection of the in vitro resistance. One must therefore proceed with a great deal of caution when using data from rodent models to predict response of human tumors to hyperthermia treatments. Such data may be useful, but interpretation of the data requires the understanding that temperature dependent responses are species and cell type specific.

Previously observed resistance of human cells to 43 and 45°C heating raised the question of whether 41°C treatment was capable of producing radiation sensitization in human cell lines and patients as had been seen in rat cells. As a first step in determining the clinical effectiveness of long duration, mild hyperthermia in thermoradiotherapy, the temperature dependence of heat induced cell killing was determined in human cells in vitro. We have used human tumor and normal cell lines to compare their low and high temperature sensitivities to that of rodent cells.

MATERIALS AND METHODS

Four human cell lines were used in these experiments. A human colon carcinoma cell line (WiDr) was obtained from Dr. Michael Borrelli [William Beaumont Hospital, Royal Oak, MI]. Human glioblastoma-astrocytoma (U-87MG), human lung carcinoma (A-S49), and normal human lung fibroblast (CCD-18Lu) cell lines were obtained from the American Type Culture Collection (Rockville, MD). These cell lines were maintained as monolayer cultures in 50% Dulbecco's modified minimal essential medium (Mediatech) and 50% Ham's F-12 medium (Mediatech) containing iron supplemented bovine calf serum (HyClone) added to 10%, 0.5% vitamins (Irvine Scientific), 0.1 mM nonessential amino acids (Mediatech), and 1.0 mM l-glutamine (Mediatech). The cells were grown in a humidified, 5% CO₂, 95% air incubator at 37°C. Fresh cultures of human tumors were restarted from frozen stock after 15 passages. The 18Lu cells were between passages 2 and 14 when used in experiments.

A rodent cell type was chosen to serve as a basis for comparing thermal sensitivities of rodent cells to the above human cell types. The rodent cell type chosen was rat 9LSF gliosarcoma, because our laboratory has a large amount of data related to mild hyperthermia and low dose rate irradiation with these cells. The rat 9L cells are relatively heat resistant at 43°C compared to Chinese hamster ovary and other commonly used rodent cell lines (7, 11, 12) that are widely used in the hyperthermia biology field. The culturing methods for the rat 9L gliosarcoma cells used in this study have previously been described by Armour et al. (1. 13). In summary, cells were maintained as monolayers in Dulbecco's modified minimum essential medium supplemented with 10% newborn bovine serum (Hazelton Biologicals, Inc.), 0.1 mM nonessential amino acids, 1% vitamins, and 2.0 mM l-glutamine.

In all experiments, cells were exposed to hyperthermia while growing exponentially in tissue culture flasks. Approximately 5 × 10⁵ cells were seeded into 25-cm² plastic tissue culture flasks (Corning) with 5 ml of medium 24 h before initiation of hyperthermia treatment, except for WiDr cells, which were seeded at approximately 3 × 10⁵ cells/flask in 5 ml of medium 48 h before initiation of treatment. Heating was carried out by sealing the tops of flasks with Parafilm and submerging in a water bath regulated to within 0.05°C of the appropriate temperature. The temperature at the cell growth surface within the flasks was measured in representative flasks with a microthermocouple. In all experiments, cells were removed from flasks with trypsin immediately after heating and the cell number in each treatment flask was determined by using a particle counter (Couler Electronics). Appropriate numbers of cells were seeded into 60-mm tissue culture dishes for colony formation. After 10 to 14 days' incubation, depending upon cell type, colonies having more than 50 cells were scored. Duration colony formation experiments, dishes were undisturbed except for those with WiDr cells. In experiments using WiDr cells, incubation medium in colony formation dishes which were seeded with more than 1 × 10⁵ cells/dish was changed once after 5 days. Preliminary investigation had demonstrated that radiated WiDr cells which were seeded at a density higher than 1 × 10⁵ cells/60-mm dish had reduced plating efficiency compared to cells plated at a lower density. This density dependent effect was eliminated if medium was changed after 5 days' incubation. In each experi-

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ment, medium on untreated control dishes was also changed and was observed to have no effect on the number of resulting colonies (data not shown). This density related toxicity was not observed with heat killed cells, but the procedure was performed in these hyperthermia experiments so the results would be consistent with experiments in which ionizing radiation was investigated.

Plating efficiencies for untreated cells, in the colony formation assay, varied from 20 to 40% for U-87MG and CCD-18Lu cells, 70 to 90% for WiDr and A-549 cells, and 40 to 60% for rat 9L cells. The data for WiDr, A549, 18Lu, and U87MG were obtained from 5, 4, 7, and 2 experiments each, respectively, at 43°C; 5, 3, 5, and 1 experiments each, respectively, at 45°C; and at least 6 experiments each at 41°C.

Samples from each exposure condition were analyzed for cell cycle distribution. An aliquot of cells from each sample used for colony formation seeding was stained with Hoechst 33258 at 3 μg/ml in a solution of 0.1 M Tris, 2 mM MgCl₂, 0.1% Triton X-100, and 9 g/liter NaCl with a final pH of 7.5. The DNA content per cell was determined by flow cytometry (Partek, GmbH) and the resultant data were analyzed by using a computer program (Phoenix Flow Systems).

RESULTS

Compared to the sensitivity of most rodent cells (7, 13), the 4 human cell lines which we tested were relatively resistant to killing by exposure to 43 or 45°C hyperthermia. Survival of the human cells at these temperatures has been compared to that for 9L cells in Fig. 1 and 2. Resistance to 43°C heating was cell line dependent for the four human cell lines tested. The human cell types arranged in order of least sensitive to most sensitive at 43°C are; WiDr, 18Lu, A549, and U87MG. Only the most heat sensitive human cell line, U87MG had survival lower than 9L cells when heating was at 43°C for 3 h or more. Human cell resistance at 43°C was manifested primarily as a plateauing of the survival curves for exposure lengths beyond 2 h. Compared to 9L cells, the relative resistance of human cells was not evident for 43°C exposures of 2 h or less. The relative sensitivity of the cell types to 45°C heating was similar to that at 43°C except for WiDr cells. For 45°C heat exposures shorter than 30 min, WiDr survival was similar to that of 18Lu, but for longer heatings the WiDr cell survival decreased rapidly, whereas the 18Lu survival curve bent upward beyond 1.5 h. A slight plateauing effect also occurred in A549 cells. The 45°C survival curves of WiDr and U87MG cells showed no sign of plateauing within the durations of heat exposures presently utilized. One difference between human and rodent cells at 45°C was that 9L cells had a distinct shoulder on their 45°C survival curve, whereas the human cells did not. Human cell resistance at 45°C was observed as shallower final slopes compared to rodent survival curves.

When the human cells were heated at 41°C for long durations, survival of some cell lines was unexpectedly lower than that for the presently tested rodent cell line (Fig. 3). The surviving fraction of 9L cells decreased to 0.6 over the first 6 h at 41°C but stabilized with longer heating times and did not drop below 0.4 for heat exposures up to 96 h. Each of the human cell types expressed a steady decrease in survival after the start of heating at 41°C. This trend continued although the curves for WiDr, A549, and 18Lu cells appeared to bend upward after 48 h at 41°C. The cell sensitivity to 41°C heating varied in the different cell types. The surviving fractions for WiDr, A549, U87MG, and 18Lu cells after 48-h heating at 41°C were 0.4, 0.2, 0.04, and 0.07, respectively. Except for WiDr cells which had sensitivity similar to that of 9L cells, heating at 41°C killed the human cell types to a greater degree than the 9L cells.

The number of cells per flask during 41°C heating, relative to the number at the start of the experiment, are plotted in Fig. 4. The number of WiDr and A549 cells per flask remained stable for 96 h. Between 96 and 168 h the WiDr cell population increased by less than a factor of 2 and the A549 population decreased by approximately 50%. The 18Lu cell population remained stable for the entire 168-h treatment at 41°C. The U87MG population remained stable for 48 h but decreased to 20% of the starting number between 48 and 120 h. The 9L cell number initially did not increase, but doubled by 48 h after the start of heating.

Cell cycle perturbations during 41°C heating were distinct in each of the cell lines (Fig. 5). Continuous movement of WiDr cells through G₁ and S was accompanied by accumulation in G₂ during the first 72 h at 41°C. Beyond 72 h the percentage of G₂ cells decreased from 80 to 50% with a rise in both G₁ and S phases. This loss of G₂ block in WiDr cells was concurrent with a rise in number of cells per flask...
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Fig. 3. Relative cellular survival after 41°C hyperthermia was markedly different than that observed at higher temperatures. Three of the human cell lines were relatively sensitive rather than resistant. The rat cell line plateaued at a relatively high survival level. Survival of the human cell lines appeared to level off after exposure durations beyond 48 h, but at significantly lower survival than the rodent cell line with the exception of WiDr which was similar to 9L. The relative sensitivity of the 3 human tumor cell lines remained constant at all three temperatures tested. The 18Lu cells were most resistant at 45°C to next to the most sensitive at 41°C. The data for WiDr, A549, 18Lu, and U87MG were obtained from at least 6 experiments each. Bars, SD.

DISCUSSION

Clinical trials utilizing hyperthermia as an adjuvant to radiation therapy have been designed with the assumption that human tumor cells respond to hyperthermia similarly to the response of rodent cells. In particular, much of the hyperthermic research from which clinical heating temperatures and durations were derived has utilized rodent cells (14). Our present data, along with results from several other investigations (7–9, 15, 16), demonstrate that in general the response of human cells to hyperthermia is substantially different from that of rodent cells. Research using rodent cells has been useful in determining qualitative responses of cells such as the general shape of time and temperature response characteristics (13), thermotolerance (17), and a variety of heat induced subcellular and molecular responses (18–21). On the other hand, using rodent cells to predict human tumor response to hyperthermia presents problems. The major issue is that of assuming quantitative similarity between rodent and human cell response. The therapeutic efficacy of hyperthermia may be over- or underesti-
Sensitivity of human cells to mild hyperthermia has yet to be learned about the temperature dependence of human cell heat sensitivity. The survival curve shape may give clues about why human cells are more heat resistant at 43 or 45°C than most rodent cell types at higher temperatures. A flattening of the survival curves beyond 3-h heating at 43°C occurred in all four human cell lines tested. Similar survival curve flattening at 42°C or lower in rodent cells has been attributed to development of thermotolerance (25). Resistance of human cells to long durations at 43°C may thus be due to their ability to develop tolerance during heating at this temperature. This presumed development of tolerance was also observed during 45°C heating with 18Lu and A549 cells but not with WiDr and U87MG cells. At 41°C the human cells showed less flattening of their survival curves compared to 9L cells. Survival of these rodent cells heated at 41°C stabilized after 6 hours and remained constant thereafter. Chinese hamster ovary cells are known to develop thermotolerance during heating at 41 or 42°C but not at 43°C or higher (26). A lack of thermotolerance development during 41°C heating in human cell lines U87MG or 18Lu could explain their sensitivity and is currently under investigation.

The temperature range over which thermotolerance appeared to develop during heating was higher in all human cells than in rodent cells, although the range was higher in some cell lines than in others. In addition to this proposed thermotolerance explanation for differences in sensitivity, it appears that individual cell lines have varying intrinsic heat sensitivities. For instance, the U87MG cells were more sensitive than the other human cell lines at all temperatures. Although human cell sensitivity to long duration, mild hyperthermia has been observed in the present and other (16, 22, 27) cell systems, a great deal has yet to be learned about the temperature dependence of human cell heat sensitivity.

Cell cycle specific interactions during heating at 41.5°C have been associated with lethality in HeLa cells (16). Mackey et al. (16) observed that during 41.5°C heating, HeLa cells in G1 continued into S phase and became blocked. This blockage in S phase corresponded with lethality. In contrast, CHO cells remained blocked in G1 during 41.5°C heating and had very high survival. Cell cycle distributions were analyzed during 41°C heating for each of the cell lines used in this investigation. Each of the cell lines had distinct changes in their cell cycle distributions. None of the cell cycle changes correlated with sensitivity to killing. In particular, failure to progress out of G1 was not associated with resistance (18Lu and A549) and S phase buildup was not related to lethality. The only cell line to have a distinct S buildup was 9L which was resistant to 41°C killing. Similar cycle perturbations were observed in the most 41°C resistant and most sensitive human cell lines. The sensitive line, U87MG, had cell cycle redistributions similar to those of resistant WiDr cells. Both cell types for rodent cells when exposure is at temperatures around 41°C for durations of 6 h or more. This response is similar to that described recently by Mackey et al. (16, 22) who compared 41°C sensitivity of HeLa and Chinese hamster ovary cells. Evidence for mild temperature

Fig. 5. Cell cycle parameters were determined on the cell samples used for survival determination. Cell cycle distributions are presented in graphs as described in Fig. 4. Each cell type went through distinct cycle perturbations. These changes could not be correlated with 41°C induced cell killing. Bars, SD. +, G1; ▲, S; ○, G2 + M.
These systems utilize interstitial heating methods which are capable of killing very few cells (33). Our observation that some human and WiDr cycle redistributions was that S phase remained stable in the be beneficial in a brachytherapy setting, development of better heating for indefinite durations with minimal supervision other than these systems are ideally suited for combination with recently been developed and are being used in clinical trials (35, 36). The only difference in U87MG cells in blocked in G2 with depletion of G1. The tumor volumes above approximately 41°C. At present, the potential benefits of hyperthermia have not been optimally tapped.

The benefits of clinical hyperthermia radiotherapy as applied presently at most institutions have been ascribed to direct heat killing rather than to synergistic sensitization of radiation (28). These treatments have typically utilized a clinical treatment goal of 43°C applied for 1 h before or after irradiation. Due in part to misunderstandings about temperature dependence of human tumor cell heat sensitivity, the potential benefits of hyperthermia have not been optimally tapped. Other factors which have limited therapeutic gains from hyperthermia include clinical toxicities and technical deficiencies (29–32). Treatment induced pain has limited clinicians’ ability to reach desired temperature elevation in many settings. Presently available clinical hyperthermia apparatus is another major problem. Institutions using the best available apparatus and techniques (29, 30) have not been able to heat 90% of tumor volumes above approximately 41°C. Attaining temperatures in the range of 43 to 45°C which are capable of killing significant fractions of human tumor cells with 1- to 2-h treatments has therefore not been feasible in tumors that are bulky or deep seated. These inadequacies have resulted in the majority of hyperthermic therapy sessions having thermal doses which are predicted to kill very few cells (33). Our observation that some human tumor cells may be sensitive to long durations (24 to 48 h) of mild hyperthermia opens a new avenue for application of hyperthermia. This mild hyperthermia probably will be tolerated well and is technically feasible. The extended duration aspect of the treatments probably will not greatly increase normal tissue toxicity, although this factor is not well understood. Normal tissue toxicity should receive further attention in light of our observation that exponentially growing normal fibroblasts are relatively sensitive to mild hyperthermia. A very distinct temperature threshold for toxicity has been observed with whole body hyperthermia (34), whereas length of treatment has not been a factor when temperature is kept below the toxic threshold. In the past, practical clinical systems capable of heating tumors over very long durations were not available. At least two such systems have recently been developed and are being used in clinical trials (35, 36). These systems utilize interstitial heating methods which are capable of heating for indefinite durations with minimal supervision other than normal clinical care used with brachytherapy patients. They do have the drawback of being invasive. Although their application is presently limited, these systems are ideally suited for combination with interstitial irradiation treatment. If long duration hyperthermia proves to be beneficial in a brachytherapy setting, development of better technology may bring about noninvasive and more broadly applicable heating systems which can be used in conjunction with external beam irradiation.

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