DNA Degradation in Chinese Hamster Ovary Cells after Exposure to Hyperthermia

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ABSTRACT

Chinese hamster ovary cells grown in suspension showed a progressive reduction in the size of their nuclear DNA to 50 to 60S fragments after hyperthermia (43–48°C). This DNA degradation was not a homogeneous response but was observed only in cells incapable of attaching to a substrate after acute heating. The DNA degradation was associated with the inability of cells to exclude the vital stain, trypan blue. The degradation process appeared to be a result of nucleolytic enzyme digestion which accompanies cell necrosis. A similar phenomenon was observed in heated monolayer cells but only after significantly greater time-temperature exposures. Our results show that cellular subpopulations can be separated after hyperthermia and that these subpopulations are biochemically distinct and characterized by different viability.

INTRODUCTION

Exposure of mammalian cells to hyperthermic temperatures has been demonstrated to result both in cytotoxicity and radiosensitization (5). The molecular rationale has not been unambiguously demonstrated for either of these phenomena. If heat-induced cytotoxicity is expected to result from the production of nuclear lesions, as is the case with radiation-induced cytotoxicity (13, 16), it is logical to expect that cell heating should result in alterations in the integrity of nuclear DNA or chromatin structure. While hyperthermic exposure has been observed to produce chromosome aberrations in cells (6, 10), neither DNA (2, 3) nor chromatin structure (17) denaturation has been observed at short time intervals after cell heating with time-temperature exposures that resulted in survival levels of $10^{-2}$ to $10^{-3}$. The purpose of this work was to examine the integrity of nuclear DNA as a function of time after exposure to hyperthermia to determine whether nuclear DNA lesions develop in heated cells at longer times after acute heating.

MATERIALS AND METHODS

Cell Culture. Asynchronous CHO² cells were maintained in exponential growth in monolayer culture with McCoy's Medium 5A (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal and 5% calf serum. Suspension cultures of CHO cells were obtained from trypsinized monolayer cultures and were maintained in exponential growth at 1 to $6 \times 10^5$ cells/ml. For labeling, suspension culture cells were exposed to whole medium containing [methyl-¹⁴C]thymidine (specific activity, 50 mCi/mmol; New England Nuclear, Boston, Mass.) at 0.01 to 0.03 μCi/ml for 18 hr.

Cell Heating. Samples containing approximately $10^7$ cells in 3 ml of whole medium were diluted in 30 ml of preheated whole medium in a precision-controlled water bath. After heating in the water bath, the cells were diluted with 37°C prewarmed medium and placed at 37°C in suspension or monolayer.

Survival Assays. Details of survival assays were reported previously (16). Briefly, cells were counted after hyperthermia with a Model B Coulter Counter (Coulter Electronics, Hialeah, Fla.), diluted and plated in 25-cm² plastic culture flasks at a cell density that would produce 50 to 200 colonies after 7 to 10 days of growth at 37°C. In some experiments, the cells that had not attached to plastic culture flasks by 24 hr after acute heating were collected, counted, and plated. After 7 to 10 days of growth, the plates were fixed with Carnoy's solution and stained with 0.1% crystal violet, and colonies containing more than 50 cells were counted.

Alkaline Elution. The alkaline elution technique used in these studies was similar to that of Kohn et al. (8). Between 1 and $5 \times 10^5$ total cells suspended in PBS were collected on filters (25-mm diameter, 2-μm pore size, polyvinyl chloride; Millipore Corp., Bedford, Mass.) that had been washed with ice-cold PBS. Cells were lysed on the filters at room temperature with 5 ml of 2% sodium dodecyl sulfate-0.02 M EDTA-0.04 M glycine, pH 10.0. The lysis solution had flowed through the filters at unit gravity, the filters were washed with 3 ml of 2% sodium dodecyl sulfate-0.02 M EDTA-0.04 M glycine, pH 10.0, and DNA was eluted with a solution consisting of 0.02 M tetrahydro-EDTA-0.05 M sodium dodecyl sulfate plus tetracyclammonium hydroxide (RSA Corp., Ardsley, N. Y.) added to give a pH of 12.2. The output tube passed through a peristaltic pump to a fraction collector. The pumping rate was $0.4 \text{ ml/min}$. Ten 3-ml fractions were collected and mixed with scintillation fluid containing 0.7% acetic acid to reduce chemiluminescence. Radioactivity remaining on the filters was determined by hydrolyzing the filter with 0.4 ml of 1 N HCl at 70°C for 60 min, followed by 0.5 ml of 2 N NaOH at room temperature for 60 min, and by counting an aliquot of the hydrolysate in scintillation fluid. Radioactivity remaining in the elution apparatus was determined by scintillation counting of a 1-ml aliquot from a 10-ml wash of the apparatus with 0.5 N NaOH.

Alkaline Sucrose Sedimentation. To determine the size of cellular DNA, alkaline sucrose gradient sedimentation was carried out similar to the method of Painter and Young (14). A 0.5-ml aliquot (0.5 to 1.0 $\times 10^6$ cells) was gently layered onto a 0.5-ml lysis solution on top of a linear 5 to 20% alkaline sucrose gradient (36 ml). Lysis was allowed to occur in the dark at 25°C for 3 hr before centrifugation at 27,000 rpm for approximately 2 hr ($ω^2 = 5.3 \text{ rad}^2/\text{sec} \times 10^{10}$) in a Beckman Model L-55 preparative ultracentrifuge (SW 28 rotor) (Beckman Instruments, Fullerton, Calif.). The gradients were fractionated from the bottom (about 2-ml fractions), and the radioactivity present in each fraction was determined by liquid scintillation counting. The gradients were calibrated with λ-phage DNA (40S).

RESULTS

We have reported previously the observation that DNA lesions, as measured by the alkaline elution technique of Kohn...
et al. (8), progressively accumulate in the genome of suspension-cultured HeLa cells during 37°C incubation after acute heating (15). Similarly, CHO cells heated at 45°C for 30 min progressively accumulate damage in nuclear DNA at 37°C postheating [e.g., progressively less DNA radioactivity was retained on polyvinyl chloride filters during elution with pH 12.2 buffer (Charts 1 and 2)]. Maximum DNA damage was expressed in the population by 12 hr with a half-time of 3 to 4 hr.

The size of the DNA, assayed by alkaline sucrose gradient sedimentation at various times after 45°C for 30 min, decreased with increasing incubation times (Chart 3a). This is shown by the progressive movement of DNA radioactivity out of the rapidly sedimenting parental DNA compartment (Fractions 16 to 20) into more slowly sedimenting fragments of DNA. The sedimentation behavior of DNA immediately after heating did not differ significantly from unheated, control cell DNA. By 2 hr, there was a broad distribution of DNA fragment sizes. By 4 to 6 hr, the majority of the DNA that did not sediment with parental DNA was found in Fractions 1 through 5 with apparent sedimentation values of 100S or less. There was little change in the DNA size distribution between 6 and 24 hr (compare Chart 3, a and b) except for an increase in that fraction of DNA that sedimented in Fractions 1 to 4 and that had an apparent sedimentation value of either 50 to 60S or less.

Chart 1. Alkaline elution of cellular DNA at various times after acute cell heating. CHO cells, grown in suspension, were labeled overnight with [3H]thymidine, washed of excess radioactivity, and resuspended in 45°C prewarmed medium for 30 min. After heating, the cells were pelleted and resuspended in whole medium at 37°C and plated in monolayer culture for 0 (O), 1.5 (A), 3 (O), or 24 (•) hr. At each time point, the heated cells and a culture of unheated control cells were pelleted, resuspended in ice-cold 0.9% NaCl solution, and up to 5 x 10⁵ cells were analyzed by alkaline elution. For each time point (0 to 24 hr), we plotted the product of the fraction of total heated cell radioactivity retained on a polyvinyl chloride filter after elution divided by the fraction of total control cell radioactivity retained on the filter after elution. The line was fitted by eye. Bars, S.E.

Chart 2. Alkaline elution of cellular DNA at various times after acute cell heating. CHO cells, grown in suspension, were labeled overnight with [3H]thymidine, washed of excess radioactivity, and resuspended in 45°C preheated medium for 30 min. After heating, the cells were pelleted and resuspended in whole medium at 37°C and plated in monolayer culture for various time periods. At each time point, the heated cells and a culture of unheated control cells were pelleted, resuspended in ice-cold 0.9% NaCl solution, and up to 5 x 10⁵ cells were analyzed by alkaline elution. For each time point (0 to 24 hr), we plotted the product of the fraction of total heated cell radioactivity retained on a polyvinyl chloride filter after elution divided by the fraction of total control cell radioactivity retained on the filter after elution. The line was fitted by eye. Bars, S.E.

Chart 3. Alkaline sucrose gradient analysis of heated cells. CHO cells were cultured in suspension and labeled overnight with [3H]thymidine. The cells were then heated at 45°C for various time periods as described in Chart 1. The cells were placed in monolayer culture for various periods of time prior to analysis by alkaline sucrose gradient sedimentation. For analysis, the cells were pelleted and resuspended in ice-cold 0.9% NaCl solution to approximately 10⁶ cells/ml. Up to 5 x 10⁵ total cells were layered gently onto a 0.5-mL lysis layer over a 36-mL linear 5 to 20% alkaline sucrose gradient. Lysis was allowed to proceed for 3 hr at 25°C in the dark before centrifugation. Centrifugation was for approximately 2 hr (u = 5.4 rad/sec x 10⁵) in an SW28 rotor. The gradients were collected from the bottom of the centrifuge tube, collected onto glass fiber filters by 10% trichloroacetic acid precipitation, and analyzed by liquid scintillation counting. The fraction of total radioactivity found in each gradient fraction is plotted. Direction of sedimentation is from left to right. The lambda phage marker (40S DNA) sedimented to Fraction 2 in the gradients. A, sedimentation profiles of cells analyzed 0 hr (O), 2 hr (A), 4 hr (O), and 6 hr (•) after acute cell heating. B, sedimentation profiles of cells analyzed 24 hr after being heated at 45°C for 0 min (O), 20 min (A), 30 min (O), and 40 min (•).
When the sedimentation behavior of nuclear DNA from heated suspension cells was assayed 24 hr after heating for various time periods of 45°, it was observed that the fraction of nuclear DNA sedimenting faster than the 40S standard decreased with increasing heating times (Chart 3b). The fraction of total DNA sedimenting in Fractions 1 to 6 corresponded reasonably well with the fractional damage estimated under similar conditions with alkaline elution.

In heated monolayer cells, alkaline sucrose gradient sedimentation after heating at 45° for up to 60 min showed a similar reduction in the size of DNA by 24 hr postheating with fragments of approximately 50 to 60S. However, considerably greater time-temperature exposures were required to produce the same level of damage in monolayer as in suspension cultures. After 30 min at 45°, 34 to 35% of total cellular DNA from suspension cells sedimented as 50 to 60S fragments, but only 10 to 12% for monolayer cells.

The extent of DNA damage that occurred by 24 hr after acute heat exposure was characterized by a dose response at various temperatures in suspension cells as measured by alkaline elution (Chart 4). The reciprocal slopes of the response curve (D0) at 45, 46, 47, and 48° were 24, 13, 8, and 3.5 min, respectively. An Arrhenius plot (7) of these data show an activation energy for DNA damage of approximately 128 kcal/mol of DNA at temperatures above 43°.

With increasing time-temperature exposures, an increasing fraction of the heated cells became permeable to the vital stain, trypan blue. Both vital dye uptake and DNA degradation are associated with cell necrosis. Another property associated with cell necrosis, the inability to become or remain attached to a plastic substratum, was also examined. Heating suspension cells at 45° for various times showed a progressive decrease in the fraction of cells able to attach to the plastic substratum within 24 hr postheating (Chart 5). Inasmuch as some cell lysis may have occurred during the course of these experiments, the extent of reattachment may be overestimated. The rate and extent of attachment for S-phase cells (cells pulsed with [3H]thymidine for 30 min immediately prior to heating) was the same as that of the whole population (cells labeled with [14C]thymidine for 18 hr prior to heating). Cells that were capable of attaching to the substratum also showed a time-temperature-dependent increase in the time needed to go from a rounded configuration to a flattened or fibroblastic shape. This latter phenomenon was not quantitated. The dose response for the loss of ability to attach to the substratum within 24 hr is shown for suspension cells in Chart 6. The D0 values at 43, 44, 45, 46, and 47° heating were 40, 23, 14, 8, and 4, respectively. An Arrhenius analysis of these data yields an activation energy for loss of cell attachment of 116 kcal/mol at temperatures above 43°. The majority of suspension cells that

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**Chart 4.** DNA degradation in heated CHO cells. CHO cells, prelabeled with [14C]thymidine, were heated at temperatures between 44 and 46° and placed into monolayer culture at 37°. The cells were assayed 24 hr after heating by alkaline elution as described in Chart 1. The fraction of DNA radioactivity retained on the filters after heating for various times at 48° (•), 47° (○), 46° (△), and 45° (□) was plotted. The average is plotted. Bars, S.D. The lines were fitted by eye.

**Chart 5.** Attachment of heated CHO cells to a substratum. CHO cells, grown in suspension, were pelleted and resuspended in medium preheated to 45° for 0 min (□), 10 min (△), 20 min (○), 30 min (△), 40 min (○), and 60 min (□) before being placed in 5 ml of whole medium at 37° in 25-sq cm flasks. At various times after acute heating, the flasks were collected, and the number of unattached cells (contained in the medium and in a 2.5-ml wash of the flask with PBS) and attached cells (collected by trypsinization of the flask) was determined. The fraction of total cells found attached at various times after heating is plotted. Values are averages of 3 to 5 separate experiments. Bars, S.D. The lines were fitted by eye.

**Chart 6.** Heat inactivation of cell attachment. CHO cells were grown in suspension and heated, as described above, at 47° (□), 46° (△), 45° (○), 44° (△), and 43° (□) for the indicated times. The cells were placed into 25-sq cm flasks and cultured at 37° for 24 hr. The fraction of cells not attached to the flask at 24 hr was determined as described in Chart 5. Values are averages of 3 to 5 separate experiments. Bars, S.D. The lines were fitted by eye.
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did not attach to the substratum after acute heating also became trypan blue stainable by 24 hr.

Monolayer cells heated at 45° showed an increasing fraction of cells that became rounded, although still attached, with increasing heating times. Few of the cells that were heated in monolayer became trypan blue positive by 24 hr, even in cultures exposed to 45° for 60 min. By 60 min at 45°, the majority of the monolayer cells could be detached easily from the flasks in the presence of 1 mM EDTA by tapping the flask. When replated into new medium at 37°, 32, 34, 38, and 42% of the monolayer cells reattached by 1, 2, 5, and 7 hr, respectively.

When the size of nuclear DNA in the floating and attached cells in suspension culture was monitored 24 hr after acute heating, the majority of the DNA damage present in the heated cells was located in the floating population, while the DNA in cells that remained attached sedimented in a manner similar to that for the DNA of an unheated, control population (Chart 7). When these cells were analyzed by alkaline elution, it was observed that the elution profiles for attached cells was similar to those for unheated control cells, while less than 5% of the DNA in the floating cells remained on the alkaline elution filter. Cell survival of heated suspension cells after 45° heating had a quasithreshold dose ($D_0$) of 12.5 and a $D_0$ of 3.3 min. The cells not attached to the plastic substratum by 24 hr postheating (i.e., cells recovered in a 5-ml PBS wash of the flasks at 24 hr) had survival values at least 10 times lower than those observed for the whole population (Chart 8).

**DISCUSSION**

We have reported previously (15) that a reduction in the size of DNA occurs progressively in suspension-cultured HeLa cells after an acute heat shock at 45°. A similar phenomenon has been observed in heated L1210 cells grown in suspension (1). This DNA degradation was not an acute heat-induced nuclear lesion, since it was not immediately observed, but required incubation at 37° for expression. We were unable to determine whether this lesion represented repairable heat-induced damage or an irreversible process in cells committed to necrosis and death.

Suspension cultures of CHO cells, like HeLa cells, show a progressive reduction in DNA size with time after cell heating (Charts 1 and 2). Thus, damage does not result from hyperthermia per se but appears to result from endogenous nucleolytic degradation of nuclear DNA. Intermediate-sized fragments of DNA are generated at short times (2 to 4 hr) after heating (Chart 3), which are ultimately converted into small fragments of DNA by 12 hr postheating in the size range of 40S (equivalent to approximately $10^7$ daltons of single-stranded DNA). The DNA degradation is accompanied by a cellular inability to attach to a plastic substratum (Chart 7). These cells (floaters) become permeable to the vital stain, trypan blue, and have at least a 10 times lower plating efficiency than do cells that are able to attach to the substratum (Chart 8).

The heat sensitivity to 45° of monolayer (7) versus suspension-cultured (Chart 8) CHO cells was similar, within experimental error, in terms of $D_0$ (5.7 and 3.3 min, respectively) but significantly different in terms of $D_0$ (2.1 versus 12.5 min, respectively). We were unable to compare directly the survival of these cells, since a comparable survival assay of monolayer-cultured cells would require a trypsinization step. One explanation for differences in the survival characteristics of suspension cells would be that the survival assay, requiring considerable manipulation of cells, is performed subsequent to cell heating. Thus, the more sensitive suspension-cultured cells (for instance the mitotic cells) may be lysed prior to cell counting and plating for the survival assay.

It seems likely, as stated above, that the DNA degradation observed in heated CHO cells is a result of endogenous nucleolytic enzyme degradation of nuclear DNA. There are a number of possible explanations for this phenomenon, including: (a) repair or excision of a heat-induced DNA lesion; (b) an
aberrant or incomplete DNA repair or replicative enzyme activity; and (c) degradation of DNA by nucleolytic enzymes released from lysosomes. The involvement of repair or excision of heat-induced DNA lesions (such as protein-DNA cross-links or depurinated regions of DNA) or of aberrant repair or replicative functions in this DNA degradation seems unlikely, since virtually no degradation is observed in either monolayer cells or in suspension cells which are able to attach. It is also possible that the DNA degradative process could be produced by nucleolytic enzymes released from heat-damaged or "activated" lysosomes (12). Although we cannot eliminate the possibility of lysosome damage in heated cells, such a phenomenon does not appear to result in the DNA degradation observed in these studies, since a similar process is not observed in reattached suspension cells or monolayer cells. It seems more probable that this DNA-degradative process is part of the macromolecular degradation which accompanies release of lytic enzymes from lysosomes during cell necrosis. In support of this conclusion is the appearance of DNA fragments with approximately the same size during necrosis resulting from exposure to a number of other cytotoxic agents (18).

The significance of the degradation of nuclear DNA to sizes roughly equivalent to the DNA replicative fragments referred to as replicons (approximately 40 to 60S) is unclear. If we assume that we are observing a typical necrotic process induced by heating, this might suggest that either lysosomal enzymes which function in necrosis have some site specificity within nuclear DNA or that the necrotic process induces nuclear enzymes with endonucleolytic functions to degrade DNA. It is clear that the presence of these fragments at short times after acute heating could interfere with DNA strand break repair studies when ionizing radiation is combined with hyperthermia. The failure to control for the presence of these biochemical lesions in cell death. Continued cell heating results in an increasing fraction of the heated suspension cells expressing an early form of cell death (morphological lysis) that is unique to these cells and involves an inability to attach to a substrate (loss of some membrane functions), degradation of nuclear DNA, and ultimately loss of the semi-permeable property of the plasma membrane of the cell (uptake of vital stains). Monolayer and suspension cells are qualitatively similar in their expression of this early type of cell death or lysis. However, quantitatively more time-temperature exposure is required to produce an equal effect in monolayer cells. At equivalent survival levels, we would expect an equivalent degree of cell necrosis to ultimately be expressed in both types of cells. However, in suspension cells, hyperthermic exposure causes cells to express necrosis at an earlier time.

In conclusion, while we observe a reduction in the size of DNA in heated cell populations, we observe no damage in the nucleic acid component of the genome of the cell for up to 24 hr after hyperthermia that cannot be attributed to cell necrosis. CHO cells in culture respond differently to hyperthermic shock, depending on whether they were cultured in suspension or monolayer prior to heating. Suspension-cultured cells have a significantly greater tendency to undergo an early morphological lysis (necrosis) after hyperthermic shock. The results show that cellular subpopulations can be separated after hyperthermia that are biochemically distinct and characterized by different viability. The generation of DNA fragments in heated populations of mammalian cells should be controlled for during any studies of DNA strand break repair when hyperthermia is combined with ionizing radiation.

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