Introduction

Ku70 was first characterized as an autoantigen and, subsequently, it was also identified as a nuclear DNA-binding component of the nonhomologous end joining (NHEJ) DNA-repair complex (1). When dimerized with Ku80, Ku70 binds to the broken end of DNA double-strand breaks (DSB; ref. 2). However, some other studies have also shown that Ku70 is also present in the cytoplasm (3). To date, 1 described function of cytoplasmic Ku70 is to bind Bax, an apoptotic protein, thereby blocking Bax-mediated cell death. The binding between Ku70 and Bax is regulated by Ku70 acetylation (4). We have previously shown that inhibiting deacetylase activity in neuroblastoma (NB) cells increases Ku70 acetylation, resulting in Bax release that triggers Bax-dependent cell death (5). Our studies further indicated that cytoplasmic Ku70 plays an important role in NB cell survival as Ku70 knock down or increased Ku70 acetylation results in Bax release, triggering cell death. Although regulating cytoplasmic Ku70 acetylation is important for cell survival, the role of nuclear Ku70 acetylation in DNA repair is unclear. Here, we showed that Ku70 acetylation in the nucleus is regulated by the CREB-binding protein (CBP), and that Ku70 acetylation plays an important role in DNA repair in NB cells. We treated NB cells with ionization radiation and measured DNA repair activity as well as Ku70 acetylation status. Cytoplasmic and nuclear Ku70 were acetylated after ionization radiation in NB cells. Interestingly, cytoplasmic Ku70 was redistributed to the nucleus following irradiation. Depleting CBP in NB cells results in reducing Ku70 acetylation and enhancing DNA repair activity in NB cells, suggesting nuclear Ku70 acetylation may have an inhibitory role in DNA repair. These results provide support for the hypothesis that enhancing Ku70 acetylation, through deacetylase inhibition, may potentiate the effect of ionization radiation in NB cells. Mol Cancer Res; 11(2); 173–81. ©2012 AACR.

DNA Damage and Repair

CREB-Binding Protein Regulates Ku70 Acetylation in Response to Ionization Radiation in Neuroblastoma

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Abstract

Ku70 was originally described as an autoantigen, but it also functions as a DNA repair protein in the nucleus and as an antiapoptotic protein by binding to Bax in the cytoplasm, blocking Bax-mediated cell death. In neuroblastoma (NB) cells, Ku70’s binding with Bax is regulated by Ku70 acetylation such that increasing Ku70 acetylation results in Bax release, triggering cell death. Although regulating cytoplasmic Ku70 acetylation is important for cell survival, the role of nuclear Ku70 acetylation in DNA repair is unclear. Here, we showed that Ku70 acetylation in the nucleus is regulated by the CREB-binding protein (CBP), and that Ku70 acetylation plays an important role in DNA repair in NB cells. We treated NB cells with ionization radiation and measured DNA repair activity as well as Ku70 acetylation status. Cytoplasmic and nuclear Ku70 were acetylated after ionization radiation in NB cells. Interestingly, cytoplasmic Ku70 was redistributed to the nucleus following irradiation. Depleting CBP in NB cells results in reducing Ku70 acetylation and enhancing DNA repair activity in NB cells, suggesting nuclear Ku70 acetylation may have an inhibitory role in DNA repair. These results provide support for the hypothesis that enhancing Ku70 acetylation, through deacetylase inhibition, may potentiate the effect of ionization radiation in NB cells. Mol Cancer Res; 11(2); 173–81. ©2012 AACR.

Databases

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doi: 10.1158/1541-7786.MCR-12-0065

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Mol Cancer Res; 11(2); 173–81. ©2012 AACR.

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doi: 10.1158/1541-7786.MCR-12-0065

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other lysine residues, K282 and K338, also take part in binding broken-end DSB DNA (16). The fact that the K539 and K542 acetylation by CBP are responsible for Bax-dependent cell death in NB cells and the same lysine residues are involved in binding to broken-end DSB DNA prompted us to investigate the role of Ku70 acetylation by CBP in response to ionizing radiation-induced DNA damage.

Our results show that ionizing radiation does not affect Ku70 expression in NB cells but ionizing radiation induces Ku70 redistribution from the cytoplasm to the nucleus. When NB cells are subjected to ionizing radiation, the more aggressive neuroblastic (N-type) NB cells undergo cell death while the less aggressive stromal (S-type) NB cells show ionizing radiation resistance. Moreover, the DNA repair activity, as measured by phosphorylated H2AX (γ-H2AX) expression and by the Comet assay, is more efficient in the S-type cells compared with the N-type cells. The possibility that increased acetylation of Ku70 might block Ku70 DNA-binding activity necessary for NHEJ repair, together with previous work showing N-type cells express higher levels of CBP, led us to hypothesize that Ku70 acetylation by CBP may play a critical role in ionizing radiation-induced killing of N-type NB cells. This model is supported by evidence that knocking down CBP in N-type cells led to a reduction of Ku70 acetylation, increased DNA-repair activity, and NB cell survival following ionizing radiation. These results suggest a critical role for CBP in Ku70 acetylation following ionizing radiation-induced DNA damage in NB cells and provide further support for the development of NB therapeutic strategies that target Ku70 acetylation.

Materials and Methods

Cell culture and irradiation

Human NB cell lines SH-SY5Y, IMR32, SH-EP1, and the fibroblast cell line IMR90 were cultured in modified Eagle’s medium (MEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, and the cells were maintained at 37°C in a humidified 5% CO2 incubator. The cells were irradiated over the clinically relevant dosage ranging from 0 to 20 Gy using Philips Broblast cell line IMR 90 was cultured in modified Eagle’s medium (MEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, and the cells were maintained at 37°C in a humidified 5% CO2 incubator. The cells were irradiated over the clinically relevant dosage ranging from 0 to 20 Gy using Philips.

Cell viability assay

The viability of the human NB cell lines SH-SY5Y, SH-EP1, IMR32, and the fibroblast cell line IMR90 was determined 24 and 48 hours after exposure to ionizing radiation by MTT or sulforhodamine assays as previously described (5). The viability of the CBP siRNA, control siRNA, and mock-transfected SH-SY5Y cells were similarly measured by MTT after ionizing radiation exposure. All experiments were carried out in triplicate and the average values and SDs were calculated.

siRNA-mediated silencing of CBP

SH-SY5Y cells were plated at a density of 2 × 10^6 cells per 10-cm plate 24 hours before transfection. The following day, the cells were transfected either with smart pool CBP siRNA, or the scrambled nontargeting siRNA (Dharmacon Inc.) using nucleofactor kit V (Lonza) according to the manufacturer’s instruction. Mock transfection as well as the nontargeting scrambled siRNA transfection served as controls. The knock down of CBP was determined 72 hours post transfection by immunoblotting using anti-CBP antibodies.

Immunoblot analysis

For immunoblot analyses, whole-cell extracts were prepared from human NB cell lines following ionizing radiation treatment. The proteins were separated by SDS-PAGE, transferred to a polyvinylidine difluoride (PVDF) membrane, and immunoblotted for γ-H2AX, CBP, Bax, or control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. The presence of proteins were visualized using Enhanced Chemiluminescence plus.

Fractionation and immunoprecipitation

The SH-SY5Y cells and the CBP-knockdown SH-SY5Y cells were fractionated into cytosolic and nuclear fractions using low-salt and high-salt buffer as previously described (17). The fractions were immunoprecipitated using an anti-acetyl-lysine antibody (Santa Cruz Biotechnology) or K103-acetylated lysine antibody (Cell Signaling) in CHAPS buffer, and complexes were immunoblotted with Ku70 antibody (3).

Clonogenic assay

To determine the survivability of NB cells after various treatments, we used a clonogenic assay (18). Cells were trypsinized and seeded in triplicate into 60-mm dishes (400 cells for unirradiated control and 40,000 for irradiated cells) and allowed to grow undisturbed for 1 week. Colonies were stained with crystal violet, and counted manually using a Leica microscope. The percentage of surviving fraction at different doses was calculated as previous described (18).

Immunofluorescent microscopy and quantification of γ-H2AX foci

NB cells and controls were seeded in chamber slides. At different time points following irradiation at 2 Gy, cells were fixed with 4% paraformaldehyde for 15 minutes, washed with PBS, and permeabilized in 0.2% Triton-X100. After blocking with 5% normal goat serum (NGS) for 1 hour, samples were incubated with anti–phospho-histone γ-H2AX (Ser139, clone JBW301) mouse monoclonal antibody (Millipore) at a 1:500 dilution in 2.5% NGS-PBS overnight at 4°C, followed by incubation with the secondary antibody Alexa Fluor 568 goat anti-mouse-IgG (1:1,000) for 1 hour. Cells were then washed with PBS and mounted using mounting solution with 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen). Images of γ-H2AX foci and nuclei were taken using Leica DMR fluorescent microscope at ×40. Quantitative analysis of foci was carried out using Image-J as previously described (19). To test for variation between experiments, at least 100 cells from 3 different experiments were scored for each data point. The mean number of foci per cell and the SD from 3 independent measurements were calculated.
The comet assay
The neutral comet assay was conducted using a Trevigen Comet Assay Kit (4250-050-K; Trevigen) according to the manufacturer’s protocol. SH-SY5Y, SH-EP1, CBF-knocked down SH-SY5Y cells, or control siRNA-treated SH-SY5Y cells were exposed to radiation dose of 10 Gy and subjected to comet assay at the indicated time points of 0, 1, or 3 hours. The comet images were captured using fluorescent microscopy after staining with SYBR green. Average comet tail moment (percentage of DNA in tail length) was scored for 3 fields (>50 comets in each field) using the Comet Score software (TriTek). The results are expressed as mean ± SD.

Results
Ku70 is acetylated in the cytoplasm and in the nucleus after irradiation

We have shown previously that acetylation of cytoplasmic Ku70 triggers Bax release and NB cell apoptosis (5). However, the role of Ku70 acetylation in NB DNA nuclear-repair responses is unknown. In this study we have determined that ionizing radiation induces Ku70 acetylation in SH-SY5Y cells, N-type NB cells (Fig. 1A, left), but not in SH-EP1 cells, S-type NB cells, (Fig. 1A, right). In addition in SH-SY5Y N-type cells, both cytoplasmic and nuclear Ku70 are acetylated 24 hours after 10 Gy ionizing radiation (Fig. 1B). Interestingly, when we determined the level of Ku70 in the cytoplasm and in the nucleus at 0, 2, 4, and 6 hours after ionizing radiation, we found the level of Ku70 is similar in the cytoplasm and in the nucleus of untreated cells (0 hour; Fig. 1C). Two hours after ionizing radiation, however, there is a visible reduction in the cytoplasmic Ku70 level, and simultaneously, nuclear Ku70 level increases. These changes of Ku70 level between the cytoplasm and the nucleus are evident for 6 hours after treatment, suggesting DNA damage induced by ionizing radiation causes Ku70 to translocate from the cytoplasm to the nucleus.

Kinetics of DNA repair in NB cells in response to irradiation

To investigate the DNA-damage response of NB cells to ionizing radiation, we used phosphorylation of histone H2AX (γ-H2AX) as a marker of DNA damage (20). Interestingly, when we compared the kinetics of γ-H2AX expression using immunoblot analyses in N-type versus S-type NB cells, we found that in SH-SY5Y N-type cells the rate of disappearance of γ-H2AX is prolonged up to 8 hours after 10 Gy ionizing radiation treatment (Fig. 2A, left). In contrast, in SH-EP1 S-type cells, γ-H2AX disappears within 2 hours of treatment (Fig. 2A, right). These results suggest that repair of DNA damage is much faster in SH-EP1 cells compared with that of SH-SY5Y cells. Similar results were obtained when we used a lower dose of ionizing radiation, 2 Gy, and counted the total foci of γ-H2AX of over 100 cells for each treatment. The immunocytochemistry staining at 0, 3, and 7 hours after 2 Gy ionizing radiation treatment is shown in Fig. 2B. The compilation of the total γ-H2AX foci counts at various times after irradiation is shown in Table 2.
Figure 2. Kinetics of DNA repair in neuroblastoma cells in response to irradiation. A, SH-SY5Y and SH-EP1 cells were irradiated at 10 Gy. At various times as indicated after ionizing radiation, lysates were collected and immunoblotted for γ-H2AX and β-tubulin. B, SH-SY5Y and SH-EP1 cells were irradiated at 2 Gy. At various times as indicated after ionizing radiation, immunofluorescence for γ-H2AX was conducted. Only data for 0, 3, and 7 hours are shown. C, compilation of the number of foci of γ-H2AX per cell after ionizing radiation as shown in B at times indicated. The results are expressed as mean ± SD (n = 3, 100 foci per sample). D and E, SH-SY5Y cells and SH-EP1 cells were irradiated at 10 Gy. At 0, 1, and 3 hours later, the cells were subjected to the Comet assay as described. The fluorescent microscopy staining with SYBR green was shown in D. The average comet tail moment (percentage of DNA in tail length) was scored in 3 different fields (at least 50 comets per field) shown in E. The results are expressed as mean ± SD (n = 3).
ionizing radiation treatment is shown in Fig. 2C for SH-SY5Y cells and SH-EP1 cells. The results are consistent with the immunoblot data (shown in Fig. 2A) when a higher dose (10 Gy) of ionizing radiation is used in that S-type NB cells have a faster kinetics of repairing DNA than that of the N-type NB cells. Similar results were also obtained when a direct measurement of DNA breaks were checked using a Comet Assay, which measures the DNA breaks at the time when the cells are lysed (21). Results shown in Fig. 2D and E show that SH-EP1 has a faster kinetics of DNA repair (measuring at 1 and 3 hours following ionizing radiation) than that of SH-SY5Y cells after 10 Gy treatment.

When we determined cell viability using MTT assay following ionizing radiation, we found that in N-type NB cells (SH-SY5Y and IMR32 cells) viability decreases proportional to the ionizing radiation dose (5, 10, or 20 Gy) used. In contrast, in SH-EP1 cells, similar to the IMR90, a fibroblast type cell line, the viability dropped minimally following (down to 85% of control) various ionizing radiation treatments (Fig. 3A). Similar results were obtained when a clonogenic assay was used to assess the survivability of SH-SY5Y cells and SH-EP1 cells following various doses of ionizing radiation (Fig. 3B).

The reduction of cell viability after ionizing radiation seen in SH-SY5Y cells, but not in SH-EP1 cells, is consistent with our previous findings showing that cytoplasmic Ku70 is acetylated in SH-SY5Y cells but not in SH-EP1 cells (Fig. 1). When we immunoprecipitated Bax after 10 Gy ionizing radiation in these 2 cell types, we found much less Ku70 is associated with Bax in the SH-SY5Y cells compared with that of SH-EP1 cells. These results indicate that Bax is released from Ku70 after ionizing radiation in SH-SY5Y cells (Fig. 3C, left) but not in SH-EP1 cells (Fig. 3C, right).

Interestingly, when we further studied SH-EP1 cells that survived ionizing radiation treatment, we found that they showed resistance to the effects of cisplatin, a chemotherapeutic agent known to induce cell death in NB cells (Fig. 4A; ref. 22). Furthermore, the SH-EP1 cells had faster growth rates in low serum conditions (0.1 or 0.5%) when compared with unirradiated cells (Fig. 4B).

CBP regulates Ku70 acetylation in response to irradiation in NB cells

We have shown previously that CBP regulates Ku70 acetylation in NB cells. Overexpression of CBP or CBP knock down will alter the response of histone deacetylase
inhibitor treatment that induces cell death in NB cells (6). In this current study, we tested whether CBP regulates Ku70 acetylation in response to DNA damage induced by ionizing radiation. Because we did not observe Ku70 acetylation in S-type cells (Fig. 1A) after ionizing radiation, we focused our studies on SH-SY5Y cells.

To test whether CBP regulates Ku70 acetylation following ionizing radiation treatment in NB cells, we knocked down CBP using CBP-specific siRNA and tested (Fig. 5A) whether Ku70 is acetylated in response to ionizing radiation (10 Gy; Fig. 5A). Twenty-four hours after ionizing radiation treatment, scrambled siRNA or CBP-siRNA-transfected cells with and without exposure to ionizing radiation were fractionated to separate the cytoplasmic and nuclear fractions. Samples were then immunoprecipitated using an anti-acetyl-lysine antibody (Santa Cruz Biotechnology) and then immunoblotted for Ku70. As shown in Fig. 5B, CBP knock down reduces the level of Ku70 acetylation in both the cytosolic and nuclear fractions following ionizing radiation.

To determine if there was a change in ionizing radiation-induced DNA-damage-repair activity following CBP knockdown, we treated scrambled siRNA control or CBP-siRNA-transfected SH-SY5Y cells with ionizing radiation (2 Gy), and the kinetics of $\gamma$-H2AX was determined by immunocytochemistry using $\gamma$-H2AX-specific antibodies (Fig. 6A). The results shown in Fig. 6B are the number of $\gamma$-H2AX foci per nucleus (100 nuclei). These results show that the CBP-knockdown cells show a faster kinetics of the disappearance of $\gamma$-H2AX compared with the scrambled siRNA control. These results suggest that CBP may be regulating the DNA-damage response, possibly by regulating Ku70 acetylation, induced by ionizing radiation. Similar results were obtained when the Comet Assay was used to determine the total DNA breaks at the time of cell lysis (Figs. 6C and D). A clonogenic assay was also carried out simultaneously to identify the survival ability after ionizing radiation-induced DNA damage when CBP is depleted. Results in Fig. 7A show CBP-knockdown SH-SY5Y cells showing significantly higher survival when exposed to various doses of ionizing radiation compared with control-transfected cells.
transfection of a Ku70 mutant which has K539 and K542 converted to arginine (K539/542R) has no effect on ionizing radiation-induced reduction in cell viability in SH-SY5Y cells (Fig. 7B), suggesting that acetylation of K539 and K542 may not be responsible in regulating SH-SY5Y cell DNA repair following ionizing radiation treatment.

**Discussion**

Of the various types of DNA damage, DSBs induced by ionization radiation is most toxic to dividing cells (23). Efficient repair of DNA DSB is critical for maintaining genomic stability and cell viability (24). In mammalian cells, DNA-repair activity in response to ionizing radiation-

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**Figure 6.** CBP depletion shortens the DNA repair time in neuroblastoma cells. A, SH-SY5Y cells transfected with CBP siRNA or control siRNA were irradiated at 2 Gy. At different times following ionizing radiation, immunofluorescence for γ-H2AX was conducted as described in the Materials and Methods section. Data for 0, 3, 5, and 7 hours after ionizing radiation are shown in A at times indicated. The results are expressed as mean ± SD (n = 3, 100 foci per sample). B, compilation of the number of foci of γ-H2AX per cell after ionizing radiation described in A at times indicated. The results are expressed as mean ± SD (n = 3).
induced DSBs occurs via 2 routes: homologous recombination (HR) and NHEJ (25). HR is an accurate form of DNA repair that is restricted to DNA repair occurring at S-phase following DNA replication. In contrast, NHEJ operates throughout the cell cycle and is considered the major S-phase following DNA replication. In Bax translocation to the mitochondria causing cell death (5). However, expression of Ku70 K539/542R mutant does not rescue SH-SH5Y cell death induced by ionizing radiation, suggesting that Bax may not regulate cell death triggered by ionizing radiation in this cell type.

Our results have also shown that N-type cells show prolonged repair activity and more apoptotic cell death when compared with the S-type NB cells following ionizing radiation, suggesting that regulation of Ku70 acetylation may play a role in NB cell survival. We have shown that the irradiated SH-EP1 cells show resistance to the chemotherapeutic drug cisplatin, and that they grow better in lower serum conditions. The results suggest that ionizing radiation induces cell death of at least survivable SH-EP1 cells, and the remaining SH-EP1 cells, having a faster DNA-repair rate and low Ku70 acetylation following ionizing radiation, adopt a better survival mechanism that is resistant to DNA-damaging compounds such as cisplatin. However, whether this survival mechanism in SH-EP1 cells is related to Ku70 acetylation affecting its DNA binding for repair remains to be tested.

Our results show that CBP acetylates Ku70 in the cytosol and in the nucleus of NB cells; depleting CBP lowers Ku70 acetylation in both compartments. Consistent with our model, the repair activity of irradiated, CBP-depleted SH-SY5Y cells is more robust than control cells, and CBP-depleted cells have higher survivability, judging by the increase of colonies of CBP-depleted cells on ionizing radiation in the clonogenic assay. These results suggest that CBP and deacetylases regulate Ku70 functions in NB cells. However, this mechanism may be cell-type specific. Ogihara and colleagues have shown that, in human lung cancer H1299 cells, depletion of CBP and p300 results in repressing DNA-repair activity, which is the opposite of what we report here (30). Together, their results as well as ours suggest that the role of CBP in DNA repair activity may be cell-context specific. What regulates this specificity remains to be determined.

Taken together, our data for the first time in NB cells identify Ku70 acetylation in response to ionizing radiation-induced DNA damage to be responsible cell death exposed to ionizing radiation. Our previous study, showing low level CBP expression in S-type cells compared with that in N-type cells, indicates that the resistance of S-type NB cells to radiation and faster DNA-repair activity may be due to low-level CBP expression (6). These results also provide novel insight into the differences between N-type and S-type NB cells in response to ionizing radiation-induced DNA damage. These findings provide a rationale for testing modulators of Ku70 acetylation in clinical trials together with radiotherapy or DNA-damaging agents for the treatment of NB.

Disclosure of Potential Conflicts of Interest

A.W. Opipari Jr. is employed as a co-founder and Senior Director of and has ownership interest (including patents) in Lycer, Inc. No potential conflicts of interest were disclosed by the other authors.
Authors' Contributions

Conception and design: C. Subramanian, A.W. Opipari, Jr., V.P. Castle, R.P.S. Kwok
Development of methodology: C. Subramanian, M. Hada, V.P. Castle, R.P.S. Kwok
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Subramanian, M. Hada, V.P. Castle, R.P.S. Kwok
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): C. Subramanian, M. Hada, A.W. Opipari, Jr., V.P. Castle, R.P.S. Kwok

Writing, review, and/or revision of the manuscript: C. Subramanian, A.W. Opipari, Jr., V.P. Castle, R.P.S. Kwok

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.P. Castle, R.P.S. Kwok

Study supervision: C. Subramanian, R.P.S. Kwok

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