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Histone H4 is cleaved by granzyme A during staurosporine-induced cell death in B-lymphoid Raji cells

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Granzyme A (GzmA) was first identified as a cytotoxic T lymphocyte protease protein with limited tissue expression. A number of cellular proteins are known to be cleaved by GzmA, and its function is to induce apoptosis. Histones H1, H2B, and H3 were identified as GzmA substrates during apoptotic cell death. Here, we demonstrated that histone H4 was cleaved by GzmA during staurosporine-induced cell death; however, in the presence of caspase inhibitors, staurosporine-treated Raji cells underwent necroptosis instead of apoptosis. Furthermore, histone H4 cleavage was blocked by the GzmA inhibitor nafamostat mesylate and by GzmA knockdown using siRNA. These results suggest that histone H4 is a novel substrate for GzmA in staurosporine-induced cells. [BMB Reports 2016; 49(10): 560-565]

INTRODUCTION

Apoptosis is a fundamental process in eukaryotic multicellular organisms. The transition between life and death is the single-most momentous event for any living entity from single cells to whole organisms (1). Not surprisingly, dramatic biochemical and morphological changes occur in apoptotic cells both outside and inside of the nucleus. The classic apoptotic features are chromatin condensation and DNA fragmentation. Apoptotic cell death is executed by a group of caspase cysteine proteases. By cleaving important cellular protein substrates, caspase activity eventually induces typical apoptotic processes such as membrane blebbing and DNA fragmentation. Apoptotic DNA fragmentation is mediated by caspase-activated DNase, endonuclease G (2, 3). During apoptosis, histone proteins are reportedly released from nucleosomes (4). The five main histone proteins, histones H1, H2A, H2B, H3, and H4, are the predominant DNA folding proteins in cells. Histones undergo a wide variety of post-translational modifications including lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, lysine ubiquitination and sumoylation, as well as ADP ribosylation, all of which are dynamically carried out by histone-modifying enzyme complexes (5, 6). Therefore, histones influence the genetic potential of DNA (7, 8) and post-translational modification of histones influence apoptotic cell death (9, 10). Previously, histones H1, H2B, and H3 were found to be cleaved by granzyme A (GzmA) in the apoptotic cells (11). GzmA, a serine protease in the cytotoxic granules of natural killer cells and cytotoxic T lymphocytes, induces caspase-independent cell death when introduced into target cells by perforin. GzmA also targets important nuclear proteins for degradation, including histones, lamins that maintain the nuclear envelope, and several key DNA damage repair proteins (12-14). Moreover, in a recent study, proteolytic processing of histones was shown to effect cellular responses. For example, histone H3 proteolysis occurs during mouse embryonic stem cell differentiation (15), histone H3 tail clipping regulates gene expression, and extracellular histones are major mediators of cell death during sepsis (16, 17). Therefore, the proteolytic processing of histones is important for cell physiology. In the present study, we demonstrated that the histone H4 N-terminal tail was cleaved by GzmA in staurosporine (STS)-induced cell death.

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RESULTS

A fast-migrating histone H4 fragment was detected in cells undergoing STS-induced cell death

Histone H4 is one of the five main histone proteins involved in regulating the structure of chromatin in eukaryotic cells. While attempting to elucidate the function of histone proteins in cell death, we observed a fast-migrating histone H4 band in the lysates of cells undergoing STS-induced cell death, but

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analogous bands were not observed for histones H2A and H2B (Fig. 1A). This histone H4 fragment was found in cells 24 h after STS treatment (Fig. 1B). Because STS induces apoptosis through a caspase-dependent pathway, we examined whether histone H4 cleavage could be prevented by treating the cells with the pan-caspase inhibitor Z-VAD-FMK (18). The pan-caspase inhibitor failed to prevent histone H4 cleavage. Interestingly, treatment with STS and Z-VAD-FMK actually increased the amount of H4 fragment that was detected. Moreover, the increase of the histone H4 fragment was not dependent on the dose of STS used (Fig. 1C). We next checked whether histone H4 was cleaved in the presence of other cell death inducers such as etoposide, TNF-α, and cycloheximide (Fig. 1D, E). These chemicals did not induce histone H4 cleavage. Therefore, we showed that histone H4 is only cleaved in response to STS treatment in Raji B-lymphoid cells.

Treatment with caspase inhibitors increased both cell death and histone H4 cleavage

Caspase inhibitors have been shown to block STS-induced apoptosis (19). We attempted to inhibit histone H4 cleavage by treating cells with caspase inhibitors; however, histone H4 cleavage was detected in cells treated with inhibitors of caspase-6 (Z-VEID-FMK) and caspase-9 (Z-LEHD-FMK) (Fig. 2A). Treatment with these caspase inhibitors also increased cell death to a greater extent than STS treatment alone (Fig. 2B). Etoposide was used as a negative control for the cleavage of histone H4 in these experiments (Fig. 2). Recently, STS was reported to induce necroptotic cell death in the presence of

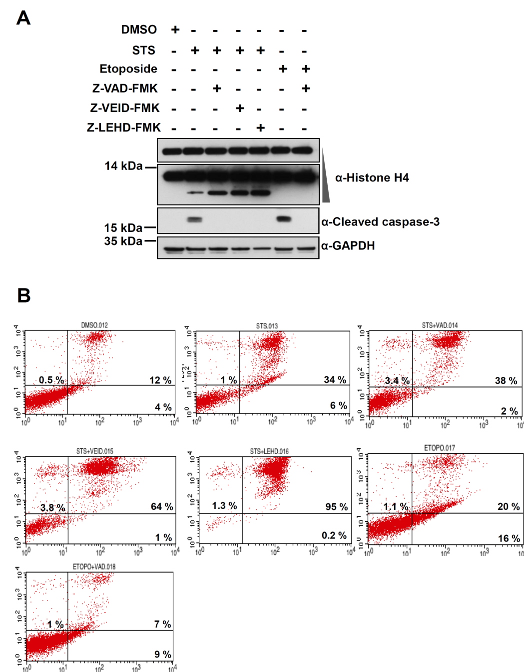

Fig. 2. The increased cell death and histone H4 cleavage in Raji cells treated with caspases inhibitors. (A) Raji cells were treated with 100 μM pan-caspase, caspase-6, or caspase-9 inhibitors and 1 μM STS for 24 h. Etoposide was used as a negative control. (B) Cell death in the same samples was analyzed using flow cytometry.

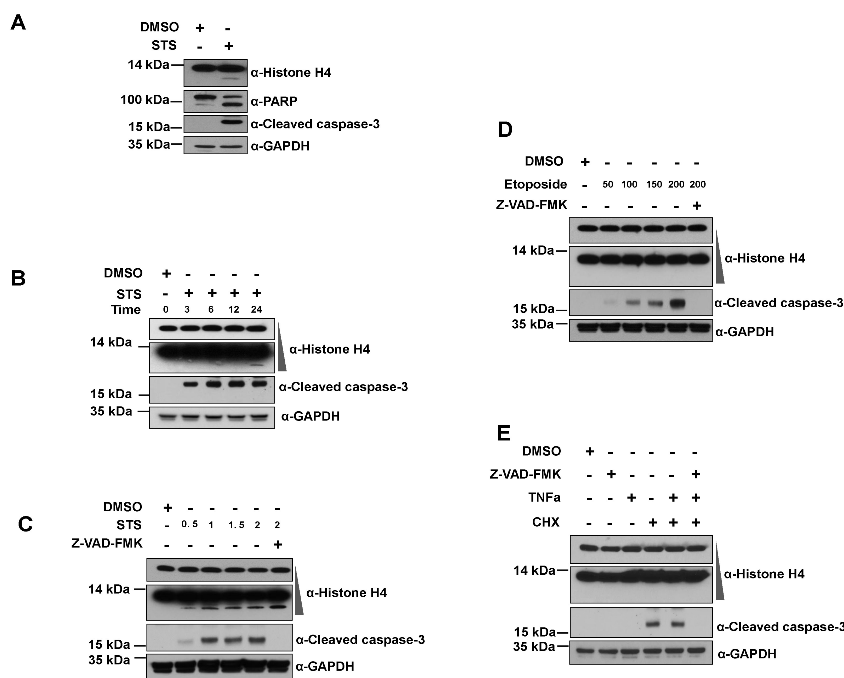

Fig. 1. Cleaving of histone H4 is during staurosporine-induced cell death in Raji cells. (A) Raji cells were treated with 1 mM staurosporine (STS) and at 24 h, histone H4 was examined by immunoblot analysis using the indicated core histone antibodies and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Histone H4 fragments were generated in response to a 24-h treatment with STS. Fragments were not observed after 3 and 6 h of treatment. (C) Cells were treated with 0.5, 1, 1.5, or 2 mM STS and 100 mM Z-VAD-FMK for 24 h. (D) Raji cell were treated with etoposide at 50, 100, 150, or 200 mM and 100 mM Z-VAD-FMK for 24 h. (E) Raji cells were treated with other inducers of cell death, TNF-α (50 ng) and cycloheximide (10 mg). Histone H4 fragments were examined by immunoblot analysis using the indicated antibodies. Poly ADP-ribose polymerase (PARP) and cleaved caspase-3 antibodies were used as a positive controls for cell death.

caspase inhibitors (19). These results indicated that STS-treated Raji cells might undergo necroptosis instead of apoptosis in the presence of caspase inhibitors.

Histone H4 was cleaved by GzmA

To identify the protease that cleaves histone H4, we treated cells with STS and various chemical protease inhibitors. STS- and STS/Z-VAD-treated cells clearly showed the presence of the histone H4 cleaved form; however, histone H4 cleavage was completely inhibited by the presence of nafamostat mesylate (NFM). At the same time, normal progress of cell death was verified throughout cleaved caspase-3 and PARP (Fig. 3A, B). Caspase inhibitors are shown to have differential effects on the extent of cell death induced by different inducers (20, 21). NFM is a highly effective tryptase inhibitor that has the ability to inhibit GzmA (22). To test whether GzmA cleaved histone H4 in STS-treated cells, we performed GzmA knockdown experiment by electroporating GzmA siRNA 24 h after STS treatment. One of the tested siRNAs clearly showed that knockdown of GzmA inhibited the cleavage of histone H4 (Fig. 3C, D).

In vitro histone H4 cleavage

We showed that STS-exposed Raji cells induced histone H4 cleavage and cell death. To confirm these results, we examined whether recombinant histone H4 monomers were cleaved by purified GzmA *in vitro*. Purified GzmA was incubated with histone H4 monomers and was found to increase histone H4 cleavage in a dose-dependent manner (Fig. 4A). We examined whether acetylation of histone H4 affected its cleavage. Recombinant histone H4 was treated with trichostatin A, an inhibitor of histone deacetylation. Only untreated histones were digested by GzmA (Fig. 4B, C).

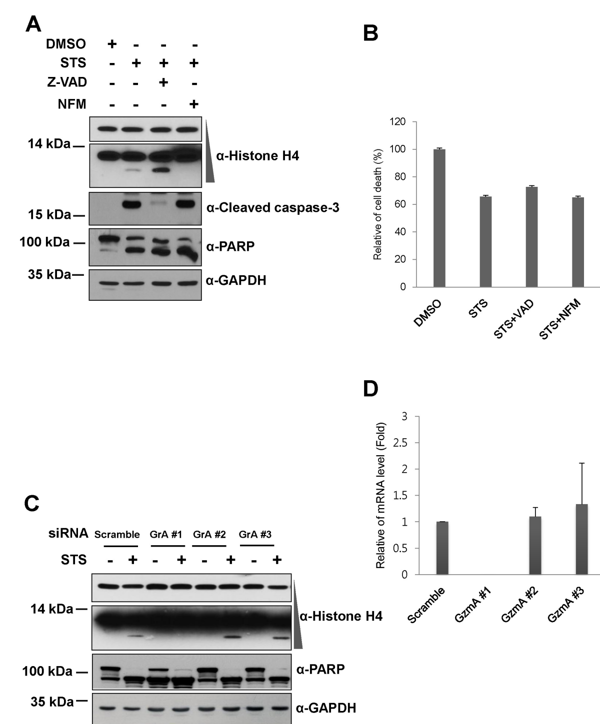

Fig. 3. Suppression of histone H4 cleavage by nafamostat mesylate. (A) Raji cells were treated STS and nafamostat mesylate (NFM; 50 μM) and then incubated for 24 h. Cells were treated with dimethyl sulfoxide (DMSO), 1 μM STS, STS + 100 μM Z-VAD-FMK, or STS + 50 μM NFM. Whole cell lysates were then separated with western blot by using antibodies for histone H4, cleaved caspase-3, and GAPDH. (B) Cell death analyses were performed using the Cell Counting Kit-8 assay, (C) Raji cells were electroporated with three different Granzyme A (GzmA) siRNAs or scrambled siRNA and were then examined by western blot using the indicated antibodies. (D) Quantification of Granzyme A mRNA levels by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

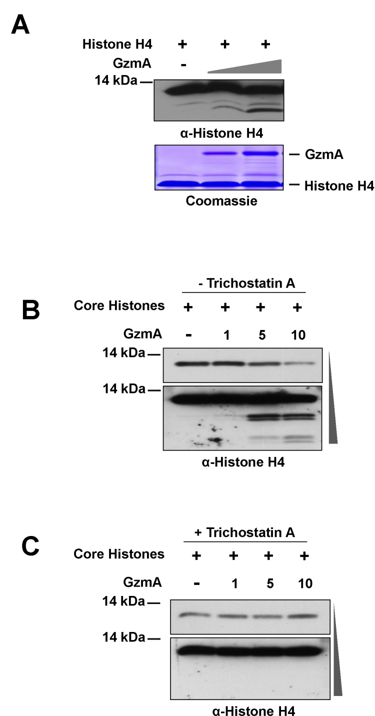

Fig. 4. Cleaving of histone H4 by granzyme A *in vitro*. (A) *In vitro* cleavage assay. Histone H4 was incubated with recombinant GzmA (10 μM) for 2 h at 37°C. Histone H4 protein cleavage was detected by western blot using a histone H4 antibody. Coomassie staining showed histone H4 and GzmA input. (B, C) Cleavage assay using core histones isolated from HeLa cell nuclei. Purified normal or acetylated core histones were incubated in the absence or presence of 1, 5, or 10 μM recombinant GzmA for 2 h at 37°C. The reaction products were separated using an 18% Tris-tricine gel and analyzed by western blot using a histone H4 antibody.

DISCUSSION

Histone proteins are known to package and organize the DNA into structural units called nucleosomes. Here, we showed that histone H4 is cleaved by GzmA during STS-induced cell death. We also showed that histone H4 cleavage was inhibited by the GzmA inhibitor. STS-treated cells contained a cleaved fragment of histone H4 and the amount of this cleavage product increased in cells treated with caspase inhibitors. The protease responsible for histone H4 cleavage was identified as GzmA, not effector caspases. These data suggested that GzmA-mediated histone H4 digestion not only occurred during apoptosis, but also under caspase-compromised conditions in STS-treated cells. STS-induced apoptosis in U937 cells reportedly occurs by secondary necrosis, while STS induces primary necrosis under caspase-compromised conditions. Necrosis in these cells is partially inhibited by necrostatin-1 and geldanamycin, two drugs that effect receptor-interacting serine/threonine-protein kinase 1 activity (19).

The SET complex is normally located in the endoplasmic reticulum, but it translocates to the nucleus in response to reactive oxygen species produced by GzmA-mediated cleavage of NADH dehydrogenase (ubiquinone) Fe-S protein 3. GzmA is mobilized in the nucleus where many of its known substrates reside. In the nucleus, GzmA digests three components of the SET complex: SET, high-mobility group protein B2, and apurinic/apyrimidinic endonuclease. SET is an inhibitor of the SET complex endonuclease NM23-H1. SET cleavage activates NM23-H1 to generate single-stranded DNA nicks. These nicks are then extended by the SET complex exonuclease three prime repair exonuclease 1. GzmA degrades the linker histone H1 and removes the tails from core histones H2 and H3, opening up the chromatin and making it accessible to nucleases (23). In normal cells, G9a histone methyltransferase and histone deacetylase 1 (HDAC1) repress p53 target genes by maintaining H3K9 methylation and histone deacetylation. Upon DNA damage, p53 target genes are activated by the competitive action of histone acetyltransferase towards G9a and HDAC1. The nuclear delivery of acetylated H4 tail peptides interferes with G9a and HDAC1 activities, leading to elevated histone acetylation and increased transcriptional activity of p53 target genes (24). Therefore, we speculate that cleavage of the histone H4 tail by GzmA removes the docking site of proteins, accelerating the disintegration of nucleosomes and digestion of DNA.

In this paper, we report for the first time that histone H4 was cleaved by GzmA under conditions of apoptosis. The amount of the cleaved histone H4 fragment was increased by the caspase inhibitor in a dose-dependent manner. We speculate that the site of cleavage was located on the histone H4 tail. The cleavage of the histone H4 tail by GzmA would contribute to the disintegration of chromosomes during the cell death process. Further studies are warranted to identify other GzmA substrates during the cell death process.

MATERIALS AND METHODS

Cell culture

HeLa and HEK 293-T cells were obtained from the American Type Culture Collection and grown in Dulbecco's Modified Essential Medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO_2 in a humidified incubator. Raji and K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO_2 in a humidified incubator.

Reagents and antibodies

Staurosporine, etoposide, phenylmethanesulfonyl fluoride (PMSF), calpain inhibitor, tumor necrosis factor (TNF)-α, cycloheximide, and enterokinase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Z-VAD-FMK, Z-DEVD-FMK, Z-VEID-FMK, Z-VDVAD-FMK, Z-IETD-FMK, and Z-LEHD-FMK were purchased from R&D systems (Minneapolis, MN, USA). Cathepsin inhibitor I and Omi/HtrA2 inhibitor were purchased from Millipore (Billerica, MA, USA). 3,4-Dichloroisocoumarin and nafamostat mesylate (NFM) were purchased from Santa Cruz (Santa Cruz, CA, USA). Bio-Gel HTP hydroxyapatite was purchased from Bio-Rad (Hercules, CA, USA). Anti-histone H4 and histone H4 modification antibodies were purchased from Abcam (Cambridge, MA, USA). Caspase-3, -8, -9, and poly ADP-ribose polymerase (PARP) antibodies were purchased from Cell Signaling (Danvers, MA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz.

Bacterial expression and purification of recombinant GzmA

Cells were grown in Luria-Bertani media containing 50 mg/ml ampicillin at 37°C. The cells were induced using 1 mM isopropyl β-D-1-thiogalactopyranoside and expression was allowed to proceed for overnight at 18°C. To purify His-fusion recombinant GzmA, cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, and 1 % NP-40) (25) and then purified using Ni-NTA chelating agarose according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). The activity of the purified protein was confirmed using a known substrate protein.

GzmA silencing by RNA interference

We prepared siRNA duplexes from synthetic RNAs to target GzmA (Bioneer, Daejeon, Korea) and numbered them in order from the 5'-end of the coding sequence as follows: siRNA #1 (sense: 5'-GUG UUG ACU GCA GCU CAC U-3'; antisense: 5'-AGU GAG CUG CAG UCA ACA C-3'), siRNA #2 (sense: 5'-GUU UCC CUA UCC AUG CUA U-3'; antisense: 5'-AUA GCA UGG AUA GGG AAA C-3'), and siRNA #3 (sense: 5'-CCU CUC UCU CAG UUG UCG U-3'; antisense: 5'-ACG ACA ACU GAG AGA GAG G-3'). Control siRNAs were as follows: sense: 5'-CCU ACG CCA CCA AUU UCG-3' and

antisense: 5'-ACG AAA UUG GUG GCG UAG G-3'. Raji cells (8×10^6/dish) were plated into 10 cm plates and transfected with siRNA (50 nM final concentration) using the Neon Transfection System (optimized protocol #16; Invitrogen, Carlsbad, CA, USA).

Flow cytometric analysis of cell death in annexin V-FITC and PI double-labeled cells

The assay was performed according to the manufacturer's instructions (BD Bioscience, San Jose, CA, USA). Cells (1×10^6 cells/ml) were washed twice with cold phosphate-buffered saline (PBS) and then resuspended in 1× binding buffer. One hundred microliters of the suspension (1×10^5 cells) were transferred to a 5-ml tube, and 5 μg/ml of propidium iodide (PI) and 500 μl of annexin V-FITC solution was added to the wells. Cells were then incubated for 15 min at room temperature (25°C) in the dark. Next, 400 μl of 1× binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) were added. Data were presented as the percentage of cells contained within the marked regions of the images. Data collection was achieved by using FACSCaliber flow cytometer (Becton Dickinson).

Purification of core histones

Nuclei under normal and acetylated conditions were isolated from HeLa cells (26). HeLa cells (200 ml at 1×10^6 cell/ml) were harvest and washed with cold PBS. Pellets resuspended in 20 pellet volumes of lysis buffer (20 mM HEPES, pH 7.5, 0.25 M sucrose, 3 mM $MgCl_2$, 0.5 % NP-40, 3 mM 2-mercaptoethanol, 0.4 mM PMSF, 1 μM pepstatin, and 1 μM leupeptin) were transferred to a Dounce homogenizer and cells were lysed. After centrifugation, nuclear pellets were resuspended in HAP buffer (50 mM sodium phosphate, pH 6.8, 0.6 M NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.5 mM PMSF) and were stirred gently. Dry BioGel HTP powder was added to the nuclear pellet suspensions, which were then poured into 2 × 15 cm open columns. The columns were washed with 10 volumes of HAP buffer and the core histones were eluted with HAP buffer containing 2.5 M NaCl. Protein concentrations were determined by measuring absorbance values at A_{230} or A_{280} and the purities of the core histones were assessed using 15% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

Cell viability assay

Cell viability in the presence of staurosporine and caspase inhibitors was determined using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories). Briefly, cells cultured in a 96-well plate were treated with the inhibitors for 24 h. After treatment, 10 μl of CCK-8 assay solution was added and the cultures were incubated for 1 h. Absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay reader. Percent viability was calculated relative to the dimethyl sulfoxide control (100% viable).

Immunoblot analysis

Generally, 24-48 h after treatment with staurosporine and several other chemicals, Raji cells were harvested, washed in PBS, and lysed in 1× tricine sample buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 20 mM DTT, 4% SDS, and 5% glycerol). Proteins separated on Tris-tricine gels were transferred to nitrocellulose membrane and immunoblotted using antibodies according to the manufacturer's recommended instructions. Primary antibodies were detected with either anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase.

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