Phospho-Nbs1 and Mre11 proteins which recognize DSBs co-localize with γ H2AX in the nucleus after heat treatment

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Abstract

Background: Critical hyperthermia targets in cancer cells have to be clearly identified.

Method: To determine if the DNA double strand break (DSB)-recognizing proteins, phospho-Nbs1 and Mre11, co-localize with phospho-H2AX (γ H2AX), immunohistochemical methods were used with multiple antibodies. After a heat treatment at 45.5°C for 20 min, human normal fibroblasts were analyzed with a laser scanning confocal microscope at 0.5 h and 8 h post-heat treatment.

Results: At 0.5 h after a heat treatment, γ H2AX formed foci in the nucleus, but phospho-Nbs1 and Mre11 were scattered over the nucleus and cytoplasm, and did not co-localize with γ H2AX. At 8 h after a heat-treatment, and also after X-irradiation, both phospho-Nbs1 and Mre11 co-localized with γ H2AX foci, which were already present, in the nucleus. Moreover, both phospho-Nbs1 and Mre11 which were observed at 0.5 h after a heat treatment in the cytoplasm, were no longer observed in the cytoplasm at 8 h after a heat treatment.

Conclusion: These findings provide support for the concept that heat, like X-rays, may lead to the induction of DSBs.

Keywords: hyperthermia, γ H2AX, phospho-Nbs1, Mre11, double strand break.

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Introduction

A number of investigators have reported that cellular DNA strand breaks can be detected in heat-treated cells by using various methods such as pulsed-fields gel electrophoresis, comet assays, and the DNA filter elution assay¹⁻⁵⁾. However, these conventional physical methods are not sensitive enough to document a relationship between cell killing and double strand breaks (DSBs) after heat treatment. The problem is that even though a single DSB can lead to cell death, these methods are insufficiently sensitive to measure low levels of DSBs. Because of this lack of sensitivity, it has been impossible to demonstrate the existence and biological effects of heat-induced DSBs until quite recently. Recent reports indicated that heat treatment could induce DSBs in cellular DNA, and these observations were made by utilizing immunohistochemical methods with antibodies capable of recognizing histone phospho-H2AX (γ H2AX)¹⁾. The induction of these γ H2AX positive foci was subsequently confirmed by another laboratory⁶⁾.

Immunohistochemical methods utilizing γ H2AX recognizing antibodies have become the gold standard

to detect the presence of DSBs^{7,8)}. These methods are known to be extremely sensitive and to be a specific indicator for the existence of a DSB^{9,10)}. There is also a possibility that the observed γ H2AX foci formation could have resulted from changes in chromatin structure, and at present, this possibility has not been directly verified, however, no γ H2AX foci have been detected after experimental chromatin-modifying treatments (hypotonic conditions, or exposure to chloroquine or trichostatin A)¹¹⁾. Although previous reports demonstrated the formation of heat-induced DSBs only during S-phase²⁾, other recent work has reported the detection of heat-induced γ H2AX foci, not only in S-phase, but also in G₁ and G₂-phases¹⁾. Observations have shown that heat stress induces ATM activation, and ATM is known to be activated by the presence of DSBs¹²⁾. In addition, heat-induced H2AX phosphorylation is mediated by an interaction between ATM and MDC1¹³⁾. However, when ATM molecules are absent, DNA-PK can act as a substitute for ATM and lead to the phosphorylation of H2AX^{6,14)}. It has also been shown that heat induces DSBs through a different pathway and in a different manner from what is seen during replication arrest with ATR activation. These results lend support to the suggestion that the formation of γ H2AX foci is dependent on the induction of a DSB⁸⁾. It has been suggested that heat-induced DSBs contribute to heat-induced cell killing, because a correlation was shown to exist between the number of heat-induced

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γ H2AX foci observed and the mean lethal heating periods^{1,15}. However, some investigators believe that recent reports suggesting that heat-induced DSBs play a role in heat induced cell killing are incorrect¹⁶. This is because the Mre11, Rad50 and Nbs1 proteins which are directly involved in DSB repair also form foci immediately after X-irradiation^{17,18}, but are not found in foci seen after heat treatment: instead, these proteins exit the nucleus after a heat treatment¹⁹. However, the behavior and localization of γ H2AX and other DSB-recognizing proteins after exposure to heat have not yet been fully examined. Therefore, the work described here was designed to observe the cellular localization of these proteins after heat treatment. The visualization of γ H2AX and two other DSB-recognizing proteins, phospho-Nbs1 and Mre11, was accomplished by using multiple antibodies with immunohistochemical staining, and observations with a laser scanning confocal microscope.

Materials and methods

(1) Cells

Normal human fibroblast cells, AG1522, were cultured and maintained in exponential growth on glass slides in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, penicillin (50 U/ml), streptomycin (50 μ g/ml) and kanamycin (50 μ g/ml), and grown at 37°C in a conventional humidified CO₂ incubator¹.

(2) Hyperthermia and irradiation

Exponentially growing cells were immersed in a water bath (Thermominder EX, TAITEC Co., Ltd., Saitama, Japan) maintained at a constant temperature. X-ray irradiation (1.0 Gy/min) was administered with a 150-kVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo, Japan). Under the present experimental conditions, no marked change of pH was detected in the medium during the treatment.

(3) Histological study of DNA damage-recognizing proteins.

After heat treatment at 45.5°C for 20 min or X-irradiation at 3 Gy, cell preparations were fixed with cold methanol for 20 min and acetone for 7 sec, dried, blocked with 3% skim milk in PBS for 10 min, and washed in TPBS (PBS containing 0.05% Tween 20). The cells were then incubated with anti-phospho-H2AX (ser139) mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA) at a 300-fold dilution, anti-phospho-Nbs1 (ser343) rabbit polyclonal antibody (Novus Biologicals, Inc., Littleton, CO, USA) at a 1,000-fold dilution and anti-Mre11 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

at a 1,000-fold dilution for 60 min at room temperature, washed with TPBS, then incubated with AlexaFluor[®] 488 donkey anti-mouse IgG, AlexaFluor[®] 555 donkey anti-rabbit IgG, and AlexaFluor[®] 594 donkey anti-goat IgG (Molecular Probes, Eugene, OR, USA) at a 400-fold dilution for 60 min at room temperature, and washed in TPBS. Nuclei were stained with 1 μ g/ml 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). The slides were mounted with SlowFade[®] Antifade (Molecular Probes). Photographs of the cells were taken with a laser scanning confocal microscope (FluoView[™] FV1000, KS Olympus Co. Ltd., Tokyo, Japan). To allow direct comparisons, all the images were captured using the same parameters. γ H2AX foci were assigned green, phospho-Nbs1 foci red, Mre11 foci gray, and the nucleus blue with analyzing software (FV10-ASW trial ver.01.02.01, KS Olympus Co. Ltd.).

Results and discussion

The formation of γ H2AX foci was not detected in untreated control cells (Fig. 1a). Typical photographs of radiation- or heat-induced γ H2AX foci in normal human fibroblast AG1522 cells are shown in Figure 1b-e. As previously described¹, the formation of γ H2AX foci was observed, not only in X-irradiated cells (Fig. 1b and c) but also in heat-treated cells (Fig. 1d and e). Although it was reported that heat shock did not induce γ H2AX foci formation in several cell lines including HeLa, CHL, HepG2, HEK 293, U2OS and et al^{20,21}, our laboratory detected the presence of heat-induced γ H2AX foci in these same cells with other more sensitive techniques using immunohistochemical methods (data not shown). From these recent observations, it was concluded that heat induction of γ H2AX foci formation might be a common phenomena.

Mre11/Rad50/Nbs1 complexes recognize DSBs in the nucleus after X-irradiation^{17,18}. Nbs1 is phosphorylated at serine 343 after X-irradiation and contributes to foci formation along with Mre11 and Rad50²². The formation of phospho-Nbs1 and Mre11 foci was not observed in untreated cells (Fig. 1f and k). Typical photographs of radiation-induced phospho-Nbs1 (Fig. 1g and h) and Mre11 foci (Fig. 1l and m) are shown. Phospho-Nbs1 and Mre11 were found only in the nucleus after X-irradiation (Fig. 1g, h, l and m). Phospho-Nbs1 was seen to co-localize with Mre11, but free Mre11 was still observed at 0.5 h after X-irradiation (Fig. 1g and l). As previously reported¹⁷, both phospho-Nbs1 and Mre11 only partially co-localized with γ H2AX at 0.5 h after X-irradiation but predominantly with γ H2AX at 8 h after X-irradiation (Fig. 1v and w).

Although it has been reported that heat treatment causes the translocation of Nbs1, Mre11 and Rad50 proteins from the nucleus to the cytoplasm, their localiza-

Fig. 1 Typical localization patterns of γ H2AX, phospho-NBS1 and Mre11. a-e, γ H2AX; f-j, phospho-NBS1; k-o, Mre11; p-t, nucleus; u-y, merging of γ H2AX, phospho-NBS1, and Mre11 in the nucleus; a, f, k, p, and u, untreated; b, g, l, q, and v, 0.5 h after X-irradiation at 3 Gy; c, h, m, r, and w, 8 h after X-irradiation at 3 Gy; d, i, n, s, and x, 0.5 h after heat treatment at 45.5°C for 20 min; e, j, o, t, and y, 8 h after heat treatment at 45.5°C for 20 min.

tion after heat treatment has not been examined in detail in relation to γ H2AX and the DSB-recognizing proteins; phospho-Nbs1 and Mre11. Thus, the association of γ H2AX with these DSB-recognizing proteins was examined here. Typical photographs of heat-induced phospho-Nbs1 (Fig. 1i and j) and Mre11 foci (Fig. 1n and o) are shown. At 0.5 h after heat treatment, there were numerous phospho-Nbs1 and Mre11 molecules in the cytoplasm (Fig. 1x). In the nucleus, at this time point, neither phospho-Nbs1 nor Mre11 were seen to co-localize with γ H2AX (Fig. 1x). However, at 8 h after heat treatment, both proteins clearly co-localized with the γ H2AX foci already present in the nucleus (Fig. 1y). These findings strongly support the concept that heat treatment may induce DSBs in cellular DNA.

Moreover, the number of γ H2AX foci was found to decrease at 8 h after X-irradiation (Fig. 1c). The residual

γ H2AX foci are considered to indicate the presence of persistent unrepaired DSBs induced by radiation²³. In contrast, numerous γ H2AX foci are present at 8 h after heat treatment (Fig. 1e). If X-irradiation was combined with a heat treatment, current thoughts were that the heat-induced inactivation of repair enzymes inhibited the repair of radiation-induced DSBs in DNA^{24,25}. If this was actually the situation, it could be difficult 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 even in wild-type cells. Alternatively, other hypotheses which could explain the damage to cellular DNA repair mechanisms induced by heat are 1) an inhibition of access to DNA damage sites for the repair enzymes, possibly due to the denaturation of nuclear matrix proteins²⁶, and/or 2) the translocation and re-

removal of repair related proteins from the nucleus^{19,27,28}. In addition, it may be invalid to compare heat sensitivity with radiosensitivity in cells with different genetic backgrounds. These considerations could help define a model for a scenario in which heat-induced DSBs could contribute to heat-induced cell killing.

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, heat induces γ -H2AX and MDC1 foci but not 53BP1 or SMC1 foci²⁹. In addition, chromosomal aberrations, associated with radiation-induced DSBs, are not found to form in some phases of the cell cycle in heat-treated cells³⁰. This phenomenon could be explained by the observation that heat-shock protein Hsp70 knockout mice displayed a high frequency of chromosomal aberrations³¹, 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 consequently be hard to identify in heat-treated cells.

It is proposed that heat-induced cell killing might be dependent on, or associated with, DSB formation in mammalian cells. However, further investigations are still necessary 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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