Histone H3 is Digested by Granzyme A During Compromised Cell Death in the Raji Cells

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Granzyme A (GzmA) was identified as a cytotoxic T lymphocyte protease protein expressed in the nucleus. A number of nuclear proteins are well known as GzmA substrates, and GzmA is related with caspase-independent apoptosis. Histones H1, H2B, and H3 were identified as GzmA substrates through in vitro experiment with purified nucleosome. Here, we demonstrated that histone H3 was cleaved by GzmA in vivo during staurosporine-induced cell death. Moreover, histone H3 cleavage was blocked by the GzmA inhibitor nafamostat mesylate and by GzmA knockdown using siRNA. Taken together, we verified that histone H3 is a real substrate for GzmA in vivo in the Raji cells treated by staurosporin.

Keywords: Caspase-independent cell death, histone H3, granzyme A

Apoptosis is a major 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 [8]. The dramatic biochemical and morphological changes occur in apoptotic cells both outside and inside of the nucleus. The fundamental apoptotic characters are chromatin condensation and DNA fragmentation. Apoptotic cell death is executed by caspases. Through cleavage of important cellular protein substrates, caspase activity eventually induces typical apoptotic processes such as membrane blebbing and DNA fragmentation. Apoptotic DNA fragmentation is executed by caspase-activated DNase and endonuclease G [1]. Histone proteins are known to release from nucleosomes during apoptosis [13].

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 [9]. Therefore, histones influence the genetic potential of DNA [7] and the post-translational modification of histones influences apoptotic cell death [6]. Previously, histones H1, H2B, and H3 were found to be cleaved by granzyme A (GzmA) in an in vitro experiment with purified nucleosome [5].

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 [4, 12]. In a recent study, proteolytic processing of histones was shown to effect cellular
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Histone H3 cleavage occurs during mouse embryonic stem cell differentiation [14], histone H3 tail clipping regulates gene expression, and extracellular histones are major mediators of cell death during sepsis [2, 3]. Therefore, the proteolytic processing of histones is important for cell physiology.

In the present study, we demonstrated that the histone H3 N-terminal tail was cleaved by GzmA in staurosporine (STS)-induced Raji cell. While attempting to elucidate the function of histone proteins in cell death, we observed a fast-migrating histone H3 band in the lysates of cells undergoing STS-induced cell death, but analogous bands were not detected for histones H2A and H2B (Fig. 1A). This histone H3 fragment was shown in cells 24 h after STS treatment (Fig. 1B). Because STS induces apoptosis through a caspase-dependent pathway, we examined whether histone H3 cleavage could be blocked by treating the cells with the pan-caspase inhibitor Z-VAD-FMK [3]. The pan-caspase inhibitor could not prevent histone H3 cleavage. Moreover, the increase of the histone H3 fragment was not dependent on the dose of STS used (Fig. 1C). We next checked for histone H3 cleaved fragments in the presence of other inducers of cell death, TNF-α (50 ng) and cycloheximide (10 μg). Histone H3 fragments were examined by immunoblot analysis using the indicated antibodies. Poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 antibodies were used as positive controls for cell death.
LEHD-FMK) (Fig. 2A). The death of cells treated with these caspase inhibitors also increased to a greater extent than STS treatment alone (Fig. 2B). Recently, STS was known to induce necroptotic cell death in the treatment of caspase inhibitors [3]. These results supposed that STS-treated Raji cells might undergo necroptosis instead of apoptosis in the presence of caspase inhibitors. To identify the protease that cleaves histone H3, we treated cells with STS and various chemical protease inhibitors. STS- and STS/Z-VAD-treated cells clearly showed the presence of the histone H3 cleaved form; however, histone H3 cleavage was inhibited by the treatment of nafamostat (NFM). Normal progress of cell death was verified throughout cleaved caspase-3 and PARP (Fig. 3A). NFM is a highly effective tryptase inhibitor that has the ability to inhibit GzmA [11]. To test whether GzmA cleaved histone H3 in STS-treated cells, we performed a GzmA knockdown experiment by electroporating GzmA siRNA (siRNA #1 sense: 5'-CCU CUC UCU CAG UUG UCGU-3'; antisense: 5'-ACG ACA ACU GAG AGA GAG G-3'), 24 h after STS treatment (Bioneer, Korea). One of the tested siRNA #1 clearly showed that knockdown of GzmA inhibited the cleavage of histone H3 (Figs. 3B and 3C). We showed that STS-exposed Raji cells induced histone H3 cleavage and cell death. To confirm these results in vitro,
we examined whether recombinant histone H3 monomers were cleaved by purified GzmA in vitro. Recombinant GzmA and the inactive mutant his-tagged at the C-terminal were expressed in the E. coli BL21 DE3 strain (Stratagene). The inactive mutant of GzmA SA was generated by mutation of the active site serine (S184) to alanine. Purified GzmA was incubated with recombinant histone H3 monomers and was found to increase histone H3 cleavage in a dose-dependent manner (Fig. 4).

In this study, we report that histone H3 was cleaved by GzmA under conditions of STS-induced cell death. The amount of the cleaved histone H3 fragment was increased by the caspase inhibitor in a dose-dependent manner. We

**Fig. 3.** Suppression of histone H3 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-VAL-FMK, or STS + 50 µM NFM. Whole-cell lysates were then separated with western blotting by using antibodies for histone H3, cleaved caspase-3, and GAPDH (The level of cleaved fragment was quantified by ImageJ software. H3 (top) was used as a loading control to normalize the data and lane 1 as a zero.). (B) Raji cells were electroporated with three different Granzyme A (GzmA) siRNAs or scrambled siRNA and were then examined by western blot assay using the indicated antibodies.
(C) Quantification of GzmA mRNA levels by quantitative reverse transcription polymerase chain reaction.

**Fig. 4.** Cleaving of histone H3 by GzmA in vitro.
(A, B) In vitro cleavage assay. Histone H3 was incubated with recombinant GzmA (5–10 µM) and GzmA inactive mutant (10 µM) for 2 h at 37°C. Histone H3 protein cleavage was detected by western blot assay using a histone H3 antibody. Histone H3 cleavage was not detected in the GzmA inactive mutant. Coomassie staining showed histone H3 and GzmA input. The bottom figure shows recombinant GzmA.
speculate that the site of cleavage was located on the histone H3 N-terminal tail. The cleavage of the histone H3 N-terminal tail by GzmA would contribute to the disintegration of chromosomes during the cell death process.

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References