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Changes in Sub-Nuclear Structures and Functional Perturbations: Implications for Radiotherapy

J.L. Roti Roti,* D. Gius, R.P. VanderWaal, and M. Xu

Washington University School of Medicine, Mallinckrodt Institute of Radiology, Radiation Oncology Center, Section of Cancer Biology, St. Louis, Missouri 63108

Abstract The eukaryotic cell nucleus is required to accomplish its functions (e.g., replicating transcription, DNA repair, hmRNA processing, etc.) within the context of a highly organized structure [Wei X, Samarabandu J, Devdhar RS, Siegel AJ, Acharya R, Berezney R. 1998. Science 281:1502–1506.], since many cancer-therapeutic modalities utilize the nucleus as target for a cytotoxic outcome. A better understanding of the organizational disruption of sub-nuclear structures and subsequent loss of nuclear function is the key to knowing both the mechanism of action of, and the basis of cellular sensitivity to, therapeutic agents such as ionizing radiation. With this prospect, we examine four examples in which changes in specific nuclear structures or functions lead to significant therapeutic end points, e.g. cell death, radiosensitization, or the intrinsic radioresistance of tumor cells. The inter-relationships delineated in these examples provide a paradigm that delineates a relationship between disruption of nuclear organization, loss of function and a point of intervention that affects a therapeutic outcome. The examples specifically address issues related to radiation and thermal therapy. However, the concepts that result from these studies are translatable to other cancer therapeutic modalities. In addition, the results echo a basic principle that proper nuclear organization is critical to the maintenance of cellular viability and genomic stability. J. Cell. Biochem. Suppl. 35:142–150, 2000. © 2001 Wiley-Liss, Inc.

Key words: nuclear matrix; nuclear proteins; radiosensitivity

The eukaryotic cell nucleus is a highly crowded, but well-organized organelle. Nuclear DNA must be compacted on the order of 40,000-fold in order to fit within the nuclear restricted volume [Getzenberg et al., 1991]. However, this compaction must be accomplished in a way that allows access to specific domains at specific points in time for replication, transcription and repair. For any given DNA domain, transcription and replication are separated in time [Wei et al., 1998]. In contrast to the separation seen between transcription and replication, certain DNA repair pathways function with transcription [Bohr et al., 1985], while other repair pathways appear to function with replication [Wilson and Sirghal, 1998]. Sites of transcription and replication must consist of large protein complexes, which require changes in DNA compaction to accomplish their DNA related functions. Thus, at any given point in time various DNA domains must be maintained in different states of compaction. However, the states of compaction of many DNA domains are changed as they undergo replication and possibly transcription and repair. Therefore, transition through S-phase, in particular, requires that nuclear organization undergo multiple time-dependent changes. Since the nucleus is the target site for the lethal effects of many anticancer agents, it is logical to assume that disruptions to the specific nuclear structures or their ability to evolve in time may be important aspects of the mechanism by which cytotoxic agents induce cell death. We are just beginning to understand the relationships between disruption of specific nuclear structures and the resulting cellular consequences. With this prospect we present four examples of nuclear structure/function disruption and the resulting cell death and/or radiosensitization, which are important cellular endpoints for radiation and thermal cancer therapy.

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*Correspondence to: Joseph L. Roti Roti, Section of Cancer Biology, Radiation Oncology Center, Mallinckrodt Institute of Radiology, Washington University Medical Center, 4511 Forest Park Boulevard, Room 404, St. Louis, MO 63108.

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Radiotherapy exploits the lethal aspects of ionizing radiation to kill tumor cells in situ. Inherent radiosensitivity varies over a modest range for normal human cells, a significant range for human tumor cells, and a wide range for human tumor cells derived from treatment failures [Hall, 1994]. This observation suggests that tumor cell radioresistance is an important determinant of treatment outcome. Further, because radiotherapy is given in five daily fractions per week for 6 to 8 weeks, small changes in the effectiveness per fraction will have a geometrically magnified effect on treatment outcome. Therefore, understanding the basis for inherent radiosensitivity and methods to increase it, have great potential to enhance therapeutic effectiveness. It has long been recognized that damage to nuclear DNA is the critical event in cell killing [Okada, 1970]. However, the number of radiation-induced DNA lesions per lethal dose is known to be 1 to 2 orders of magnitude greater than the number thought to be needed per lethal event [Kraft et al., 1995]. Thus, post-irradiation events, such as DNA repair must also contribute to the ultimate survival of the cell. Recently, there have been numerous advances in the characterization of several DNA repair pathways [Nickoloff and Hoekstra, 1998]. However, both DNA damage and its repair occur in the context of the nuclear organization of any given cell, which is very likely to have a modulating effect on both DNA damage and its repair [Oleinick et al., 1995].

HEAT-INDUCED DISRUPTION OF DNA-REPLICATION COMPLEXES AND CELL KILLING

DNA replication is one of the most heat-sensitive cellular processes [Dewey et al., 1978]. Additionally, S-phase cells are significantly more heat sensitive than either G_1 or G_2 cells [Bhuyan et al., 1977]. Further, heat-induced cell death is manifested following cell-cycle progression through S-phase [VanderWaal et al., 1997] and delaying S-phase progression prevents or reduces cell killing by hyperthermia [VanderWaal et al., 1999]. Because of these considerations and the fact that DNA replication occurs in discrete complexes, we will use heat-induced S-phase cell killing and the effects of heat on DNA replication complexes as our first illustration. Heat shock induces stabilization of DNA replication complexes via aberrant nuclear protein binding or aggregation [VanderWaal et al., 1999], as shown by three observations. (1) the S value of isolated complexes containing polymerase α increased with increasing heat shocks [Roti Roti et al., 1998]; (2) heat shock of 45°C for 15 and 30 min prevented the progressive loss of the type I foci as seen in progressive steps of the nuclear matrix isolation process [VanderWaal et al., 1999]; and (3) DNA polymerase α, replication protein A, PCNA, and cyclin A, proteins associated with DNA replication complexes, are recovered in greater abundance in isolated nuclear matricies from heat-shocked cells. The increase in association of these proteins with the nuclear matrix is dependent on the duration of the heat shock [VanderWaal et al., 1999].

The heat-shock induced stabilization of DNA replication factories via alterations in protein–protein interactions inhibits subsequent DNA replication and S-phase progression as measured by the relative amount of DNA synthesized and the shift from type I to type II DNA replication patterns. For unheated S-phase cells (obtained by blocking cells with 3 μM aphidicolin for 17 hr), the relative fraction of DNA synthesized reached 0.5 by 2.5 hr and the transition from type I to type II DNA replication patterns (i.e., the time at which there is 50% of each type) occurred at 2.5 hr. For cells heated at 45°C for 15 min the relative fraction of DNA synthesized reached 0.5 by 6 hr and the transition from type I to type II DNA replication patterns occurred at 3.75 hr. For cells heated at 45°C for 30 min., the relative fraction of DNA synthesized had not reached 0.5 by 12 hr, but the transition between type I and type II DNA replication patterns occurred at 4.75 hr [see VanderWaal et al., 1999]. Thus, the heat-induced delay in DNA replication was 4–10 hr depending on the severity of the heat shock. In contrast, the transition between type I and type II DNA replication patterns demonstrated a heat induced delay of only 1.25–2 hr. Thus, it cappears that heat shock uncouples the relationship between the amount of DNA made and the transition to new DNA replication factories.

If heat shock uncouples the relationship between the amount of DNA made and the transition to new DNA replication factories, then there should be a longer time period in which DNA synthesis will be ongoing in DNA

replication factories. It has been reported that DNA synthesis remains ongoing in typical DNA replication factories in control cells for 45 min [VanderWaal et al., 1999]. Very little overlap between type I and type II factories is seen in dual label CldU and IdU experiments in unheated cells if the time between the labels in more than 1.5 hr [Manders et al., 1992]. However, in heat-shocked cells (45°C for 30 min), in which the CldU and IdU labels were separated by 6.5 hr, showed that cells synthesizing DNA in type II - III foci at the time of the IdU label were also still synthesizing DNA in the type I foci labeled with CldU 6.5 hr earlier [VanderWaal et al., 1999]. This observation confirms the conclusion that heat shock uncouples the relationship between the amount of DNA synthesized and the transition to new DNA replication foci. If, as has been suggested, that cells maintain a separation in time and space between replicating DNA domains of different types and transcribing domains (Wei et al., 1998), then this uncoupling should have important cellular consequences.

One of the important cellular consequences of uncoupling the relationship between the amount of DNA synthesized and the transition to new DNA replication foci appears to be cell killing. Delaying S-phase progression post-heat-shock reduces the excess lethality of S-phase cells to that observed for exponentially growing cells [VanderWaal et al., 1999], suggesting that reducing unbalanced S-phase progression post-heat-shock rescues cells from heat-induced lethality. The observation that blocking the progression of cells through S-phase can protect them from at least some of the lethal effects of heat shock suggests that there is an interaction between ongoing DNA synthesis and heat-induced changes presumably in DNA replication factories. Further, evidence for this idea comes from studies targeting mutant proteins to DNA replication factories. Two groups, [Liu et al., 1998], report toxic effects of over-expressing methyltransferase mutants that retain targeting sequences for DNA replication complexes. It has been suggested [Liu et al., 1998] that the toxic effect is caused by the over-expressed mutant methyltransferase sequestering critical components of the DNA replication machinery. Further, it has been suggested that the formation of a complex between RPA and nucleolin contributes to heat-induced inhibition of DNA replication [Daniely and Borowiec, 2000]. These suggestions are similar to our observations that several nuclear proteins become more tightly bound to the nuclear matrix following heat shock. Also, the descriptions of cell death due to mutant methyltransferase over-expression in Liu et al. [1998] suggest that it is similar to that which occurs for moderate heat shocks, raising the possibility that the two modes of cell death have the same target.

To gain further insight regarding the subnuclear target of the lethal effects of heat on S-phase cells we quantified the extent of clonogenic cell survival and the binding of specific nuclear proteins with nuclear matricies from heated cells under conditions in which S-phase progression was inhibited for various durations. The binding of proteins, associated with DNA replication complexes, with the nuclear matrix at the time S-phase progression resumed followed a single correlation curve under four different experimental conditions. In contrast, the binding of proteins, associated with other nuclear structures, showed no correlation at all under the same conditions. (The full description of these experiments has been submitted for publication.) These results are consistent with the conclusion that heat-induced altered protein binding within DNA replication complexes represents a potentially lethal lesion that can be fixed by progression through S-phase.

PERTURBATION OF DNA REPAIR FOCI AND HEAT-INDUCED RADIOSENSITIZATION

It has long been thought that unrepaired or misrepaired DNA double-strand breaks are critical lesions causing radiation-induced cell death [Dikomey et al., 1998] and that heat-induced inhibition of DNA double strand break repair is a major cause of heat-induced radiosensitization. As more components of various DNA double-strand repair pathways are identified, it becomes possible to characterize and study repair complexes in situ. Recently, foci containing the DNA double-strand break repair protein, Mre 11, have been reported 8 hr following irradiation with 12 Gy [Carney et al., 1998]. Prior to irradiation Mre 11 is dispersed through out the nucleus [Maser et al., 1995]. Following irradiation, the Mre 11 foci uniquely appear in the irradiated parts of nuclei [Petrini, 1999]. However, the exact role of Mre 11 in DNA double-strand break repair remains uncertain.

Also, it is not clear whether these foci represent sites of repair or sites of reprocessing of the repair enzymes prior to being returned to storage sites. Since these foci appear to assemble after radiation, we determined if mild heat shocks, i.e., 41.1°C for 1–6 hr, affected the formation of these foci. Prior work, [Seno and Dynlacht, 2000], suggested that Mre 11 became dispersed from the nucleus into the cytoplasm following 45.5°C heat shocks. We found that dispersion of Mre 11 from the nucleus to the cytoplasm occurred following 1 hr at 41.1°C, but the effect was more dramatic at 6 hr (Fig. 1A). To determine if dispersal of Mre 11 into the

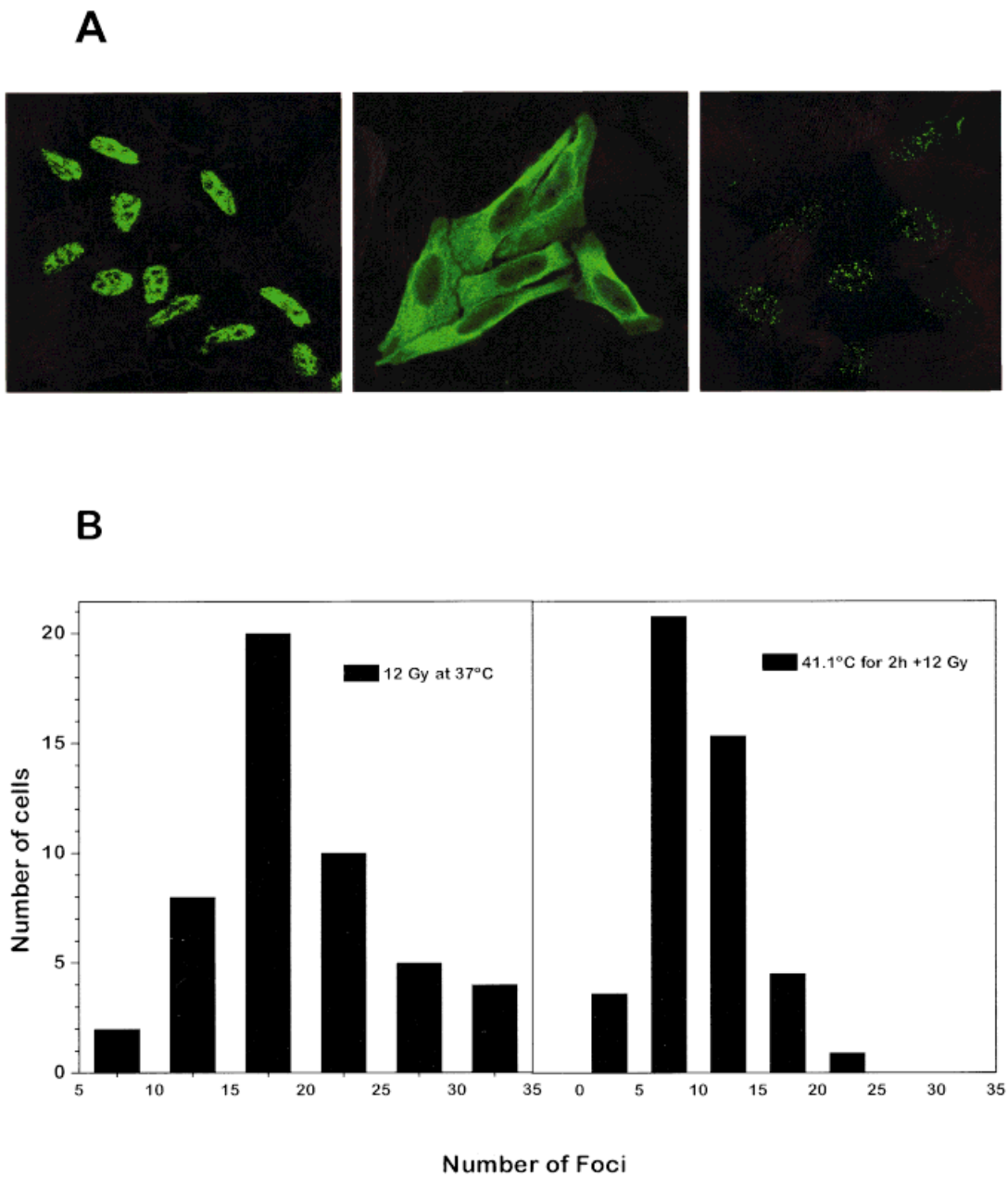

Fig. 1. The effects of heat shock on DNA repair foci. **A:** Typical repair foci containing Mre II. Upper panel: control cells immunostained with Mre II antibodies. Middle panel: HeLa cells heat shocked for 6 hr at 41.1°C and immunostained with Mre II antibodies. Note that the fluorescence intensity is reduced in this panel relative to the upper two in order to show the foci more clearly. **B:** The numbers of Mre II foci per cell 7 hr after irradiation. Upper panel: a typical distribution of foci numbers for unheated NSY cells. Lower panel: a typical distribution of

cytoplasm inhibited the induction of foci by ionizing radiation (see Fig. 1A for typical foci), we counted the number of foci per nucleus at 7 hr following 12 Gy of X-rays in heat-shocked and control cells. Typical distributions of the numbers of foci per cell are shown in Figure 1B. It can be seen that in the NSY cells heated at 41.1°C for 2 hr, an exposure in which the cells become approximately twofold more radiosensitive, the number of foci per cell was reduced twofold. Next, we compared heat shocks that give a twofold radiosensitization ($D_0 = 0.8$ Gy) with those that do not radiosensitize ($D_0 = 1.5$ Gy). In cells with $D_0 = 0.8$ Gy, the mean number of foci per cell was 7.5 ± 2.5 and the percentage of cells with 15 or more foci was $8.0 \pm 6.0\%$. In contrast, in cells with $D_0 = 1.5$ Gy, the mean number of foci per cell was 15.5 ± 3.5 and the percentage of cells with 15 or more foci was $32.5 \pm 5.5\%$. Thus, when cells are radiosensitized by heat shock there is a significant reduction on the number of DNA repair foci, possibly due to the dispersion of Mre 11 into the cytoplasm (see Fig. 2). That this effect may contribute to heat-induced radiosensitization is supported by the observation that certain DNA double-strand break repair deficient cells are not radiosensitized by heat shock [Iliakis et al., 1990].

DNA-NUCLEAR MATRIX ANCHORING WITH INCREASING RADIORESISTANCE

DNA compaction with in the nucleus is maintained in part via negative supercoiling [reviewed in Roti Roti et al., 1997]. DNA supercoiling status is maintained in separate domains by periodic DNA nuclear-matrix anchor regions. One approach to studying changes in DNA supercoiling status and the stability of DNA anchoring is the nucleoid halo assay, which was introduced by Vogelstein et al. [1980] and developed further [reviewed in Roti Roti et al., 1997]. Studies of DNA supercoiling in situ show that heat-shock causes a reduction in DNA loop size and an increase in loop rewinding efficiency, suggesting that the nuclear matrix-DNA attachment has become strengthened presumably by the increased binding of proteins to the nuclear matrix [reviewed in Roti Roti et al., 1997]. Conversely, DNA-nuclear matrix attachments appear to be loosened when nucleoids are isolated in the presence of 1-mM dithiothreitol (DTT), as shown by an enhanced unwinding of the DNA loops and an inhibition of loop rewinding [Roti Roti et al., submitted]. The conclusion that DTT affected the protein anchor is supported by the difference between the effects of DTT and ionizing radiation and

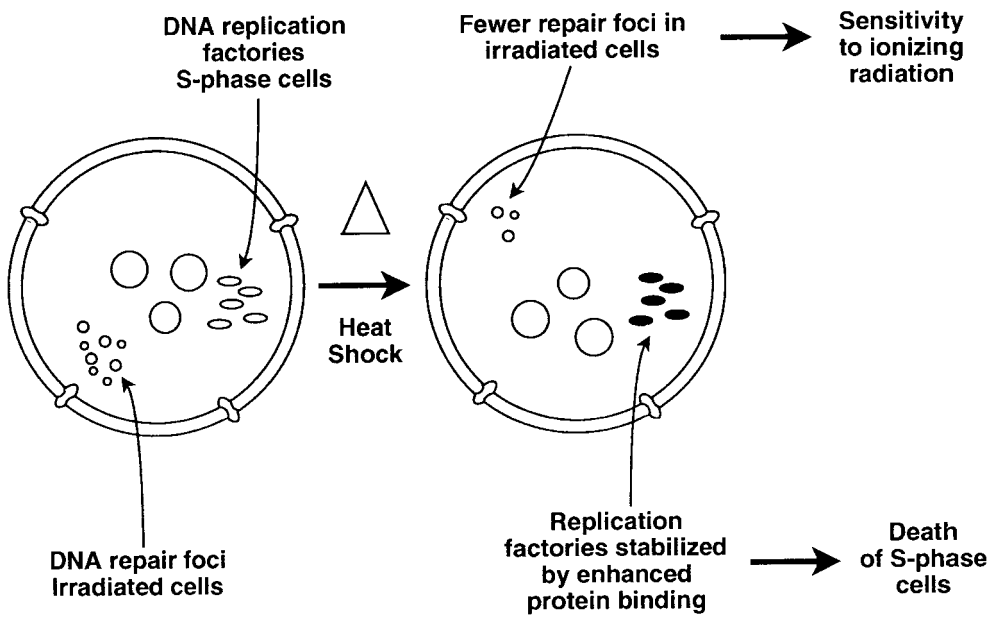

Fig. 2. Heat-induced alterations of specific nuclear structures and subsequent cellular effects. As described in the text it is becoming possible to characterize the effects of heat shock on specific nuclear structures and relate these changes to certain effects of heat on eukaryotic cells.

topoisomerase I inhibitors, which cause DNA single-strand breaks. Specifically, both the latter agents inhibit DNA loop rewinding, but significantly alter loop unwinding. Further, when nucleoids were prepared from irradiated cells, the effects of radiation and DTT were additive implying that the two agents act on separate parts of the complex, i.e., ionizing radiation on the DNA and, by implication, DTT on the protein portion of the anchoring region. Interestingly, the overall loop size was not significantly larger in the presence of DTT suggesting that it did not alter the number of anchor points. Thus, the anchoring region appears to consist of a redox sensitive component and a component that is stable in the presence of changes in the oxidative environment (see Fig. 3).

Considerable evidence has accumulated that DNA nuclear-matrix anchoring regions are different in radiosensitive cells [Malyapa et al., 1995]. Specifically, the radiosensitive cells show a greater inhibition of DNA supercoil rewinding for a given radiation dose and level of DNA damage. This effect has been observed in radiosensitive mutants of CHO cells, in spontaneous variants of L5178Y cells and in rat embryonic cells transfected with the ras oncogene [reviewed in Roti Roti et al., 1997]. As described above, when nucleoids are prepared from irradiated cells the effects of radiation and DTT were additive. In contrast, in radiosensitive cells the extent of inhibition of DNA loop rewinding following 5 Gy or in the presence of 1 mM DTT was not significantly different from that observed when the two agents were used together. Further, while 1mM DTT solubilized proteins of nucleoids from radioresistant cells, those proteins were already soluble in the radiosensitive cells. This result implies that the redox sensitive component of nuclear matrix-DNA anchoring is compromised in radiosensitive cells.

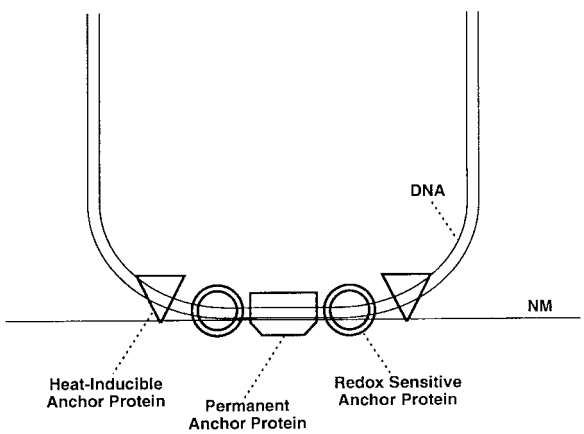

Fig. 3. A DNA-nuclear matrix anchoring region inferred by DNA supercoiling studies. This cartoon illustrates the anchoring components necessary to explain the results from DNA supercoiling experiments described in the text. The arrangement of the components explaining both changes in DNA loop size and in supercoil unwinding and rewinding efficiency. The redox sensitive anchoring components appear to be compromised in radiosensitive cells.

HEAT-INDUCED INHIBITION OF SIGNALING PATHWAYS INVOLVING NUCLEAR TRAFFICKING

The cellular response to the elevated temperature is remarkably well conserved across all species and is primarily mediated at the transcriptional level by pre-existing transcriptional activators, known as heat-shock factors (HSFs) [Jurivich et al., 1992]. In addition to HSFs, it now appears that other transcriptional and signal transduction factors are activated in response to heat including certain signal transduction pathways, such as p38/HOG1 kinase, *jun* N-terminal kinase, MAPK1 (mitogen-activated protein kinase), and protein kinase C, previously known to have regulatory roles in the cellular response to other forms of cell stress, are also induced by heat [Curry et al., 1999]. There is also considerable evidence that several of the same cytoplasmic signaling and/or nuclear transcription factors that play a protective role in tumor cells responding to damage from ionizing radiation [Hallahan et al., 1993], are altered by heat shock [Diamond et al., 1999]. Thus, it is conceivable that heat shock induces signaling pathway(s) that alter and/or prevent the induction of specific transcription factor(s) post-irradiation that may be acting to protect tumor cells against cytotoxic effects of radiation.

To test the hypothesis that heat disrupts or alters the regulation of signaling factors activated by radiation, the effect of heat shock on the activation of NF-κB was determined. The importance of the role of NF-κB in the cellular response to ionizing radiation is supported by several reasons. First, clinical experience shows that one of the first side effects of IR is an inflammatory response first detected on the skin and oral mucosa of head and neck patients receiving

therapeutic irradiation [Gius et al., 1999]. Second, the DNA-binding of NF-B appears to be transiently induced in a wide range of irradiated tumor cell lines [Gius et al., 1999]. Third, the regulation of NF-κB involves sub-cellular compartmentalization via a physical interaction with its inhibitor protein, I-κB that prevents the nuclear uptake of the DNA-binding sub-units through the masking of nuclear localization signals [Gius et al., 1999]. Lastly, the activation of NF-κB induces the expression of specific target genes involved in the cellular defenses against the cytotoxic effects of radiation [Curry et al., 1999]. Following irradiation there are transient increases in NF-κB DNA-binding activity, as well as NF-κB protein nuclear localization, and I-κB cytoplasmic degradation, corresponding temporally with the increase of NF-κB DNA-binding (Fig. 4). Heat shock prior to irradiation inhibited the radiation-induced increase in NF-κB DNA-binding, nuclear localization, as well as the phosphorylation and subsequent degradation of I-κB [Curry et al., 1999]. Furthermore, pretreatment with cycloheximide, to block de novo protein synthesis, did not alter the ability of heat shock to inhibit the induction of NF-κB by ionizing radiation [Curry et al., 1999]. These experiments demonstrate that heat shock transiently inhibits the induction of NF-κB by I-κB preventing degradation by a mechanism independent of protein synthesis. Heat-induced inhibition of NF-κB activation may contribute to radiosensitization of tumor cells by hyperthermia by preventing the expression of downstream genes [Sukhatme et al., 1988] that are capable of reducing the lethal effects of radiation [Datta et al., 1992]. Finally, preventing nuclear translocation of a specific transcription factors, may be a paradigm for understanding changes in radiation-induced signaling pathways that are altered by thermal stress.

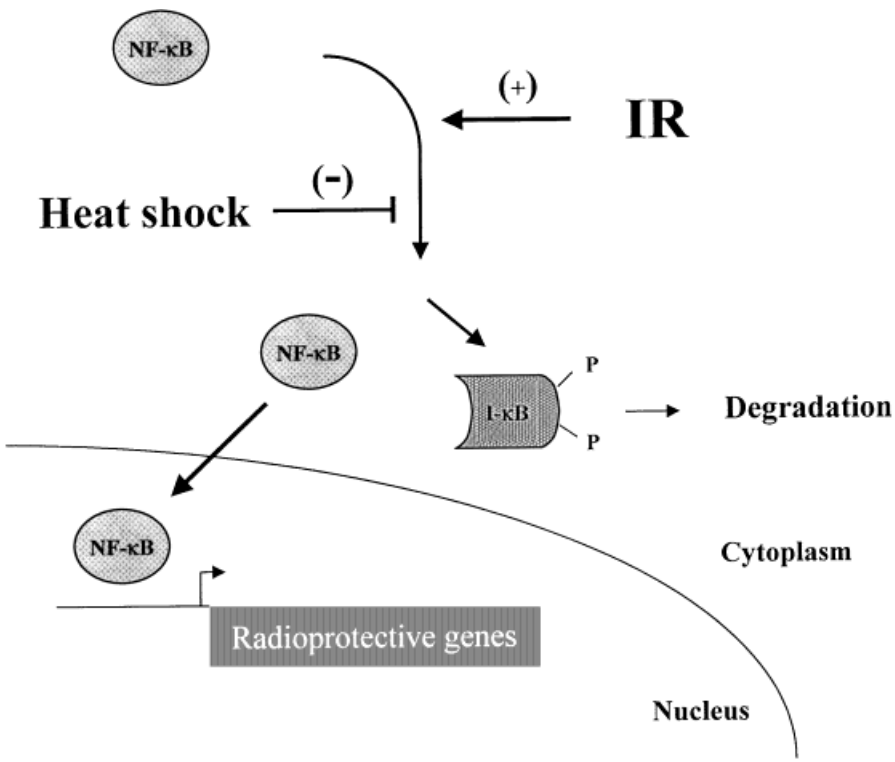

Fig. 4. IR-induced activation of NF-κB is inhibited by heat shock by preventing nuclear localization. Exposure to IR induces phosphorylation of I-κB leading to I-κB degradation via proteasome dependent mechanism. Following degradation, NF-κB is subsequently transported into the nucleus via a 19 nuclear localization sequence and once in the nucleus binding to specific *cis*-acting upstream enhancer elements that induce the expression of a series of downstream genes. It has been suggested that these downstream genes play a role in multiple cellular processes including a radioprotective process. Exposure to thermal stress prior to IR inhibits radiation-induced phosphorylation and degradation of I-κB, and as a result heat shock prevents NF-κB nuclear localization by inhibiting the activation of the upstream I-κB kinase complex [Curry et al., 1999].

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