Review

What is the Critical Hyperthermia Target in Cancer Cells ?

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Abstract : Currently, the molecular mechanisms involved in heat-induced cell killing are not completely understood, although protein denaturation is known to be the major target for hyperthermia in cancer cells. A new hypothesis is described here which proposes that heat-induced cell killing is associated with cellular DNA double-strand break (DSB) formation. This review provides details of this model for hyperthermia cell killing, and a possible mechanism to explain heat-induced DSB formation.

Key Words : hyperthermia, cell killing, protein denaturation, γH2AX, double-strand break

Introduction

Interest in hyperthermia has grown tremendously over the past three decades, and molecular biology has contributed to the understanding of hyperthermia. In response to new knowledge, new methods of applying hyperthermia are being constantly proposed[1,2]. Heat induces protein denaturation through the breakage of hydrogen bonds in protein molecules which results in cell killing. It has been thought that heat-induced cell killing was not dependent on the formation of DSBs, although X-ray-induced cell killing does depend on creating DSBs. This conclusion was reached because of data showing that there was no correlation between thermosensitivity and radiosensitivity after exposure to various conditions such as hypoxia[3], low pH[4], and in response to variations in cell cycle position[5] and DNA repair activity[6]. In addition, hyperthermia induce chromosomal aberrations only when cells are heated in S-phase cells[7], and does not cause cell transformation like radiation.

Recently, however, it was reported that exposure to heat could induce cellular DNA double-strand breaks (DSBs)[8]. In the light of this report, reconsideration of the critical events involved in heat-induced cell killing seem appropriate, and this review describes observations of DSBs which likely result from the direct cytotoxic effect of heat.

Protein denaturation

In general, it is now accepted that proteins are the major target in the clinically relevant temperature range (Table I) for hyperthermia. One reason is that heat sensitivity is induced by dysfunction in protein activity because hydrogen bonds and non-polar hydrophobic interactions are heat-labile. Denaturation

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Table I. Observations supporting protein denaturation as the major hyperthermic target in cancer cells.

Observations	References
Relationship of heat-induced cell killing and protein stabilization	9-18
Correlation of the energy of cell killing with protein denaturation	20
Important heat-labile proteins for cell viability	21, 22
Heat sensitivity under various conditions such as hypoxia, low pH, cell cycle and DNA repair activity	3-6
Heat-induced chromosomal aberrations	7

of proteins disrupts normal α-helix and β-sheet structures in proteins, allowing a protein to uncoil into a random shape, and leads to the disruption and possible destruction of both secondary and tertiary protein structure. Glycerol can protect cells from heat-induced cell killing, by preventing heat induced protein denaturation[9-11]. To prevent protein denaturation, cells can also express a set of proteins called heat shock proteins (HSPs)[12,13]. HSPs are known to be molecular chaperones and can protect proteins from the deleterious effects of acute or chronic stress by stabilizing and refolding proteins, or by facilitating protein degradation[14]. Overexpression of HSPs in cells has been reported to induce heat resistance[15,16], and conversely, downregulation of HSPs increases heat sensitivity[17]. Furthermore, thermotolerance requires the chaperone activities of HSPs to protect heat-labile proteins[18].

It has been reported that the retention of the essential structural and functional properties of proteins in species adapted to different temperatures is achieved through variations in amino acid sequences, and by the accumulation of small organic solutes which can stabilize protein properties[19]. Interestingly, there is an inflection point at about 42°C in the Arrhenius plot of cell killing and protein denaturation in mammalian cells, and the thermal activation energies of both, cell killing and protein denaturation, are almost the same above and below this inflection point[20]. These results indicate that an irreversible change in proteins could be regarded as a cause of heat-induced cell killing. In support of this observation, important heat-labile proteins are necessary for cell survival and functioning, for example the elongation factor Ts proteins. The aggregation of nuclear proteins has also been linked to the inhibition of numerous nuclear matrix-dependent functions (*e.g.,* DNA replication, DNA transcription and mRNA processing) and can result in cell death[21]. Heat-induced centrosome denaturation in chromosomes can be linked to mitotic catastrophe after hyperthermia, and thus to the loss of the cell's reproductive capacity[22]. Based on these examples and on other observations, the conclusion that protein denaturation is a major target for hyperthermia action in cancer cells is described in textbooks and has been well accepted.

DSBs

Another possible target involved in hyperthermic cell killing is cellular DNA, because heat-induced DSBs were found using recently developed techniques to detect foci of γH2AX (histone H2AX phosphorylated at serine 139) by immunocytochemical staining[8]. DSBs represent a significant DNA damage event; one DSB remaining unrepaired in a cell can potentially result in cell death. Recently, the measurement of γH2AX foci formation has attracted considerable attention because this method can

provide a very sensitive and specific indicator for the existence of a single DSB ; specifically, one γH2AX focus correlates to one cellular DSB[23–25]. A recent report indicated that heat induces the formation of γH2AX foci[8], which possibly associates the effects of heat with the generation of cellular DSBs, and these results were subsequently confirmed by another laboratory[26].

A number of investigators have reported that cellular DNA strand breaks can be detected in heat-treated cells using various methods[27–30] (Table II), however, these conventional physical methods are not sensitive enough to document a relationship between cell killing and DSBs after heat treatment. The problem is that although a single DSB can lead to a cell death, the sensitivity of these methods is insufficient to measure such a very low level of DSBs. Because of this lack of sensitivity, it has been impossible to demonstrate the existence and biological effects of heat-induced DSBs until very recently.

Table II. Studies of heat-induced cellular DNA strand breaks.

Methods used	References
alkaline elution	27
alkaline unwinding	28
in situ nick translation	29
pulse-field gel electrophoresis	30
neutral single-cell electrophoresis	8
immunocytostaining of γH2AX foci	8, 26

DSBs may not be present, even if heat-induced γH2AX foci are observed, although radiation-induced DSBs do lead to the formation of γH2AX foci. It is likely that γH2AX foci formation results from the disorganization of chromatin structure without DSB formation, although, to date, this has not been directly shown to occur since no γH2AX foci have been detected after chromatin-modifying treatments (hypotonic conditions, or exposure to chloroquine or tricostatin A)[31]. Though previous reports demonstrated the formation of DSBs only when cells are heated in S-phase[30], other recent work has reported the detection of heat-induced γH2AX foci, not only in S-phase, but also in G_1 and G_2-phase[8]. Observations have shown that heat stress induces ATM activation, and ATM is known to be activated by the presence of DSB lesions[32]. In addition, MDC1 amplifies the ATM-signaling pathway by accumulating active ATM to areas adjacent to the sites of DNA damage and then promotes the expansion of H2AX phosphorylation[33]. But when ATM molecules are absent, DNA-PK can contribute to the phosphorylation of H2AX without the presence of ATM[26,34]. It has also been confirmed that heat induces DSBs through a different pathway and not in the same manner as seen in replication arrest with ATR activation[26]. These results still support the idea that the formation of γH2AX foci is dependent on the induction of a DSB[25]. Using pulsed field gel electrophoresis[30], observation of heat induced DSBs was observed, and the data was then extrapolated to the region where very low numbers of heat induced DSBs could be generated. These low numbers of DSBs were found to correspond with the number of heat-induced γH2AX foci[8]. This result suggests that it is possible to examine the biological effects of heat-induced DSBs, which has been overlooked because of their small amount, by using immunocytostaining for the detection of γH2AX foci. In the plateau area of

heat-induced DSBs detected by pulse-field gel electrophoresis[30] and neutral single-cell electrophoresis[8], it appears likely that protein denaturation plays a major role in heat-induced cell death in accord with current concepts. When cells are heated at 41.5-45.5°C, the number of γH2AX foci increases in a time-dependent manner[8]. An inflection point was observed at 42.5°C, and the thermal activation energies above and below the inflection point were almost the same for cell killing and foci formation according to Arrhenius plot analysis. During periods when cells were exposed to heat, the cell-cycle-dependent pattern for cell killing was the same as the cell cycle pattern observed for γH2AX foci formation[8]. It was also found that thermotolerance was due to a depression in the number of γH2AX foci formed after heating, when the cells were pre-treated or pre-conditioned with a heat exposure[8]. These findings suggest that cell killing could be associated with DSB formation.

It is possible that the biological responses which lead to the formation of DSBs are different after exposure to X-rays or heat. For example, chromosomal aberrations, associated with radiation-induced DSBs, are only found to form when cells are heated in S-phase[7]. This phenomenon can be explained by the observation that heat-shock protein Hsp70 knockout mice displayed a high frequency of chromosomal aberrations[35], suggesting that Hsp70 can protect cells against such aberrations. Because inducible Hsp70 is more abundant in heat-treated cells than in X-irradiated cells, chromosomal aberrations may be hard to identify in heat-treated cells. DSB-recognizing proteins (Nbs1 and Mre11) clearly co-localize with the γH2AX already present in the nucleus late in their formation, though the observed translocation behavior of these DSB-recognizing proteins from the nucleus immediately after heat treatment appears to be different from that observed after exposure to X-rays[36,37]. It also may not be possible to make a valid comparison between heat sensitivity and radiosensitivity in DNA repair-deficient cells and wild-type cells, because DNA repair enzymes could be inactivated by heat treatment[38] even in wild-type cells. In addition, it may be invalid to compare heat sensitivity with radiosensitivity in cells with different genetic backgrounds.

These observations and examples collectively provide the support for the concept that heat-induced DSBs contribute to heat-induced cell killing.

Possible mechanisms of heat-induced DSB formation

Possible mechanisms for the formation of heat-induced DSB through a cellular response *via* protein denaturation[8] (Fig. 1) have been proposed. Heat might not form DSBs directly in the same manner as ionizing radiation does. PCR methods are commonly used to study molecular biology at relatively high temperatures, but few DSBs are observed to form, even at high temperatures. However, heat induces base modifications such as oxidative base damage[39], abasic DNA sites[40], deamination of cytosine[41] and other types of damage through reactive oxygen species[39,42] and reactive nitrogen species[43]. Incision enzymes such as glycosylases and AP-endonucleases may incise DNA bases containing these modifications, but DNA synthesis enzymes may not operate at these nick sites because DNA polymerase β, for example, is heat sensitive when compared with incision enzymes used for base excision repair[44]. Thus, there is a theoretical mechanism which can be used to explain how hyperthermia can induce nicks through base repair processes. DSBs could be generated if nicks in DNA form in close proximity to each other on opposite strands. In fact, the inhibition of poly (ADP) ribose polymerase, which is involved

in base excision repair and single-strand break repair, induces γH2AX foci[45]. It has also been hypothesized that a nick is converted to a DSB at a DNA replication fork. This explanation was supported by recent findings that more γH2AX foci were formed when cells were heated in S-phase than in G_1- or G_2-phase[8].

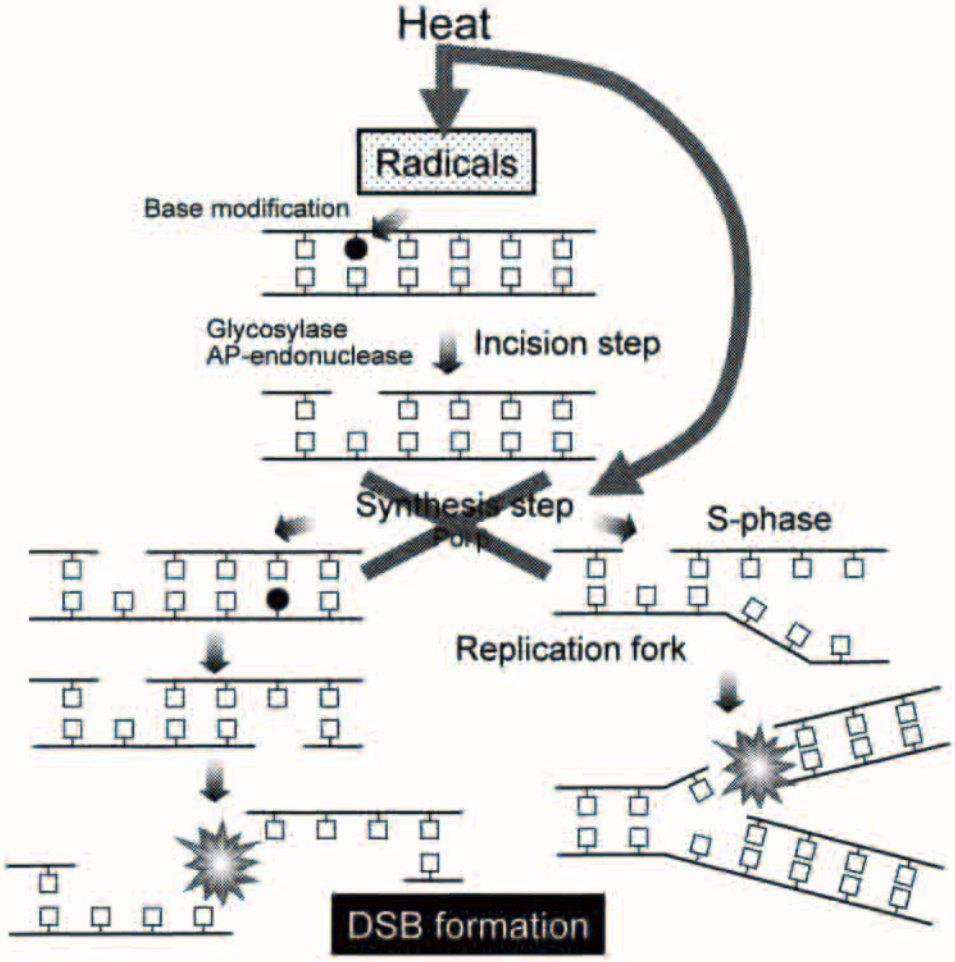

Fig. 1. Schematic drawing showing possible mechanisms of heat-induced DSB formation.

If a sufficient number of base modifications, which can lead to the formation of DSBs, are not induced by hyperthermia, the depression of cellular repair capacities of DSB damage generated spontaneously during the process of transcription and replication could also offer a mechanism to explain the formation of heat-induced DSBs ; such a mechanism could act through the denaturation of repair related proteins such as Ku70 and Ku80[46]. Such possible mechanisms could also involve the inhibition of access for the repair enzymes to damage sites due to the denaturation of nuclear matrix proteins[21], and/or the translocation and removal of repair related proteins from the nucleus[36,37,47,48].

Closing comments

It is proposed that heat-induced cell killing might be dependent on, or associated with, DSB formation in mammalian cells. Further investigations are required to define the exact mechanism leading to heat-induced DSB formation. Such studies could contribute to new concepts and further understanding of hyperthermic biology and oncology.

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ハイパーサーミアのターゲットは何か？

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要　　旨：温熱による細胞死の分子機構については多くの不明な点が残されている．従来，温熱による細胞死の原因がタンパク質変性と考えられてきた．最近我々はDNA二本鎖切断 (DSB) こそが細胞死の原因とする実験結果を報告した．ここでは，温熱による細胞死の原因について，従来のタンパク質変性が主因と考えられる論拠を概説し，次にDSBが細胞死の主因と考えられる論拠を紹介する．さらに，温熱によるDSB生成機構について考察する．