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DR396, an apoptotic DNase γ inhibitor, attenuates high mobility group box 1 release from apoptotic cells

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ABSTRACT

High mobility group box1 (HMGB1) is a non-histone chromatin chromosomal protein playing an important role in chromatin architecture and transcriptional regulation. Recently, HMGB1 has been shown to be secreted into extracellular milieu in necrosis and apoptosis, and involved in inflammatory responses. However, the mechanism by which apoptotic cells release HMGB1 is unclear. In this study, to investigate the mechanism of HMGB1 release, we searched inhibitors of HMGB1 release from apoptotic cells. As a result, three compounds, 4-(4,6-dichloro-[1,3,5]-triazin-2-ylamino)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid (DR396), Pontacyl Violet 6R (PV6R), and Fmoc-D-Cha-OH (FDCO) in our in-house chemical library were found to inhibit HMGB1 release from staurosporine (STS)-induced apoptotic HeLa S3 cells. Interestingly, these three compounds have been previously categorized into apoptotic DNase γ inhibitors. Therefore, we examined whether apoptotic nucleosomal DNA fragmentation is involved in the release of HMGB1 during apoptosis. Expectedly, DR396, which is the most potent and specific inhibitor of DNase γ, was found to almost completely inhibit both HMGB1 release and internucleosomal DNA cleavage in HeLa S3 cells transfected with DNase γ expression vector and stably expressing DNase γ (HeLa S3/γ cells). These results clearly suggest that nucleosomal DNA fragmentation catalyzed by DNase γ is critical in the release of HMGB1 from apoptotic cells.

1. Introduction

High mobility group box 1 (HMGB1) is a well-known non-histone chromosomal protein that binds into the minor groove of DNA and plays important roles in the modulation of chromatin structure, and thereby regulates DNA transaction, such as transcription, replication, and repair in various cellular processes during cell growth and differentiation.[1–7] In addition to its role in the nucleus, HMGB1 can be released from necrotic and apoptotic cells into the extracellular space. HMGB1 is also secreted by monocytes and macrophages in the late stage of inflammation and response to various stimuli including lipopolysaccharide and tumor necrosis factor-α (TNF-α).[8,9] The released HMGB1 acts as a inflammatory ligand through interactions with receptor for advanced glycation end-products (RAGE) and toll-like receptors (TLR)-2 and -4, and activates inflammation responses.[10,11]

Recently, HMGB1 has been reported to be elevated in the serum of patients with not only sepsis but also cerebral and myocardial ischemia, rheumatoid arthritis, and diabetes.[12–17] HMGB1 is also involved in the pathogenesis of disorders of lung, liver and gut, and tissue transplantation.[14,18,19,20] Furthermore, HMGB1 elicits proinflammatory responses in endothelial cells by increasing the expression of vascular adhesion molecules as well as secretion of TNF-α and chemokines.[21] Therefore, inhibitors of HMGB1 release may provide a novel therapeutic strategy for treatment of many inflammatory diseases. Also, the identification of novel inhibitors for HMGB1 release could provide an important clue for elucidation of the molecular mechanism by which HMGB1 is released from apoptotic cells.

In this study, we screened inhibitory compounds of HMGB1 release from our in-house chemical library using staurosporine (STS)-induced apoptotic HeLa S3 cell system. We show here for the first time that 4-(4,6-dichloro-[1,3,5]-triazin-2-ylamino)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid (DR396), which has been already identified as a specific and potent inhibitor of an apoptotic DNA endonuclease, DNase γ,[22] almost completely attenuates HMGB1 release from apoptotic HeLa S3 cells. Furthermore, close association of the suppressive effect of DR396 on HMGB1 release with the inhibition of apoptotic nucleosomal cleavage of DNA was clearly shown in STS-induced apoptotic HeLa S3 cells transfected with DNase γ expression vector and stably expressing DNase γ (HeLa S3/γ cells). Taken together, these results suggest that nucleosomal DNA fragmentation catalyzed by DNase γ is critical for the release of HMGB1 from apoptotic cells. Thus, it might be expected that DNase γ inhibitors like DR396 become

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novel lead compounds for development of new pharmaceuticals for HMGB1 release-related inflammatory diseases, such as sepsis and cerebral ischemia.

2. Results

2.1. Discovery of inhibitors of HMGB1 release from apoptotic cells

HeLa S3 cells are known to be induced apoptotic cell death by STS-treatment ranging from 0.3 to 3 μM.[22] In this conditions, the release of HMGB1 occurred in the late stage of apoptosis as judged by the restriction cleavage of poly(ADP-ribose) polymerase (PARP), which is the characteristic of apoptosis (Fig. 1). Using this apoptotic cell system, we searched inhibitors of HMGB1 release in our in-house chemical library. HeLa S3 cells were pretreated with 100 μM of each screening compound for 1 h, and treated with 0.5 μM of STS for 24 h. As a result, three compounds, 4-(4,6-dichloro-[1,3,5]-triazin-2-ylamino)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid (DR396), Pontacyl Violet 6R (PV6R), and Fmoc-D-Cha-OH (FDCO) (Fig. 2A), were found to inhibit the release of HMGB1 (Fig. 2B). DR396 almost completely suppressed the STS-induced apoptotic HMGB1 release. On the other hand, PV6R and FDCO were inhibited approximately 50% and 20% HMGB1 release, respectively.

2.2. Novel HMGB1 release inhibitors inhibit DNase γ activity in vitro

Interestingly, the three compounds screened belong to the category of inhibitors of DNA endonucleases, especially apoptotic DNase γ, in our chemical library. So, we compared the degrees of DNase γ inhibitory activities of these three compounds using in vitro DNase γ assay system. As shown in Fig. 3, all three compounds inhibited recombinant human DNase γ (rhDNase γ) activity in a dose-dependent manner. Among them, DR396 showed the most effective inhibition on rhDNase γ with the IC_{50} value of 3.2 μM. On the other hand, the IC_{50} values of PV6R and FDCO were 16.1 and 34.5 μM, respectively. It is noted that the degrees of their inhibitory effects on DNase γ activity showed a good correlation with those of their attenuation of HMGB1 release from apoptotic HeLa S3 cells. These results indicate that nucleosomal DNA fragmentation catalyzed by DNase γ during apoptosis plays an important role in the release of HMGB1 into extracellular milieu.

2.3. HMGB1 release is triggered by nucleosomal DNA fragmentation catalyzed by DNase γ in apoptotic cells

To confirm the above idea, we used HeLa S3 cells transfected with DNase γ expression vector and stably expressing DNase γ, named HeLa S3/γ cells, because expression levels of endogenous DNase γ and other DNases, such as DNase I family endonucleases, DNase II, and CAD, are quite low in parental HeLa S3 cells.[23] Using HeLa S3/γ cells, we investigated the effect of the most potent DNase γ-inhibitor, DR396, on both nucleosomal DNA fragmentation and HMGB1 release after STS-treatment. As shown in Figure 4A, DR396 was able to inhibit nucleosomal DNA fragmentation in a dose-dependent manner. At 30 μM DR396, approximate 50% inhibition of nucleosomal DNA fragmentation was observed. As expected, DR396 also clearly inhibited the release of HMGB1 in the same dose-dependent manner; the IC_{50} value was approximately 30 μM (Fig. 4B). Thus, the degrees of the inhibition of nucleosomal DNA fragmentation and HMGB1 secretion into the culture medium by DR396 are almost the same. These results strongly suggest the positive correlation of nucleosomal DNA fragmentation catalyzed by DNase γ with HMGB1 release in apoptosis.

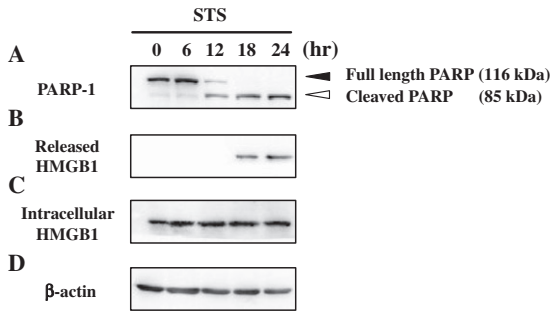

Figure 1. The release of HMGB1 from STS-induced apoptotic HeLa S3 cells. HeLa S3 cells were treated with 0.5 μM STS for the indicated times. The cleavage of PARP-1 (A), released HMGB1 (B), intracellular HMGB1 (C), and β-actin for loading control (D) were analyzed by Western blot as described under 'Section 4'. Closed and open arrowheads indicate the intact PARP-1 (116 kDa) and its cleaved fragment (85 kDa), respectively.

3. Discussion

HMGB1 is a member of HMG family which is expressed in the nucleus of eukaryotic cells and plays an important role in chromatin architecture and transcriptional regulation.[1–7,24–26] Recently, HMGB1 has been found to be implicated as a inflammation mediator.[27–31] HMGB1 is released passively during necrosis and actively secreted during apoptosis. However, there is little information how HMGB1 release extracellular milieu during apoptosis. It is possible that identification of inhibitors for HMGB1 release allow us to elucidate the molecular mechanism and physiological significance of HMGB1 release into extracellular space. Thus, in this study, we searched inhibitors of HMGB1 release in our in-house chemical library using apoptotic cellular system.

Interestingly, among the various categories of our chemical library tested, only DNase γ inhibitors, DR396, PV6R, and FDCO, were found to suppress HMGB1 release during apoptosis. It is noteworthy that there is a good correlation of the degree of suppression effects on HMGB1 release (Fig. 2B) with that of the inhibitory activities for DNase γ activity in vitro (Fig. 3). The inhibitory effect of DR396 was greater than that of PV6R and FDCO, suggesting the tight interaction of DR396 with DNase γ. Although the differences in the mechanisms of inhibition are not yet clear, the stereochemical compositions of functional groups may be important for interaction with DNase γ molecule.

This is the first report that DNase γ inhibitors are able to suppress apoptosis-mediated HMGB1 release. Especially, DR396, a potent and specific inhibitor of DNase γ [22] (Fig. 3) almost completely attenuated HMGB1 release during STS-induced apoptosis in HeLa S3 cells (Fig. 2B). These observations suggest that nucleosomal DNA fragmentation catalyzed by DNase γ is involved in the HMGB1 release. To assess the HMGB1 release triggered by DNase γ-mediated apoptotic DNA fragmentation, we used DNase γ stably expressing HeLa S3/γ cells. As a result, the close association of inhibition of apoptotic nucleosomal DNA fragmentation with suppression of HMGB1 release was clearly observed (Fig. 4). Taken together, these results suggest that nucleosomal DNA fragmentation catalyzed by DNase γ plays a pivotal role in HMGB1 release. Furthermore, these date indicate that nucleosomal DNA fragmentation serves as an important initiator of HMGB1 transport from nucleus to cytoplasm and ultimately to extracellular milieu (Fig. 5). The novel inhibitors of DNase γ like DR396 will, therefore, be useful in studies on the molecular mechanism and physiological significance of nucleosomal DNA fragmentation in apoptosis. Since HMGB1 is involved in the pathogenesis of many inflammatory diseases,[12–20] suppression of HMGB1 release by DNase γ inhibitors may be a potential therapeutic strategy for the treatment of diseases, such as sepsis and cerebral and myocardial ischemia. Thus,

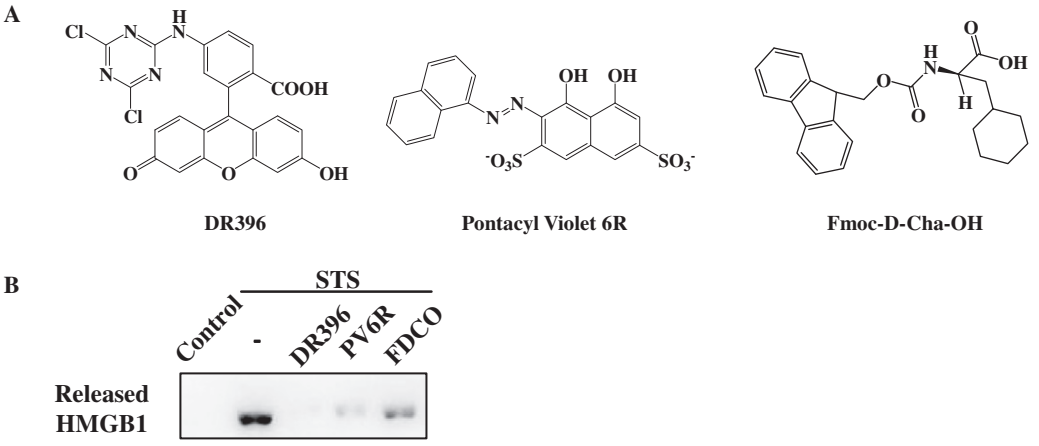

Figure 2. Chemical structures of DR396, PV6R and FDCO (A). Effects of DR396, PV6R and FDCO on the release of HMGB1 in apoptotic HeLa S3 cells. HeLa S3 cells (3×10^5) were pretreated with 100 μM of DR396, PV6R and FDCO for 1 h and treated with 0.5 μM STS for 24 h (B). Cell lysate was prepared as described under 'Section 4'. The released and remaining cellular HMGB1 were analyzed by Western blot as described under 'Section 4'.

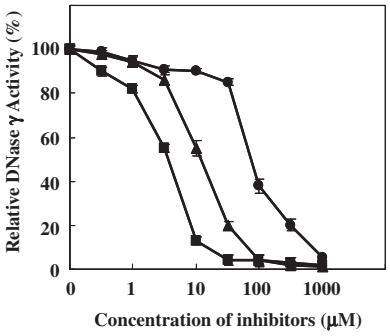

Figure 3. Inhibitory effects of HMGB1 release inhibitors on DNase γ activity. The dose-dependencies of HMGB1 release inhibitors, DR396 (■), PV6R (▲), and FDCO (●), on DNase γ activity were measured by in vitro DNase γ assay as described under 'Section 4'. Values are averages of three independent experiments and shown with standard deviations.

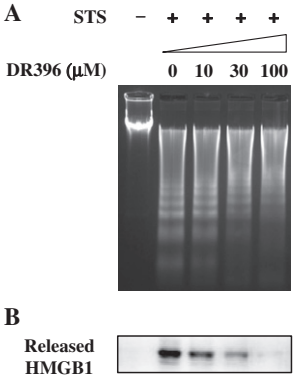

Figure 4. Effects of DR396 on nucleosomal DNA fragmentation and HMGB1 release in apoptotis-induced HeLa S3/γ cells. HeLa S3/γ cells (3×10^5) were pretreated with 10, 30 and 100 μM of DR396 for 1 h and treated with 0.5 μM STS for 24 h. Cell lysate was prepared as described under 'Section 4'. DNA extracted from the cells was analyzed by 1.8% agarose gel electrophoresis as described under 'Section 4' (A). The released HMGB1 was analyzed by Western blot as described under 'Section 4' (B).

it might be expected that DNase γ inhibitors become novel lead compounds for development of new pharmaceuticals for HMGB 1 release-related disorders.

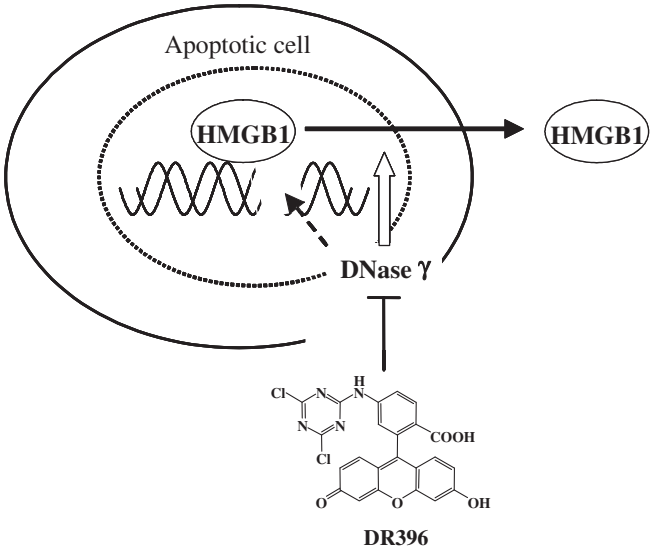

Figure 5. The link of HMGB1 release and nucleosomal DNA fragmentation in apoptosis. DR396 suppresses HMGB1 release via inhibition of nucleosomal DNA fragmentation catalyzed by DNase γ during apoptosis.

4. Experimental

4.1. Materials

4-(4,6-Dichloro-[1,3,5]-triazin-2-ylamino)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid (DR396) and Fmoc-D-Cha-OH were purchased from Sigma–Aldrich Library of Rare Chemicals. Staurosporine (STS) and mouse anti-PARP antibody were Wako Pure Chemical Industries, Ltd. Pontacyl Violet 6R was parched from Tokyo Kasei Kogyo Co., Ltd. Monoclonal anti-HMGB1 antibody was purchased from R&D Systems.

4.2. Cell culture

HeLa S3 and HeLa S3/γ cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin as described previously.[22,23]

4.3. Establishment of HeLa S3/γ cells

HeLa S3/γ cells were established as described previously.[22,23] The DNase γ expression vector, pOPRSVr γ, was transfected to HeLa S3 cells by using lipofection. Stable HeLa S3 transfectants (HeLa S3/γ cells) were selected by adding G418 to the culture medium.

4.4. Detection of DNA fragmentation

Cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.8), 10 mM EDTA, and 0.5% (w/v) sodium *N*-lauroylsarcosinate] and incubated with 0.5 mg/ml proteinase K overnight at 50 °C. Then 0.5 mg/ml RNase A was added, and the lysates were incubated for 30 min at 50 °C. DNA thus prepared was subjected to 1.8% agarose gel electrophoresis. The DNA was visualized by UV illumination after ethidium bromide staining.[22,23]

4.5. Western blot analysis

Harvested cell samples were lysed in SDS sample buffer [50 mM Tris–HCl (pH 6.8), 2% SDS, 5% glycerol, 1% 2-mercaptoethanol, and 0.1% bromophenol blue] and boiled for 5 min. The culture medium was collected by centrifugation at 2500g for 5 min and added 30 μl of 50% slurry heparin Sepharose 6 Fast Flow (GE Healthcare). After rotated for 1 h at 4 °C, supernatants were discarded and 1× SDS sample buffer was added to the beads pellet and boiled for 5 min. Protein samples thus obtained were separated by 12% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 2% skim milk and 0.25% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, and then probed with appropriate primary antibodies for overnight at 4 °C or for 1 h at room temperature. After the membranes were washed with TBS-T and incubated with the appropriate secondary antibody for 1 h at room temperature. After washing the membranes with TBS-T, the blotted proteins were visualized by Light Capture System (ATTO) using Immunostar LS (Wako).[22,23]

4.6. Preparation of human recombinant DNase γ

The cDNA fragment encoding the mature DNase γ protein was obtained by PCR using 5′-ATATAT**TCATGA**GAATCTGCTCCTTCAACG-3′ (sense) and 5′-TATAT**GGATCC**CTAAGTGACAGATTTTTTG-3′ (anti-sense) primers, with the BspHI (sense) and BamHI (anti-sense) sites flanking the coding sequences shown in bold letters. After the fragments were excised by BspHI and BamHI digestion, they were subcloned into the NcoI/BamHI sites of pET15b vector. The recombinant human DNase γ protein was expressed in Rosetta (DE3) cells, and refolded and purified from inclusion bodies. Briefly, inclusion bodies containing recombinant DNase γ were dissolved in buffer [40 mM Tris–HCl (pH 7.5), 10 mM Dithiothreitol] containing 8 M urea and then refolded by dilution to 0.08 M urea. The recombinant DNase γ was purified by affinity column chromatography on Heparin-5PW (Tosho) as described previously.[22]

4.7. Assay of DNase γ activity

DNase γ activity was assayed by measuring the increase in acid-soluble DNA that remains unprecipitated in the presence of 5% perchloric acid (PCA) as described previously.[22] Briefly, 0.8 Kunitz U/ml of DNase γ was incubated with inhibitor in the standard assay buffer [50 mM Mops–NaOH (pH 7.2), 3 mM $CaCl_2$, 3 mM $MgCl_2$, and 0.1 mg/ml BSA] at 37 °C for 30 min. Then, 0.5 mg/ml of double-stranded salmon testis DNA (dsDNA) was added, and the mixture was incubated at 37 °C. The reaction was terminated by the addition of 5% PCA and leaving the mixture on ice for 20 min. After centrifugation at 800g for 15 min at 4 °C, the absorbance of the supernatant at 260 nm was measured.

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