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Increased PARP-1 Levels in Nuclear Matrix Isolated from Heat Shock Treated Rat Liver

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Abstract—Poly(ADP-ribose) polymerase-1 (PARP-1), a chromatin-associated enzyme that catalyzes the NAD^+-dependent addition of ADP-ribose polymers onto a variety of nuclear proteins, has been shown to be associated with the nuclear matrix. PARP-1 levels in the nuclear matrix vary depending on the matrix isolation method used. The nuclear matrix appears to be the most thermosensitive nuclear structure during heat shock. Here we provide evidence for the extensive translocation of PARP-1 from chromatin to the nuclear matrix during heat shock. This translocation is accompanied by inhibition of PARP activity in the nucleus and elevation of PARP activity in the nuclear matrix. Our data suggest that thermal destabilization of the nuclear matrix is less likely to contribute to the translocation of PARP-1 to the nuclear matrix.

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Poly(ADP-ribose) polymerase-1 (PARP-1) is a 113 kDa DNA-dependent enzyme found in eukaryotic cells. Using NAD^+ as a substrate, PARP-1 catalyzes the formation and attachment of linear or branched ADP-ribose polymers to numerous nuclear proteins, including PARP-1 itself [1]. PARP-1 is thought to play a role in the modulation of chromatin structure, transcription, DNA repair, etc. [2-4]. Numerous studies have indicated that PARP-1 is involved in the heat shock response. It has been shown that PARP activity is inhibited by hyperthermia [5]; PARP-1 and poly(ADP-ribose) are involved in the regulation of Hsp70 gene expression [6, 7]; thermosensitivity is higher in PARP-$1^{-/-}$ than in PARP-$1^{+/+}$ murine fibroblasts [8]; and PARP-1 also associates with the ATPase domain of Hsp70 [9].

It has been reported that PARP-1 is present in the nuclear matrix isolated from different tissues [10-13]. The amount of PARP-1 in the nuclear matrix depends on the tissue type, matrix preparation conditions, and the level of PARP-1 automodification [14, 15]. The nuclear matrix is thought to be a dynamic protein framework within the nucleus to which chromatin fibers, which are folded into loops, are attached. Various nuclear processes, such as DNA replication, transcription, and repair, have been reported to be associated with the nuclear matrix [16-18]. Although the techniques used for visualization of the nuclear matrix have improved over the years, it is still unclear as to what extent the isolated nuclear matrix corresponds to the *in vivo* structure, and many researchers consider the nuclear matrix to be an artificial structure [19, 20].

Proteins of the nuclear matrix are among the most thermolabile proteins in the nucleus and undergo denaturation at temperatures as low as 43-45°C [21]. Heat shock-induced protein denaturation results in the aggregation of denatured and/or native proteins to the nuclear matrix, leading to an increase in protein mass in isolated nuclear matrix fraction. This event is associated with the disruption of many nuclear matrix-dependent processes and cell death [22-24].

The aim of this study was to measure PARP enzymatic activity in subnuclear fractions and to determine the extent of PARP-1 translocation between the nuclear matrix and non-matrix regions that were isolated from the livers of heat-shocked rats.

MATERIALS AND METHODS

Reagents. PARP-1 (A-20) goat polyclonal IgG, rabbit anti-goat IgG-HRP, and Luminol reagent for Western

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blot analysis were obtained from Santa Cruz Biotechnology (USA). DNase I, β-NAD^+, phenylmethylsulfonyl fluoride (PMSF), 3-aminobenzamide, 1,5-isoquinolinediol, and protease inhibitor cocktail were purchased from Sigma-Aldrich Co. (USA). [^{14}C]NAD^+ was obtained from Amersham (England). All reagents used in this study were of the highest purity commercially available.

WBH (whole body hyperthermia) treatment of rats. For WBH treatment, male Wistar rats weighing approximately 200 g were placed in a dry air incubator at 42°C. After 1 h, the rats were decapitated. All experiments were conducted in accordance with the European Communities Council Directive of November, 1986 (86/609/EEC).

Preparation of nuclei and subnuclear fractions. Rat liver nuclei were isolated according to the method of Chauveau et al., with some modifications [25]. Purity of the nuclei was determined by phase-contrast microscopy and by measuring the activity of glucose 6-phosphatase, cytochrome oxidase, and NADH-cytochrome *c* reductase as cytoplasmic markers. The DNA/protein ratio of isolated liver nuclei was approximately 1 : 4. Nuclear extract containing PARP-1 was prepared as described previously [26]. Nuclear matrix was prepared using the high-salt extraction method [14]. To obtain the nuclear matrix, nuclei (2 mg of nuclear protein/ml) were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M sucrose, 50 mM NaCl, and 5 mM $MgCl_2$ and then treated with pancreatic DNase I (40 units/mg DNA) for 15 min at 25°C. The suspension was quickly cooled and centrifuged at 3000*g* for 10 min. The supernatant was recovered as the DNase-extractable fraction, and the pellet was further extracted for 15 min with 10 mM Tris-HCl buffer (pH 7.4) containing 2 M NaCl and 0.2 mM $MgCl_2$ and then centrifuged at 10,000*g* for 10 min. This nuclei extraction procedure was repeated twice. The supernatant was saved as the high salt-extractable fraction. The precipitate remaining after extraction was resuspended in buffer containing 10 mM Tris-HCl (pH 7.4), 0.2 mM $MgCl_2$, and 0.5% Triton X-100, incubated for 15 min, and then centrifuged at 3000*g* for 10 min. The pellet was washed three times with the same buffer but without detergent. The DNase-extractable and high salt-extractable fractions were considered the chromatin bound fractions, and the final pellet was considered the nuclear matrix fraction. Nuclear matrix fractions isolated from control and WBH-treated animals contained 8% and 16-20% of total nuclear protein, respectively.

All solutions used for the isolation of nuclei, nuclear extract, and nuclear matrix fractions contained 0.1 mM PMSF and protease inhibitor cocktail.

Western blotting analysis. Material corresponding to 10^6 nuclei or nuclear matrix isolated from $(1\text{-}5)\cdot10^6$ nuclei were separated by 7% SDS-PAGE according to the Laemmli protocol [27] and transferred onto a nitrocellulose membrane using an electroblotting apparatus. The membrane was first incubated with anti-PARP-1 antibody at a dilution of 1 : 500 and subsequently with HRP-conjugated secondary antibody at a dilution of 1 : 10,000. The bands labeled with the antibodies were visualized using the chemiluminescence Luminol reagent by exposure to X-ray films. The blots were quantitatively analyzed using Scion Image software.

Nuclear and nuclear matrix PARP activity assay. PARP activity in the nuclei and nuclear matrix fractions was determined by incorporation of [^{14}C]NAD^+ in acid-insoluble material as described before [28].

Nuclear matrix DNA preparation. DNA from nuclear matrix fractions was extracted using the phenol–chloroform method as described previously [29].

PARP-1 binding to isolated nuclear matrix fractions. Nuclear matrix (M) and nuclear extract (E) fractions were prepared in buffer containing 10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, and 3 mM $MgCl_2$. Samples of M and E, at a concentration of 0.1 mg protein/0.1 ml and 0.9 mg protein/ml, respectively, were left at room temperature or heated in a water bath at 41°C for 10 min. The resulting samples were referred to as nuclear matrix-heated (M^H), nuclear extract-heated (E^H), nuclear matrix-non-heated (M^{NH}), and nuclear extract-non-heated (E^{NH}). After being heated, the samples were mixed in the following manner: $M^H + E^{NH}$, $M^{NH} + E^{NH}$, $M^{NH} + E^H$, and $M^H + E^H$. In one case, M and E samples were mixed prior to heating (ME^H). Next, all the samples were left at room temperature for 15 min before collection of the nuclear matrix fractions by centrifugation at 8000 rpm for 5 min. The pellets were resuspended in 1 ml of the above-mentioned buffer, centrifuged again, and then the pellet was resuspended in 50 μl of Laemmli sample buffer for Western blot analysis.

Protein and DNA assay. Protein concentration was determined by the Bradford protein assay using bovine serum albumin as a standard [30]. DNA was measured using the diphenylamine reaction and calf thymus DNA as a standard [31] and also spectrophotometrically at 260 nm.

RESULTS

Influence of hyperthermia on PARP-1 levels in rat liver nuclear matrix. After the WBH treatment of rats at 42°C for 1 h, the amount of PARP-1 in liver nuclear matrix was estimated by Western blot analysis. In nuclear matrix fractions isolated from 10^6 nuclei, the PARP-1 immunoreactive band was barely detectable. In some cases, for better detection and quantitation, nuclear matrix samples prepared from $5\cdot10^6$ nuclei and 10^6 nuclei were loaded onto the gel. As shown in Fig. 1a, nuclear matrix isolated from control animals contains ~4% nuclear PARP-1, while PARP-1 level in the matrix isolated from WBH-treated rats significantly increases to up to

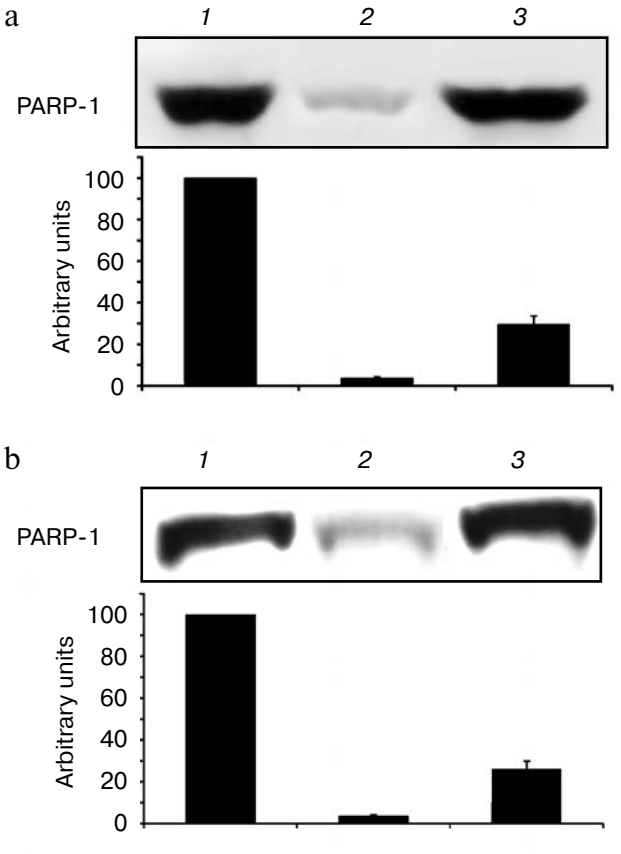

Fig. 1. Detection of PARP-1 in nuclear matrix fractions isolated from liver of WBH-treated rats and preheated nuclei. a) Approximately 10^6 purified rat liver nuclei (*1*) and nuclear matrix corresponding to $5 \cdot 10^6$ nuclei isolated from untreated and WBH-treated (42°C, 1 h) animals (*2* and *3*, respectively) were separated by 7% SDS-PAGE, and the PARP-1 level was determined by Western blot analysis. The data are presented as the mean ± SD of five separate experiments. b) Approximately 10^6 purified rat liver nuclei (*1*) and nuclear matrix corresponded to $5 \cdot 10^6$ nuclei isolated from untreated (*2*) and preheated nuclei (42°C, 10 min) (*3*) were separated by 7% SDS-PAGE, and the PARP-1 level was determined by Western blot analysis. The amount of PARP-1 is expressed in arbitrary units as a percentage of total nuclear PARP-1 levels (100%). The data are presented as the mean ± SD of three independent experiments.

~30% nuclear PARP-1. Similar results were obtained when isolated nuclei were pre-incubated at 42°C for 10 min before nuclear matrix preparation (Fig. 1b).

To identify the specific nuclear fraction from which PARP-1 was translocated to the nuclear matrix, isolated nuclei were heated at 42°C for 10 min before or after DNase I treatment. During isolation of the nuclear matrix, all the fractions were collected, and PARP-1 level in each fraction was measured (Fig. 2). Our results show that the DNase-sensitive nuclear fraction prepared from nuclei heated before DNase I treatment contained much less PARP-1 than the DNase-sensitive fraction extracted from non-heated nuclei. At the same time, the PARP-1 level in high salt-extracted fractions isolated from nuclei treated with DNase I before or after hyperthermia was almost equal. The difference appeared again between nuclear matrix fractions: PARP-1 content was higher in the matrix fraction obtained from nuclei heated before DNase I treatment than in the matrix fraction obtained from nuclei heated after DNase I treatment.

Effect of high temperature on PARP-1 translocation from nuclear extract to nuclear matrix. It is well known that hyperthermia induces structural changes of the nuclear matrix and may even bring about conformational changes of non-matrix proteins, including PARP-1. Therefore, we decided to investigate whether one of these changes or both are the cause of increased PARP-1 translocation to the nuclear matrix during high-temperature conditions.

To test this, non-heated and heated (at 41°C for 10 min) nuclear matrix and nuclear extract samples were mixed as described in "Materials and Methods". PARP-1 binding to the nuclear matrix was detected by Western blot analysis. As shown in Fig. 3, PARP-1 translocation

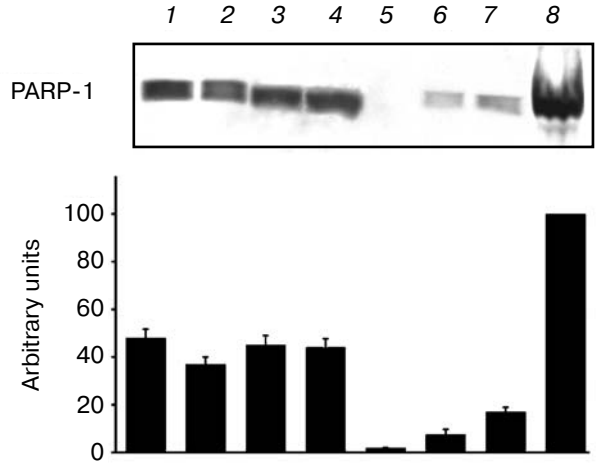

Fig. 2. PARP-1 translocates from chromatin to the nuclear matrix during heat treatment of isolated nuclei. Isolated nuclei were digested with DNase I before or after incubation of the nuclei at 42°C. PARP-1 levels in the DNase-extractable and DNase-non-extractable (high salt-extractable) chromatin fractions, as well as nuclear matrix isolated from non-heated and heated nuclei treated with DNase I before or after incubation of nuclei at 42°C were analyzed. DNase I-extractable chromatin prepared from non-heated nuclei (*1*), DNase I-extractable chromatin obtained from nuclei heated before DNase I treatment (*2*), high salt-extractable chromatin prepared from nuclei heated after DNase I treatment (*3*), high salt-extractable chromatin obtained from nuclei heated before DNase treatment (*4*), nuclear matrix prepared from non-heated nuclei (*5*), nuclear matrix prepared from nuclei heated after DNase I treatment (*6*), and nuclear matrix prepared from nuclei heated before DNase I treatment (*7*) equivalent to $2 \cdot 10^6$ purified rat liver nuclei (*8*) were separated by 7% SDS-PAGE. PARP-1 levels were determined by Western blot analysis. The amount of PARP-1 is expressed in arbitrary units as a percentage of total nuclear PARP-1 level (100%). The data are presented as the mean ± SD of four independent experiments.

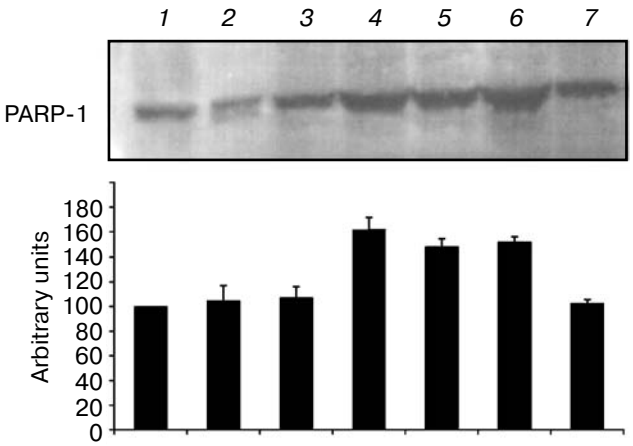

Fig. 3. PARP-1 translocation from nuclear extract to isolated nuclear matrix. Non-heated (M^{NH}) or heated (M^{H}) purified nuclear matrix fractions, and non-heated (E^{NH}) or heated (E^{H}) nuclear extract fractions were mixed as described in "Materials and Methods". Nuclear matrix (*1*), nuclear extract (*2*), E^{NH} + M^{NH} (*3*), E^{H} + M^{NH} (*4*), E^{H} + M^{H} (*5*), $(ME)^{H}$ (*6*), and E^{NH} + M^{H} (*7*) were separated by 7% SDS-PAGE. PARP-1 levels were determined by Western blot analysis. The amount of PARP-1 is expressed in arbitrary units as a percentage of total nuclear matrix PARP-1 level (100%). The data are presented as the mean ± SD of five independent experiments.

to the nuclear matrix took place only in the samples containing heated nuclear extract mixed with non-heated or heated nuclear matrix. In the samples where nuclear matrix and nuclear extract were heated together, the extent of PARP-1 translocation was comparable to that in the samples containing separately heated nuclear extract.

Influence of hyperthermia on PARP activity in nucleus and nuclear matrix. Next, we investigated how the redistribution of PARP-1 as a result of heat shock affects PARP activity in the nuclei and nuclear matrix fractions. As shown in Fig. 4a, PARP activity in WBH-treated rat liver nuclei was decreased by 30% compared to that of non-treated animals. In contrast, in the nuclear matrix fractions isolated from WBH-treated animals, both specific (Fig. 4b) and total activities (Fig. 4c) of PARP increase by ~1.5- and ~2.4-fold, respectively. Similar results were obtained when isolated nuclei were heated to 42°C (data not shown).

DISCUSSION

The protein content in nuclear matrix fractions isolated from the liver of WBH-treated rats increased 2-2.5-fold in comparison to nuclear matrix isolated from untreated rats. However, in contrast to previously published data [32], there was no detectable increase in total protein content in nuclei isolated from WBH-treated rats.

Our results show that PARP-1 level in the nuclear matrix dramatically increases after hyperthermia and is accompanied by the inhibition of PARP activity in the nucleus. It has been shown that caspase-mediated cleavage of PARP-1 occurs during heat shock [33]. However, we did not detect any bands for cleaved PARP-1 on immunoblots of nuclei isolated from WBH-treated rats, thus ruling out the possibility of caspase-mediated cleavage of PARP-1. This conclusion is further supported by

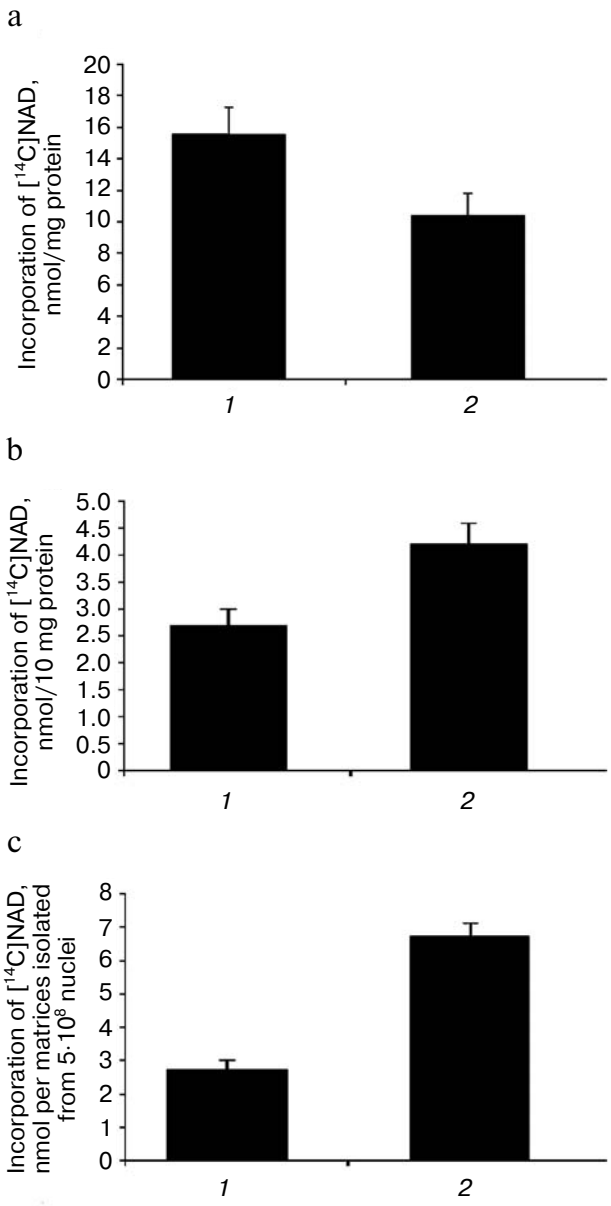

Fig. 4. Effect of WBH treatment of rats on PARP activity in liver nuclei and nuclear matrix fractions. a) PARP activity in the nuclei of untreated (*1*) and WBH-treated (*2*) rats. The data are presented as the mean ± SD of four independent experiments. b) Specific activity of PARP in nuclear matrix fractions isolated from the nuclei of untreated (*1*) and WBH-treated (*2*) rats. The data are presented as the mean ± SD of four independent experiments. c) Total activity of PARP in nuclear matrix fractions isolated from $5 \cdot 10^8$ nuclei of untreated (*1*) and WBH-treated (*2*) rats. The data are presented as the mean ± SD of four independent experiments.

the fact that inhibition of PARP activity is also observed when isolated nuclei are heated, where the presence of active forms of caspases is excluded. Therefore, inhibition of PARP activity in the nuclei is likely due to heat inactivation of PARP-1 resulting in increased affinity of destabilized PARP-1 to the nuclear matrix, and/or the relocation of native PARP-1 molecules from chromatin to the nuclear matrix, where PARP-1 exhibits only partial activity. The latter scenario is more likely because in nuclear matrix fractions obtained from WBH-treated rats there is an increase PARP activity. This suggests that upon translocation to the nuclear matrix, PARP-1 retains its enzymatic activity, at least partially.

The measurement of PARP-1 levels in different nuclear fractions showed that the DNase-extractable fraction isolated from non-heated nuclei contained the amount of PARP-1 equivalent to the difference between PARP-1 levels in nuclear matrix obtained from nuclei heated before or after DNase I treatment and PARP-1 levels of the DNase-extractable fraction isolated from nuclei heated before DNase I treatment. Based on our results, we can conclude that PARP-1 translocation to nuclear matrix occurs from both the DNase-extractable and high salt-extractable chromatin fractions.

As mentioned previously, the nuclear matrix is the most thermolabile nuclear structure to which soluble nuclear proteins, both native and denatured, aggregate during heat shock [20]. However, our *in vitro* investigation indicates that PARP-1 translocation to the nuclear matrix is more likely due to the effect of heat on nuclear extract proteins, including PARP-1, rather than the structural destabilization of the nuclear matrix. When nuclear extracts were added to nuclear matrix samples with equal amounts of DNA (~0.1% of total nuclear DNA), PARP-1 binding to the nuclear matrix took place only in those samples to which pre-heated nuclear extract was added, suggesting that the presence of excess DNA in the nuclear matrix fractions isolated from WBH-treated rats does not contribute to increased PARP-1 binding to the nuclear matrix.

The involvement of cytoplasmic factors in the process of PARP-1 translocation can be excluded because similar amounts of PARP-1 were detected in nuclear matrix isolated from WBH-treated animals and heated nuclei. Based on these experiments with isolated nuclei, the role of PARP-1 automodification in its own translocation to the nuclear matrix can also be excluded, as the isolated nuclei did not contain NAD^+. This latter observation was supported by the fact that intraperitoneal injection of PARP-1 inhibitors, 3-aminobenzamide and 1,5-isoquinolinediol, prior to WBH treatment of animals did not change the extent of PARP-1 translocation to the nuclear matrix (data not shown).

Our data demonstrate that the changes in the nuclear PARP activity at the early stages of heat shock are accompanied by PARP-1 translocation from chromatin to the nuclear matrix. However, it remains unclear whether this process is a specific response of eukaryotic cells to heat shock or whether it is the result of nonspecific protein aggregation. Regardless, the changes in PARP-1 subnuclear distribution as well as the inhibition of nuclear PARP activity during heat treatment are thought to be involved in the process of normal and/or tumor cell killing by hyperthermia.

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