Journal of Cellular Biochemistry 103:1488–1496 (2008)

Histone H1.2 Is Translocated to Mitochondria and Associates With Bak in Bleomycin-Induced Apoptotic Cells

Hirohiko Okamura,* Kaya Yoshida, Bruna Rabelo Amorim, and Tatsuji Haneji

Department of Histology and Oral Histology, Institute of Health Biosciences, The University of Tokushima Graduate School, Kuramoto, Tokushima 770-8504, Japan

Abstract Bleomycin induces single- and double-stranded breaks in DNA, with consequent mitochondrial membrane aberrations that lead to the apoptotic cell death. It is poorly understood how DNA damage-inducing apoptotic signals are transmitted to mitochondria, from which apoptotic factors are released into the cytoplasm. Here, we investigated the localization of histone H1.2 in the bleomycin-treated human squamous carcinoma SCCTF cells. The presence of DNA double-strand breaks in the bleomycin-treated cells was examined by Western analysis using antibody against phosphorylated histone H2AX (γ-H2AX). Incubation of SCCTF cells for 48 h with 10 μM bleomycin induced apoptosis, as determined by cleavage of lamin B1 to 28 kDa fragment and DNA ladder formation. The mitochondrial permeabilization causing apoptotic feature was also detected with MitoCapture in the bleomycin-treated cells. Histone H1.2 was translocated from the nucleus to the mitochondria after treatment with bleomycin and co-localized with Bak in mitochondria. Our present results suggest that histone H1.2 plays an important role in transmitting apoptotic signals from the nucleus to the mitochondria following double-stranded breaks of DNA by bleomycin. J. Cell. Biochem. 103: 1488–1496, 2008. © 2007 Wiley-Liss, Inc.

Key words: apoptosis; Bak; bleomycin; H1.2; mitochondria

Histone H1 binds to the linker DNA between nucleosomes, sealing off two turns of DNA around a histone octamer, and is involved in the formation of higher-ordered chromatin structures and in the inhibition of transcription [Vignali and Workman, 1998]. To date, at least 8 subtypes of histone H1 have been identified. Among them, all of the somatic H1s (H1.1–H1.5) are ubiquitously expressed in all body tissues throughout development [Franke et al., 1998]. It was reported that in response to some apoptosis-inducing stimuli histone H1.2, a specific isoform of the linker histone, was translocated from the nucleus into the cytoplasm, where it induced the release of cytochrome *c* from the mitochondria and subsequent apoptosis [Konishi et al., 2003; Yan and Shi, 2003]. Recombinant H1.2 is able to induce conformational changes and activation of Bak in a concentration-dependent manner. Histone H1.2-deficient mice also exhibited increased cellular resistance in thymocytes and small intestine to X-ray-induced apoptosis [Konishi et al., 2003]. These findings showed that histone H1.2 plays an important role in transmitting apoptotic signals from the nucleus to the mitochondria following DNA double-strand breaks.

Mitochondria play a key role in anticancer drug-induced apoptosis, because deficiency of apoptosis-related proteins in mitochondria decreases the sensitivity of cancer cells to anti-cancer drugs [Kim et al., 2006; Wallach-Dayan et al., 2006]. The involvement of the mitochondria in apoptosis is manifested by the release of cytochrome *c*, a resident protein in mitochondrial intermembrane space. Members of the Bcl-2 protein family are critical death regulators

*Correspondence to: Hirohiko Okamura, DDS, PhD, Department of Histology and Oral Histology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8504, Japan. E-mail: okamura@dent.tokushima-u.ac.jp

Received 31 May 2007; Accepted 16 July 2007

DOI 10.1002/jcb.21537

© 2007 Wiley-Liss, Inc.

that reside upstream of mitochondria and might have proapoptotic or antiapoptotic functions. Bcl-2 and Bcl-xL are potent inhibitors of apoptosis, whereas other family members, such as Bak and Bax, promote apoptosis [Adams and Cory, 2007]. In response to the proapoptotic stimuli, Bak or Bax causes the permeabilization of the outer mitochondrial membrane, allowing cytochrome *c* in the mitochondrial intermembrane space to escape into the cytoplasm. Released cytochrome *c* triggers apoptosome assembly from apoptotic protease-activating factor-1 (Apaf-1), ATP, and procaspase-9, which activates effecter caspase-3 and caspase-7 [Baliga and Kumar, 2003].

Bleomycin (BLM) is a water-soluble antibiotic used in the chemotherapy of carcinomas and lymphomas. This antibiotic is internalized by a receptor-mediated endocytosis. BLM is required to bind to its membrane receptor and then the complex is transferred into endocytotic vesicles [Pron et al., 1999; Yamamoto, 2006]. BLM is known as one of the most famous anti-cancer drugs that induce DNA fragmentation [Kaufmann and Earnshaw, 2000]. BLM and peplomycin, a BLM derivative antibiotic, induce apoptosis in oral squamous carcinoma cells [Urade et al., 1994; Okamura et al., 2001]. Cytotoxicity of BLM toward mammalian cells is considered to be due to its ability to induce DNA damage and the sequential release of cytochrome *c* and proapoptotic small molecules from the mitochondrial membrane [Kim et al., 2006; Wallach-Dayan et al., 2006]. Although some previous reports showed the function and the translocation to mitochondria of histone H1.2 in genotoxic regents-induced apoptosis [Konishi et al., 2003; Yan and Shi, 2003], cytochemical and biochemical analysis about histone H1.2 and Bak has not been done in the BLM-induced apoptotic cells. In the present study, we investigated the localization and association of H1.2 and Bak together with other apoptotic events in the BLM-treated SCCTF cells by utilizing Western blot and immunohistochemistry.

MATERIALS AND METHODS

Materials

Dulbecco's minimal essential medium (D-MEM) was purchased from GIBCO BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Bleomycin hydrochloride was purchased from Nippon-kayaku (Tokyo, Japan). Stock solution (10 mM) was prepared in phosphate-buffered saline (PBS) and diluted to the appropriate concentrations with medium. Anti-histone H1.2 antibody was purchased from Abcam (Cambridge, UK). Anti-Bak monoclonal antibody was from Merck (Darmstadt, Germany). Anti-phospho-H2AX antibody was from Cell Signaling (Danvers, MA). Anti-lamin B1 monoclonal antibody was from ZyMED (South San Francisco, CA). MitoCapture mitochondrial apoptosis detection kit was obtained from BioVision (Mountain View, CA). Alexa Fluor-labeled second antibodies were from Invitrogen (Carlsbad, CA). Plastic dishes were from Iwaki (Chiba, Japan). DNase-free RNase and proteinase K were purchased from Sigma (St. Louis MO). Other materials used were of the highest grade commercially available.

Cells and Culture Conditions

Human squamous carcinoma cell line, SCCTF cells, was purchased from Riken Cell Bank (Tsukuba, Japan). These cells have minimal sensitivity to BLM and peplomycin [Urade et al., 1994; Okamura et al., 2001]. SCCTF cells were cultured in plastic dishes containing D-MEM. Each medium contained 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For cytochemistry, the cells were plated on 18-mm round coverslips placed in 60-mm plastic dishes and cultured. Cell modification was monitored with the use of an Olympus IMT-2 phase-contrast microscope and microphotographs were recorded on a computer (Olympus, DP70-WPCXP).

DNA Isolation and Agarose Gel Electrophoresis

Cultured cells were washed twice in PBS followed by lysis in cold 10 mM Tris-HCl buffer (pH 7.5), 10 mM EDTA, and 0.5% Triton X-100. After cell lysis, debris was removed by centrifugation at 15,000*g* for 20 min. DNase-free RNase was added to the lysates at a final concentration of 20 μg/ml, and incubated for 1 h at 37°C with gentle shaking. Then proteinase K was added to the samples at a final concentration of 20 μg/ml for another 1 h at 37°C. DNA was precipitated with 50% 2-propanol and 0.5 M NaCl overnight at −20°C. After centrifugation and drying, the DNA was dissolved in TE-buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA). Agarose

gel electrophoresis of DNA was performed through a 2.0% agarose gel. One hundred bp DNA markers (New England BioLabs, Beverly, MA) were run in the same gels. The gels were stained for 10 min with 10 μg/ml ethidium bromide. To visualize apoptotic alterations to DNA integrity, we observed the DNA bands on an UV transilluminator (Vilber Lourmat, Marnela Vallee, France). Photographs were taken with a Polaroid DS-300 camera.

Cytochemistry

After having been cultivated for various periods, living cells were labeled for 45 min with 100 nM Mito-tracker. For immunocytochemistry, the cells on coverslips were washed three times with PBS and fixed with 3.7% formaldehyde for 10 min at ambient temperature followed by methanol-permeabilization for an additional 20 min at −20°C. Subsequent procedures were conducted at ambient temperature. Nonspecific binding sites were blocked with 4% BSA and 5% normal goat serum or rabbit serum in PBS for 10 min in a humidified atmosphere. After having been rinsed with cold PBS, the coverslips were incubated with 5 μg/ml of the IgG fraction of anti-histone H1.2 antibody and/or anti-Bak antibody in 4% BSA for 45 min. After 3 washes with 0.05% Tween-20 in PBS (PBS-Tween) over a 15-min period, they were next incubated for another 45 min with Alexa Fluor-labeled second antibodies diluted 1:300 in 4% BSA in PBS. Finally, the cells were incubated with Hoechst 33342 (10 μg/ml) for 10 min to stain the nuclei, washed as described above, and mounted with Gel/Mount aqueous mounting medium (Biomeda, Foster City, CA). For detection of mitochondrial permeabilization, cells were treated for 48 h with or without BLM at the final concentration of 10 μM followed by incubation with MitoCapture for 45 min at 37°C. The samples were examined under an Olympus BX50 microscope equipped with epifluorescence illumination with a U-MNIBA filter for green fluorescence and a U-MWIB filter for red fluorescence. Photomicrographs were recorded on a computer (Olympus, DP70-WPCXP).

SDS–PAGE and Immunoblotting

After appropriate periods of cultivation, cells were washed twice with PBS and scraped into lysate buffer containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 2 μg/ml aprotinin, and 5 mM EGTA in PBS. The cells were sonicated for 10 s with a sonifier cell disrupter, and the sonicated samples were then centrifuged for 10 min at 10,000*g*. The supernatants were denatured in sample buffer and heated in boiling water for 5 min. Equal amounts of proteins estimated with the protein assay kit (Bio-Rad, Richmond, CA, USA) and prestained molecular weight markers (GIBCO BRL) were separated by SDS–PAGE and transferred electrophoretically from the gels to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were incubated in a blocking solution consisting of 5% skim milk in PBS-Tween and incubated for 2 h at ambient temperature. The membranes were washed briefly in PBS-Tween and incubated at 4°C overnight with each antibody diluted (1:100–1:500). Subsequent procedures were conducted at ambient temperature. The membranes were washed four times within 30 min in PBS-Tween by using a rotary shaker. The washed membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-rabbit or -mouse IgG (diluted 1:5,000) in PBS-Tween. The membranes were washed as described above and the proteins recognized by the antibodies were visualized by using an ECL detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions.

RESULTS

Bleomycin Stimulated the Phosphorylation of H2AX in SCCTF Cells

To examine whether treatment with BLM could alter the status of phosphorylation of H2AX in SCCTF cells, the cells were incubated with 10 μM BLM for various time periods and subjected to Western analysis. Figure 1A shows that the anti-phospho-H2AX antibody interacted with a major band having an estimated molecular weight of 45 kDa in the proteins prepared from the BLM-treated cells. The phosphorylated H2AX appeared after 0.5 h of BLM-treatment, and the level of staining intensity remained unchanged up to 2 h. This band was not detected in the extracts prepared from the control cells. The immunoreaction was not observed in the control cells with anti-phospho-H2AX antibody (Fig. 1Ba). In contrast, intense fluorescence for phospho-H2AX was

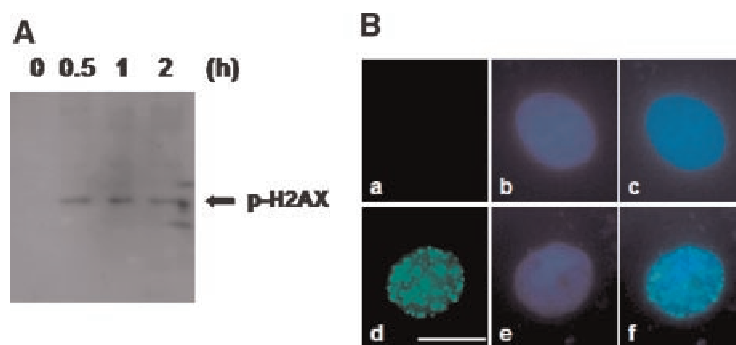

Fig. 1. Phosphorylation of H2AX in BLM-treated SCCTF cells. **A**: SCCTF cells were treated with 10 μM BLM for the variable time periods indicated and cell lysates were prepared from each type of culture. Ten microgram samples were separated on a 12.5% of SDS-PAGE gel, transferred to a PVDF membrane, and analyzed for phospho-H2AX by Western blotting. **B**: Immunostaining of SCCTF cells with anti-phospho-H2AX antibody. Cultured SCCTF cells were treated for 1 h without (**a**–**c**) or with 10 μM BLM (**d**–**f**). The treated cells were fixed with 3.7% formaldehyde, and then permeabilized with methanol. After having been washed with PBS-Tween, the cells were incubated with anti-phospho-H2AX antibody. Nuclei were stained with Hoechst 33342. The cells were examined under a fluorescence microscope. a,d: Phosphor-H2AX; (b,e), Hoechst 33342; and (c,f), merged view of phosphor-H2AX and Hoechst 33342. White bar indicates 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

observed in the nucleus in the cells treated for 1 h with 10 μM BLM (Fig. 1Bd). The control and BLM-treated cells were also stained with Hoechst 33342 (Fig. 1Bb,Be, respectively). The merged views of the control and BLM-treated cells were also shown in Figure 1Bc,Bf, respectively. These findings taken together with the results of Western analysis indicate that phosphorylation of H2AX occurred in the nucleus in the BLM-treated SCCTF cells.

Bleomycin Induced Apoptosis in SCCTF Cells

Lamin B1 is major component of nuclear envelope and cleaved into 28 kDa fragment in the apoptotic cells [Bortul et al., 2001]. Figure 2A shows Western analysis of lamin B1 in the cell lysates obtained from the control and BLM-treated SCCTF cells. In the control cells, a major immuno-positive band with an estimated molecular weight of 60 kDa was detected. However, the intensity of the 60-kDa band decreased in the cells treated with BLM. Furthermore, an additional band of 28-kDa was detected in the cell lysate prepared from the BLM-treated cells. This band was not detected in the proteins prepared from the control cells. Figure 2B shows that treatment of SCCTF cells with BLM caused a DNA fragmentation pattern forming a ladder of multiples of 180 bp. This result together with lamin B1 cleavage indicates that BLM induced apoptosis in SCCTF cells.

Bleomycin Caused Alteration of Mitochondrial Membrane Stability

The loss of mitochondrial membrane potential is a hallmark for apoptosis. Mitochondrial transmembrane potential can be determined by using JC-1 dye known as MitoCapture. In the cells with intact mitochondria this dye accumulates and aggregates inside the mitochondria, giving a red fluorescence. Whereas in the cells with damaged mitochondria or altered mitochondrial transmembrane potential, the dye cannot aggregate and remains in the cytoplasm in its monomeric form, giving a green fluorescence [Reers et al., 1995]. After treatment with BLM for 48 h, cell rounding and shrinking were obvious in SCCTF cells and these cells easily detached from the culture dishes (data not shown). In shrinking cells, we can hardly distinguish mitochondria from nucleus and other intracellular organelles. To detect clearly the changes in mitochondrial membrane potential in the BLM-treated SCCTF cells, we observed the cells that received the damage but did not shrink absolutely. The cells were treated for 45 min with MitoCapture at the end of BLM treatment. Figure 3 shows the representative images of the mitochondria labeled with MitoCapture. MitoCapture aggregated in the mitochondria, giving a bright red fluorescence in the control cells (Fig. 3b) however, only weak green fluorescence was observed in these cells (Fig. 3c). In the BLM-treated cells (lower panel), no red fluorescence was observed

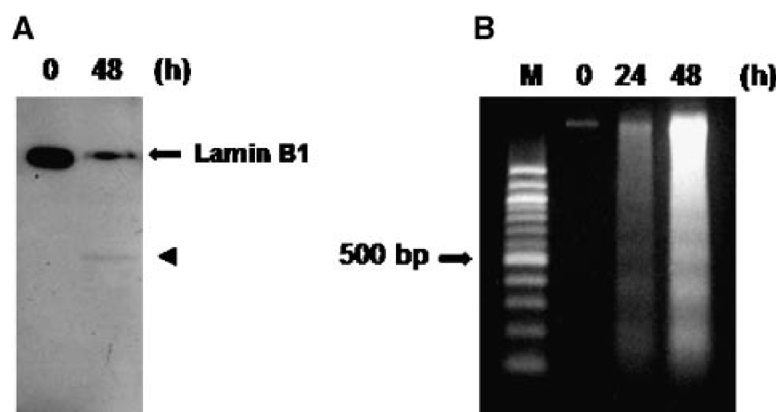

Fig. 2. Cleavage of lamin B1 and DNA fragmentation in SCCTF cells treated with BLM. **A**: Proteins prepared from SCCTF cells incubated for 48 h with 10 μM BLM were loaded on a 12.5% of SDS–PAGE gel, transferred to a PVDF membrane, and analyzed lamin B1 expression by Western blotting. Arrowhead indicates fragmented form of lamin B1. **B**: DNA ladder formation in the BLM-treated SCCTF cells. SCCTF cells were exposed to 10 μM BLM for 24 and 48 h. DNA was extracted and analyzed on a 2% agarose gel. Lane M, standard DNA markers; Arrow indicates 500 bp.

(Fig. 3e) whereas intense green fluorescence was detected in the cytoplasm (Fig. 3f). These results indicate that BLM increased mitochondrial permeability in SCCTF cells. The phase contrast microphotographs of the same fields were also shown (Fig. 3a,d).

Translocation of Histone H1.2 in BLM-Treated SCCTF Cells

After treatment for 48 h with 10 μM BLM, we labeled SCCTF cells with Mito-tracker for 45 min, fixed, permeabilized, and stained with anti-histone H1.2 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG as a second antibody. Histone H1.2 was detected in the nucleus in the control cells, whereas no staining was observed in the cytoplasm (Fig. 4a). Histone H1.2 was distributed in the nucleus and cytoplasm as dot-like bodies in the BLM-treated cells (Fig. 4d). In the cells that had been labeled with Mito-tracker, numerous dot-like particles with red fluorescence were observed in the cytoplasm both in the control and BLM-treated cells (Fig. 4b,e). The merged view confirmed

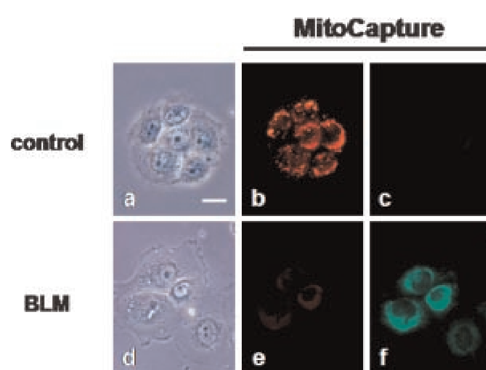

Fig. 3. Alteration of mitochondrial membrane in BLM-treated SCCTF cells. SCCTF cells were treated for 48 h without (**a**–**c**) or with 10 μM BLM (**d**–**f**). After BLM treatment, SCCTF cells were exposed to MitoCapture for mitochondria staining. Phase-contrast microphotographs of control SCCTF cells (a) or SCCTF cells treated with 10 μM BLM (d) are shown. Red fluorescence (healthy) is detected by using a U-MWIG filter (b,e). Green fluorescence (apoptotic) is detected by a U-MNIBA filter (c,f). Bar represents 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

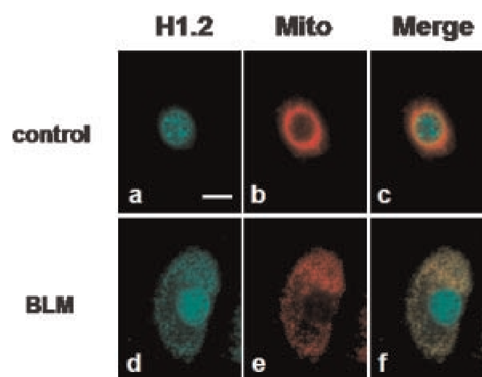

Fig. 4. Localization of H1.2 in SCCTF cells. Cultured cells were treated for 48 h without (**a**–**c**) or with 10 μM BLM (**d**–**f**) followed by incubation for 45 min with 100 nM Mito-tracker. The treated cells were fixed with 3.7% formaldehyde, and then permeabilized with methanol. After having been washed with PBS-Tween, the cells were incubated with anti-H1.2 antibody. The cells were examined under a fluorescence microscope. a,d: H1.2; (b,e), Mito-tracker; and (c,f), merged view of H1.2 and Mito-tracker. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that histone H1.2 was localized in mitochondria in the BLM-treated cells (Fig. 4f), because the reaction was visible as yellow fluorescence. However, histone H1.2 was not stained in Mito-tracker positive sites in the control cells (Fig. 4c), indicating that histone H1.2 did not locate in mitochondria.

Bak is Located in Mitochondria in SCCTF Cells

With the anti-Bak antibody, mitochondria-like bodies were intensely stained in the control and BLM-treated cells. These positive staining was visible as green fluorescence because an Alexa fluor 488-labeled second antibody was used (Fig. 5a,d). Mito-tracker stained the same sites of the Bak-positive regions in the untreated (Fig. 5b) or BLM-treated cells (Fig. 5e), which gave a red fluorescence. The merged view confirmed that Bak and Mito-tracker were localized in the same sites in the control or BLM-treated cells, because the reaction was visible as yellow fluorescence (Fig. 5c,f). Because Mito-tracker stains mitochondria, these results indicate that Bak is located in mitochondria in SCCTF cells regardless of BLM-treatment. Nuclei were also stained with Hoechst 33342.

Association of Histone H1.2 and Bak in BLM-Treated SCCTF Cells

To confirm the mitochondrial localization of histone H1.2 in the BLM-treated cells, we labeled SCCTF cells with anti-histone H1.2 and anti-Bak antibodies. Figure 6 shows the immunostaining of histone H1.2 and Bak in the control (upper panel) and BLM-treated (lower panel) cells. The distribution of red fluorescence (histone H1.2) was observed only in the nucleus in the control cells (Fig. 6a), whereas dot-like green fluorescence (Bak) was detected in mitochondria in the control cells (Fig. 6b). The merged view shows that histone H1.2 and Bak distributed in different sites in the control cells because no yellow fluorescence was detected in these cells (Fig. 6c). In contrast, in addition to the nucleus, red fluorescence of histone H1.2 was detected in the cytoplasm as dot-like bodies in the BLM-treated cells (Fig. 6d). The dot-like green fluorescence of Bak was observed in the cytoplasm in the BLM-treated cells (Fig. 6e). The merged view of histone H1.2 and Bak revealed that these proteins were co-localized in cytoplasm in the BLM-treated cells (Fig. 6f), indicating mitochondrial distribution of histone H1.2 and Bak. Cell fractionation and Western analysis also showed that histone H1.2 was translocated from the nucleus to the mitochondria and associated with Bak in the BLM-treated cells (data not shown).

DISCUSSION

Various apoptotic stimuli including treatment of anticancer drugs initially activate individual stimulus-specific signaling pathways, which subsequently focus on mitochondrial dysfunction mediated by mitochondrial membrane permeabilization [Green and

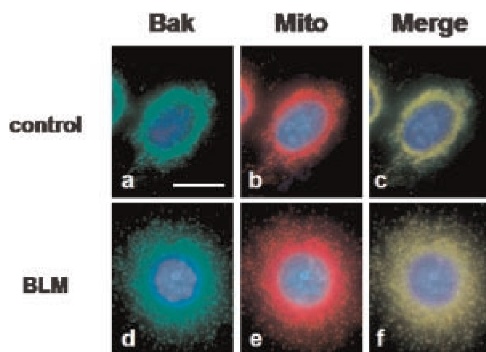

Fig. 5. Localization of Bak in SCCTF cells. Cultured cells were treated for 48 h without (**a**–**c**) or with 10 µM BLM (**d**–**f**) followed by incubation for 45 min with 100 nM Mito-tracker. The treated cells were fixed with 3.7% formaldehyde, permeabilized, and immunostained with anti-Bak antibody. The cells were examined under a fluorescence microscope. a,d:, Bak; (b,e), Mito-tracker; and (c,f), merged view of Bak and Mito-tracker. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

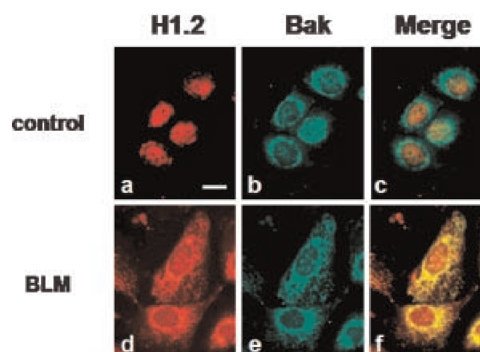

Fig. 6. Immunocytochemical identification of H1.2 and Bak in SCCTF cells. The cells were treated for 48 h without (**a**–**c**) or with 10 µM BLM (**d**–**f**). The cells were interacted with anti-H1.2 and anti-Bak antibodies as described in the text. a,d: H1.2; (b,e), Bak; and (c,f), merged view of H1.2 and Bak. Bar represents 10 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Kroemer, 2004; Spierings et al., 2005]. In the present study, we focused on histone H1.2 as a signaling candidate for transmitting the information of BLM-induced DNA double strand brakes from nuclei to mitochondria. H2AX in association with replication protein A contributes to both DNA damage checkpoint control and repair in response to DNA strand breaks [Balajee and Geard, 2004]. The phosphorylation of histone H2AX at serine 139 is one of the earliest responses to DNA breaks in mammalian cells [Rogakou et al., 2000; Tanaka et al., 2006]. Phosphorylation of histone H2AX was observed within 30 min, which should be one of the initial steps of BLM-induced apoptosis in SCCTF cells. Lamin B1 is major component of nuclear envelope and cleaved into 28 kDa fragment in the apoptotic cells, whereas in the necrotic cells lamin B1 is cleaved into 50 kDa fragment [Bortul et al., 2001]. The cleavage of lamin B1 to 28 kDa fragment indicates that BLM induced apoptosis in SCCTF cells. The BLM-induced apoptosis was also determined by DNA ladder formation.

Because histone H1.2 is a linker protein which interacts with DNA between the nucleosomes [Zong, 2004], BLM-mediated DNA breaks are thought to allow histone H1.2 to release from the nucleus and translocate to cytoplasm. Disruption of the mitochondrial transmembrane potential is one of the intracellular events that occur following induction of apoptosis. MitoCapture utilizes a cationic dye whose fluoresces is differently stained in the healthy (red) or apoptotic (green) cells [Reers et al., 1995]. In agreement with other report [Kobayashi et al., 2004], alteration of mitochondrial membrane permeabilization was detected in SCCTF cells treated with BLM. Mitochondria undergo major changes in membrane integrity before classical signs of apoptosis become manifest [Loeffler and Kroemer, 2000]. Mitochondrial membrane permeabilization induces the membrane transition pore to release small molecules including cytochrome *c* from the intermembrane space [Reed, 2006]. Members of the Bcl-2 family are major regulators of mitochondrial apoptotic events. They can be divided into three main subclasses; (1) anti-apoptotic members such as Bcl-2, (2) pro-apoptotic proteins that share homology only at the BH3 domain, such as Bid and Bim, and (3) pro-apoptotic molecules, such as Bax and Bak, with sequence homology at BH1, BH2, and BH3. Expression of Bcl-2 and Bcl-xL prevents the redistribution of cytochrome *c* in response to multiple death-inducing stimuli. However, Bid, Bax, and Bak promote cytochrome *c* release from mitochondria. Bak and Bax exist as monomers in viable cells, however, when death signals are received, Bax is inserted into the mitochondrial outer membrane as homo-oligomerized multimers [DeGiorgi et al., 2002]. Cells deficient of both Bak and Bax were demonstrated to be resistant to diverse intrinsic death pathway stimuli [Wei et al., 2001]. Bak also resides in an inactive status in the mitochondria and undergoes an allosteric conformational alternation in response to death signals, leading to its oligomerization and permeabilization of the mitochondrial outer membrane for the release of small molecules from the intermembrane space [Kim et al., 2006]. In correspondence with these reports, localization of Bak in mitochondria did not change in SCCTF cells treated with BLM as determined by immunocytochemistry and cell fractionation assay. Bak-deficient cells have lower sensitivity for various anti-cancer drugs including BLM, which suggest that Bak plays an important role in the release of apoptotic factors from mitochondria in BLM-induced apoptosis [Wang et al., 2001].

A variety of proteins have been described as molecules that transmit DNA-damage-induced apoptotic signals, including p53, Bax, Noxa, Puma, and Prep [Erster et al., 2004; Ihrie and Attardi, 2004; Roos and Kaina, 2006]. Apoptosis induced by BLM appeared to depend on the presence of a wild-type p53 gene [Enokido et al., 1996]. However, it was reported that induction of apoptosis in squamous carcinoma cells by BLM was p53-independent [Patel et al., 2000]. Furthermore, it was reported that BLM induced apoptosis in HL-60 cells that is known to be deficient in p53 gene [Gimonet et al., 2004] and activated a mitochondria-dependent caspase cascade in these cells. Cisplatin, a DNA-damaging drug, induces Bak and Bax modulation and mitochondrial depolarization in the cells harboring mutant p53 [Viktorsson et al., 2003]. These findings suggest that other factors except p53 should be involved in BLM-induced apoptosis by transmitting DNA damage to mitochondria. By searching molecules that could stimulate cytochrome *c* release, histone H1.2 was identified as one of the key factors that transmit apoptotic signals to the mitochondria in thymocytes [Konishi et al., 2003]. Histone

H1.2 is involved in apoptosis induced by X-ray irradiation and etoposide-treatment. In contrast, histone H1.2 was not related to other forms of apoptosis induced by TNF-α, staurosporine, paclitaxel, and UV irradiation [Konishi et al., 2003; Yan and Shi, 2003]. Both X-rays and etoposide cause DNA damage that primarily involves double-strand breaks. DNA double-strand breaks trigger a megabase range of chromatin remodeling, which leads to release of a significant amount of H1s from the chromatin. Thus histone H1.2 seems to be specifically involved in apoptosis induced by DNA double-strand breaks. These observations support the hypothesis that histone H1.2 also involved in apoptosis induced by BLM. In the present study, we investigated the intracellular localization of H1.2 and Bak during apoptosis by using biochemical and cytochemical approaches in BLM-treated SCCTF cells. We demonstrated that histone H1.2 was released from the nucleus by BLM treatment. The released histone H1.2 was translocated into the mitochondria in which histone H1.2 associated with proapoptotic protein Bak. Co-localization of H1.2 and Bak in mitochondria suggests that histone H1.2 transmits DNA breaks occurred in the nucleus to the mitochondria and that Bak in mitochondria receives its information to elicit the release of proapoptotic factors. Although further studies are necessary to elucidate the function and regulatory mechanisms of histone H1.2 in apoptotic cells, our results suggest that H1.2 promotes the activation of proapoptotic Bcl-2 family proteins, mitochondrial cytochrome *c* release, and ultimately, apoptotic cell death in the cells treated with BLM as well as other DNA double-strand inducing stimulation.

ACKNOWLEDGMENTS

We thank Mrs. E. Sasaki for her skilful technical assistance. This study was supported in part by grants from the Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

Adams JM, Cory S. 2007. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 26:1324–1337.

Balajee AS, Geard CR. 2004. Replication protein A and γ-H2AX foci assembly is triggered by cellular response to DNA double-strand breaks. Exp Cell Res 300:320–334.

Baliga B, Kumar S. 2003. Apaf-1/cytochrome c apoptosome: An essential initiator of caspase activation or just a sideshow? Cell Death Differ 10:16–18.

Bortul R, Zweyer M, Billi AM, Tabellini G, Ochs RL, Bareggi R, Cocco L, Martelli AM. 2001. Nuclear changes in necrotic HL-60 cells. J Cell Biochem 81:19–31.

DeGiorgi F, Lartigue L, Bauer MK, Schubert A, Grimm S, Hanson GT, Remington SJ, Youle RJ, Ichas F. 2002. The permeability transition pore signals apoptosis by directing Bax translocation and multimerization. FASEB J 16:607–609.

Enokido Y, Araki T, Tanaka K, Aizawa S, Hatanaka H. 1996. Involvement of p53 in DNA strand break-induced apoptosis in postmitotic CNS neurons. Eur J Neurosci 8:1812–1821.

Erster S, Mihara M, Kim RH, Petrenko O, Moll UM. 2004. In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. Mol Cell Boil 24:6728–6741.

Franke K, Drabent B, Doenecke D. 1998. Expression of murine H1 histone genes during postnatal development. Biochim Biophys Acta 1398:232–242.

Gimonet D, Landais E, Bobichon H, Coninx P, Liautaud-Roger F. 2004. Induction of apoptosis by bleomycin in p53-null HL-60 leukemia cells. Int J Oncol 24:313–319.

Green DR, Kroemer G. 2004. The pathophysiology of mitochondrial cell death. Science 305:626–629.

Ihrie RA, Attardi LD. 2004. Perpetrating p53-dependent apoptosis. Cell Cycle 3:267–269.

Kaufmann SH, Earnshaw WC. 2000. Induction of apoptosis by cancer chemotherapy. Exp Cell Res 256:42–49.

Kim R, Emi M, Tanabe K. 2006. Role of mitochondria as the gardens of cell death. Cancer Chemother Pharmacol 57:545–553.

Kobayashi T, Ogawa Y, Watanabe Y, Furuya M, Kataoka S, del Saz EG, Tsunawaki S, Dinauer MC, Seguchi H. 2004. Mitochondrial transmembrane potential is diminished in phorbol myristate acetate-stimulated peritoneal resident macrophages isolated from wild-type mice, but not in those from gp91-phox-deficient mice. Histochem Cell Biol 122:323–332.

Konishi A, Shimizu S, Hirota J, Takao T, Fan Y, Matsuoka Y, Zhang L, Yoneda Y, Fujii Y, Skoultchi AI, Tsujimoto Y. 2003. Involvement of histone H1.2 in apoptosis induced by DNA double-strand breaks. Cell 114:673–688.

Loeffler M, Kroemer G. 2000. The mitochondrion in cell death control: Certainties and incognita. Exp Cell Res 256:19–26.

Okamura H, Morimoto H, Haneji T. 2001. Peplomycin-induced apoptosis in oral squamous carcinoma cells depends on bleomycin sensitivity. Oral Oncol 37:379–385.

Patel V, Ensley JF, Gutkind JS, Yeudall WA. 2000. Induction of apoptosis in head-and-neck squamous carcinoma cells by γ-irradiation and bleomycin is p53-independent. Int J Cancer 88:737–743.

Pron G, Mahrour M, Orlowski S, Tounekti O, Poddevin B, Belehradek J Jr, Mir LM. 1999. Internalisation of the bleomycin molecules responsible for bleomycin toxicity: A receptor-mediated endocytosis mechanism. Biochem Pharmacol 57:45–56.

Reed JC. 2006. Proapoptotic multidomain Bcl-2/Bax-family proteins: Mechanisms, physiological roles, and therapeutic opportunities. Cell Death Differ 13:1378–1386.

Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M, Chen LB. 1995. Mitochondrial membrane potential monitored by JC-1 dye. Methods Enzymol 260:406–417.

Rogakou EP, Nieves-Neira W, Boon C, Pommier Y, Bonner WM. 2000. Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. J Biol Chem 275:9390–9395.

Roos WP, Kaina B. 2006. DNA damage-induced cell death by apoptosis. Trends Mol Med 12:440–450.

Spierings D, McStay G, Saleh M, Bender C, Chipuk J, Maurer U, Green DR. 2005. Connected to death: The (unexpurgated) mitochondrial pathway of apoptosis. Science 310:66–67.

Tanaka T, Halicka HD, Traganos F, Darzynkiewicz Z. 2006. Phosphorylation of histone H2AX on Ser 139 and activation of ATM during oxidative burst in phorbol ester-treated human leukocytes. Cell Cycle 22:2671–2675.

Urade M, Ogura T, Uematsu T, Takahashi Y, Kishimoto H, Yoshioka W. 1994. Induction of bleomycin resistance in a human oral squamous carcinoma cell line and characterisation of bleomycin-resistant and -sensitive clones. Oral Oncol 30:409–414.

Vignali M, Workman JL. 1998. Location and function of linker histones. Nat Struct Biol 5:1025–1028.

Viktorsson K, Ekedahl J, Lindebro MC, Lewensohn R, Zhivotovsky B, Linder S, Shoshan MC. 2003. Defective stress kinase and Bak activation in response to ionizing radiation but not cisplatin in a non-small cell lung carcinoma cell line. Exp Cell Res 289:256–264.

Wallach-Dayan SB, Izbicki G, Cohen PY, Gerstl-Golan R, Fine A, Breuer R. 2006. Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway. Am J Physiol Lung Cell Mol Physiol 290:L790–L796.

Wang GQ, Gastman BR, Wieckowski E, Goldstein LA, Gambotto A, Kim TH, Fang B, Rabinovitz A, Yin XM, Rabinowich H. 2001. A role for mitochondrial Bak in apoptotic response to anticancer drugs. J Biol Chem 276:34307–34317.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. 2001. Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. Science 292:727–730.

Yamamoto T. 2006. Bleomycin and the skin. Br J Dermatol 155:869–875.

Yan N, Shi Y. 2003. Histone H1.2 as a trigger for apoptosis. Nat Struct Biol 10:983–985.

Zong WX. 2004. Histone, H1.2: Another housekeeping protein that kills. Cancer Biol Ther 3:42–43.