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Reversible Changes in the Nuclear Lamina Induced by Hyperthermia

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Abstract The nuclear matrix (NM) has been identified as a potential target for heat-induced cell killing. Previous studies have shown that heat-shock may significantly modulate lamin B content. Since changes in NM structure have often been accompanied by changes in protein composition, we investigated whether hyperthermia induced changes in nuclear lamina (NL) structure in non-tolerant and thermotolerant cells, and the implications of these changes on cell survival. Using indirect immunofluorescence techniques and confocal microscopy, we found that heating cells at 42 or 45.5°C caused invaginations and other distortions of the peripheral NL. While hyperthermia did not alter the number or structure of internal lamin B foci, heat-induced alterations to the peripheral NL were dose-dependent. Interestingly, NL structure recovered with time after heating in cells that were destined to live or die. Thermotolerant cells heated at 45.5°C showed similar initial changes in the NL compared to non-tolerant cells, but recovery occurred much faster. Taken together, these results suggest that the amount of initial damage to the peripheral NL is not correlated with heat-induced cell killing. However, the possibility that an increased rate of recovery might confer a survival advantage cannot be discounted. J. Cell. Biochem. 86: 451–460, 2002. © 2002 Wiley-Liss, Inc.

Key words: heat shock proteins; nuclear matrix; hyperthermia; confocal microscopy; lamin B

The nuclear matrix (NM) is a lattice of intermediate filament-like proteins which serves to maintain nuclear shape and chromatin architecture as well as provide a framework for the temporal and spatial regulation of DNA replication, transcription, and at least some modes of DNA repair [Berezney et al., 1995]. Many of the polypeptides comprising the NM still await characterization, although many that have been identified to date are believed to be involved in the maintenance of nuclear structure or nucleic acid metabolism.

Despite its central role in the maintenance of nuclear structure and function, the NM has received little attention as a potential target for heat-induced cell killing and heat-radiosensitization. The NM is very heat-labile compared to other organelles. Recent data suggest that a subset of nuclear matrix proteins (NMPs) is subject to denaturation after heat-shock [Lepock et al., 2001]. Upon denaturation, an increased exposure of hydrophobic domains may render NMPs more susceptible to binding and aggregation of both native and denatured nuclear proteins. Though direct evidence is still lacking, several reports suggest both direct or indirect roles for the NM or specific NMPs in heat cytotoxicity [Roti Roti et al., 1997, 1998]. For example, hyperthermia causes dose-dependent changes in NM composition and NMP content [Dynlacht et al., 1999, 2000; Zhu et al., 1999] and architecture [Wachsberger and Coss, 1993]. Recovery of ultrastructural alterations of the NM may also occur, and appears to be correlated with cell killing and resumption of RNA synthesis [Wachsberger and Coss, 1994]. In addition, while it is well known that heat causes enhanced binding of nuclear proteins to the NM, VanderWaal et al. [2001] recently showed that enhanced binding of specific proteins involved in DNA replication correlated well with lethality of heat-sensitive S-phase cells. Hyperthermia inhibits DNA synthesis and transcription [Henle and Leeper, 1979; Wong and Dewey,

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1982], and it is conceivable that structural changes in the NM, as well as changes in the abundance and conformation of specific NMPs, could be critical for the continuation or resumption of DNA metabolism and RNA processing after heat shock.

The nuclear lamina (NL) anchors the internal NM to the inner nuclear membrane [Schindler et al., 1985), and dictates the overall shape and size of the NM. The NL is principally composed of lamins A, B, and C. The lamins are self-polymerizing proteins that account for up to 73% of the protein mass of the NL [Krachmaraov et al., 1986]. Recent studies implicate lamins (particularly lamin B, 67 kDa) in chromatin organization, nuclear growth, anchorage, and spatial arrangement of nuclear pore complexes [Stuurman et al., 1998]. Lamin B has been identified as a MAR binding protein [Luderus et al., 1992; Belmont et al., 1993], in turn implicating the NL as a key structural component of chromatin architecture.

In addition to their localization at the nuclear periphery, lamins may be localized in a diffuse or spotted pattern within the nucleoplasm [Goldman et al., 1992; Bridger et al., 1993; Moir and Goldman, 1993; Moir et al., 1994, 2000a], as components of filamentous "cable" structures aligned with regions of heterochromatin, or assembled into transnuclear tube-like structures that extend deep into the nucleoplasm [Hozak et al., 1995; Fricker et al., 1997; Broers et al., 1999]. Nucleoplasmic foci are more prevalent in G_1 and S-phase cells, but they decrease in number as cells progress toward S phase. There has been some speculation that A- or B-type lamin foci appearing in early G_1 represent sites of assembly of newly synthesized lamins, which will be required for nuclear growth after division [Goldman et al., 1992; Bridger et al., 1993; Moir et al., 1994, 2000a]. The foci may represent clusters of lamins awaiting various post-translational modifications prior to becoming incorporated into the lamina, such as isoprenylation, phosphorylation, and methylation [Lutz et al., 1992]. Nucleoplasmic lamin B foci colocalize during mid-late S phase with sites of DNA replication as visualized by BrdU incorporation, and co-align with proliferating cell nuclear antigen (PCNA) [O'Keefe et al., 1992; Moir et al., 1994]. Recently, the nuclear lamins were found to be required for the progression of DNA replication during S phase [Moir et al., 2000b].

Lamin B may account for over 1% of total nuclear protein [Verheijen et al., 1988; Paddy et al., 1990]. Because of its abundance and importance in maintaining NM structure and function, and because the protein was recently identified as a prompt heat shock protein [Dynlacht et al., 1999], lamin B has been investigated extensively in our laboratory as a determinant for heat-induced cell killing. In the present confocal microscopic study, we used an antibody against lamin B as a probe to determine the effects of hyperthermia on NL structure and whether changes in NL structure represent a critical step in thermal killing.

MATERIALS AND METHODS

Cell Culture and Heat Treatments

Human U-1 melanoma cells were maintained in monolayer in 75-cm^2 tissue culture flasks containing McCoy's 5A medium supplemented with 10% iron-supplemented calf serum (Hyclone, Inc., Logan, Utah). Stock cultures were grown in a humidified incubator (95% air, 5% CO_2) at 37°C. Two or 3 days prior to each experiment, 2×10^4 or 10^4 cells, respectively, were plated into each well of a 4-well sterile covered tissue culture chamber slide (Lab-Tek, #1 borosilicate coverglass, Nalgene Nunc Int., Naperville, IL) containing 1 ml of McCoy's 5A medium per well and returned to the incubator. On the day of the experiment, medium in the wells of each sample was aspirated and replaced with fresh medium (equilibrated to 37°C) 1 h prior to heating. Cells were then heated for various times in either a 45.5 or 42°C water bath using a specially designed stainless steel support which allowed the slide and half of the chamber to be submerged in the water. The temperature of the medium within each of the wells (as determined using a thermistor probe) reached 45.5°C ($\pm$ 0.2°C) within 3 min or reached 42°C ($\pm$ 0.4°C) within 4.5 min after the chamber slides were placed into the water bath, after which time the medium remained steady at that temperature for the duration of heating. Following heating, the cells were either returned to the incubator or fixed immediately. For treatment of thermotolerant cells, 2 days prior to the induction of thermotolerance, 2×10^4 cells were plated into each well of the chamber slides and returned to the incubator. The medium was aspirated and replaced with fresh 37°C medium and the slides returned to

the incubator 1 h prior to heating cells for 5 min at 45.5°C. Cells were then returned to the incubator for 15 h before the second heat challenge. Methods for measuring clonogenic survival using a colony-forming assay have been described previously [Zhu et al., 1999].

Fixation, Staining, and Confocal Microscopy

After heating and/or incubating the cells for the desired length of time, the medium was aspirated and the wells immediately flooded with ice-cold 57% ethanol in phosphate buffered saline (PBS). Slides were then placed in a 4°C refrigerator overnight. The following day, while the cells were on ice, the fixative was aspirated and the cells were washed twice with TGT buffer [containing 20% goat serum (JRH Biosciences, Lenexa, KS) and 0.01% Triton X-100 in Tris buffered saline (TBS; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl)]. Following the second TGT wash, the wells were filled with TGT buffer and the slides placed on ice for 10 min. The TGT buffer was aspirated and 200 μl of fresh buffer was added back to each well. Then 4 μl of lamin B primary antibody (clone 101-B7, 100 μg/ml, Oncogene Research Products, Boston, MA) was added to each well and the slides were placed on ice for 1 h. Slides were agitated gently every 15 min. The buffer containing the primary antibody was then aspirated and the cells were washed twice with TGT buffer. Then, 200 μl of TGT buffer containing 4 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Caltag Laboratories, Burlingame, CA) was added back to each well, and the slides were placed on ice for 1 h in the dark. Slides were agitated every 15 min. The cells were washed three times with cold TBS and finally resuspended in 500 μl of TBS. Slides were covered with foil and held on ice during the few minutes preceding analysis of the NL and lamin B foci, based on lamin B immunofluorescence.

The cells were examined using a Bio-Rad 1024 MRC laser-scanning confocal microscope equipped with a krypton argon laser set at 488 nm. Midplane and z-series sections were obtained. Sections were imaged using a 60× or 100× 1.4 NA oil immersion objective, and an FITC filter set (522 nm/35-nm bandpass). Sequential z-series images were obtained with a step size of 0.5 μm. Each optical section was the result of four scans.

Criteria for Scoring Aberrant NL

On an average, at least 100 representative cells were scored from 9 to 10 different representative fields per treatment. The NL was scored as abnormal if the NL displayed evidence of any of the following characteristics: rough or rippled peripheral appearance, discontinuous borders, invaginations, herniations, bulges, blebs, or buds. The NL was also deemed abnormal if it was lobulated, adopted a "kidney bean" or otherwise irregular shape, or if it appeared to be collapsed from the nuclear membrane. For each experiment, the NL was observed in an unheated control population of cells. Using these strict criteria, we found that a significant number of U-1 melanoma cells were scored as abnormal even in unheated control populations. While some control cells were dysmorphic or irregularly shaped, in general the abnormalities were less severe compared to heated cells. On an average, approximately 20% of unheated control cells were deemed to contain an abnormal NL. In every experiment, data were normalized to take into account the percentage of cells containing an abnormal NL in untreated cells. For all figures in which the percentage of cells with a normal NL in treated samples is plotted relative to the percentage in untreated control cells as a function of time during or after heating, the number of cells with a normal NL from each sample was divided by the total number of cells scored to obtain the percentage of cells with a normal NL. The percentage of cells with a normal NL for each sample was then divided by the percentage of unheated control cells with a normal NL. In control (unheated) cells, an entire continuous peripheral NL was usually found to be visible in the mid-plane optical section when examined with the confocal microscope. However, in order to observe a continuous peripheral NL in heated cells, it was often necessary to examine four–five sections above or below the mid-plane section.

RESULTS

Normally, the NL of unheated U-1 human melanoma cells, when probed with a monoclonal antibody against lamin B, is readily distinguished by distinct perinuclear rim staining (Fig. 1A). Nucleoplasmic foci are often evident, and peripheral staining density may appear uneven or heterogeneous in some cells. These characteristics of the normal NL have been

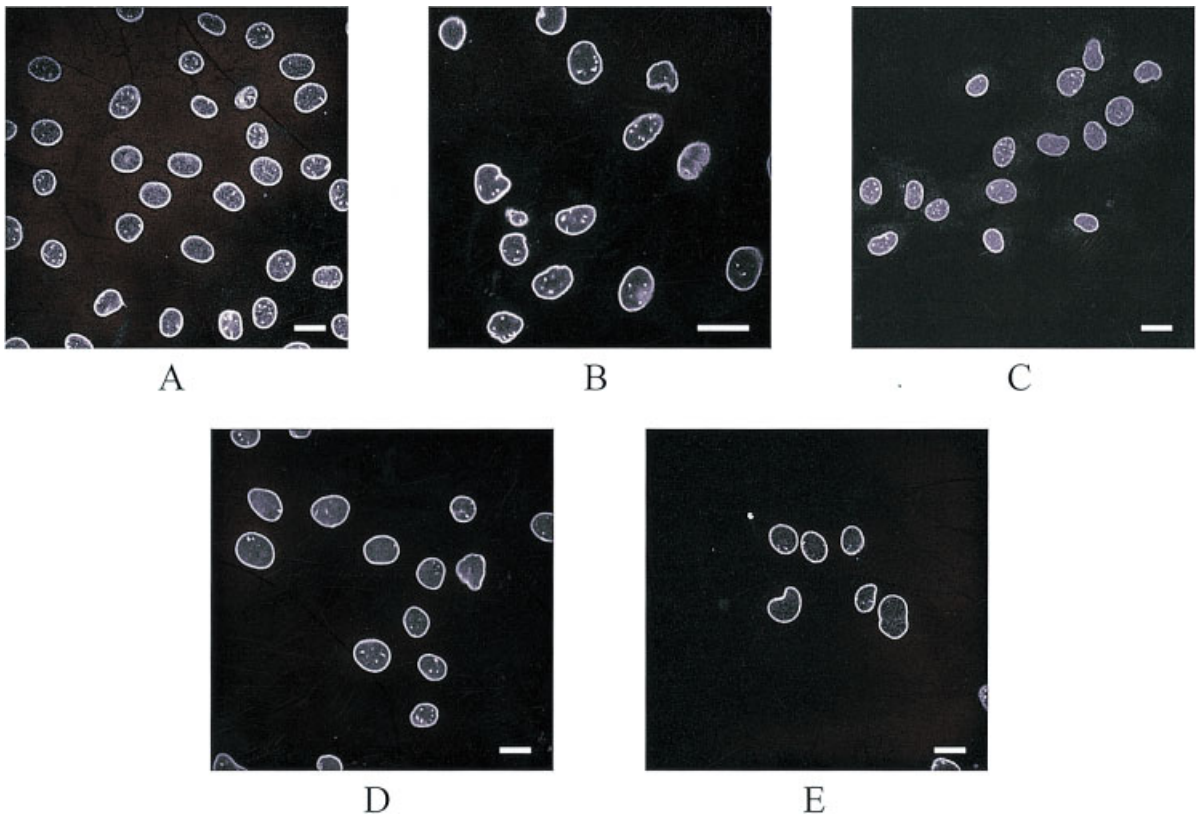

Fig. 1. Heat-induced abnormalities in the nuclear lamina (NL) structure of U-1 melanoma cells. Cells were heated for 20 min at 45.5°C and then incubated for various times at 37°C prior to fixation, staining, and visualization of the NL using monoclonal antibodies against lamin B as described in Materials and Methods. (**A**) Unheated control; (**B**) heat only; (**C**) heat + 3 h at 37°C; (**D**) heat + 4.5 h at 37°C; and (**E**) heat + 7 h at 37°C. Images in panels A, C, D, and E were acquired using a 60 × objective, while the image in panel B was acquired using a 100 × objective. Bar, 20 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

documented by others studying mammalian cells [Goldman et al., 1992; Bridger et al., 1993; Moir and Goldman, 1993; Moir et al., 1994, 2000a].

In unheated samples of U-1 cells grown in monolayer, we found that about 20% of nuclei have an aberrant NL (see Materials and Methods, for a more detailed description of criteria for distinguishing between a normal and an abnormal NL). We observed that heat induced an increased frequency of abnormalities in the NL of cells. Shown in Figure 1 are mid-plane optical sections of cells that were heated for 20 min at 45.5°C and incubated at 37°C for various times thereafter, prior to fixation and staining. Several cells with an abnormal NL are apparent immediately after heating (Fig. 1B) and up to 7 h after treatment (Fig. 1C–E). Examples of cells with an aberrant NL are shown in Figure 2, which contains a complete series of optical sections of a single representative field of cells which had been heated for 20 min at 45.5°C and allowed to incubate for 1 h at 37°C. Inspection of consecutive sections of individual fields of heated cells, obtained using a 0.5-μm step size, was often necessary to confirm the existence of heat-induced invaginations of the peripheral lamina and distinguish them from nucleoplasmic foci distributed along the nuclear periphery. The predominant deformity evident in the NL of heated cells was the irregular lobulated "kidney shape," but a significant fraction of cells possessed one or more invaginations of the lamina (see Figs. 1and 2). In a small percentage of cells, invaginations appeared to bisect the nucleus.

Using the criteria mentioned previously, we performed a quantitative analysis to determine the effects of heat shock on the NL. After the cells were exposed to a mild hyperthermia treatment (5 min at 45.5°C), the fraction of cells with a normal NL decreased by approximately 20% (Fig. 3) compared to unheated cells. The percentage of cells with a normal NL continued

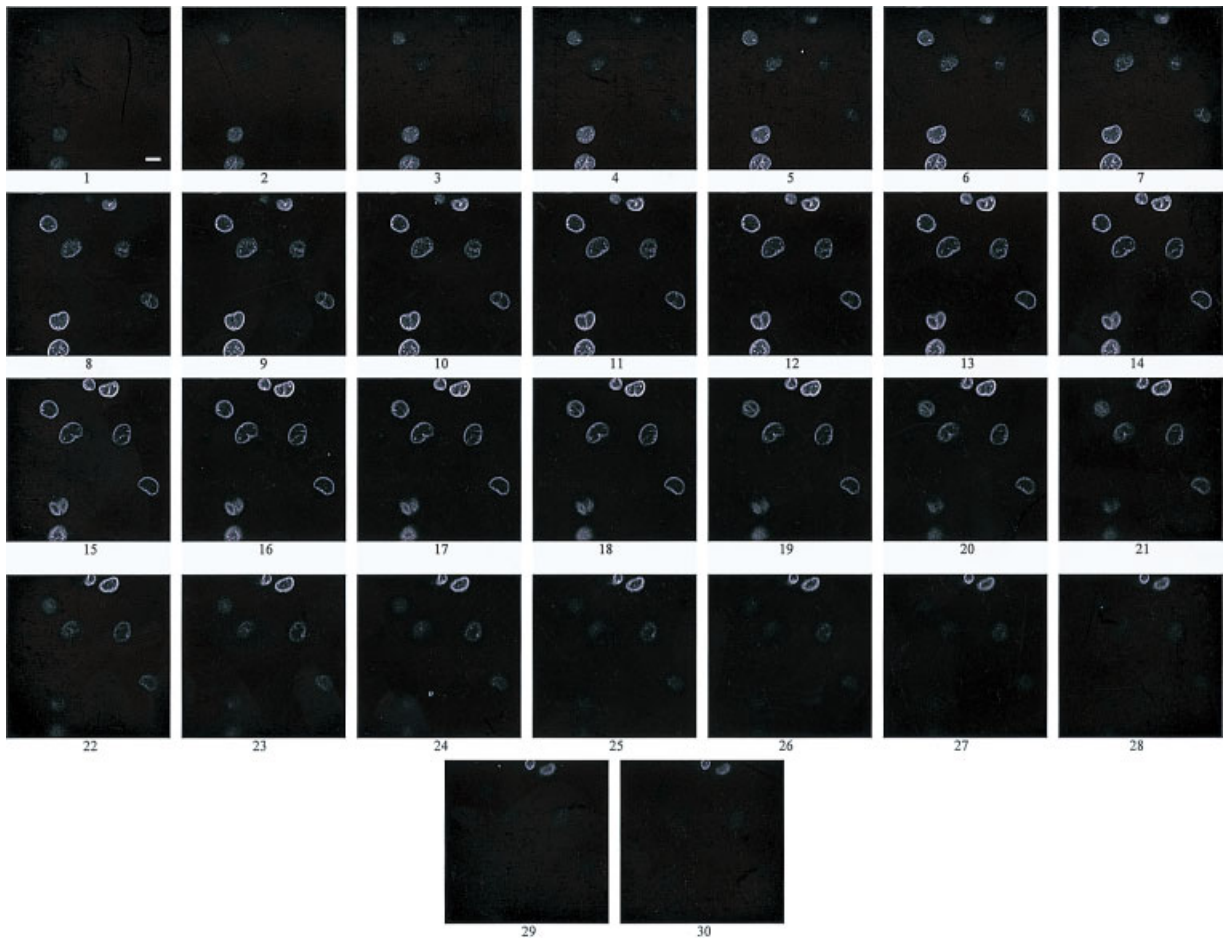

Fig. 2. A complete series of optical sections of a single representative field of cells heated for 20 min at 45.5°C and allowed to incubate for 1 h at 37°C prior to fixation and staining. Consecutive sections of the field were obtained using a 0.5-μm step size. Bar, 20 μM (see first panel). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to decrease in a dose-dependent manner when cells were heated for an additional 5–15 min. However, there was no significant difference in the number of cells with a normal NL after 20–30 min of heating (37–40% of cells contained a normal NL, respectively).

Interestingly, cells heated for 20 min at 45.5°C showed a recovery of NL structure with time after heating (Fig. 4). Within 12 h after heating, only 30% of cells had an abnormal NL, compared to approximately 63% of cells when fixed immediately after heating. Thus, recovery of normal NL structure was observed to occur in approximately 50% of cells that contained an abnormal NL. This finding was somewhat unexpected, since the surviving fraction was reduced nearly 100-fold after a 20-min heat dose (Fig. 5).

Cells in which thermotolerance had been induced were more resistant to heat killing (Fig. 5). Cells rendered thermotolerant and then heated for 20 min at 45.5°C showed a similar decrease in the percentage of cells with a normal NL compared with non-tolerant cells (Fig. 4). While recovery of NL structure was observed to occur in both non-tolerant and thermotolerant cells, and ultimately to the same extent during the 12 h period of observation, the kinetics of NL recovery was different. Recovery of NL structure began immediately after the heat treatment in thermotolerant cells, and no further increase in the percentage of cells with a normal NL was observed after 3 h of incubation at 37°C. In contrast, the same extent of recovery of the NL of non-tolerant cells did not occur until approximately 12 h after the heat treatment.

Chronic heating at a lower temperature also induced an increased frequency of abnormalities in the NL (Fig. 6). After 3 h of heating at 42°C, the number of cells with a normal NL had

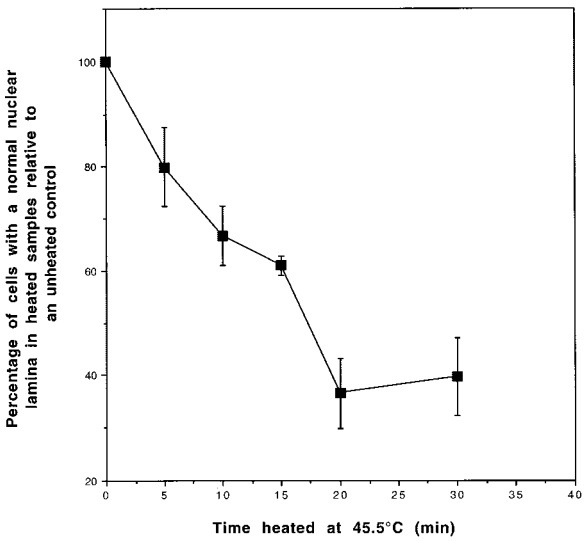

Fig. 3. Morphometric analysis of heat-induced changes in the NL. Cells were heated for various times at 45.5°C and then immediately fixed, stained, and prepared for confocal microscopy as described in Materials and Methods. Criteria for distinguishing cells with a normal NL from those with an abnormal NL are also described in the Materials and Methods. Error bars represent the SE of the mean (SEM) for four–five individual experiments.

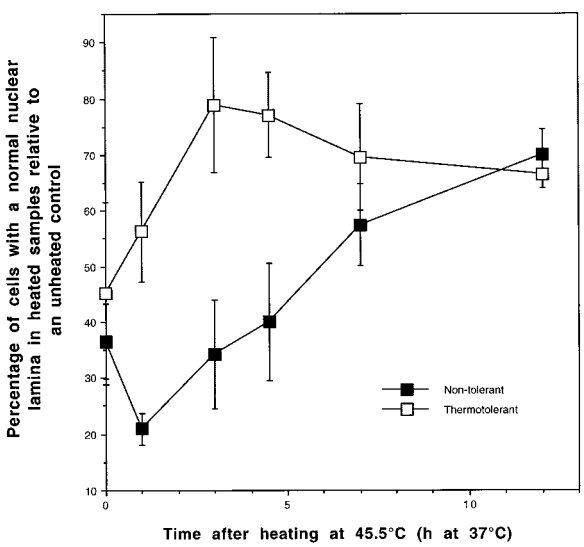

Fig. 4. Recovery of the NL in non-tolerant and thermotolerant cells after heat-shock. Cells were heated for 20 min at 45.5°C and then incubated for various times at 37°C prior to fixation and staining, and confocal microscopic observation as described in Materials and Methods. Thermotolerance was induced by heating cells for 5 min at 45.5°C and then incubating them for 15 h at 37°C prior to re-heating for the times indicated. Error bars represent the SEM for four–eight individual experiments.

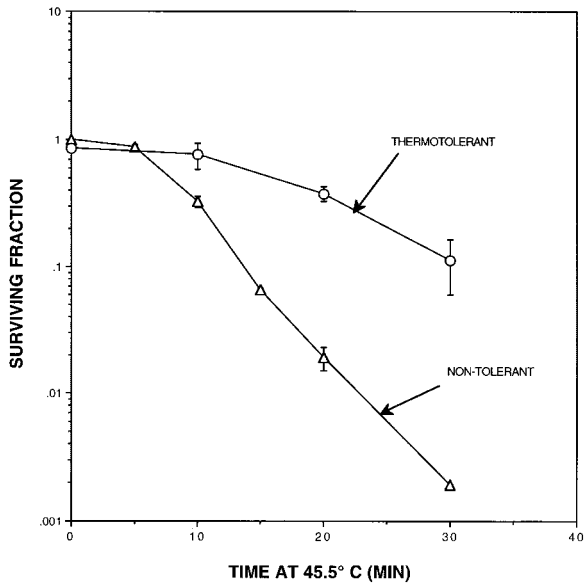

Fig. 5. Survival of asynchronous non-tolerant or thermotolerant U-1 cells heated in monolayer at 45.5°C. Thermotolerance was induced by heating for 5 min at 45.5°C followed by incubation at 37°C for 15 h prior to re-heating.

decreased to approximately 68% relative to unheated control cells. After 5 h at 42°C, the percentage of cells with an abnormal NL (~ 44%) was only slightly higher compared to

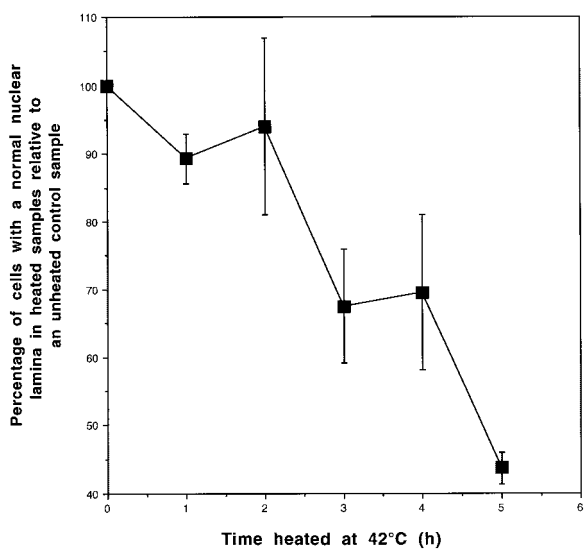

Fig. 6. Morphometric analysis of changes in the NL of cells heated at 42°C. Cells were heated for various times at 42°C and then immediately fixed, stained, and prepared for confocal microscopy as described in Materials and Methods. Criteria for distinguishing cells with a normal NL from those with an abnormal NL are described in the Materials and Methods. Error bars represent the SE of the mean (SEM) for four–five individual experiments.

the percentage of cells that had an abnormal NL after treatment for 20 min at 45.5°C (37%), despite the fact that there was a 10-fold difference in the surviving fraction for the two heat treatments [surviving fraction = 0.02 for 20 min at 45.5°C (see Fig. 5) versus a surviving fraction of 0.2 for cells heated for 5 h at 42°C; data not shown].

The number of lamin B foci scored per cell varied considerably in unheated cell populations. The number of foci per cell ranged from 0–10, possibly due to the fact that the cells were asynchronous. Cells were categorized as having either 0–3, 4–6, or 7–10 foci. In unheated samples, ~84% of cells contained up to three foci, while ~14% contained four–six foci. Only ~3% of cells contained 7–10 foci. While the percentage of cells with a normal NL decreased as a function of time of heating at 42 or 45.5°C, the number or size of foci in each cell did not change when cells were heated up to 5 h at 42°C or 30 min at 45.5°C and fixed immediately thereafter, nor did their distribution within nuclei. Furthermore, when cells were heated at 45.5°C and then incubated as long as for 12 h at 37°C after heating, no significant differences in the number or distribution of foci were observed when compared to the unheated control (not shown). There was also no difference in the number of foci observed between non-tolerant and thermotolerant heated cells.

DISCUSSION

It has been well established that the NM plays an integral role in the structural and functional aspects of nuclear organization and nucleic acid metabolism. Thus, any biochemical or structural alterations of the NM might be expected to interfere with continued cellular viability. The NL appears to be involved in maintenance of nuclear structure, cell-cycle progression, DNA replication, and gene expression [Moir et al., 2000a]. Previous studies indicated that one of the main protein components of the NL, lamin B, increases abruptly when cells are heated at 45.5°C [Dynlacht et al., 1999; Zhu et al., 1999]. Since the NL is a dynamic entity that undergoes growth and/or restructuring during the different phases of the cell cycle, we speculated that changes in NL structure might occur concomitantly with the increase in lamin B content during or after heat shock, possibly as a result of incorporation of newly synthesized lamin B. We reasoned that such changes could readily be observed by staining cells with an antibody against lamin B. Thus, in order to gain a better understanding of the effects of hyperthermia on the NL and determine whether the NL is a target for heat-induced cell killing, we used confocal microscopy to study alterations in the distribution of lamin B as an indicator of changes in the peripheral NL induced by heat-shock, and correlated our results with cell survival. We also sought to determine whether hyperthermia would modulate the number of nucleoplasmic lamin B foci in cells.

Heating at 42 or 45.5°C induced a dose-dependent increase in the number of cells with an aberrant NL (Figs. 3 and 6). Though the frequency of cells with an aberrant NL was increased, it is noteworthy that heat did not induce any aberrations that were unique and which could not be found in a minority of unheated control cells. However, we generally noted that the invaginations were more severe in heated cells than in unheated cells. Since lamin polymerization has been implicated in the maintenance of nuclear shape during interphase, it is attractive to speculate that heat-induced changes in NL structure, at least in part, may be a result of localized depolymerization of the NL, possibly attributed to misregulation of filament length or width, or bundling [see Moir et al., 2000a], or denaturation/aggregation of NMPs themselves [Lepock et al., 2001]. Conversely, however, as there is a correlation between lamin expression and polymerization and both irregularities in nuclear shape and mechanical stability of the nucleus [Moir et al., 2000c], the heat-induced modulation of lamin B content itself could also conceivably have an impact on NL structure.

When thermotolerant or non-tolerant cells were heated for 20 min at 45.5°C, both populations contained approximately the same percentage of cells with structural alterations of the NL (Fig. 4). Interestingly, the heat-induced aberrations in the NL of both non-tolerant and thermotolerant cells appeared to be reversible, as the number of cells with an abnormal NL decreased with time if the cells were allowed to incubate at 37°C after treatment (Fig. 4). However, recovery of NL structure in thermotolerant cells occurred more rapidly as compared to non-tolerant cells. Within 3 h after the heat treatment, the percentage of thermotolerant cells with an abnormal NL was equivalent to

the percentage of cells found in the unheated control (~20% abnormal). Recovery of NL structure in non-tolerant cells continued throughout the 12-h period of observation. A comparison of the data in Figures 4 and 5 suggest that while recovery of the NL will occur in ~50% of cells heated for 20 min at 45.5°C, only one in 100 cells will survive such a severe heat dose. Thus, it is readily apparent that NL recovery can and does occur in non-clonogenic cells.

Using resinless section electron microscopy, Wachsberger and Coss [1994] noted that a recovery of NM ultrastructure occurred in cells after alterations were induced by heating at either 43 or 45°C. They postulated that "repair" of NM ultrastructure is dependent upon the removal of denatured and/or aggregated nuclear protein that precipitates onto the NM after heat shock. By measuring the number of "fiber anastomosing points per unit area per NM," and the length of fibers between points of anastamoses within individual NMs, they found that recovery within 20 h after heating correlated with the magnitude of cell killing. However, in contrast to our findings, no recovery was noted when the surviving fraction was lower than ~0.02.

There is a prevalent view that new lamin substructures can be formed in a stage-specific manner, depending on the functional requirements of the cell [Jagatheesan et al., 1999]. For example, an increased synthesis in S phase may be related to the expansion of the nucleus concomitant with DNA synthesis. A role for lamin B in the assembly of replication foci has been proposed, as nucleoplasmic lamin B foci colocalize with PCNA and sites of DNA replication [Ellis et al., 1997; Moir et al., 2000b]. Indeed, it has been proposed that the lamins, notably lamin B, may act as a scaffold upon which replication factors are organized and the elongation and replication complexes assembled [Moir et al., 1994; Spann et al., 1997]. Thus, the formation and maintenance of nucleoplasmic foci may be critical for restructuring of the peripheral NL as well as S-phase progression. Data from Goldman and co-workers indicate that nucleoplasmic lamin focus formation may be a prerequisite for the association of lamins with the endogenous NL [Moir and Goldman, 1993; Moir et al., 1994, 2000b].

DNA replication is severely inhibited in heated cells [Wong and Dewey, 1982; Warters and Stone, 1983; Warters, 1988; Wong et al., 1989a,b; Warters et al., 1993]. Delaying S-phase progression enhances the survival of cells heated in S phase [VanderWaal et al., 2001]. Disruption of the nucleoplasmic organization of lamins may lead to the reversible arrest of DNA synthesis, specifically at the elongation phase. We wished to determine whether hyperthermia induced changes in the number of nucleoplasmic foci per cell, and whether there was a significant change in the size or appearance of the foci, since we reasoned that a heat-induced disruption of lamin foci could confer a survival advantage [Moir et al., 2000b] by delaying S-phase progression. We found that the number of cells with either 0–3, 4–6, or 7–10 nucleoplasmic lamin B foci did not change during or after heating cells from 5–20 min at 45.5°C, nor did the overall size of the foci (data not shown). Thus, we could not attribute the heat-induced inhibition of DNA synthesis to disruption of foci or inhibition of foci formation.

In summary, our data indicate that heating at 42–45.5°C induces changes in the NL of non-tolerant and thermotolerant human melanoma cells, but these changes are reversible and the rate of recovery is enhanced in thermotolerant cells. Because recovery is also noted in non-viable cells, there appears to be no correlation between changes in NL structure and heat cytotoxicity. However, an association between an increased rate of recovery of NL structure (as noted in thermotolerant cells) and enhanced survival cannot be discounted.

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