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Post-translational modification and mitochondrial relocalization of histone H3 during apoptosis induced by staurosporine

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ABSTRACT

Post-translational modifications (PTMs) of histones such as phosphorylation, acetylation, and ubiquitination, collectively referred to as the "histone-code", have been known to regulate gene expression and chromatin condensation for over a decade. They are also implicated in processes such as DNA repair and apoptosis. However, the study of the phosphorylation of histones has been mainly focused on chromosome condensation and mitosis. Therefore, the phosphorylation of histones in apoptosis is not fully understood. It was recently demonstrated by Tang et al. that histones are released from nucleosome during apoptosis, an observation that is in agreement with our findings. In addition to the release of histones, the dephosphorylation of histone H3 at Thr-3 and Ser-10 was observed during apoptosis in some cancer cells. Our data suggest that the modification and release of histones could serve markers of apoptosis in human cancer cells. We also suggest that the released histones, especially H3, could be translocated to mitochondria during apoptosis.

1. Introduction

Histones, small basic proteins composed of a globular domain and an N-terminus tail, are the fundamental units of eukaryotic chromatins. Histone octamers, formed by two H2A–H2B dimers and an H3–H4 tetramer, are wrapped by an approximately 146 bp portion of DNA to form a nucleosome, the major repeating unit of chromatin. In addition, histone H1 binds to the linker DNA between nucleosomes to form chromatin [1].

Post-translational modifications (PTMs) of histones—such as phosphorylation, acetylation, and ubiquitination—have been known to regulate gene expression and control chromatin condensation [2–4]. It has also been demonstrated that the highly organized structure of chromatin is remodeled following PTMs in order to recruit transcriptional machinery. This indicates that the PTMs of histones contribute to change chromatin functionally as well as structurally [5]. PTMs are also implicated in the processes of DNA repair and apoptosis [6], making the PTMs very essential in cellular survival and death. However, the phosphorylation of histones has been studied mainly in relation to chromosome condensation and mitosis [7,8]. Specifically, the Ser-10 of H3 is known to be a major target for phosphorylation during mitosis, which accompanies chromatin condensation. During the cell cycle, alterations in the phosphorylation level at Ser-10 are commonly observed. At the G2/M phase, this residue is highly phosphorylated. Dephosphorylation at this position is observed after anaphase [9]. Recently, several reports demonstrated that the phosphorylation of histones could be implicated in apoptosis, but the mechanism is still controversial [10,11]. While chromatin condensation accompanies apoptosis, both phosphorylation and dephosphorylation of histone H3 could be observed at the Ser-10 residue. Cisplatin, an anti-cancer drug, is reported to induce apoptosis following the phosphorylation of Ser-10 [10]. However, several topoisomerase inhibitors were shown to dephosphorylate H3 at Ser-10 in addition to inducing apoptosis [11].

Apoptosis is programmed cell death, a fundamental process to eliminate damaged cells, especially in higher organisms [12]. From embryonic development to tissue homeostasis, apoptosis is a tightly regulated process. Therefore, malfunctions in the regulation of apoptosis could be a hallmark for many diseases, including cancer and neurodegenerative diseases [13–15]. However, the process of apoptosis is still not fully understood, especially as it relates to histones. Mitochondria are not only energy-generating systems in eukaryotes, but also the central organelles responsible for the

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initiation of apoptosis. For example, the release of cytochrome c from mitochondria is one of the major events of apoptosis. This release leads to the activation of caspases which mediate the activation of other enzymes that eventually result in cell death [15].

In this study, we show that H3 phosphorylation is involved in apoptosis and suggest that the histone H3 released from nucleus could be relocalized into the mitochondria.

2. Material and methods

2.1. Cells and materials

Jurkat cells were grown in RPMI-1640 (Mediatech, Inc., USA) and HeLa/HEK293T cells were grown in DMEM (Mediatech, Inc.) with 10% FCS and penicillin/streptomycin at 37 °C with 5% CO_2 in a humidified atmosphere. Staurosporine (STS), streptonigrin (SR), phosphatase inhibitor cocktail, and protease inhibitor cocktail were purchased from Sigma–Aldrich (St. Louis, USA).

2.2. Cytotoxicity assays

Cellular cytotoxicity was measured using WST- (Roche, Switzerland). Approximately 5×10^3 cells were seeded in 96 well plates. After the cells attached, SR or STS was treated. Following an incubation period of 5–24 h, 10 μl/well of WST-1 solution was added, and incubation was continued for an additional 1–2 h. Absorbance was measured at 450 nm against a 650 nm reference using a Microplate Reader (Bio-Rad, USA).

2.3. Histone extraction

Cells were incubated for 5 h with STS to induce apoptosis. They were then washed with PBS and lysed in a hypotonic lysis buffer (10 mM Tris–HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, phosphatase and protease inhibitor cocktails) to isolate the nuclear fraction. The nuclear pellet was resuspended in 0.4 N H_2SO_4, incubated for 1 h at 4 °C, and centrifuged at 14,000*g* for 5 min at 4 °C. Histone proteins partitioned into the supernatant were precipitated with acetone at −80 °C overnight. Finally, the pellets were collected by centrifugation at 14,000*g* for 5 min at 4 °C and redissolved in water containing phosphatase and protease inhibitor cocktails.

2.4. Western Blot analysis

The cells were washed with PBS and lysed in a lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.1–1% SDS, phosphatase and protease inhibitor cocktails). Protein concentrations were determined by BCA assay (Pierce, USA) using bovine serum albumin (BSA) as standard. Samples (20 μg) were electrophoresed on 10% or 16% SDS–PAGE. For the Western blotting assay, the membrane was blocked for 3–5 h in Tris-buffered saline (TBS) buffer containing 0.1% Tween-20 and 5% (w/v) dry skim milk powder and incubated overnight at 4 °C with the primary antibody. The bound antibody was detected by chemiluminescence using the SuperSignal® West Pico kit. The antibodies for tubulin, nuclear matrix protein p84, histone H3, histone H4, H3-pho-Thr3, and H3-pho-Ser10 were purchased from Abcam (Cambridge, USA); the antibodies for phospho-Ser and phosphor-Thr were purchased from Sigma; and the antibodies for caspases-3, -7, -9 and PARP were purchased from Cell Signaling (Danvers, USA).

2.5. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis

For isoelectric focusing (IEF), proteins (400 μg) were precipitated with acetone. Bio-Rad IPG strips (7 cm, pH 3–10) were rehydrated overnight (16 h) with DeStreak rehydration solution from Amersham (Piscataway, USA). IEF was performed using Ethan IPG-phor II (Amersham) at 300 V for 2 h, at 1000 V for 30 min, and 5000 V for 100 min, with total focusing for 6.5 kVh. For the second dimension, IPG strips were electrophoresed on a16% SDS–PAGE and the proteins on the gel were transferred onto PVDF membranes for Western blot analysis.

2.6. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

The proteins of interest visualized on gels were identified by LC–MS/MS. Briefly, the protein samples (20 μg) obtained by lysis of cells were separated on a 16% SDS–PAGE gel and stained with Coomassie Blue. The bands chosen for protein identification were excised and subjected to in-gel digestion by trypsin. Samples containing the tryptic peptides were analyzed by LC–MS/MS, which consisted of a Surveyor Micro AS auto sampler, a Surveyor MS Pump, and an LTQ linear ion trap mass spectrometer (Thermo, USA). Proteins were identified by database searching the peptide product ion spectra against protein databases using Mascot (Matrix Science, USA).

2.7. Immunofluorescence and confocal microscopy

HeLa cells were plated on slide glass and treated by STS. After fixing and permeabilization, cells were incubated for 15 min with the blocking solution containing 3% BSA in PBS, anti-histone H3 antibody in blocking solution for 1 h, and secondary antibody that was fluorescent conjugated (Alexa fluor®488 goat anti-rabbit, Molecular probes, USA). Cells were stained with DRAQ5 (Biostatus Limited, UK) for nuclear staining and MitoTracker Red CMXRos (Molecular probes) for mitochondria staining, respectively. Images were taken by a confocal microscope (Olympus, USA).

3. Results

3.1. Release of histones into cytosol during apoptosis

We used a non-selective protein kinase inhibitor (STS) and a selective topoisomerase inhibitor (SR) to induce apoptosis. They are well-known inducers of apoptosis in several cells [16,17]. Both were cytotoxic to Jurkat (human T cell leukemia) and HeLa (human cervical cancer) cells at 1 μM as determined by WST-1 assay (Fig. 1A). A significant effect was observed after 5 h treatment in both cell lines. Therefore, this concentration was used in all the subsequent experiments. Activation of several caspases and PARP was observed in Jurkat (Fig. 1B) and HeLa cells (data not shown). A significant change in the activation of caspases due to treatment with STS was seen compared to the treatment with SR. However, HEK293T (human embryonic kidney) cells required much higher concentrations (10 μM) and longer treatment (24 h) to induce cytotoxicity and the apoptosis through activation of caspases was not observed in these cells with either STS or SR treatment (Fig. 1B).

Protein expression profiles of the cell lines studied were compared using SDS–PAGE gels stained with Coomassie. In STS-treated Jurkat (Fig. 1C), there were obvious changes in protein levels. However, neither of the reagents used resulted in any alteration in protein expression in HEK293T (Fig. 1C), which was in agreement with our findings from caspase activation experiments. LC–MS/MS

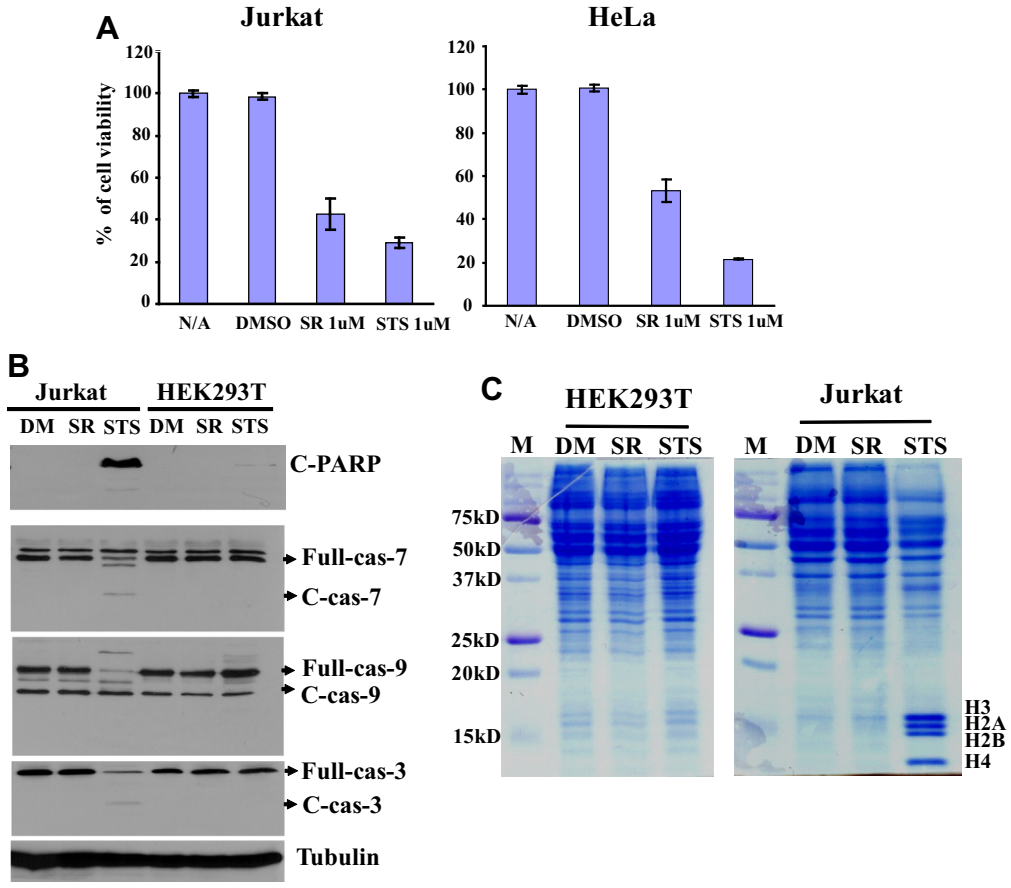

Fig. 1. Induction of apoptosis by STS. (A) Cell viability assays (WST-1) were performed after treatment with STS and SR with the indicated concentrations and incubation for 5 h. Untreated cells were represented as N/A. DMSO was used as vehicle to resolve STS and SR and as a negative control. The results were expressed as a percentage of control (DMSO). The mean ± SD was calculated from three independent experiments. Values of $^{*}P < 0.01$ were considered statistically significant. ANOVA's *t*-test was used to compare the significance of values. (B) The cleavage of caspases and PARP was shown as an indicator of apoptosis by Western blot analysis. Detection of PARP and caspase cleavage in cells treated with STS was observed using anti-cleaved PARP (C-PARP), anti-caspase-3, anti-caspase-7, and anti-caspase-9 antibodies. Cleaved caspases and full-length caspases are indicated with arrows and represented as C-cas and Full-cas, respectively. Western blot analysis probing with anti-tubulin antibody was employed to ensure equal loading amounts in the gel for loading control. (C) After treatment with STS, an increased level of histones in the cytosol was observed in Jurkat, but not in HEK293T. To visualize the bands, the gel was stained with Coomassie blue. LC–MS/MS was performed to analyze the specific bands that were observed by STS treatment in Jurkat cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nalyses were performed to identify the major proteins whose levels changed in response to STS treatment in Jurkat and HeLa cells. The major proteins were identified to be histone proteins H2A, H2B, H3, and H4. We thought of two possible scenarios to explain the increase in these proteins. One explanation is that the highly expressed histones in the cytoplasm are not transported to nucleus due to apoptosis and remain there at high levels. Another possibility is that histones are released from the nucleus as part of the apoptosis process, which was referred to as "histone release" in a previous study [18]. To address these possible scenarios, lysis buffers containing varying amount of SDS were used to lyse cells after the induction of apoptosis. The mild lysis buffer, which contained 0.1% SDS, removed only the cellular membrane, leaving the nucleus compartment intact. This allowed us to detect cytosolic and nuclear histones separately. We were able to detect the histones released from the nucleus within the cell lysates (supernatant) after treatment of STS. The buffer that contained 0.5% or 1% SDS resulted in complete lysis of cellular compartments, including the nucleus, so all the histones were detected in the cell lysates (Fig. 2A). A nuclear matrix protein (p84) was used as control to ensure that detection of H3 was not the result of nuclear contamination. The p84 level did not change between control (DMSO treated Jurkat cell) and STS-treated Jurkat cells in 0.1% SDS-containing lysis buffer, but the H3 level significantly increased with STS treatment (Fig. 2A). In the case of 0.5% and 1% SDS-containing lysis buffer, the similar levels of H3 and p84 were measured between control (DMSO) and STS-treated Jurkat cells. This suggested that histones were released from nucleus during apoptosis. It also showed that the release of histones, especially H3, increased in a time- and dose-dependent manner in Jurkat and HeLa cells treated by STS (Fig. 2B). However, the level of H3 in the cytosol did not change by STS treatment in HEK293T cells (Fig. 1C), which agreed with the findings from the activation of caspases and PARP (Fig. 1B). This supported the notion that the elevated concentrations of histones in cytosol, possibly due to the breakdown of chromatin in the nucleus, could be an indicator of apoptosis.

3.2. Modification of histone H3

Besides their well-established role in the regulation of gene expression, the PTMs of histones have also been proposed to be involved in chromatin condensation and apoptosis [6,19]. Phosphorylation, a well-known modification, adds ~2 negative charges around the physiologic pH by replacing neutral hydroxyl groups on serines, threonines, or tyrosines. We utilized this change in the isoelectric point (pI) of proteins to detect phosphorylation using 2D analysis. As shown in Fig. 3A and B, samples were separated by 2D-gel electrophoresis, and multiple trailing spots with slight

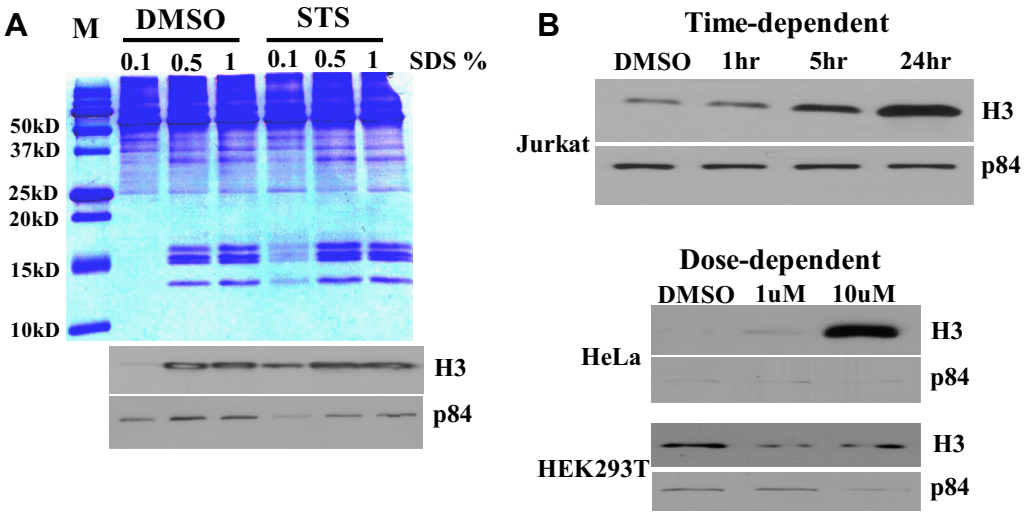

Fig. 2. Histones are released from the nucleus during apoptosis. (A) Several different lysis buffers that contained 0.1% to 1% SDS were used to confirm the release of histones into the cytosol. Jurkat cells were lysed with each lysis buffer after apoptosis was induced with 1 μM STS for 5 h. The H3 level was determined with anti-H3 antibody. The nuclear matrix protein p84 was used to ensure that detection of H3 was not the result of nucleus contamination. (B) Time-dependent histone release was observed in Jurkat cells. After induced by 1 μM STS for the indicated time, Jurkat cells were lysed with 0.1% SDS contained lysis buffer. The level of released H3 was determined with anti-H3 antibody. Dose-dependent histone release was observed in HeLa. After induced by STS at the indicated concentration for 5 h, each cell was lysed with 0.1% SDS-containing lysis buffer. The level of released H3 was determined by anti-H3 antibody. HEK293T cells were used as a negative control, as their H3 level was not changed by STS treatment.

changes in pI, which are typical of differential phosphorylation, were observed. In Fig. 3A, the lysis buffer with 1% SDS was used to obtain whole cell lysate that included nucleus. The H3 levels in control and STS-treated Jurkat cells were the same; however, the distribution of H3 changed compared to control, implying a change in the phosphorylation level of H3 as a result of STS treatment. In Fig. 3B, the mild buffer with 0.1% SDS was used to obtain cytosolic histones. Much higher level of H3 was apparent in STS-treated Jurkat cells, suggesting that H3 had been released from the nucleus. The distribution of H3 also changed compared to control, implying a change in the phosphorylation level of H3 caused by STS treatment. To confirm the phosphorylation of specific residues, Western blot analyses were performed using H3-p-Ser10, which demonstrated phosphorylation of H3 residues at Ser10. In Fig. 3C, the cytosolic fraction (Cyto) was used to show the changes in phosphorylation of released H3. STS treatment induced the release of H3 into the cytosol; however, the released H3 had decreased phosphorylation at Ser10 based on with 2D-gel electrophoresis (Fig. 3B and C). The anti-H3 antibody was used to confirm the release of H3 into the cytosol. To confirm the phosphorylation of H3, Western blot analyses were performed with histone extract from Jurkat cells using anti-H3-p-Thr3 and H3-p-Ser10. To show the overall phosphorylation of H3, Western blot analysis was performed using p-Ser and p-Thr antibodies. This analysis showed dephosphorylation of H3 at both Thr3 and Ser10 (Fig. 3D). H3 and H4 levels were determined by anti-H3 and H4 antibodies after stripping the membrane to ensure equal loading.

3.3. Re-localization of released H3 on mitochondria

Localization of H3 was traced by immunofluorescence assay under a confocal microscope after STS treatment. In control cells treated by DMSO as vehicle, H3 was not released from nucleus and overlapped with DRAQ5, which was a marker for nucleus (data no shown). In contrast, in then cells treated by STS, H3 was released from the nucleus (Fig. 4A) and overlapped this time with mitochondria, as evidence by H3 being merged with MitoTracker Red CMXRos (Fig. 4A). To confirm the translocation of H3 into mitochondria, Western blot analysis was performed. Fig. 4B shows that H3 was detected in mitochondria after treatment with STS. This supported our confocal data showing the localization of released H3 in mitochondria. The porin, which is an outer membrane mitochondrial protein, was used as an internal control and an indicator of the mitochondrial fraction. Porin was not detected in the cytoplasmic fraction (Cyto), but the mitochondrial fraction (Mito) showed the same level of porin in control and STS-treated Jurkat cells, assuring the equality of the amount of mitochondria from both cell lines. The release of cytochrome c, which was originally located in mitochondria, occurred during the initial step of apoptosis. Some released cytochrome c was detected in the cytosol (Cyto) after treatment with STS, which confirmed that apoptosis had been induced by STS treatment. These results supported the hypothesis that STS treatment induced apoptosis and that the induction of apoptosis released H3, which was translocated to the mitochondria.

4. Discussion

Involvement of histones in apoptosis has been established in recent reports [1,2]. Activation of caspases and DNA fragmentation are among the well-established hallmarks of apoptosis. We have seen a strong correlation between these signs and high cytosolic concentrations of histones, previously referred to as "histone release". Even though cytotoxicity due to STS treatment was observed in human embryonic kidney cells (HEK293T), the activation of caspases and the release of histones were not observed. This suggests that the release of histones could be a specific marker for apoptosis in some cancer cells.

In this study, we have also shown that the elevated levels of histones in cytosol during apoptosis induced by STS in human T cell leukemia (Jurkat) and human cervical cancer (HeLa) cell lines were accompanied by reduced phosphorylation of histone H3 at Ser-10.

Mechanism behind so called "histone release" from nucleosomes or increase in relative concentrations of histone in cytosol is still not clear. Changes in phosphorylation of H3 as observed in this work suggest that PTMs have a role in this process. They in fact are known to induce changes in chromatin structure and function. Histones are rich in basic residues that are positively charged at physiologic pH. These charged moieties are utilized in binding to

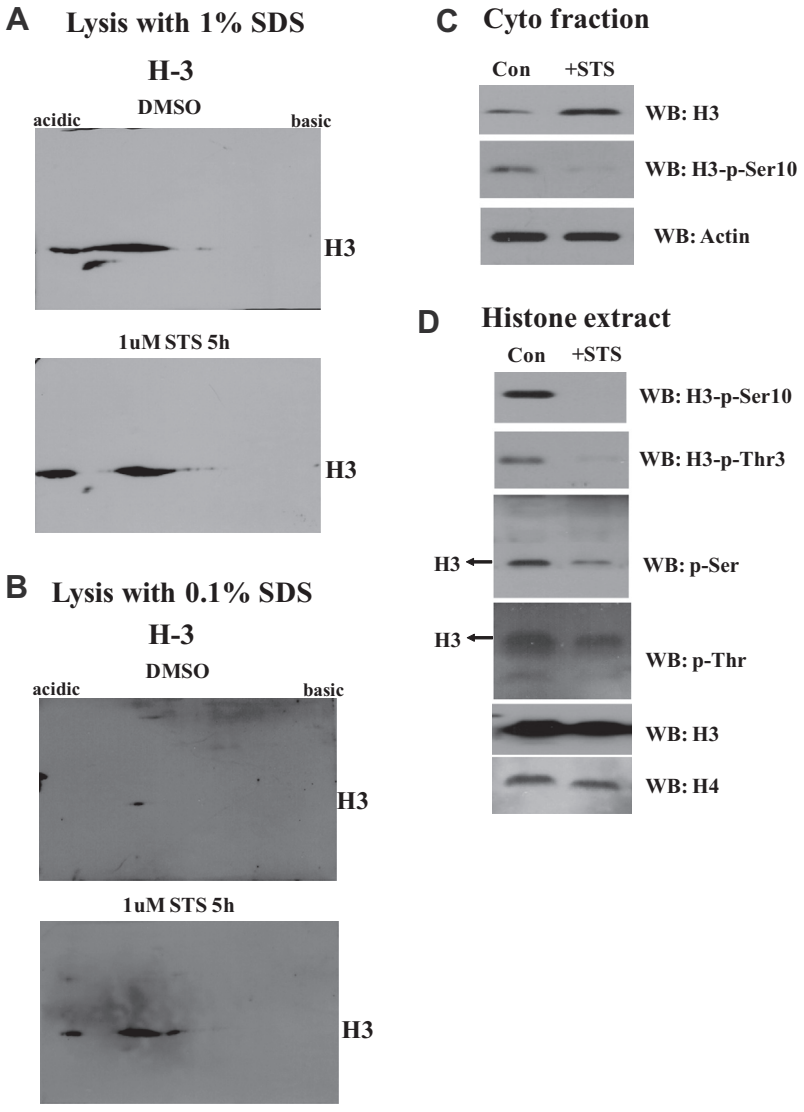

Fig. 3. Modification of H3 during apoptosis. (A) 2D-gel analysis was performed to show the modification of H3. Jurkat cells were treated with 1 μM STS for 5 h. For the cell lysis, 1% SDS-containing lysis buffer was used to break down the nucleus completely and to get whole cell extract. Western blot analysis was performed using anti-H3 antibody to show the change in molecular weight and/or pI which was indicators of change of phosphorylation level of H3. (B) To isolate the cytosolic fraction, 0.1% SDS-containing lysis buffer was used. (C) After treatment with 1 μM STS for 5 h in Jurkat cells, the cytosolic fraction (Cyto) was prepared as described to show the changing level of phosphorylation of released H3. H3-p-Ser10 showed specific phosphorylation of H3 residues at Ser10. The H3 level was determined with anti-H3 antibody after membrane stripping. Actin was used as a loading control. (D) After treating Jurkat cells in 1 μM STS for 5 h, histone extract was prepared as described. Western blot analysis was performed to confirm the phosphorylation of H3. H3-p-Thr3 and H3-p-Ser10 showed specific phosphorylation of H3 residues at Thr3 and Ser10, respectively. The overall phosphorylation level of H3 at Thr and Ser confirmed by anti-phospho-Thr and anti-phospho-Ser antibodies, respectively. H3 and H4 levels were determined with anti-H3 and anti-H4 antibodies after stripping membranes to ensure equal loading amounts in the gel.

and interacting with the negatively-charged DNA. Modification of histones, especially phosphorylation and acetylation, results in dramatic changes in the charge of a given moiety and cause conformational changes in chromatin structure. For example, phosphorylation introduce negative charges to N-terminus tails and can cause dissociation between histones and DNA. Similarly a lysine residue that carries a positive charge will be neutral when acetylated. Unbalanced charges between DNA and histone proteins can result in distortion as a consequence. These modifications can therefore be implicated in the release of histones from nucleus due to the changes in chromatin structural changes during apoptosis.

Our data supports the involvement of decreased phosphorylation of this specific residue in apoptosis induced by STS in some cancer cells. The acetylation of lysine residues, another common PTM in histones, has been well studied in gene regulation. It is established that acetylation induces the conformational change of chromatin to make it accessible for the transcriptional machinery. Some studies showed that the acetylation of lysine in histones is another important modification for apoptosis [6]. To investigate the possibility of acetylation during apoptosis, Western blot analysis was performed using anti-acetylated-lysine antibody. However, STS did not result in any detectable changes in the acetylation in this study (data not shown), suggesting that the phosphorylation is more critical in regulation of apoptosis at least in some cell lines than acetylation.

The sequence of the cleavage site of H3 was determined using MitoProt, which calculated the N-terminal protein region that could support a Mitochondrial Targeting Sequence (MTS) and the cleavage site [20]. The possibility of localization of H3 on mitochondria was over 99%, much higher than for other histones (H2A, H2B, and H4). The mitochondrion is well known to be the energy-generating system of the cell as well as an important

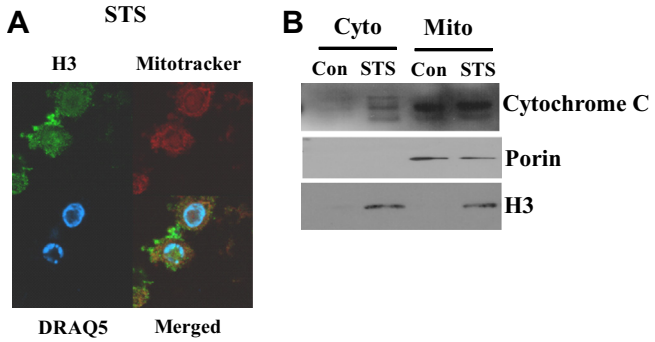

Fig. 4. Re-localization of released H3 into mitochondria. (A) HeLa cells were stained with MitoTracker Red CMXRos (red coloring) and DraQ5 (blue coloring) to visualize mitochondria and nuclei, respectively. H3 was stained with anti-H3 and was visualized with Alexa488-conjugated fluorescent antibody (green coloring). After induction of apoptosis with 1 μM STS treatment for 5 h, H3 released from the nucleus was observed, and released H3 overlapped with mitochondria (orange coloring). (B) Western blot analysis was performed to confirm the localization of H3 on mitochondria. Mitochondria were isolated from the Jurkat cells. After induction of apoptosis in Jurkat cells with 1 μM STS treatment for 5 h, released H3 was detected in the cytosolic fraction (cyto) and the mitochondrial fraction (mito). Porin was used as a marker of the mitochondrial fraction. Re-localization of released H3 into mitochondria was detected by anti-H3 antibody. Cytochrome c was used as an indicator of apoptosis, as it was released by mitochondria during apoptosis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regulator of apoptosis. Therefore, this translocation indicates a further piece of the apoptotic mechanism. Mitochondrial networks are dynamic, and their morphology can be changed, depending on the fate of the cell. For this reason, many proteins are involved in regulating mitochondrial morphology and function [21]. Mitochondrial proteins such as cytochrome c, Smac/Diablo, AIF, and EndoG, which reside in the intermembrane space, have been identified to regulate apoptotic processes directly and/or indirectly by being releasing to the cytosol in response to a variety of apoptotic stimuli [15]. In addition, Bcl-2 families translocate to mitochondria from other cellular organelles in response to apoptotic stimuli [22]. These pieces of evidence support regulation of apoptosis by not only release of apoptotic factors into cytosol, but also translocation of proteins into mitochondria. Therefore, there is still a possibility that H3 is bound to the mitochondria to regulate further procedures during apoptosis, such as occurs with Bcl-2 families (Bax, Bad, and Bcl-2) [23]. Positively charged nature of histones would also be expected to facilitate their transport into mitochondrial matrix that is negatively charged relative to other cell compartments due to proton pumping into the inner membrane. H3 could be involved in triggering the activation of pro-apoptotic proteins or the inhibition of anti-apoptotic proteins such as activation of pro-apoptotic proteins like Bad and Bax and inhibition of anti-apoptotic proteins line Bcl-2 or Bcl-XL. Additional studies are needed to investigate the mechanism for the involvement of histone PTMs in apoptosis.

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