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Heat-Induced Phosphorylation of NBS1 in Human Skin Fibroblast Cells

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Abstract NBS1 is known to be involved in DNA damage-induced cellular responses after exposure to ionizing radiation (IR). Phosphorylation of NBS1 contributes to cell-cycle checkpoints. The aim of this study was to determine whether heat exposure induces or stimulates cellular responses mediated by the phosphorylation of NBS1 in human skin fibroblast cell lines. The results of immunofluorescent staining and Western blot analysis showed that NBS1 proteins are phosphorylated after exposure to heat in the nucleus of a normal skin fibroblast cell line (82-6 cells). This suggests that the NBS1-mediated signal transduction could be induced by heat. We further examined whether a deficiency in the NBS1 protein modifies heat sensitivity in human skin fibroblast cell lines. A skin fibroblast cell line (Gmtert), derived from a Nijmegen breakage syndrome (NBS) patient containing mutant *NBS1*, showed higher sensitivity to heat than the same cell line transfected with the wild-type copy of the *NBS1* gene. We also showed that transfection of a DNA cassette expressing small interference RNA (siRNA) targeted to *NBS1* into 82-6 cells enhanced cell sensitivity to heat. These results suggest that NBS1 is involved in cellular responses to DNA damage which is induced by heat exposure as well as by radiation exposure in human skin fibroblast cells. J. Cell. Biochem. 99: 1642–1650, 2006.

Key words: NBS1; siRNA; RNAi; heat; DNA repair

Nijmegen breakage syndrome (NBS), a human genetic disorder characterized by a high incidence of cancer [Tauchi et al., 2002a,b], is caused by a deficiency in NBS1 protein function. NBS1 plays an important role in the repair of DNA double strand breaks (DSBs), cell-cycle checkpoints, and telomere stability after ionizing radiation (IR) [Tauchi et al., 2002b; Howlett et al., 2006; Zhang et al., 2006]. After exposure of mammalian cells to IR, NBS1 contributes to recruitment of a complex of MRE11 and RAD50 into the nucleus. The complex binds to H2AX histone which has been phosphorylated by the ataxia telangiectasia mutated protein (ATM), and then moves to the vicinity of any DNA DSB. The MRE11/RAD50/NBS1 (MRN) complexes can be detected as foci in the irradiated regions of the nucleus [Nelms et al., 1998] as a consequence of DNA damage, and the number of foci present depends on the radiation dose (Ito et al., 1999]. These foci generate the nuclease activity necessary to produce 3′ single-stranded DNA tails, and remain present until DNA repair is complete [Maser et al., 1997; Mirzoeva and Petrini, 2001]. NBS1 phosphorylated by ATM functions in signal transduction events for cell-cycle checkpoints [Lim et al., 2000; Wu et al., 2000; Zhao et al., 2000]. It also leads to the activation of CHK2 which leads to G1 and G2/M arrest after irradiation [Buscemi et al., 2001].

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NBS1 is possibly a key signaling modifier through its phosphorylation in a process of cross-talk between cell-cycle checkpoints and DNA repair.

It has been reported that ATM mediates the heat-induced signal transduction pathway [Miyakoda et al., 2002], and since ATM is involved in NBS1 phosphorylation, this implies that heat could be involved in NBS1 phosphorylation like radiation. In the present study, we examined whether heat exposure leads to NBS1 phosphorylation that could possibly activate signal transduction after DNA damage. We recently reported that heat exposure causes γH2AX foci formation that serves as an indicator for the presence of DSBs [Takahashi et al., 2004]. We propose that heat as a DNA damaging agent could activate cellular responses induced by DNA damage that depends on *NBS1* status, similar to ones activated by irradiation. In the present study, we examined whether heat exposure leads to NBS1 phosphorylation that activate signal transduction events after DNA damage. Furthermore, in order to examine the biological significance of the heat-induced phosphorylation of NBS1, we measured heat sensitivity in a skin fibroblast cell line derived from a NBS patient deficient in NBS1 protein expression (NBS1 deficient cells). We also examined the effect of a DNA cassette expressing small interference RNA (siRNA) targeted for *NBS1* on the heat sensitivity of normal skin fibroblast cells (82-6). The results reported here indicate that cellular *NBS1* status can modify the heat sensitivity of human cells in culture.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Human skin fibroblast cell lines, 82-6, Gmtert (GM07166, Coriell, Camden, NJ), Gmtert + *NBS1*, AT2KYSV, and AT2KYSV/*neo* cells (the AT2KYSV cell lines were a gift from Dr. Yosuke Ejima, Hiroshima Prefectural College of Health Sciences) were used in this study. A skin fibroblast cell line (Gmtert) was established from an NBS patient and immortalized with the pBABE-neo-hTERT retrovirus vector [Ranganathan et al., 2001]. This cell line is homozygous for the Slavic 657del5 mutation in the *NBS* gene, which introduces a premature termination signal at codon 218, and results in the production of a severely truncated NBS1 polypeptide [Varon et al., 1998]. Gmtert + *NBS1* cells are Gmtert cells stably transfected with a pMMP-NBS1 retrovirus vector, carrying a full-length *NBS1* cDNA. The human 82-6 cultured skin fibroblast cell line (containing wild-type *NBS1*) was established from a control subject and immortalized with the pBABE-puro-hTERT retrovirus vector [Oshima et al., 1995]. The AT2KYSV cell line is a human SV40 immortalized fibroblast cell line derived from a Japanese female A-T patient [Ejima et al., 1990]. AT2KYSV/*neo* cells contain a complete wild-type chromosome 11. These cell lines were cultured at 37°C in Dulbecco's Modified Eagle's medium (ICN Biomedicals, Aurora, OH) containing 10% (v/v) fetal bovine serum (ICN Biomedicals), penicillin (50 U/ml, Sigma, St. Louis, MO), streptomycin (50 μg/ml, Meiji, Tokyo, Japan), and kanamycin (50 μg/ml, Meiji) (DMEM-10). Heating was performed by immersing 25 cm^2 flasks containing the cells in a circulating water bath (TAITEC, Saitama, Japan) at 44°C and then incubating them for the indicated periods at 37°C. X-ray irradiation was performed with a 150 kVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo, Japan).

Immunofluorescent Staining

The cells (approximately 10^4 cells) were plated on glass slides and heated in the same manner as described. After the heating and the incubation for 2 h at 37°C, the cells were rinsed twice with PBS, fixed with cold 100% methanol for 1 min, washed with PBS three times, and then permealized for 2 min with a solution containing 0.1% Triton X-100. Next, the cells were washed with PBS and incubated with anti-serine 343-phosphorylated NBS1 polyclonal antibody (Novus Biologicals, Littleton, CO), anti-Mre11 polyclonal antibody (Novus Biologicals) or anti-Rad50 polyclonal antibody (GeneTex, San Antonio, TX), for 1 h at 37°C. After the incubation, the cells were washed with PBST three times and then incubated with Alexa 488 (Nacalai Tesque, Kyoto, Japan) for 1 h at 37°C. After this incubation, the cells were washed with PBST and covered with a glass cover slip.

Preparation of Cytoplasmic and Nuclear Extracts

Cytoplasmic and nuclear protein fractions were extracted from the cells according to the protocol described for the TransFactor Extraction Kit (BD Biosciences, Palo Alto, CA). Briefly, 4×10^6 cells were washed once with PBS and harvested with a sterile cell scraper (IWAKI,

Tokyo, Japan). The collected cells were resuspended in 200 μl of lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.001 M DTT, and Protease inhibitor cocktail (Sigma)), and homogenized on ice with 10 strokes of a syringe with a 27G needle. The homogenates were centrifuged at 10,000*g* for 20 min to precipitate the nuclei while leaving the cytoplasmic fractions in the supernatant. To obtain nuclear extracts, the precipitated nuclei were resuspended in 100 μl of extraction buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.42 M $NaCl_2$, 0.2 mM EDTA, 25% glycerol, 0.001 M DTT, and Protease inhibitor cocktail (Sigma)), and then homogenized on ice with 10 strokes of a syringe with a 27G needle. The homogenates were centrifuged at 20,000*g* for 5 min to precipitate the chromatin and cell debris and the supernatants containing the nuclear extracts were then collected. Total cellular proteins were extracted from the cell suspension in RIPA buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.05% SDS) and collected after centrifugation (15,000*g*).

Western Blots

Total cellular, cytoplasmic or nuclear proteins were quantified with a BIO-RAD protein assay kit (Bio-Rad Labs, Richmond, CA). Aliquots of proteins (20 μg) were subjected to Western blot analyses. After electrophoresis on 10% or 15% polyacrylamide gels containing 0.1% SDS, the proteins were transferred electrophoretically onto Poly Screen PVDF membranes (Dupont/Biotechnology Systems, NEN Research Products, Boston, MA). The membranes were then incubated with anti-NBS1 polyclonal antibody (Novus Biologicals), anti-serine 343-phosphorylated NBS1 polyclonal antibody, anti-Mre11 polyclonal antibody, anti-Rad50 monoclonal antibody or anti-actin polyclonal antibody (Santa Cruz Biotechnology, Inc.). For visualization of the bands, we used a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) for NBS1, serine 343-phosphorylated NBS1, Mre11 and Rad50 antibodies and used a horseradish peroxidase-conjugated anti-goat IgG antibody (Zymed Laboratories, Inc.) for anti-actin were employed with the BLAST®: Blotting Amplification System (DuPont/Biotechnology Systems, NEN Research Products).

Preparation and Transfection of SEC

To target different regions of the *NBS1* mRNA, four *NBS1*-siRNAs were designed using Dharmacon's siRNA program (www.dharmacon.com). They are:

(1) 5′-AACATACGTAGCTGACACAGA-3′ (1,135–1,155 bp);
(2) 5′-AACTCCATCAGAAACTACTTT-3′ (1,409–1,429 bp);
(3) 5′-AACAAACACAACCTGCTACAC-3′ (1,524–1,544 bp); and
(4) 5′-AATAGCTCATCATGCTCGAAA-3′ (2,194–2,214 bp).

Searches of the human genome database (BLAST) were also carried out to ensure that the chosen sequences would not target other gene transcripts. Four different human U6 (hU6) promoter constructs were synthesized and used to transcribe four different hairpin siRNAs using a siRNA Expression Cassette (SEC) Kit (Silencer™ Express, Ambion, Austin, TX). These constructs were designated as SEC1, SEC2, SEC3, and SEC4, and were used to target each of four *NBS1*-nucleotide sequences listed above. Briefly, each hU6 promoter construct was PCR amplified using sense and anti-sense primers provided in the kit. Using the manufacturer's protocol, the synthesized SECs were transfected into the cells with liposomes and an enhancer method (Targeting Systems, Santee, CA) 2 days before heat treatment or irradiation. The scrambled sequence as a negative control for SEC1 is as follows: AAACGCGACTAACCGATATAG. BLAST (siPRECISE™) was carried out to ensure that the scrambled sequence would not target other gene transcripts.

Transfection Efficiency of SECs

To measure the transfection efficiency of the SEC constructs, the SECs were labeled using the Silencer siRNA Labeling Kit (Ambion). Following the manufacturer's protocol, the labeling mixtures, nuclease-free water, 10× labeling buffer, SEC and Cy3 (a fluorescent dye), were incubated at 37°C for 1 h in the dark, and then precipitated with ethanol. For quantitative estimation of the transfection efficiency, 40–70 cells were evaluated in three independent random microscope fields, and a total of 120–210 cells were evaluated for each concentration of the SEC constructs used for trasfection.

Colony Formation Assay

The fraction of surviving cells after heat exposure or X-ray irradiation was determined using a clonogenic assay. The plating efficiency was 50–60% in 82-6, Gmtert, and Gmtert + *NBS1* cells. A total of 5×10^2 or 1×10^3 cells were plated in two T25 flasks per experiment, and three independent experiments were performed for each survival point. Different fractions of cells were heated at 44°C, for 30, 60, 90, or 120 min. Other cells were irradiated with 0, 1, 3, or 6 Gy X-ray dose. All cells, after heat exposure or irradiation, were incubated for 14 days and visible colonies composed of more than 50 cells were detected. Colonies were fixed with 100% methanol for a few minutes, stained with a 2% Giemsa solution (Merck & Co., Inc., Rahway, NJ) and scored.

RESULTS

Detection of Phosphorylated NBS1, MRE11, and RAD50 Proteins by Immunofluorescent Staining

Immunofluorescent staining was used to detect NBS1 phosphorylation and MRE11 and RAD50 foci formation after heating or X-ray exposure (as a control) in 82-6 cells. Figure 1 shows that heating as well as irradiation, in our system, produces groups of representative cells positive for foci of phosphorylated NBS1, MRE11, or RAD50 antibody in 82-6 cells. Control cells that were not treated with heat or X-rays were negative (phosphorylated NBS1) or weakly positive (MRE11 and MRE11) for each antibody (Fig. 1, Control).

Heat or X-rays Causes Accumulation of NBS1 and Phosphorylated NBS1 Proteins

Cytoplasmic and nuclear fractions were extracted from 82-6, Gmtert, and Gmtert + *NBS1* cells and analyzed on Western blot. Accumulation of NBS1 was observed in the nuclear portion of the cellular extracts at 1 h after heating (Fig. 2a, lane 8, top) or at 0.5 h after X-ray exposure (Fig. 2a, lane 18, top), but very little NBS1was observed in the cytoplasmic fractions of 82-6 cells (Fig. 2a). Elevated NBS1 protein was still observed at 6 h after heating (Fig. 2a, lane 10, top) or X-ray irradiation (Fig. 2a, lane 20, top). The accumulation of phosphorylated NBS1 was observed in the nuclear portion of the extracts from 82-6 cells at 2 h after heating (Fig. 2a, lane 9, middle), and 0.5–2 h after X-ray irradiation (Fig. 2a, lanes 17–19, middle). NBS1 was not detected in the cytoplasmic or nuclear extracts from Gmtert cells, whether or not they were exposed to heat or X-rays (Fig. 2b). In contrast, NBS1 was detected in nuclear extracts of Gmtert + *NBS1* cells whether or not they were exposed to heat or X-rays (Fig. 2b).

To determine whether ATM was involved in the heat-induced phosphorylation of NBS1, whole-cell protein extracts were purified from AT2KYSV (*ATM*-deficient) and AT2KYSV/*neo*

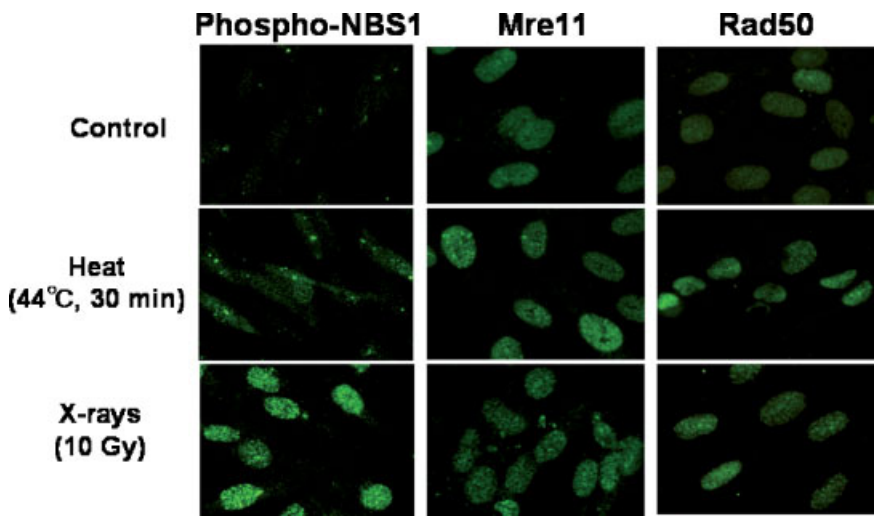

Fig. 1. Immunofluorescent staining of 82-6 cells with phospho-NBS1, MRE11, or RAD50 antibodies. Cells were incubated at 37°C for 2 h after being heated at 44°C for 30 min, or after exposure to 10 Gy of X-rays. Control cells were not exposed to heat or radiation.

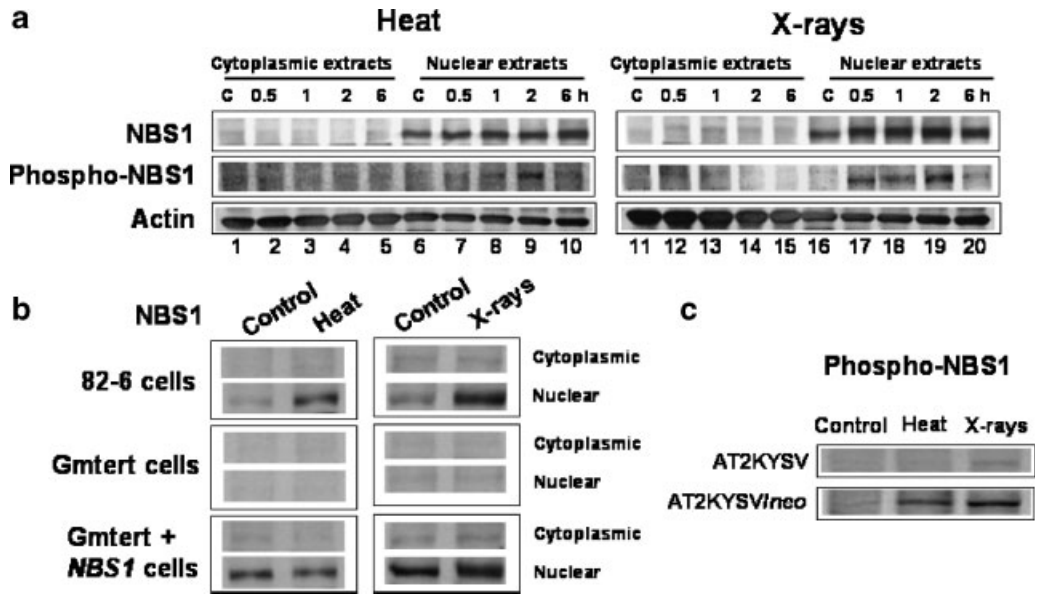

Fig. 2. Western blot analysis of NBS1 and phospho-NBS1 in nuclear and cytoplasmic extracts from heat-treated or X-ray irradiated cells. **a**: NBS1 or phospho-NBS1 observed at 0.5, 1, 2, or 6 h after heating (44°C for 30 min), or after exposure to X-rays (10 Gy) in 82-6 cells. C, non-heated control. **b**: NBS1 2 h after heating (44°C for 30 min) or X-ray irradiation (10 Gy) in 82-6, Gmtert, and Gmtert + *NBS1* cells. **c**: phospho-NBS1 1 h after heating (44°C for 30 min) or X-ray irradiation (3 Gy) in AT2KYSV and AT2KYSV/neo cells. Actin was used as a control to ensure that the same amount of protein was loaded on each gel.

cells and analyzed on Western blots using phospho-NBS1 antibody. Phosphorylated NBS1 was found to accumulate after exposure to heat or to X-rays in AT2KYSV/*neo* cells, but not in AT2KYSV cells (Fig. 2c). These results show that NBS1 is phosphorylated by ATM after heating.

Heat Does Not Cause Translocation of Mre11 and Rad 50 proteins

Significant accumulation of Mre11 and Rad50 was not observed after heating in the cytoplasmic or nuclear extracts from 82-6 cells over a period of time after exposure (Fig. 3). However, a marginal translocation of Mre11 from the cytoplasm (Fig. 3, lanes 1–5, top) to the nucleus (Fig. 3, lanes 6–10, top) was observed.

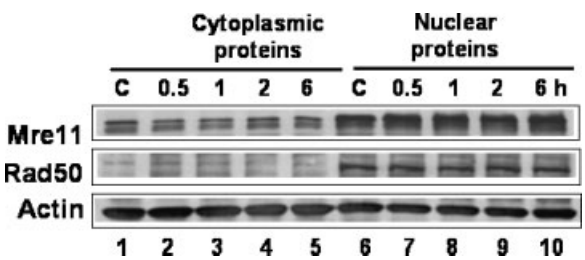

Fig. 3. Western blot analysis of Mre11 and Rad50 in nuclear and cytoplasmic extracts from heat-treated 82-6cells. The protein extraction was performed at 0.5, 1, 2, or 6 h after heating (44°C for 30 min). C, non-heated control.

SEC1 (NBS1-siRNA) Transfection Reduces the Cellular Concentration of NBS1

Transfection of SEC1 into cells resulted in a strong inhibitory effect on NBS1 expression, and was far more effective than the other SEC constructs (SEC2, SEC3, and SEC4) (data not shown). The transfection efficiency of the SEC1 construct was estimated using Cy3-labeled SEC1. In Cy3-labeled SEC1 (2 μg/ml) transfected 82-6 cell population, 85% of the cells were observed to be Cy3 labeled, 48 h after transfection (Fig. 4a). When 4 μg/ml of SEC1 was used, the efficiency of transfection was almost the same as previously observed with the lower SEC1 concentration. In view of this result, 2 μg/ml of SEC1 was used for transfection in all subsequent experiments. Under these conditions, the level of NBS1 expression was clearly decreased (Fig. 4b, right panel, lane 3, top). However, decrease of MRE11 and RAD50 amounts was not observed (Fig. 4b, right panel, lane 3, the second and third rows). When the scrambled SEC was transfected into the cells, the amounts of NBS1, MRE11, and RAD50 were not decreased (Fig. 4b, left panel).

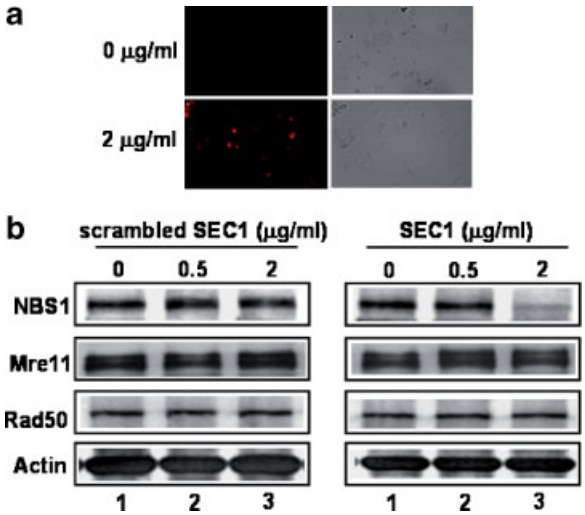

Fig. 4. Transfection of a DNA cassette expressing siRNA targeted for *NBS1* (SEC1). **a**: Photographs of 82-6 cells transfected with 0 μg or 2 μg of Cy3-labeled SEC1 in 1 ml of medium; (**b**) cellular levels of NBS1, MRE11, and RAD50 at 48 h after transfection with SEC1 (0, 0.5, or 2 μg/ml) in total protein extracted from 82-6 cells.

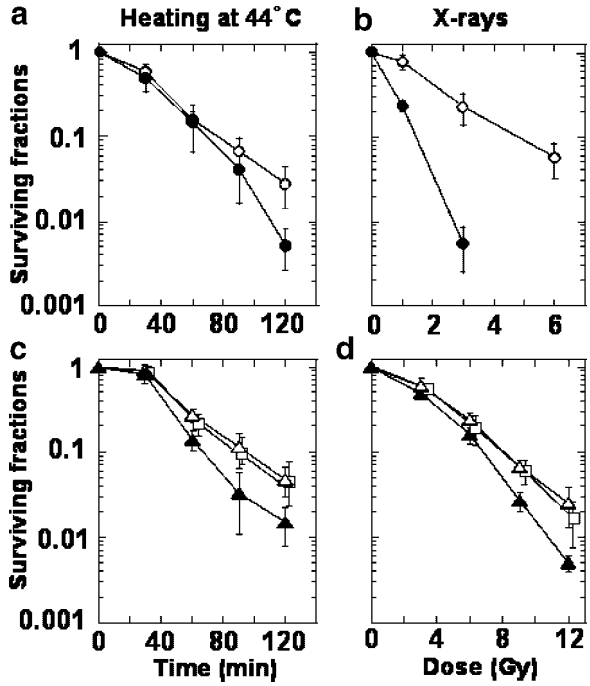

Fig. 5. Clonogenic surviving fractions after heating at 44°C or X-ray irradiation. **a**: heat sensitivity; (**b**) X-ray sensitivity. Open circles, Gmtert + *NBS1* cells; closed circles, Gmtert cells. **c**: heat sensitivity; (**d**) X-ray sensitivity. Open squares, non-transfected 82-6 cells; open triangles, 82-6 cells transfected with scrambled SEC (2 μg/ml); closed triangles, 82-6 cells transfected with SEC1 (2 μg/ml). Cells were heated for 20, 60, 90, or 120 min. For radiation, cells were irradiated with 0, 1, 3, 6, 9 or 12 Gy dose. All cells, heated or irradiated, were incubated for 14 days and colonies composed of more than 50 cells were scored.

SEC1 Transfected Cells Are Sensitive to Heat and Radiation

Clonogenic survival assays were used to determine the heat (Fig. 5a) and X-ray sensitivities of Gmtert and Gmtert + *NBS1* cells (Fig. 5b). After heat exposure, Gmtert cells were more heat sensitive than Gmtert + *NBS1* cells, especially after the exposure for 120 min (Fig. 5a). When Gmtert and Gmtert + *NBS1* cells were exposed to X-rays, Gmtert cells displayed much higher sensitivity to X-rays than Gmtert + *NBS1* cells (Fig. 5b). Next, clonogenic survival assays were measured to reveal whether heat or X-ray sensitivity could be triggered by *NBS1*-siRNA in normal human fibroblast 82-6 cells (carrying wild-type *NBS1*). As shown in Figure 5c,d, 82-6 cells transfected with SEC1 became more sensitive to heat and X-rays than 82-6 cells transfected with scrambled SEC.

DISCUSSION

Focus formation of γH2AX protein recently developed into an indicator for detecting the presence of cellular DSBs using immunocytochemical analysis [Rogakou et al., 1999; Sedelnikova et al., 2002]. We previously reported that γH2AX focus formation was detected after exposure to heat [Takahashi et al., 2004]. Heat-induced γH2AX focus formation has also recently been reported in mouse embryo fibroblasts by others [Kaneko et al., 2005]. Analysis by pulse-field gel electrophoresis showed DNA double-strand breaks induced by heat [Wong et al., 1995]. Those reports may imply that heat exposure leads to cellular responses induced by DNA damage. Although, when compared to proteins, DNA molecules are known to be quite stable to heat exposure, it is possible that DSBs are indirectly induced by radicals generated by the heat [Hall et al., 1994; Bruskov et al., 2002], or generated during base excision repair processes which might be active in repairing base damage induced by heat-generated radicals [Roti Roti, 1982]. During the excision repair process, heat-labile exonucleases and/or DNA polymerases could possibly be inactivated by heat [Spiro et al., 1982; Bodell et al., 1984], and this inactivation of enzymes during a repair process could possibly result in the production of DSBs in the heat-treated cells. The phosphorylation of NBS1 and the focus formation of the

MRN complex are important cellular responses to the presence of DSBs particularly after irradiation. In the present study, X-ray-induced phosphorylation of NBS1 was observed rapidly at 0.5 h after irradiation. In contrast, the phosphorylation of NBS1 after heat exposure was observed at 2 h after heating (Fig. 2a). This temporal lag could possibly result from DSBs being indirectly formed in a delayed fashion after heat exposure.

MRN foci are formed in the nuclei of cells exposed to radiation [Tauchi et al., 2002b]. Defects in NBS1 expression lead to failure in the formation of MRN complexes and induce cellular radiosensitization [Cerosaletti et al., 2000]. NBS1 proteins lacking the C-terminus region cannot interact with MRE11 [Cerosaletti et al., 2000], and the formation of damage-inducible foci is abrogated in cells with this mutation. Restoration of the NBS1 expression also restores the ability of MRE11, RAD50, and NBS1 to form complexes and redistribute within the nuclei after irradiation, and complements normal radiosensitivity [Cerosaletti et al., 2000]. There was no clear difference in heat sensitivity between scrambled siRNA-transfected and non-transfected 82-6 cells. On the contrary, *NBS1*-siRNA-transfected 82-6 cells were shown to be more sensitive to heat as well as to radiation than scrambled siRNA-transfected 82-6 cells (Fig. 5). Therefore, the specific enhancement of heat sensitivity might be dependent on the expression of *NBS1*-siRNA. As in the case of *NBS1*-siRNA-transfected 82-6 cells, Gmtert cells were more sensitive to heat as well as to radiation than Gmtert + *NBS1* cells. These results imply that MRN foci are formed in the nuclei of heated cells in a manner similar to that reported for irradiated cells, and these complexes are involved in cellular heat sensitivity. Additional evidence supporting the possible formation of MRN foci after heating was the detection of phosphorylated NBS1 in the nuclei 2 h after heating (Fig. 2a, lane 9). Elsewhere, it has been reported that NBS1, MRE11, and RAD50 are translocated from the nucleus into the cytoplasm shortly (0.5–1 h) after heating [Zhu et al., 2001; Seno and Dynlacht, 2004]. In the present study, however, the translocation of NBS1 was not detected with Western blot analysis of cytoplasmic and nuclear fractions extracted from heated or irradiated cells over a period of time after exposure (0.5–6 h) (Fig. 2a). The translocation of MRE11 or RAD50 from the nucleus into the cytoplasm was also not detected after heating in the present study (Fig. 3). It is interesting to note that NBS1 was accumulated in the nucleus after heating or irradiation. To our knowledge, heat-induced accumulation of NBS1 has not been previously reported. A recently published study showed gamma-irradiation-induced accumulation of NBS1 in 82-6 and Gmtert + *NBS1* cells [Howlett et al., 2006]. Since NBS1 was accumulated rapidly (0.5–1 h after heating or irradiation), it is likely that the accumulation is due to the delay of degradation of NBS1. Accumulated NBS1 which remains localized in the nuclei after heating or irradiation may be associated with cell sensitivity to such treatment.

ATM is known to be activated by genotoxic stresses such as radiation and DNA-damaging agents [Yang et al., 2003; Kurz and Lees-Miller, 2004], and to be a kinase which phosphorylates NBS1 [Gatei et al., 2000]. In addition, a recent study has shown that ATM is involved in heat-induced signal transduction [Miyakoda et al., 2002]. In view of this report [Miyakoda et al., 2002], it appears to be possible that NBS1 is phosphorylated by ATM after heating. In support of this model, the results reported here using AT2KYSV cell lines showed that NBS1 is phosphorylated by ATM after heating (Fig. 2c). ATM is thus likely to contribute to the phosphorylation of NBS1 after heat treatment in a manner similar to the phosophorylation of NBS1 after exposure to genotoxic stresses.

Sensitivity to X-rays was considerably higher in Gmtert cells than that in Gmtert + *NBS1* cells (Fig. 5b). This high sensitivity to X-rays seen in Gmtert cells is considered to be due to the functional deficiency of the mutant NBS1 expressed in these cells. In contrast, SEC1-transfected 82-6 cells were only slightly sensitive to X-rays when compared to scrambled siRNA-transfected or non-transfected 82-6 cells (Fig. 5d); since there was no clear difference in X-ray sensitivity between scrambled siRNA-transfected and non-transfected 82-6 cells, the enhanced X-ray sensitivity is surely due to expression of *NBS1*-siRNA. This result suggests that the transfection of SEC1 did not completely suppress the expression of *NBS1*. Since about 20% of the cells exposed to SEC1 did not take up SEC1, the observed trace amounts of NBS1 protein observed in the Western blots (Fig. 4b) probably originated from the

sub-population of 82-6 cells which did not take up the SEC1 construct, or from incomplete suppression of *NBS1* expression by the SEC1 construct. Under these circumstances, residual *NBS1* expression could contribute to DNA repair, and partial radio-resistance. On the other hand, sensitivity to heat was observed to be only slightly higher in Gmtert cells than that in Gmtert + *NBS1* cells (Fig. 5a). This slight difference may be due to a relatively small number of heat-induced DSBs present in these studies when compared with the large number of radiation-induced DSBs [Takahashi et al., 2004]. Another possible cause of the slight difference in heat sensitivity between these cell lines is the fact that heat-induced DNA repair might be partially defective or repressed due to possible heat-induced degradation of DNA repair-related proteins. The slight difference in heat sensitivity between *NBS1*-siRNA-transfected 82-6 cells and control 82-6 cells may be also due to this reason.

In conclusion, the present study shows that NBS1 is involved in sensitivity to heat as well as to radiation in human skin fibroblast cells. These results can be used to support the suggestion that NBS1-mediated cellular responses after DNA damage can be driven by heat as well as by radiation.

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REFERENCES

Bodell WJ, Cleaver JE, Roti Roti JL. 1984. Inhibition by hyperthermia of repair synthesis and chromatin reassembly of ultraviolet-induced damage to DNA. Radiat Res 100:87–95.

Bruskov VI, Malakhova LV, Masalimov ZK, Chernikov AV. 2002. Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA. Nucleic Acids Res 30:1354–1363.

Buscemi G, Savio C, Zannini L, Micciche F, Masnada D, Nakanishi M, Tauchi H, Komatsu K, Mizutani S, Khanna K, Chen P, Concannon P, Chessa L, Delia D. 2001. Chk2 activation dependence on Nbs1 after DNA damage. Mol Cell Biol 21:5214–5222.

Cerosaletti KM, Desai-Mehta A, Yeo TC, Kraakman-Van Der Zwet M, Zdzienicka MZ, Concannon P. 2000. Retroviral expression of the *NBS1* gene in cultured Nijmegen breakage syndrome cells restores normal radiation sensitivity and nuclear focus formation. Mutagenesis 15:281–286.

Ejima Y, Oshimura M, Sasaki MS. 1990. Establishment of a novel immortalized cell line from ataxia telangiectasia fibroblasts and its use for the chromosomal assignment of radiosensitivity gene. Int J Radiat Biol 58:989–997.

Gatei M, Young D, Cerosaletti KM, Desai-Mehta A, Spring K, Kozlov S, Lavin MF, Gatti RA, Concannon P, Khanna K. 2000. ATM-dependent phosphorylation of nibrin in response to radiation exposure. Nat Genet 25:115–119.

Hall DM, Buettner GR, Matthes RD, Gisolfi CV. 1994. Hyperthermia stimulates nitric oxide formation: Electron paramagnetic resonance detection of .NO-heme in blood. J Appl Physiol 77:548–553.

Howlett NG, Scuric Z, D'Andrea AD, Schiestl RH. 2006. Impaired DNA double strand break repair in cells from Nijmegen breakage syndrome patients. DNA Repair 3: 251–257.

Ito A, Tauchi H, Kobayashi J, Morishima K, Nakamura A, Hirokawa Y, Matsuura S, Ito K, Komatsu K. 1999. Expression of full-length NBS1 protein restores normal radiation responses in cells from Nijmegen breakage syndrome patients. Biochem Biophys Res Commun 265: 716–721.

Kaneko H, Igarashi K, Kataoka K, Miura M. 2005. Heat shock induces phosphorylation of histone H2AX in mammalian cells. Biochem Biophys Res Commun 328: 1101–1106.

Kurz EU, Lees-Miller SP. 2004. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair 3:889–900.

Lim DS, Kim ST, Xu B, Maser RS, Lin J, Petrini JH, Kastan MB. 2000. ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. Nature 404:613–617.

Maser RS, Monsen KJ, Nelms BE, Petrini JH. 1997. hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks. Mol Cell Biol 17:6087–6096.

Mirzoeva OK, Petrini JH. 2001. DNA damage-dependent nuclear dynamics of the Mre11 complex. Mol Cell Biol 21:281–288.

Miyakoda M, Suzuki K, Kodama S, Watanabe M. 2002. Activation of ATM and phosphorylation of p53 by heat shock. Oncogene 21:1090–1096.

Nelms BE, Maser RS, MacKay JF, Lagally MG, Petrini JH. 1998. *In situ* visualization of DNA double-strand break repair in human fibroblasts. Science 280:590–592.

Oshima J, Campisi J, Tannock TC, Martin GM. 1995. Regulation of c-fos expression in senescing Werner syndrome fibroblasts differs from that observed in senescing fibroblasts from normal donors. J Cell Physiol 162:277–283.

Ranganathan V, Heine WF, Ciccone DN, Rudolph KL, Wu X, Chang S, Hai H, Ahearn IM, Livingston DM, Resnick I, Rosen F, Seemanova E, Jarolim P, DePinho RA, Weaver DT. 2001. Rescue of a telomere length defect of Nijmegen breakage syndrome cells requires NBS and telomerase catalytic subunit. Curr Biol 11:962–966.

Rogakou EP, Boon C, Redon C, Bonner WM. 1999. Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. J Cell Biol 146:905–916.

Roti Roti JL. 1982. Heat-induced cell death and radiosensitization: Molecular mechanisms. Natl Cancer Inst Monogr 61:3–10.

Sedelnikova OA, Rogakou EP, Panyutin IG, Bonner WM. 2002. Quantitative detection of (125)IdU-induced DNA double-strand breaks with gamma-H2AX antibody. Radiat Res 158:486–492.

Seno JD, Dynlacht JR. 2004. Intracellular redistribution and modification of proteins of the Mre11/Rad50/Nbs1 DNA repair complex following irradiation and heat-shock. J Cell Physiol 199:157–170.

Spiro IJ, Denman DL, Dewey WC. 1982. Effect of hyperthermia on CHO DNA polymerases α and β. Radiat Res 89:134–149.

Takahashi A, Matsumoto H, Nagayama K, Kitano M, Hirose S, Tanaka H, Mori E, Yamakawa N, Yasumoto J, Yuki K, Ohnishi K, Ohnishi T. 2004. Evidence for the involvement of double-strand breaks in heat-induced cell killing. Cancer Res 64:8839–8845.

Tauchi H, Kobayashi J, Morishima K, van Gent DC, Shiraishi T, Verkaik NS, vanHeems D, Ito E, Nakamura A, Sonoda E, Takata M, Takeda S, Matsuura S, Komatsu K. 2002a. Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells. Nature 420:93–98.

Tauchi H, Matsuura S, Kobayashi J, Sakamoto S, Komatsu K. 2002b. Nijmegen breakage syndrome gene, *NBS1*, and molecular links to factors for genome stability. Oncogene 21:8967–8980.

Varon R, Vissinga C, Platzer M, Cerosaletti KM, Chrzanowska KH, Saar K, Beckmann G, Seemanova E, Cooper PR, Nowak NJ, Stumm M, Weemaes CM, Gatti RA, Wilson RK, Digweed M, Rosenthal A, Sperling K, Concannon P, Reis A. 1998. Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. Cell 93:467–476.

Wong RS, Dynlacht JR, Cedervall B, Dewey WC. 1995. Analysis by pulsed-field gel electrophoresis of DNA double-strand breaks induced by heat and/or X-irradiation in bulk and replicating DNA of CHO cells. Int J Radiat Biol 68:141–152.

Wu X, Ranganathan V, Weisman DS, Heine WF, Ciccone DN, O'Neill TB, Crick KE, Pierce KA, Lane WS, Rathbun G, Livingston DM, Weaver DT. 2000. ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. Nature 405:477–482.

Yang J, Yu Y, Hamrick HE, Duerksen-Hughes PJ. 2003. ATM, ATR and DNA-PK: Initiators of the cellular genotoxic stress responses. Carcinogenesis 24:1571–1580.

Zhang Y, Zhou J, Lim CUK. 2006. The role of NBS1 in DNA double strand break repair, telomere stability, and cell cycle checkpoint control. Cell Res 16:45–54.

Zhao S, Weng YC, Yuan SS, Lin YT, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EY. 2000. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. Nature 405:473–477.

Zhu WG, Seno JD, Beck BD, Dynlacht JR. 2001. Translocation of MRE11 from the nucleus to the cytoplasm as a mechanism of radiosensitization by heat. Radiat Res 156:95–102.