Published in final edited form as:

Crit Rev Eukaryot Gene Expr. 2009 ; 19(3): 235–251.

Molecular Parameters of Hyperthermia for Radiosensitization

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Abstract

Hyperthermia is a potent sensitizer of cell killing by ionizing radiation (IR), however, the precise mechanism of heat-induced cell death is not yet clear. Radiosensitization can be attributed to the fact that heat is a pleiotropic damaging agent, affecting multiple cell components to varying degrees by altering protein structures, thus influencing the DNA damage response. Hyperthermia alone induces several steps associated with IR signaling in cells. For example, hyperthermia enhances ATM kinase activity and increases cellular ATM autophosphorylation. This prior activation of ATM or other components of the IR-induced signaling pathway by heat interferes with the normal IR-induced signaling required for chromosomal DNA double-strand break repair, thus resulting in increased cell killing post irradiation. Hyperthermia also induces heat shock protein 70 (HSP70) synthesis and enhances telomerase activity. HSP70 expression is associated with radioresistance. Inactivation of HSP70 and telomerase increases residual DNA DSBs post IR exposure, which correlates with increased cell killing, supporting the role of HSP70 and telomerase in IR-induced DNA damage repair. Thus, hyperthermia influences several molecular parameters involved in sensitizing tumor cells to radiation and can enhance the potential of targeted radiotherapy.

Keywords

heat shock; HSP70; transcription; ATM; DNA damage repair; signaling

I. INTRODUCTION

Hyperthermia is a therapeutic procedure used to raise the temperature of a region of the body affected by cancer. It is a potent radiosensitizer that has been under clinical investigation as a means to improve the response to ionizing radiation (IR)-based cancer treatments that acts to improve the local tumor control.¹ In applications involving external tumors, hyperthermia has shown promising and positive results in phase II–III trials. Hyperthermia itself has several cellular effects that should be synergistic with IR-induced tumor cell killing.^{2,3} For example, unlike the IR response, neither hypoxic nor plateau-phase cells are resistant to heat-induced cell killing.^{4,5} Despite the complementary nature of hyperthermia- and IR-induced cell killing, the precise mechanism of heat-induced cell death is not yet clear, and the synergistic interaction between heat and IR in cell killing is even less well understood. Because phase III clinical trials have shown significant benefits from adding hyperthermia to radiotherapy regimens for a number of malignancies,^{6,7} understanding the mechanisms involved in heat-mediated IR sensitization has become

clinically important. This review will focus on the possible role of hyperthermia in DNA damage response and transcriptional regulation of heat shock proteins in the context of enhancing the potential of targeted radiotherapy.

II. HEAT SHOCK AND DNA DAMAGE RESPONSE

Heat is a pleiotropic damaging physical agent that affects several cellular components to varying degrees; altering protein metabolism, thus affecting the assembly and stability of critical macromolecular complexes. Because of such effects, heat is such a potent radiosensitizer, presumably by interfering DNA damage response (DDR).

II.A. Heat Activates Ataxia-Telangiectasia Mutated (ATM) Protein and its Effectors

The ataxia-telangiectasia mutated gene product (ATM), whose loss of function is responsible for ataxia-telangiectasia disease, is a protein kinase that interacts with several substrates and is implicated in mitogenic signal transduction, chromosome condensation, meiotic recombination, cell-cycle control, and telomere maintenance. The ATM protein kinase is primarily activated in response to DNA DSBs caused by IR or radiomimetic drugs. Unstressed cells contain inactive ATM in a dimer or higher-order multimer form. Several studies suggest that chromatin alterations induce rapid autophosphorylation of ATM at Ser1981 in human cells, which causes dimer dissociation and initiates cellular ATM kinase activity.^{8,9} Heat is known to induce chromatin alterations¹⁰⁻¹³ and recent findings reveal that heat-induced chromatin alterations are similar to those induced by IR exposure in context of ATM autophosphorylation and some of the ATM-regulated signaling pathways.¹⁴ Heat shock induced ATM autophosphorylation in conserved as it was found both in human and mouse cells.¹⁴ Interestingly, deficiency of Hsp70.1/3 in mouse cell lines had an insignificant effect on heat- or IR-induced ATM autophosphorylation. Furthermore, Hunt and coworkers reported that heat treatment combined with IR, did not affect ATM autophosphorylation in any obvious fashion.¹⁴

In addition, heat-induced ATM autophosphorylation was reported to be independent of Mre11 status because the Mre11 complex has been shown to facilitate ATM activation on DNA damage.^{15,16} This was evident by the fact that heat shock was able to induce ATM autophosphorylation in A-TLD1 cells, which are deficient for Mre11 function.¹⁴ These results suggest that heat-induced ATM autophosphorylation is independent of the Mre11-transduced DNA damage signal. Consistent with the heat-induced autophosphorylation of ATM, heat treatment also enhanced ATM kinase activity as was determined by a cell-free kinase assay using either glutathione S-transferase (GST)-p53 or GST-Abl (HP) as substrates.¹⁴ It has been further established that heat treatment could activate ATM only in cellular context because when the purified ATM was heated at 43° C for 15 min in a cell-free system, the purified ATM resulted in the loss of its ability to phosphorylate a p53 substrate,¹⁴ arguing that heat does not directly enhance ATM activation.

Activation of ATM is correlated with DNA damage response.^{17,18} Several major key components of the genome surveillance network are activated by DNA double-strand breaks. Both 53BP1 and SMC1 become progressively, yet transiently, immobilized on chromatin adjacent to double-strand break within minutes of DNA damage.¹⁹ Although heat enhances ATM kinase activity and autophosphorylation, the phosphorylated ATM did not form discrete foci.¹⁴ Heat shock resulted in the foci formation for H2AX and MDC1 but not for 53BP1 and SMC1, suggesting that heat may not have a uniform effect on all the downstream targets of ATM.¹⁴

II.B. Heat-Induced γ -H2AX Foci Is ATM Dependent

Several studies have shown that heat shock induces γ -H2AX foci similar to IR exposure. γ -H2AX foci formation after IR exposure occur in both *Atm*^{+/+} and *Atm*^{-/-} mouse cells; however, IR-induced phosphorylation of H2AX is delayed in *Atm*^{-/-} cells as was determined by both foci appearance and Western blot analysis.¹⁹ In the absence of ATM, there is a relative delay in both the initial formation and subsequent disappearance of γ -H2AX foci. The kinetics, but not the overall level, of IR-induced γ -H2AX foci formation is therefore dependent on ATM status. Although heat was shown to induce phosphorylation of histone H2AX in mammalian cells,²⁰ such phosphorylation has been shown to be dependent upon ATM.¹⁴ The Hsp70 status did not affect γ -H2AX foci formation following heat treatment or IR exposure.¹⁴

II.C. Moderate Hyperthermia Does Not Induce Detectable DNA Strand Breaks

Cell killing has been linked with the failure in DNA DSB repair. There has been a great debate on whether heat induces DNA strand breaks. Hunt and coworkers used three separate techniques to examine whether heat, like IR, causes direct production of chromosomal DNA strand breaks.¹⁴ Pulsed-field gel electrophoresis analysis of cells treated with heat at 43° C for 30 or 60 min did not show any induction of chromosomal DNA strand breaks.¹⁴ Heat treatment before radiation exposure also had no significant effect on chromosomal DNA strand break induction by IR.¹⁴ Hunt and coworkers also used the DNA unwinding-rewinding (halo) assay to determine if heat shock can cause a change in the halo (nucleoid) diameter, an indirect measure of DNA damage.²¹ The halo diameter was maximum at 7.5 Ag/mL concentration of propidium iodide.¹⁴ Halo diameter increased due to the unwinding of the DNA supercoils. Using the Halo assay, the authors observed that heat shock caused a change in the nucleoid halo diameter, but no inhibition of DNA rewinding was observed, which is consistent with an increase in nuclear matrix-DNA anchoring and the absence of DNA strand breaks induced by hyperthermia.¹⁴ In contrast, IR exposure results in a significant inhibition of DNA loop rewinding, but heat shock actually enhances DNA loop rewinding. Moreover, the nucleoid diameter (which reflects the length of the DNA loops at maximum relaxation) was reduced after heat shock, whereas IR exposure resulted in an increase in nucleoid diameter. These results are consistent with the previous reports that heat does not induce chromosomal single-strand breaks as measured by the alkaline elution technique.²² Furthermore, Hunt and coworkers did examine whether heat shock induces G2-type chromosomal aberrations or inhibited repair of DNA strand breaks or effected both processes.¹⁴ Hunt and coworkers first established the conditions to measure the chromosome aberrations in cells with and without functional ATM in the presence and absence of repair inhibitors.¹⁴ Cells with functional ATM treated with okadaic acid and caffeine had ~4-fold higher chromosome aberrations as compared with untreated cells after IR exposure. Hunt and coworkers reported that the number of chromosome aberrations detected after irradiation with 0.15 Gy was statistically significant as compared with unirradiated cells.¹⁴ Cells deficient in ATM treated with okadaic acid and caffeine displayed a minimum increase in IR-induced chromosome aberrations, suggesting that such treatment in ATM proficient cells resulted in the inhibition of DNA strand break repair. These results showed a striking difference in the ability of *Atm*^{+/+} and *Atm*^{-/-} cells to repair their damaged DNA. Hunt and coworker exposed cells to IR doses as low as 0.15 Gy and immediately analyzed for G2-type aberrations.¹⁴ Whereas irradiated cells had detectable levels of chromosomal aberrations after a 0.15 Gy exposure, no aberrations have been reported in cells that were treated with heat alone, which otherwise induce the number of γ -H2AX foci equivalent to that induced by 1.7 Gy.¹⁴ Furthermore, heat shock in combination with IR did not result in increased production of chromosomal aberrations as compared with IR alone. Together these results all support the argument that moderate heat does not induce DNA double-strand breaks. However, heat shock has been shown to enhance S-phase-specific

chromosomal aberrations.²³ Hunt and coworkers¹⁴ tested whether γ -H2AX foci induction by heat occurred predominantly in S-phase cells undergoing replication. The findings from such studies revealed that heat shock induced higher frequency of γ -H2AX foci in S-phase cells as compared with non-S-phase cells.¹⁴ In contrast, IR exposure resulted in a similar frequency of γ -H2AX foci in S-phase as in non-S-phase cells. These results support the argument that heat shock induces more chromatin modifications in S-phase cells than the cells in other phases of the cell cycle, which abrogates the DNA repair process, resulting in higher chromosome aberrations observed at metaphase.

II.D. Are Heat-Induced γ -H2AX Foci Similar to Those Induced by IR?

Perusal of the literature suggests that γ -H2AX foci induced by heat may not be the same as those induced by IR. This assumption is based on the following observations: (i) heat-induced, but not IR-induced, γ -H2AX foci formation is ATM dependent; (ii) heat induces γ -H2AX and MDC1 foci, but not 53BP1 or SMC1 foci; (iii) heat induces ATM autophosphorylation independent of Mre11 function; (iv) heat, in contrast to IR, does not produce chromosomal breaks as was determined by three independent assays¹⁴; and (v) heat treatment induces a relatively higher number of γ -H2AX foci in S-phase than in non-S-phase cells. Such observations suggested that heat-induced γ -H2AX foci could be different from those induced by IR exposure, and heat-induced γ -H2AX foci formation does not require chromosomal DNA strand breaks. These results are consistent with the dogma that moderate heat treatment itself does not induce chromosomal DNA strand breaks but can alter chromatin structure as evident from DNA halo (nucleoid) assay, thus influencing DNA repair and enhancing cellular radiosensitization.¹⁴ Such results further addressed an important issue about whether or not chromatin alterations alone are sufficient to activate ATM, as proposed by Bakkenist and Kastan.⁹ Hunt and coworkers demonstrated that heat can induce chromatin changes as evidenced by phosphorylation of H2AX and MDC1 foci formation; however, heat shock itself did not induce DNA damage because no induction of 53BP1 or SMC1 foci or chromosomal DNA strand breaks were observed.¹⁴ Whereas heat treatment does induce ATM autophosphorylation or enhance its kinase activity, it is possible that heat-activated ATM may not be functionally equivalent to the species induced by IR exposure and thus could impair the signaling pathway associated with DNA damage repair. Such an assumption is consistent with the long-standing observation that heat-induced inhibition of DNA repair is due to an alteration in higher-order chromatin structure.²⁴ Although the detailed mechanism is not yet known, the studies reported by Hunt et al.¹⁴ support a working model for heat-induced chromatin alterations that correlate with activation of ATM in the absence of DNA damage. Further studies are required to determine whether and how heat abrogates ATM sensing to make cells more sensitive to cell killing by IR.

III. ROLE OF HSP70 IN CELL GROWTH AND GENOMIC STABILITY

Across a wide range of species from *Escherichia coli* to humans, HSP70 is the most highly conserved HSP at the sequence level and displays the largest, most consistent increase in expression following heat shock.²⁵ Increased levels of HSP70 protein are cytoprotective.²⁶ Cells subjected to a nonlethal heat shock increase cellular HSP70 levels, and those levels correlate with a transient resistance to higher, normally lethal temperatures.²⁷ Perusal of the literature suggests that inactivation of either of the two genes (*Hsp70.1* and *Hsp70.3*) results in deficient maintenance of acquired thermotolerance and increased sensitivity to heat stress-induced apoptosis.^{28–30} The synthetic or protein rescue functions of HSP70 are counterbalanced by participation in the ubiquitin pathway that clears the cell of unstable or damaged proteins by proteasome degradation.³¹ The heat shock protein 70 family is a family of multifunctional repair/removal agents for denatured and damaged proteins that can enhance cell survival following injury caused by many different agents including heat,

radiation, and chemotherapeutic agents. Basal level cellular expression of inducible HSP70 (HSPA1A and A1B) is upregulated in many cancer cells and confers a high level of resistance to radiation and chemotherapeutic agents in the absence of heat. HSP70 (HSPA1A, A1B, A6) can be further upregulated by heat and thermometric agents and would compromise the effectiveness of fractionated radiation doses delivered subsequently to the initial heat shock or heat mimic agent. Because of such functions, HSP70 also influences cell death and the cell transformation process.³²

Hunt et al.²³ exploited a more direct genetic approach to determine whether HSP70 proteins play a role in genomic instability and DNA damage response by generating a mouse model system in which HSP70 was knocked out. The murine *Hsp70* gene family contains two major genes induced by heat stress stimuli, *Hsp70.1* and *Hsp70.3*.³³ The only major difference between *Hsp70.1* and *Hsp70.3* is a lack of *Hsp70.3* expression in liver tissue.²³ The two genes are identical except for one amino acid difference³⁴ and have nearly identical expression in different tissues; HSP70 functional studies require both genes to be inactivated. Depletion of HSP70 had an effect on heat- and IR-induced cell killing and chromosomal repair. Consistent with the differences in growth and genomic instability, *Hsp70.1/3*^{-/-} cells were radiosensitive compared to parental cells.²³ *Hsp70.1/3*^{-/-} cells treated with heat had a more significant effect on IR-induced cell killing than did *Hsp70.1/3*^{+/+} cells,²³ suggesting a critical role of *Hsp70.1/3* in the heat-modulated IR-induced cell killing. Thus, HSP70 plays a critical role in karyotypic stability, population doubling times, cell survival after heat and/or IR treatment, and cellular phenotypes that are linked to defective chromosomal repair. Consistent with such a function of HSP70, Hunt and coworkers found that *Hsp70.1/3*-null mice are lighter in weight and have elevated levels of spontaneous genomic instability.²³ Two possible and not mutually exclusive reasons for the lighter weight of *Hsp70.1/3*^{-/-} mice could be the loss of cells, increased population doubling time, and/or both. Hunt et al.²³ reported that *Hsp70.1/3* affects the cell growth, as *Hsp70.1/3*^{-/-} mouse embryonic fibroblasts (MEFs) had a longer doubling time than *Hsp70.1/3*^{+/+} MEFs. The difference in population doubling is not attributable to the cell cycle differences, as no major difference was found in the distribution of cells in different phases of the cell cycle among cells with and without *Hsp70.1/3*.²³ One possible mechanism contributing to the increase in population doubling could be genomic instability. Hunt et al. reported that both in vivo and in vitro studies revealed the role of *Hsp70.1/3* in genomic stability.²³ Other investigators have reported that HSP70 interacts with human apurinic/apyrimidinic endonucleases and enhances the specific endonuclease activity of HAP1,³⁵ thus supporting the idea that *Hsp70.1/3* play a role in the repair of DNA damage. *Hsp70.1/3*^{-/-} mice have a higher ratio of normochromatic to polychromatic erythrocytes than do *Hsp70.1/3*^{+/+} mice. Furthermore, *Hsp70.1/3*^{-/-} mice also have a higher frequency of micronuclei in bone marrow erythrocytes than do *Hsp70.1/3*^{+/+} mice. Both the ratio of normochromatic to polychromatic erythrocytes and the frequency of micronuclei in erythrocytes were reported to be significantly increased after heat shock treatment in *Hsp70.1/3*^{-/-} mice compared with *Hsp70.1/3*^{+/+} mice. The in vivo results were consistent with the in vitro studies of residual chromosome damage analysis after heat or heat and IR treatment, supporting the argument that *Hsp70.1/3* play a role in combating spontaneous or heat-induced genotoxic stress. Thus, based on the fact that heat treatment significantly enhanced the frequency of micronuclei in *Hsp70.1/3*^{-/-} mice compared to that in *Hsp70.1/3*^{+/+} mice, in vivo studies were thus in agreement with the role of HSP70 in repair of DNA damage. The role of HSP70 in DNA damage repair was further strengthened by the fact that MEFs from *Hsp70.1/3*^{-/-} mice were reported to have a higher frequency of spontaneous as well as heat-modulated and IR-induced chromosome aberrations than do *Hsp70.1/3*^{+/+} cells.

The significance of HSP70 is further substantiated by the observations that inactivation of Hsp70.1/3 led to enhanced heat-modulated IR-induced cell killing, and the enhanced cell killing correlates with higher S-phase-specific chromosome residual damage.²³ Furthermore, a role for Hsp70.1/3 in S phase was evident from the fact that deficient cells demonstrate radioresistant DNA synthesis after IR exposure. Interestingly, expression of Hsp70.1 in *Hsp70.1/3*^{-/-} cells had rescued the radioresistant DNA synthesis phenotype in such cells, thus supporting the role of Hsp70.1/3 in the IR response in S phase.²³ HSP70 family members transiently associate with key molecules of the cell cycle control systems, including p53, Cdk4, pRb, p27/Kip1, cMyc, Wee-1, and some others, which affect cell growth.³⁶⁻³⁸ Cell growth is also affected by several other factors, for example, defective telomere metabolism.³⁹

HSP70 is known to interact with the catalytic unit of telomerase (TERT), which is involved in telomere metabolism.⁴⁰ Evidence is mounting to support the argument that telomerase may have functions other than the synthesis of telomeric repeats of the G strand.⁴¹ Ectopic expression of TERT prevents replicative senescence in several cell types including fibroblasts and epithelial cells.^{39,42,43} It may also exert an antiapoptotic action at an early stage of the cell death process prior to mitochondrial dysfunction and caspase activation.⁴⁴ In support of this idea, Wong et al.⁴⁵ reported that telomere dysfunction in mTerc-null mice impairs DNA repair and subsequently leads to cell growth arrest. Goytisolo et al.⁴⁶ reported radiosensitivity of the late-generation telomerase-KO mice. Choi et al.⁴⁷ demonstrated that telomerase expression suppresses senescence-associated genes in Werner syndrome cells. Sharma et al.⁴¹ reported that hTERT interacts with the telomeres, influences the interaction of telomeres with the nuclear matrix, and leads to transcriptional alteration along with increased genomic stability and enhanced DNA repair. Thus, some of the effects of TERT and HSP70 seem to be similar. Hunt et al.²³ reported that the inactivation of Hsp70.1/3 does influence telomerase activity, as *Hsp70.1/3*^{-/-} cells have 2.5-fold-less telomerase activity than do *Hsp70.1/3*^{+/+} cells. Cells deficient in Hsp70.1/3 also showed loss of telomeric signals and chromosome end associations, which are known to contribute to the genomic instability. In addition to HSP70's unique function in protecting the cells from stress-related damage, HSP70s have attracted attention in the cancer field by their aberrant expression in most human tumors in general and their physical interaction with cellular proteins of vital biological importance including tumor suppressors like p53.³⁶ Although it is well established that tumor cells have a higher expression of HSP70, in contrast its absence leads to genomic instability and higher IR-induced cell killing, both phenomena, which are linked, with oncogenic transformation. Consistent with such a hypothesis, *Hsp70.1/3*^{-/-} cells have a higher frequency of oncogenic transformation, suggesting that the absence of such gene products is essential to suppress tumor formation. Hunt et al.²³ reported that inactivation of Hsp70.1/3 resulted in reduced telomerase activity with telomere instability and reduced growth potential as well as increased radiosensitivity. Although it is likely that these different effects are the result of inactivation of Hsp70.1/3 and, therefore, of independent origin, it remains possible that interference with DNA repair and telomere functions could contribute to the overall growth defects. The chromosome end-to-end associations observed in *Hsp70.1/3*^{-/-} cells could induce cell-cycle arrest and genomic instability. Therefore, such results suggest that the overall growth phenotypes and radiosensitivity observed in *Hsp70.1/3*-null cells may be the result of a combination of effects. Thus, inactivation of Hsp70.1/3 influences cell growth and cell survival after IR treatment, telomere stability, chromosome repair, and oncogenic transformation. These observations are consistent with a model that predicts that Hsp70.1/3 has a critical role in stress response and is involved during the process of oncogenesis.

III.A. HSP70 Interacts with the Catalytic Unit of Telomerase

The reverse transcriptase motifs of the protein component of telomerase are conserved among diverse organisms.⁴⁸ Several human chaperone proteins have been found to be associated with hTERT. HSP70 has been found to be associated with hTERT prior to its assembly with the RNA component of telomerase (hTR); however, p23 and HSP90 are important for the assembly of telomerase activity in vitro as well as in vivo.⁴⁹ It is likely that HSP70 may be important for the stability of hTERT prior to its assembly to remain functionally active. Barker et al.⁵⁰ reported a link between the telomerase activity and HSP70 expression, as they found that the autonomous cells constitutively expressed telomerase activity, whereas the nonautonomous cells expressed telomerase activity only transiently. Interestingly, northern analysis of HSP70 indicated that like telomerase, *HSP70* gene expression was constitutive in autonomous cells and transient in nonautonomous cells.⁵⁰ These results suggest that hTERT expression may partly be regulated by heat shock elements. Heat shock transcription regulatory elements have been identified in the telomeric sequences in *Chironomus thummi*.⁵⁰ Hunt et al.²³ reported reduced telomerase activity in *Hsp70.1/3*^{-/-} cells as compared to *Hsp70.1/3*^{+/+} cells. However, it remains to be determined whether HSP70 knock down in human cells results in the reduced telomerase activity.

III.B. Hyperthermia Transiently Enhances Telomerase Activity

Hyperthermia induces HSP70 synthesis and HSP70 expression is associated with radioresistance. HSP70 interacts with the telomerase complex and expression of the telomerase catalytic unit (hTERT) extends the life span of the human cells.⁴³ Agarwal and coworkers⁵¹ examined telomerase activity in exponentially growing (293, HeLa, and BJ +hTERT) cells after heat shock (at 41° C–47° C for 1 h). Cells treated with heat at 41° C did not show any change in basal (37° C) telomerase activity. Maximum telomerase activity was observed in cells heat shocked at 43° C, whereas acute heat shock of 45° C or above resulted in decreased telomerase activity.⁵¹ HeLa as well as 293 cells showed the maximum enhancement of telomerase activity within 1 h of heat treatment at 43° C. A modest increase in telomerase activity was reported in ectopically expressed hTERT in cells with ATM function (BJ+hTERT) and without ATM function (GM5823+hTERT). The increase in telomerase activity after heat shock was followed by a gradual decrease in telomerase activity during the recovery period.⁵¹ Heat shock treatment did not induce any telomerase activity in primary fibroblast cells (HFF and GM5823) or in cells with alternative lengthening of telomeres (ALT; GM847). Heat-induced change in telomerase activity was not due to altered transcription of hTERT and hTR RNA. Because HSP70 interacts with hTERT before its assembly with hTR and other telomerase protein components,⁴⁰ such an association of HSP70 with TERT is not required for the functional activity of hTERT in vitro⁴⁰; however, cells deficient for HSP70 had reduced basal level telomerase activity in mouse embryonic cells.⁴ Heat shock was reported to have a modestly greater enhancement of telomerase activity in *Hsp70.1/3*^{+/+} cells than *Hsp70.1/3*^{-/-} cells. The transient heat-induced increase in telomerase activity was not a direct effect of heat on the telomerase complex. This is because as the cell extracts or immunoprecipitated telomerase complex when treated with different temperatures (37° C, 43° C, and 47° C) for 1 h, a significant drop in telomerase activity was observed at or above 43° C.⁵¹ These results suggested that the transient increase in telomerase activity observed in cells after heat shock is not the direct consequence of the effect of heat on the telomerase complex.

III.C. Effect of hTERT on Heat-Induced and IR-Induced Cell Killing

Ectopic expression of hTERT in telomerase-silent cells is sufficient to overcome senescence by extending cellular life span.^{42,43} This leads to transcriptional alterations in a subset of genes and changes the interaction of telomeres with the nuclear matrix.⁴¹ These alterations are associated with a reduction in spontaneous chromosome damage, enhancement of DNA

repair kinetics, and increased nucleotide triphosphate levels.⁴¹ These effects of hTERT have been reported to occur rapidly before any significant lengthening of telomeres was observed.⁴¹ Such functions of telomerase are distinct from its known effect on telomere length and have potentially important biological consequences. Moderate heating enhances telomerase activity, suggesting that telomerase may have a role in cellular protection during stress conditions. Because heat shock has been shown to activate ATM independent of DNA double-strand breaks,¹⁴ Aggarwal et al.⁵¹ reported that ectopically expressing hTERT cells with (BJ+hTERT) and without (GM5823+hTERT) ATM exhibited an increased survival after heat shock treatment compared with cells without hTERT expression (BJ and GM5823), and ATM-deficient cells with ectopic expression of hTERT (GM5823+hTERT) had a higher survival after heat and IR exposure than ATM-deficient cells without hTERT expression (GM5823). These results again suggested that expression of telomerase does significantly reduce heat-mediated IR-induced cell killing in both cells with and without ATM, although the effect of heat on cells deficient in ATM was more dramatic. These reports further supported the argument that the presence of telomerase influences heat-mediated radiosensitization.

III.D. Hyperthermia Enhances R-Induced Cell Killing in Telomerase Inactivated Cells

Hyperthermia is used to treat external tumors such as sarcoma,^{52–54} breast cancer at the chest wall,^{55,56} cervix,^{57–59} or with surgically accessible tumors such as prostate⁶⁰ and liver,^{61,62} which are mostly telomerase positive. Agarwal and coworkers used multiple approaches to determine the effect of telomerase inactivation on heat- and IR-induced cell killing. They used genetic approaches for telomerase inactivation as well as transient inactivation of telomerase to determine its influence on heat-mediated IR-induced cell killing. Because telomerase activation has been linked with extension of cell life span,^{14,33} it is possible that inactivation of telomerase may enhance cell death after heat and IR exposure. Mouse embryonic cells, cells deficient for Tert or Terc, had increased heat-mediated IR-induced cell killing compared with their wild-type control cells. These results suggested that the presence of Tert or Terc plays a role in heat-mediated IR-induced cell killing. Agarwal and coworkers also established that transient inactivation of telomerase effects heat-mediated IR-induced cell killing. Transient inactivation of telomerase was achieved by targeting the hTR, which contains an 11-nucleotide long template region for binding to and extending telomeric substrates that is easily accessible for hybridization with complementary oligonucleotides. No difference in the heat-mediated IR-induced cell killing was reported in primary fibroblasts (BJ GM5823 or GM857 cells: both lack telomerase activity) with or without GRN163L (potent inhibitor of telomerase) treatment; however, a more significant difference in heat-mediated IR-induced cell killing was reported in GRN163L-treated BJ+hTERT cells than untreated BJ+hTERT cells. Such studies suggest that telomerase activity may protect against heat-mediated IR-induced cell killing.

IV. PROTEINS LINKED WITH HEAT SHOCK RESPONSE

In most mammalian cells, hyperthermia induces a profound alteration at transcriptional and translational levels resulting mainly in the preferential synthesis of four specific proteins of approximately 27 kD, 70 kD, 90 kD, and 110 kD, which are referred to as heat shock proteins (HSP). Over-expression of HSP70s and HSP90s in many tumors⁶³ correlates with the cellular proliferation and survival of oral,⁶⁴ gastrointestinal,⁶⁵ colorectal,⁶⁶ histiocytic lymphoma,⁶⁷ and breast cancers.^{36,68} Because of these observations, HSPs have recently gained attention and have become of interest as targets for anticancer therapy.⁶⁹

Hyperthermia is a potent radiosensitizer that has been under clinical investigation as a means to improve the response to ionizing radiation (IR)-based cancer treatments, and acts to improve the local tumor control.¹ The most highly induced and conserved HSPs in all

organisms from *E. coli* to man is HSP70. The evolutionary conserved members of the HSP70 family prevent the disruption of normal cellular processes that involve mitosis, meiosis, or differentiation by environmental stressors. Members of the HSP70 family play essential roles in preventing misfolding and aggregation of newly synthesized or unfolded proteins.^{4–6} HSP70 holds unfolded substrates in an intermediately folded state to prevent irreversible aggregation and then catalyzes the refolding of unfolded substrates in an energy and co-chaperone-dependent reaction. HSP70s interact with co-chaperones through the N-terminal ATPase domain and with substrates at the C-terminal substrate domains.

Among the HSPs, HSP70 is the most evolutionarily conserved, being the only member present in all organisms from *E. coli* to humans,²⁵ and also the most highly conserved at the level of amino acid sequence. Over-expression of HSP70 or the less highly conserved HSP27 protein increases cellular resistance to hyperthermia and other forms of stress by both maintaining normal cellular functions and blocking apoptotic cell death.^{70,71} HSP110 expression has also been shown to result in a small increase in thermoresistance, although the mechanism involved is unknown.⁷² All three HSPs have chaperone activity, as demonstrated by the ability to re-fold damaged proteins or maintain the proteins in a soluble state.^{73,74} In addition to stress-inducible HSPs, mammalian cells contain constitutively expressed proteins that are structurally related to specific HSPs. The HSP70 family has five constitutively expressed members homologous to the heat-inducible HSP70 protein — two family members expressed only in germ cells and three that specifically localize to either the mitochondria, endoplasmic reticulum, or cytoplasm (Table 1). Both the cytoplasmic member, heat shock cognate protein 70 (HSC70), and newly synthesized HSP70 translocate into the nucleus following heat shock and over-expression of HSC70, which can result in protection from heat or other forms of stress.^{75,76} Constitutively expressed relatives of HSP27 and HSP110 may also be present in some cells but their potential contribution to thermoprotection is not well defined.⁷⁷ Low-level synthesis of HSP70 can occur in cultured human cells and is also detected in specific tissues from both mice and humans,^{29,78} but it appears to be upregulated in many cancer cell lines. Regulation of basal level synthesis in human cells is mediated through serum response elements present in the HSP70 promoter,⁷⁹ whereas the substantial increase in cellular levels of HSP70 and other HSPs following heat treatment is mediated by heat shock transcription factor 1 (HSF1). Heat induction and regulation of HSP70 is a rather complicated process, involving initial release of the heat shock transcription factor 1 (HSF1) from HSP90, followed by trimerization, and binding to the heat shock element (HSE) DNA sequences in the HSP gene promoters. For transcription to occur the trimer must first be activated by phosphorylation at serine 230 with calcium/calmodulin kinase II (CamKII)⁸⁰ and/or threonine 142 by casein kinase 2 (CK2).⁸¹ In contrast, phosphorylation at other sites, downregulates the transcriptional activity of HSF1. For example, phosphorylation at 121 by mitogen-activated kinase 2 (MK2) causes unfolding of HSF1 and rebinding to HSP90.⁸² Likewise, phosphorylation of serine 307 by ERK or serine 363 by JNK inactivates HSF1.⁸³ Binding of the transcription factor to promoter DNA takes place by a “winged” helix-turn-helix motif.⁸⁴ Heat shock elements consisting of four to five multiple tandem inverted repeats of the core sequence NGAAN are absolutely required for heat- and stress-inducible gene expression.^{85,86}

IV.A. Transcriptional Regulation of Hsp70

Hsp70 is a highly conserved protein in all organisms, and is composed of three domains, namely N-terminal ATPase domain, substrate-binding domain, and C-terminal lid.⁸⁷ It assists in the folding of a large variety of proteins via the association of its substrate binding domain with hydrophobic peptide segment within its substrate proteins in an N-terminal ATPase domain-dependent manner. Furthermore, Hsp70 function is highly regulated by co-chaperones through its interactions with the C-terminal lid. Hsp70 thus has housekeeping

functions in cells for protein folding and quality control, and repairing misfolded conformers. However, the broad spectrum of cellular functions of Hsp70 is achieved by its interaction with co-chaperones, and co-operation with other chaperones. Therefore, with co-chaperones and co-operating chaperones, Hsp70 constitutes a complex network of protein folding and quality control machines. Thus, Hsp70 is central to the cellular networks of molecular chaperones, and, hence, is an important proteostatic regulator for normal cellular functions.

The gene encoding Hsp70 is induced under stress conditions, and thus, leads the cells to survive the stress. Hsp70 interacts with a variety of key regulatory proteins.⁸⁷ Through these interactions, Hsp70 regulates signal transduction, cell cycle progression, and differentiation and programmed cell death, and thus, plays crucial roles in developmental and pathological processes such as oncogenesis, neurodegenerative and autoimmune diseases, viral infections, and aging.^{38,88–94} Hsp70 has also been shown to protect myocardium from ischemic injuries by preventing protein aggregation.^{95–97} Thus, considering the key role of Hsp70 to protect the cells against stress-induced damage, it is crucial to understand the regulatory mechanisms of *HSP70* expression under stress conditions.

The heat-shock response enhances the expression of the *HSP70* gene primarily at the level of transcription to protect the cells against proteotoxic and other stresses. However, the heat-shock response on *HSP70* expression also occurs post-transcriptionally at the levels of mRNA stability,⁹⁸ translation,⁹⁹ and subcellular localization and activity.¹⁰⁰ The heat-shock transcription factor (HSF) mediates the heat-shock response by binding to the heat-shock element (HSE) at the promoter of the *HSP70* gene, and subsequently initiating transcription through formation of active transcription complex assembly.^{101–105} HSF is expressed under non-stress conditions, and exist as a non-DNA binding state. Under stress conditions, HSF forms trimer and binds to the promoter of the *HSP70* gene to initiate transcription. Several HSFs are expressed in mammals (HSF1, HSF2, and HSF4) and avians (HSF1–4). However, only HSF1 is expressed in *Drosophila*, *Caenorhabditis elegans*, and yeast.^{106–109} HSF1 is the ubiquitous stress-responsive activator in all organisms,¹¹⁰ whereas other HSFs in higher eukaryotes are cell-type specific or developmentally regulated.^{111–114}

The HSFs are composed of several functional modules.¹⁰⁸ These are N-terminal helix-turn-helix DNA-binding domain, hydrophobic heptad repeat (HR), and transcription activation domain. The DNA-binding domain is the most common functional domain of HSFs. Activation-induced trimerization occurs by three arrays of hydrophobic heptad repeats (HR-A/B).¹¹⁵ These repeats are characterized by helical coiled-coil structure. However, hydrophobic heptad repeat, HR-C, is attributed to suppress HSF trimerization.¹¹⁶ HR-C is well conserved among vertebrate HSFs. But, it is poorly conserved in plant and *Saccharomyces cerevisiae*.^{106,117} Thus, such a poorly conserved HR-C may be the cause of constitutive trimerization of HSF1 in *S. cerevisiae*.^{106,117}

A large variety of stress signals induces the transcriptional activity of HSF1. These stress signals include a broad range of perturbations of physiological states and diseases.¹¹⁸ However, how these signals induce HSF1 and heat-shock response has not been thoroughly investigated. The previous studies indicate that misfolded and damaged proteins serve as the primary signal to derepress HSF1.¹¹⁹ Under non-stress conditions, HSF1 remains transiently bound to Hsp90, Hsp70 and Hsp40,^{120–122} making it incompetent for binding to HSE of the *HSP70* gene. However, the presence of excessive unfolded and damaged proteins perturb the chaperone equilibrium, thus inducing the dissociation of Hsp90/70/40 from HSF1. Such a process derepresses HSF1 to undergo conformational changes to an active state, which involves the formation of HSF1 homotrimer via an extended heptad repeat (HR-A/B) located between the DNA-binding and transcription-activation domains. The formation of

the HSF1 homotrimer releases the DNA-binding domain to interact with HSE of the *HSP70* gene to initiate transcription.¹¹⁵ A high level of *HSP70* transcription is maintained when HSF1 trimer remains bound to HSE under stress conditions. However, when the stress signal disappears, Hsp70 and other chaperones associate with HSF1, and hence, heat-shock response attenuates rapidly.^{123,124} Further, the stress-activated HSF1 undergoes post-translational modification by phosphorylation, sumoylation, and acetylation.^{80,125–130} Such post-translational modifications have multiple regulatory consequences in stimulating or repressing the transcriptional activity of HSF1. Thus, the combination of post-translational modifications of HSF1 and its interaction with Hsp70 or other chaperones provide the fine tuning of the *HSP70* expression and its chaperonin activity to protect cells against proteotoxic and other cellular stresses.

Although the heat-shock response has been portrayed as a universal response to various stress stimuli, there are cases where heat-shock response is poorly or not at all activated. For example, human neuroblastoma Y79 cells respond to heat shock by expression of *HSP90* through the activation of HSF1.¹³¹ However, *HSP70* is not expressed, even though HSF1 is activated in response to heat shock in the neuroblastoma Y79 cells.¹³¹ Furthermore, in the case of the intact primary hippocampal neurons from neonatal rat embryos, HSF1 is not expressed until later stages of development,¹³² and thus, primary hippocampal neurons do not respond to heat shock. Similarly, primary motor neurons do not exhibit heat-shock response due to defect in the activation of HSF1.¹³³ Therefore, heat-shock response does not occur in all cell types. However, the molecular mechanisms for the defect of heat-shock response in certain cell types are not clearly known.

Like many genes, *HSP70*, undergo changes in chromatin structure upon activation. Petesch and Lis¹³⁴ have mapped the nucleosome landscape of Hsp70 after an instantaneous heat shock at high spatial and temporal resolution. They found an initial disruption of nucleosomes across the entire gene within 30 seconds after activation, which is faster than the rate of Polymerase II transcription, followed by a second further disruption within 2 min. It has been reported that initial change occurs independently of Polymerase II transcription.¹³⁴ Furthermore, the rapid loss of nucleosomes was found to extend beyond *HSP70* and halts at the scs and scs' insulating elements. Interestingly, an RNAi screen of 28 transcription- and chromatin-related factors has revealed that depletion of heat shock factor, GAGA Factor, or Poly(ADP)-Ribose Polymerase or its activity abolishes the loss of nucleosomes upon *HSP70* activation in *Drosophila*.¹³⁴

V. FUTURE STUDIES AND DIRECTIONS

Hyperthermia has enormous clinical applications, specifically in heat-mediated IR-induced tumor control. How heat sensitizes cells to IR-induced killing is under intensive investigation. Heat does not induce DNA double-strand breaks but rather appears to inhibit repair of DNA damage. Especially in the case of IR-induced DNA damage, which produces complex closely clustered damage on opposed DNA strands, heat shock pre-irradiation results in delays in completing multi-step repair processes that have single-strand break intermediates and increases the probability for production of lethal double-strand DNA breaks.^{135,136} Perusal of the literature supports that heat inhibits base excision and single-strand break repair; the mechanism seems to be unrelated to direct effects on the repair enzymes, but is more likely an indirect result of altered access to DNA damage sites.²⁴ It is known that heat causes protein unfolding, which can lead to aggregation. Precipitation or binding of protein aggregates to cellular structures such as the nuclear matrix could block access to sites of DNA damage repair enzymes. Alternatively or in addition, heat may also cause protein aggregates to sequester the proteins involved in DNA damage repair. Heat shock proteins modulate the effects of heat by binding unfolded protein domains and

maintaining the protein in a soluble form until it can be refolded or degraded, thereby minimizing protein aggregation. Moreover, HSPs have been shown to enhance the function of DNA repair enzymes such as human apurinic/apyrimidinic endonuclease.³⁵ Inactivation of HSP70 by gene targeting as well as HSP70 antisense increases the thermosensitivity of human and mouse cell lines.^{23,137} How heat modulates the transcription and translational aspects of heat shock proteins in the context of stress DNA damage response needs thorough investigation.

There is growing indirect evidence suggesting that heat treatment modifies the chromatin structure and thus enhances cell kill by IR.^{138,139} As discussed previously, hyperthermia results in the autophosphorylation of ATM along with the ATM-dependent phosphorylation of H2AX. However, there is no direct biochemical evidence to substantiate the idea that heat alters chromatin structure in vivo or in vitro. Chromatin structure is an important factor for determining protein-DNA interactions, with consequences for DNA metabolism and transcription control.^{140–143} The literature suggests a link between chromatin structure and IR sensitivity.^{144–166} Nackerdien et al.¹⁵⁶ suggested that packing and accessibility of DNA in chromatin appear to be the major factors that influence radiation sensitivity. Intensive research is ongoing to determine whether heat treatment influences the accessibility of the repair machinery at the site of DNA DSB. Therefore, targeting a combination of tumor-specific and DNA repair pathways will not only enhance heat-induced radiosensitization, but will also decrease the overall level of normal tissue toxicity occurring during radiotherapy. Such information could eventually help to improve sequencing of the heat and IR treatment to obtain a better clinical outcome.

Acknowledgments

The work in the laboratory of Tej K. Pandita is supported by NIH (CA123232, CA129537, and CA10445). Thanks are due to John-Stephen Taylor, Clayton Hunt, Andrei Laszlo, and laboratory members for their comments and suggestions.

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TABLE 1

HSP70 Family and Properties

Name	Alternate names	Homology	HSP locus	Cellular localization	Stress inducible
HSP70-1a	70,72,70-1	100	A1A	Cytoplasmic, nuclear, lysosomal	Yes
HSP70-1b	Same	99	A1B	Cytoplasmic, nuclear, lysosomal	Yes
HSP70-1c	70-hom	91	A1L	Cytoplasmic, nuclear	No
HSP70-2	70-3, A2	84	A2	Cytoplasmic, nuclear	No
HSP70-5	BiP, Grp78	64	A5	Endoplasmic reticulum	Yes
HSP70-6	70B'	85	A6	Cytoplasmic, nuclear	Yes
HSP70	70-8,73	86	A8	Cytoplasmic, nuclear	No