Cycle Progression and Division of Viable and Nonviable Chinese Hamster Ovary Cells following Acute Hyperthermia and Their Relationship to Thermal Tolerance Decay

Glenn C. Rice, Joe W. Gray, and William C. Dewey

ABSTRACT

Cell cycle progression delays and subsequent growth kinetics of viable and nonviable Chinese hamster cells following acute (45.5°C) hyperthermia were documented in an attempt to correlate these changes with the decay of thermal tolerance.

Following heating for various lengths of time, cells exhibited a delay in subsequent division which was related to cell survival by a power function relationship. A cell was considered to be viable if it retained the ability to divide to form a colony of 50 or more cells. The components of the delay in cycle transit for viable cells heated in G1 for a treatment of 20 min at 45.5°C were approximately 28 hr in G1 and 20 hr in S and G2-M. This represents a 7-fold decrease in the rate progression through G1 and a 2-fold decrease through S and G2-M relative to control rates. The doubling times of viable cells, in subsequent generations, were significantly decreased to a rate 61% of that of control up to 120 hr after heating. This reduction was in part due to lethal sectoring, i.e., a division which produces only one daughter that is capable of forming a viable subclone, within the viable progeny. Within a viable subclone, up to 30% of the cells that divided from 48 to 91 hr after a heat treatment of 20 min at 45.5°C were found to be nonclonogenic. Following resumption of division, nonviable cells slowly lost their capacity for proliferation. Nearly all thermal tolerance development induced by a 20-min pretreatment occurred while the viable cells remained in G1. Subsequent progression into heat-sensitive S and G2-M phases modulated thermal tolerance only slightly. Finally, maximal loss of thermal tolerance was exhibited at the time corresponding to the resumption of viable cell division.

INTRODUCTION

Evidence supporting the use of hyperthermia in the treatment of cancer has been accumulating from biological studies both in vitro and in vivo (for reviews, see Ref. 3) and from preliminary work on the treatment of spontaneous tumors in domestic animals (9), both of which are establishing the foundation for use of hyperthermia in human cancer therapy (15, 20). The potential importance of hyperthermia is predicted partly on its ability to increase selectively the killing of cells that are resistant to X-irradiation and to have a potential of imparting a differential cytotoxic effect on the tumor relative to normal tissues. This is due mainly to the complementarity of the cell cycle age response function of heat and X-irradiation cell killing (2, 28, 32) and the greatly increased heat sensitivity of cells under acidic pH or nutritional deprivation (6, 22), conditions which are more likely to be found in tumors than in normal tissues.

Hyperthermia as used in the clinical protocols will almost certainly be contingent on fractionated regimens of heat, either alone or combined with other modalities. In split-dose experiments in vitro and in vivo, resistance to thermal killing at elevated temperatures (43°C) has been observed, which is defined as acute thermal tolerance. Chronic thermal tolerance is also a phenomenon of increased resistance to heat toxicity and is manifest at temperatures between 41.5 and 42.5°C as a transient plateau in the heat cell survival curve (29). In this paper, we will be concerned only with acute (45.5°C) thermal tolerance. It is defined as the increased D0 of the heated cell survival curve obtained at a given time after a preheating treatment relative to the D0 of the nonpreheated control survival curve. Although there have been several studies lately dealing with induction kinetics of acute thermal tolerance (10, 22, 23), there have been very few investigations quantifying the kinetics of in vitro thermal tolerance decay (8, 19).

In efforts to elucidate the mechanisms of heat-induced cell killing and thermal tolerance, it is of importance to discriminate and quantitate the response of those cells destined for clonogenic sterility from those retaining reproductive integrity. Many biochemical studies of hyperthermically perturbed populations take place over extended periods of time following the heat treatment. Alterations in the fraction of viable and nonviable subpopulations within the experimental duration can lead to difficulties in the interpretation of changes in either cellular or molecular parameters which may be relevant to mechanisms of heat-induced toxicity or to the expression of thermal tolerance within the surviving fraction.

In this study, we have attempted to quantitate cell cycle progression and growth kinetics of both nonviable cells and viable clonogenic cells within a heat-treated population. We then attempted to study the relationship between the decay of thermal tolerance and cell cycle progression of viable cells in the hope of obtaining information on the mechanism of expression and decay of thermal tolerance. Such information may be of potential use in the manipulation of thermal tolerance within a clinical context.

MATERIALS AND METHODS

CHO cells were cultured as monolayers in Falcon tissue culture flasks or in suspension in Bélico spinner flasks or in Corning 15-ml disposable centrifuge tubes on a rotating roller wheel at 37°C in a humidified atmos-
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To eliminate heat-trypsin interaction (Ref. 25; this paper), log-phase cells were obtained from spinner cultures, diluted, and plated into 4 ml of medium in Falcon T-25 flasks to appropriate cell numbers. After a 2-hr attachment period at 37°C, the necks of flasks were sealed with Parafilm and heated by horizontal submersion into temperature-controlled water baths (±0.02°C). After the hyperthermic treatment, the flasks were rapidly transferred to a 37°C water bath. All thermometers were calibrated by standardization against a National Bureau of Standards certified thermometer. Measured temperature transitions (half-times of 20 sec) were identical to values reported previously (24, 30). Thermal doses shown were not corrected for temperature transients.

Afterwards, the flasks were either transferred into a 37°C incubator for 6 to 10 days for macroscopic colony development, viewed over several days with phase-contrast microscopic optics to view the cells plated on a gridded Falcon T-flask, or placed under a phase-contrast optical field for extended time-lapse photography at 37°C. Macroscopic colonies were stained with crystal violet dissolved in 95% ethanol. The survival data were corrected for multiplicity at the time of treatment (4, 31).

Individual cells were followed on a gridded Falcon T-flask under ×40 magnification to distinguish viable from nonviable cells. Viability was operationally defined as the ability of a cell to form a colony of 50 cells or more (4). Typically, over 100 cells were followed per point for up to 10 days after the treatment. All of the data shown are averages of 2 to 4 experiments. Regression analysis of the generated growth curves was performed with an HP-67 calculator.

Time-lapse microphotography was performed using a Bolex 16M camera utilizing Kodak Plus X reversal film at ×40. Photomicrographs were taken with a timing circuit, set at 60-sec intervals, coupled to a shutter that eliminated continuous illumination of the cells. Fields were chosen to encompass approximately 30 cells and viewed at intervals up to 6 days following the hyperthermic treatment. The photographic records were viewed with a 16-mm variable-speed cine projector. Resolution was sufficient to distinguish individual cells, precluding errors due to misidentification. Single cells and their progeny were followed in successive frames, while a temporal record corresponding to elapsed real time was made of cell division, fusion, detachment, pyknosis, and other relevant events. Division was scored as the first appearance of a cleavage line at telophase following the treatment; however, the rate of division decreased exponentially with time following a hyperthermic treatment of 20 min at 45.5°C. This treatment protocol reduced survival to 0.241. The unheated controls exhibited exponential growth with a doubling time of 12.6 hr. The heated cells suffered an initial delay in division which was 48.3 ± 3.6 hr.

The division delay was calculated from the intercept of the regression lines of the growth curves with the regression lines of the multiplicity after heating (Chart 1, D1). Data were analyzed both in this manner and by calculating the division delay as the intercept of the regression line of the growth curve with N = 2 (Chart 1, D1). There appeared to be no significant difference in calculating delays by either of these 2 methods (see Chart 4). Therefore, all delay data are presented as analyzed by the former method.

In addition, there was an apparent increase in viable cell-doubling time even after recovery from the division delay, to 20.3 ± 2.5 hr, a 61% increase over that of the control. Many of the nonviable cells were capable of a limited number of divisions following the treatment; however, the rate of division decreased continuously within this subpopulation. Both the apparent innate sensitivity of CHO cells to hyperthermia and the variable phenotypic changes were modified by the presence of feeder cells. Irradiated feeder cells were seeded into the T-25 treatment flasks 24 hr prior to the plating of the test cells to allow for sufficient conditioning of the medium (13).

Cell cycle progression was evaluated by bivariate flow cytometric analysis of propidium iodide-stained cells in suspension. Studies published elsewhere (28) have shown similar alterations in cycle progression kinetics after heating for cells cultured in either monolayer or suspension. Briefly, approximately 10^6 cells were fixed in 70% ethanol, treated with RNase (1 mg/ml) at 37°C for 1 hr, and then stained with propidium iodide (10 μg/ml). DNA content was measured with excitation on the Livermore flow cytometer at 488 nm. Size was measured simultaneously by right-angle light scatter analysis. In all 2-parameter contour plots, the lowest contour plotted was at 5% of the largest peak height, the remainder being separated by a factor of 2.

Synchronous G1 cells were obtained by centrifugal elutriation of an asynchronous population on a Beckman J2-21 as described (27). Approximately 10^6 cells were loaded at 20°C in full medium, and fractions were collected at 0.5 (mm/hr-g) sedimentation velocity intervals on ice. The labeling index (percentage of cells labeled with [3H]thymidine (5 μCi/ml), 2 Ci/mmol for 10 min) of the pooled G1 fractions was 5.31 ± 0.08.

RESULTS

Division Delay of Viable and Nonviable Cells. Chart 1 shows the increase in cellular multiplicity (Nt), i.e., the number of cells/copy Saturday colony-forming unit, as a function of time following a hyperthermic treatment of 20 min at 45.5°C. This treatment protocol reduced survival to 0.241. The unheated controls exhibited exponential growth with a doubling time of 12.6 hr. The heated cells suffered an initial delay in division which was 48.3 ± 3.6 hr.

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Division Probability. We further investigated the growth kinetics of those cells sterilized by the various heat treatments. The histograms in Chart 5 depict the size distributions of the nonviable colonies scored 5 days after the hyperthermia. There was good evidence of decreasing probability of more than one division in the sterilized cells with increasing heat duration. Chart 6 indicated that the relationship could be described by the power function

\[
S = 4.97 \times 10^4 X^{-2.13}, \quad r^2 = 0.96
\]

where \(S\) is survival level, and \(X\) is the percentage of nonviable cells with a multiplicity of 1 at 120 hr after the heat treatment.

Lethal Sectoring. Heated cells and their progeny were examined through pedigree analysis of time lapse films for at least

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\( S = 4.76 \times 10^2 D_2^{-1.99}, \quad r^2 = 0.93 \) (A)

\[ S = 4.97 \times 10^4 X^{-2.13}, \quad r^2 = 0.96 \] (B)
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In addition, we observed examples of lethal sectoring, i.e., a division which produced only one daughter that was capable of forming a viable subclone. For instance, one daughter cell of the heated parent in Chart 7C produced only normally dividing progeny. The other sister divided normally for one generation but then either lysed or underwent fusion attempting the subsequent division. Lethal sectoring was evident not only in the first generation (Chart 7C), but also the second (Chart 7B), and possibly even the third (Chart 7D).

We confirmed that lethal sectoring occurred in viable clones following 20 min at 45.5 °C through the experiment depicted in Chart 8. Replicate plates were heated and either scored on a gridded flask for viable colony multiplicity (i.e., number of cells/viable colony) as before or gently trypsinized and dispersed to single cells, which were then placed in a 37 °C incubator for colony growth. The upper curve is the product of the survival of the cells without trypsinization multiplied by the multiplicity of viable colonies. The lower curve is survival calculated from the dispersed replicate flasks.

A number of important implications are evident from Chart 8. (a) There is a large sensitivity of the heated cells to trypsin up to 24 hr after the treatment. This has been reported previously by several investigators (25). (b) It supported the observation of

Chart 5. The percentage of nonviable cells with the multiplicity indicated at 120 hr after heating at 45.5 °C for the durations indicated. The average multiplicity for control, nonheated, nonviable cells was 2.4.

Chart 6. The percentage of the total nonviable population with a multiplicity of 1.0 indicated at 120 hr following various levels of 45.5 °C hyperthermia. Cells were heated and either plated for survival or analyzed for microcolonies on a gridded T-flask as described in "Materials and Methods." The value for control cells (survival = 1.0) was 0.36.

120 hr after a hyperthermic treatment of 20 min at 45.5 °C. Sterilized cells were classed as either "nondividing," "pyknotic," or "multinucleate." As seen, the actual expression of damage could be delayed from the first generation (Chart 7E), to the second (Chart 7F), or third (Chart 7A). Even within the same colony (Chart 7E), there were differences in the number of divisions a daughter was capable of undergoing before becoming reproductively inactivated. Therefore, the behavior of progeny during the first several divisions does not necessarily portend a subsequent inactivation. Parenthetically, we observed very little detachment of the cells from the plastic substrate over the course of our observations, in contrast to previous reports (18). Even those cells which become pyknotic and presumably lysed remained attached to the T-flasks in both the time-lapse and manual observations.

Chart 7. Representative pedigrees as obtained by phase-contrast time-lapse cinematography. The numbers correspond to the time in hr to division; PYK, pyknotic; MN, multinucleate; ND, nondividing. All others were judged to be viable (i.e., eventual division to a colony of 50 cells or larger). All cells were heated in the first generation.
Survival of trypsinized replicates

Maximal thermal tolerance developed in cells surviving the first heat treatment at approximately 24 hr. Thereafter, and up to 120 hr, thermal tolerance decreased. The time of the greatest decrease in tolerance (48 to 72 hr) corresponded to the time at which viable cells resumed division. Between 48 and 72 hr posttreatment, thermal tolerance decreased by a factor of 3.4, while the viable cells had divided approximately once. This suggested that the more important element of thermal tolerance decay may be associated with division of the viable cells and not cell cycle redistribution into a heat-sensitive phase (Chart 1). Indeed, data in Chart 2 suggested that between 24 and 48 hr posttreatment, when thermal tolerance was maximal, many of the ultimately cloningogenic cells were in heat-sensitive S and G2-M phases of the cell cycle.

DISCUSSION

The growth kinetics of individual CHO cells were assessed through periodic colony size measurements following the hyperthermic treatment and were retroactively evaluated for clonogenicity, i.e., the ability to proliferate to a colony of 50 cells or more. The data indicated that nearly all viable cell division was arrested for some period of time following hyperthermia at 45.5° for even short doses. For instances, the delay before the resumption of division was 19 hr for a 5-min treatment or 68 hr for a 20-min treatment. These 2 heating durations reduced survival to 0.97 and 0.15, respectively. The division delays reported were comparable to those obtained by others (33) using CHO cells heated at 45.5° which successfully completed division at the end of the first generation. However, the division delays for viable cells (using the ability for unlimited proliferation as the viability criteria) presented here were longer than those reported previously for viable CHO cells heated at 44° (19) for a normal human diploid WI-38 fibroblast or for a virus-transformed subline at 43° at comparable survival levels (14). This may suggest that division delay is both temperature and cell line dependent. The former is

lethal sectoring, since every survival level of the trypsinized replicates beyond 48 hr was less, by approximately 20 to 30%, than that calculated by using viable colony multiplicities. This suggested that an average of approximately 30% of the subclones within a viable colony following 20 min at 45.5° was not clonogenic, i.e., not capable of forming a clone of 50 cells or larger. (c) From 48 through 96 hr, the curves were basically parallel, with a slope of approximately 20 hr. From this could be inferred that the rate of lethal sectoring was essentially constant within this time period and that the viable subclones had an increased generation time of 20 hr, compared to 12.4 hr for controls.

Viable Cell Division and Thermal Tolerance Decay. Finally, we attempted to study the relationship between cell division and temporal loss of thermal tolerance induced by 20 min at 45.5°. Briefly, cell survival curves were generated as a function of time after the priming treatment, and the D0 (a measure of thermal tolerance) was calculated as described in "Materials and Methods." Chart 9 depicts the changes in the D0 and in viable colony multiplicity as a function of time following the heat treatment.
suggested by work by Landry and Marceau (16), who found that colonies arising from Hela cells treated at temperatures above 49° did not differ in size from those developed from nonheated controls, whereas at intermediate temperatures (43-45°), single cells developed into colonies smaller than those of nonheated controls. However, colony size at some interval following treatment is a function of both delay and subsequent proliferation rate which were not separated in their study.

The dose-response relationship between division delay and survival at 45.5° was fit by a power function given in Equation A. This indicates that the division delay of viable cells was very sensitive to decreases in cell survival. For instance, we found a 4-fold increase in the division delay of viable cells for a 10-fold decrease in cell survival. Division delay used as an end point with asynchronous cell populations is actually a heterogeneous measurement of the cell cycle progression delays to cytokinesis of cells heated in various compartments of the cell cycle. Therefore, even though there appeared to be a given delay in division for an asynchronous population heated to a given survival, it was really a complicated function of differential delays and subsequent progression rates of cells heated in various phases of the cell cycle. In addition, the slight upward slope of the viable and nonviable multiplicity curves in Chart 1 suggested that a small amount of division, probably in cells heated in G2-M phases, was possible immediately following heating. We evaluated by flow cytometry the progression of viable cells through the cell cycle following 20 min at 45.5°. Westra and Dewey (32) showed previously that S and G2-M cells are more sensitive to hyperthermic cell killing than are G1 cells; data to be published elsewhere (28) indicated virtually all surviving cells given this heat treatment were in the G1 phase at the time of heating. Conversely, the nonviable cells consisted largely of S- and G2-M-phase fractions. Chart 2 indicates that the ultimately clonogenic cells, which were heated in G1, were delayed in G1, to approximately 28 hr posttreatment (7 times greater than control). Subsequently, progression through S and G2-M was slowed to about 20 hr before division (2 times greater than control). Therefore, the main component of division delay of ultimately clonogenic cells at asynchronous survival levels of <10% was delay in G1.

Area integration in Chart 2 indicated that there was a cohort of nonviable cells which progresses from G0 into S and G2-M, in addition to the viable subpopulation, since by 42 hr, 55.1% of the total G0 population (of which only 24.1% of the total were clonogenic) had moved into S and G2-M.

It has been shown previously that nonviable cells are capable of a limited number of divisions before cessation of cell proliferation (14, 33). Chart 6 indicates that division delay of nonviable cells was a strong function of survival. For instance, 33% of the ultimately nonviable cells had not divided 5 days following a hyperthermic treatment which reduced survival to 0.97. In contrast, nearly 68% had not divided when the treatment duration was increased to reduce survival to 0.113. Again, this does not differentiate the cell cycle specificity of division probability of nonviable cells, since asynchronous populations were used. Also, time lapse cinematography indicated that, even though most division had ceased in nonviable cells, they were capable of sustained metabolic activity beyond even 5 days following the treatment.

Unlike postirradiation growth kinetics (5), progression of viable cells through the cell cycle (i.e., generation time) after heating may be delayed in subsequent generations as well as in the first (33). Charts 1 and 7 depict the increased doubling time associated with the viable cell subpopulation as long as 5 days posttreatment. Others (12, 14, 16, 19, 33) have reported previously increased viable population doubling times following various amounts of heat, and all found slower rates from that of control after the division delay.

If the probability of a viable cell to carry out another successful division were 1.0, then the increased doubling time of the viable cells in Chart 1 would actually represent an increased generation time. This would indicate that cell cycle traverse rates were decreased to approximately 20 hr in viable cells. However, the data of Charts 7 and 8 suggest the phenomena of lethal sectoring may take place in viable subclones following hyperthermia. In particular, Chart 8 indicates the phenomena were significant in both proportion of total cells within the clone and duration of occurrence during treatment. If the probability of each viable cell undergoing a successful division to produce 2 viable daughters was reduced to 0.70 as is suggested in Chart 8, then this alone could explain the increased doubling time of the viable population as seen in Chart 1. Straightforward mathematical analysis of viable cell growth equations (see Ref. 5 for derivation) indicated that, even though the population doubling times were approximately 20 hr, the generation time of viable cells may be equal to that of the control, given that the probability of successful division is 0.70 and not 1.0. This appears to be the most probable explanation, since our data (Chart 8) indicated that not only had lethal sectoring occurred but that it occurred at a rate consistent with a division probability of 0.70.

Few data exist on the role viable cell division may play in modulating thermal tolerance decay. Gerner et al. (7) first postulated that tolerance was not directly heritable in HeLa cells, since viable progeny harvested 13 days after a heat treatment exhibited survival responses identical to those of nonpreheated controls. However, Majima and Gerweck (19) have shown recently that CHO cells divided 3 to 5 times before tolerance completely decayed and that, even in the absence of cell division, a certain amount of decay was possible. Also, Nielson (21) found that tolerance decay rates in unshed plateau-phase mouse lung L1, A2 cells essentially parallel to exponentially dividing cultures. Our results suggest that thermal tolerance decay may be modulated slightly by progression of viable cells into heat-sensitive cell cycle phase compartments (Charts 9 and 12, 24 to 48 hr) but that a major role in decay was associated with actual viable cell division (Charts 2 and 9, 48 to 72 hr). Chart 2 indicates that ultimately viable and nonviable cells heated in G1 entered S phase at approximately 28 hr after 20 min at 45.5°. Maximal thermal tolerance was reached at about 24 hr and decreased only slightly by 48 hr. Therefore, most of the development of thermal tolerance under these conditions occurred while the viable cells remained in G1, with only a small decrease corresponding to viable cell redistribution into the S and G2-M phases. The time of greatest decay in thermal tolerance occurred between 48 and 72 hr when the viable cells had resumed division, as seen from Chart 9. These results would be compatible with the idea of protection of heat-sensitive sites by a mechanism which is segregated during cytokinesis, such as a specific protein(s) (17), or that events in mitosis, such as condensation of the chromatin, may play a role in loss of tolerance.

These findings suggest possibilities in formulating treatment
protocols with maximal cytotoxic effects on cycling clonogenic cells. For example, therapeutic advantages may be possible based on exploitation of differential thermal tolerance decay rates between rapidly proliferating tumor tissue and slowly dividing normal tissue.

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