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MDC1 cleavage by caspase-3: a novel mechanism for inactivating the DNA damage response during apoptosis

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Abstract

Recently, we identified the “apoptotic ring”, containing phosphorylated histone H2AX (γ -H2AX), as an early chromatin modification during apoptosis. Because γ -H2AX initiates the DNA damage response (DDR), we tested whether the apoptotic H2AX response leads to the full recruitment of the DDR factors that normally coordinate DNA repair and cell cycle checkpoints. We show that the apoptotic H2AX response does not recruit the DDR factors because MDC1 (mediator of DNA damage checkpoint protein 1), which normally binds to γ -H2AX in response to DNA damage and amplifies the DDR, is cleaved by caspase-3. This cleavage separates the BRCT and FHA domains of MDC1 and constitutes a novel mechanism for the inactivation of DNA repair in apoptotic cells. Also, we show that downregulation of MDC1 increases the apoptotic response to TRAIL. Together, these results implicate MDC1 in the cellular apoptotic response.

Keywords

apoptosis; MDC1; caspase-3; H2AX; histone; DNA repair; TRAIL

Introduction

Apoptosis (programmed cell death) is required in multicellular organisms for organ formation, elimination of damaged or harmful cells and maintenance of tissue homeostasis. Disruption of the equilibrium between cell death and proliferation leads to tumor formation when cells divide faster than they die (1). Caspases, a family of cysteine proteases that cleave and inactivate a broad range of cellular proteins have been identified as the central effectors of apoptosis (2,3).

One promising approach for cancer therapy is the development of targeted agents that selectively kill tumor cells by activating caspases. One such agent is recombinant human Apo2L/TRAIL (Tumor necrosis factor [TNF]-Related Apoptosis-Inducing Ligand), a pro-apoptotic agonist that activates the two membrane death receptors (DR4 and DR5). Human endogenous TRAIL is expressed by immune cells as a 281 amino acid polypeptide involved in the innate immune response, autoimmunity and tumor immunosurveillance (4-6). Recombinant Apo2L/TRAIL is presently in phase II clinical trials against lymphomas and non-small cell lung cancers (7).

We recently identified the “apoptotic ring” as the nuclear peripheral staining of phosphorylated histone H2AX (γ -H2AX) (8,9). The γ -H2AX apoptotic ring forms in the

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early phase of the apoptotic response to a broad range of stimuli including TRAIL. γ -H2AX (histone H2AX phosphorylated on serine 139 by ATM, ATR and DNA-PK) is otherwise a fundamental chromatin response to DNA double-strand breaks (10), serving as a molecular platform for the docking of protein complexes to chromatin and the amplification the DNA damage response (DDR) and DNA repair (10,11). Direct binding of MDC1 (mediator of DNA damage checkpoint protein 1) to γ -H2AX *via* the MDC1 BRCT (breast cancer C-terminal) domain (12) is essential for DDR because it leads to the subsequent recruitment and assembly of DDR protein complexes including 53BP1 that repair DNA and regulate cell cycle checkpoints (13,14). Recruitment of MDC1 at DNA damage sites is critical for the efficient activation of the intra S-phase checkpoint (15). MDC1 couples DNA double-strand break recognition by Nbs1 (a member of the MRN complex containing Mre11, Rad50 and Nbs1) with its H2AX-dependent chromatin retention (16). Accordingly, MDC1^{-/-} mice recapitulate the phenotype of the H2AX^{-/-} mice, including growth retardation, male infertility, immune defects, chromosome instability, DNA repair defects, and radiation sensitivity (17).

The focus of the present study was to determine whether the apoptotic γ -H2AX response (apoptotic γ -H2AX ring) (8,9) is associated with the recruitment of the DDR complexes and the role of MDC1 and phosphorylation of H2AX at its C-terminal tyrosine 142 (18-20) in that process.

Materials and Methods

Chemicals

Recombinant human soluble TRAIL was obtained from Alexis (Axxora, San Diego, CA). Anti-Fas was purchased from Upstate (Temecula, CA). Camptothecin and cisplatin were from Sigma-Aldrich (St. Louis, MO). The broad-spectrum caspase inhibitor Z-VAD-fmk (z-Val-Ala-DL-Asp-fluoromethylketone) was from Bachem (Torrance, CA) and the proteasome inhibitor MG132 from Sigma-Aldrich.

Cell lines

Human colon carcinoma HCT116 and leukemic Jurkat cell lines were obtained from ATCC (Rockville, MD). Normal human prostate epithelial PrEC cell line was obtained from Lonza (Walkersville, MD).

Western blotting and antibodies

Cells were washed twice in PBS (Phosphate Buffered Saline) and lysed at 4°C in buffer containing 1% SDS (sodium dodecyl sulphate), 10 mM Tris-HCl pH 7.4, supplemented with protease inhibitors (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitors (Sigma-Aldrich). Viscosity of the samples was reduced by brief sonication. Equal amounts of proteins were boiled for 5 minutes in Tris-glycine-SDS sample buffer (Invitrogen, Carlsbad, CA) or heated at 70°C for 10 minutes in LDS (lithium dodecyl sulphate) sample buffer (Invitrogen), separated by Tris-glycine or Tris-acetate polyacrylamide gels (Invitrogen) and electroblotted onto nitrocellulose membranes (Biorad, Hercules, CA). The membranes were saturated with milk, incubated overnight at 4°C with primary antibodies, washed, then incubated for 45 minutes with secondary antibodies: peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were revealed by autoradiography using the Enhanced Chemiluminescence detection kit (Pierce, Rockford, IL).

Primary antibodies used were: anti-caspase-2 (551094, BD Biosciences, San Jose, CA), anti-pT²⁶⁰⁹-DNA-PK (ab18356, Abcam, Cambridge, MA), anti-GAPDH (2118, Cell Signaling,

Danvers, MA), anti- γ -H2AX (05-636, Upstate, Temecula, CA), anti-P^{Y142}-H2AX (07-1590, Millipore, Temecula, CA), anti-H2AX (07-627, Upstate), anti-MDC1 (ab11169, Abcam) and anti-tubulin (MS-581, Lab Vision, Fremont, CA).

Short interfering RNA (siRNA)

siRNA targeting MDC1 was obtained from Dharmacon, Chicago, IL (SMARTpool, catalogue number M-003506-04-0005). Negative control siRNA was obtained from Ambion, Austin, TX (catalogue number AM4635). Cells were seeded in 6-well plates, at a density of 50,000 cells per well 16 hours before transfection. For each sample, 500 pmoles of siRNA were mixed with 250 μ L Optimem (Invitrogen) (mix A). Five μ L lipofectamine 2000 (Invitrogen) were mixed with 250 μ L Optimem and incubated for 5 minutes at room temperature (mix B). After combining mixes A and B, reactions were further incubated for 20 minutes at room temperature before adding the siRNA/lipofectamine complexes in 2 mL culture medium. After 5 hours, the medium was replaced with regular medium and cells were incubated for a further 96 hours.

Plasmid vector

The GFP-MDC1 construct used has been previously described [T. Ozaki *et al.*, *DNA Cell Biol* **19**, 475 (Aug, 2000)].

Immunofluorescence microscopy

Cells were washed with PBS, fixed with 2% formaldehyde in PBS for 20 minutes, washed with PBS, postfixed and permeabilized with cold (-20°C) 70% ethanol for 20 minutes, washed with PBS, blocked with 8% bovine serum albumin (BSA) in PBS for 1 hour, washed with PBS, incubated with the first antibody (P^{T2609}-DNA-PK, 1/250 dilution; γ -H2AX, 1/500 dilution; P^{Y142}-H2AX, 1/250 dilution; MDC1, 1/200 dilution) in 1% BSA in PBS for 2 hours, washed with PBS, incubated with secondary antibody conjugated with Alexa Fluor 488 or 568 for 1 hour, washed with PBS and mounted by using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Confocal images were sequentially acquired with Zeiss AIM software on a Zeiss LSM 510 NLO Confocal system (Carl Zeiss Inc, Thornwood, NY).

Sub-G1 analysis

Cells were washed with PBS, fixed, and permeabilized with cold (-20°C) 70% ethanol overnight. The next day, cell pellets were washed again with PBS, resuspended in PBS buffer containing 0.2% NP40 and 0.5 mg/mL RNase A, incubated at room temperature for 15 minutes, and put on ice 10 minutes prior the addition of 50 μ g/mL of propidium iodide. DNA content was determined with a FACScan flow cytometer (Becton Dickinson) and quantified with CellQuest software (Becton Dickinson).

Site-directed mutagenesis

Point mutations were introduced into the MDC1 expression vector with the “QuickChange Lightning Site-Directed Mutagenesis kit” (Stratagene, La Jolla, CA) according to the manufacturer's instructions. MDC1 expression vector was used with the relevant oligonucleotides and PCR was performed for 18 cycles. Mutations were confirmed by sequencing. Primers used for mutagenesis were as listed below.

	Sequence (5' to 3')
D173A sense	ctcggaggaggagtagctttctttctgaaagc
D173A antisense	gccttcagaaagaaagctactctcctccgag

D219A sense	ccttcaattgaacagtgccacagatgtggaagaagg
D219A antisense	ccttctccacatctgtggcactgttcaattgaagg
D261A sense	gaaatccagcttgaaggctcagccttttagtgaaggag
D261A antisense	ctccttactaaaggctgagcctttcaagctggatttc
D683A sense	gtgggtgggaccaaggcctctgaagacaactat
D683A antisense	atagttgtcttcagaggccttggtccaccac
D686A sense	gaccaaggactctgaagccaactatggtgattctg
D686A antisense	cagaatcaccatagttggctcagagtccttggtc
D690-693-695A sense	gaagacaactatggtgttctgaagctctggccctacaagctaccag
D690-693-695A antisense	ctgggtagctttagggccagagcttcagaagcaccatagttgtcttc
D695A sense	tgattctgaagatctggccctacaagctaccagt
D695A antisense	actgggtagctttagggccagatcttcagaatca
D716A sense	ccagagcatggaggctgaacctaccagg
D716A antisense	cctgggtaggttcagcctccatgctctgg
D744A sense	acaacaggtagcctagctgaacctgggaggtc
D744A antisense	gacctccatggttcagctaggtacctgttgt
D762A sense	gagagagtctgaggcctctgagaccagc
D762A antisense	gtgggtctcagaggcctcagactctctc
D769A sense	gaccagccttttggccagcaccttgagg
D769A antisense	cctcaagggtcgtggcaaaaggctgggtc
D788A sense	gggcaataccaggagcccaacatccagagag
D788A antisense	ctctctgtagttgggtcctctgattgccc
D811A sense	agggaggcagactgtggctaaagtcatgggtatac
D811A antisense	gtataccatgacttttagccacagtctgcctcct
D845A sense	accagaaagacagacagctgtgacaggagaggaag
D845A antisense	cttctctctgtcacagctgtctgtctttctggt
D858A sense	taaccaaggggaaacaggccagagaaacaaacagttg
D858A antisense	caactgttttctctctggtgtttcccttgggtta
D868A sense	aacagttgttagctagagccaccagagacaagaatc
D868A antisense	gattctgtctctgggtggtctagctaacaactgtt
D875A sense	cccagagacaagaatctccaaaaatgggaaagtgc
D875A antisense	gcactttcccattttggcagattctgtctctggg
D1035A sense	gatcaggaaatctccagctgctgtctgcctcct
D1035A antisense	aggaggcagacaagcagctggagattcctgatc

In vitro translation – In vitro caspase cleavage assay

In vitro translation was performed with a coupled transcription/translation reticulocyte lysate system (Promega, Charbonnières, France) according to the manufacturer's protocol using [35S]methionine (Amersham, Pittsburgh, PA). We mixed 6 µL of lysate, 6 µL of buffer, (+/-) 1 µL of recombinant caspase (R&D Systems, Minneapolis, MN), (+/-) 1 µL of caspase inhibitor 2 mM, and buffer (50 mM HEPES, pH 7.2, 50 mM NaCl, 0.1% CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate}, 10 mM EDTA, 5% glycerol, 10 mM dithiothreitol) for a total volume of 12 µL. Ten microliters of reticulocyte lysate were boiled for 5 min in Tris-glycine-SDS sample buffer, separated by 4-20% Tris-glycine polyacrylamide gel. The gel was then dried, and the radioactive signals were detected by autoradiography.

Results

Mutual exclusion between the apoptotic ring and phosphorylation of H2AX on tyrosine 142

Whether the apoptotic γ -H2AX response (8-10) is coupled with the full recruitment/activation of the DDR factors has recently been questioned (9,19). Indeed, endogenous phosphorylation of H2AX on its last residue (tyrosine 142) (P^{Y142} -H2AX) has been shown to prevent the binding of MDC1 to the neighboring phosphorylated serine 139 (γ -H2AX) (18,20), and retention of Y142 phosphorylation (P^{Y142} -H2AX) proposed to drive the apoptotic response (18). Because this explanation was apparently consistent with our prior observation that 53BP1 (p53 binding protein 1: one of the DDR chromatin binding factors downstream from γ -H2AX and MDC1) fails to associate with the apoptotic γ -H2AX ring (9), we tested phosphorylation of H2AX at tyrosine 142 (P^{Y142} -H2AX) during TRAIL-induced apoptosis. Unexpectedly, we found that tyrosine 142 phosphorylation (P^{Y142} -H2AX) decreases after TRAIL treatment, while γ -H2AX increases (Fig. 1A). The changes in opposite direction of γ -H2AX and P^{Y142} -H2AX were confirmed by immunofluorescence (Fig. 1B). To avoid interference between the γ -H2AX antibody and the P^{Y142} -H2AX antibody due to the proximity of the epitopes (residues 139 and 142), we used phospho-DNA-dependent protein kinase (P^{T2609} -DNA-PK) antibody instead of the γ -H2AX antibody, knowing that P^{T2609} -DNA-PK has the same (ring and diffuse) staining as γ -H2AX during apoptosis (8,9). Figure 1B demonstrates that apoptotic cells that stain for P^{T2609} -DNA-PK are negative or very weak for P^{Y142} -H2AX, further demonstrating the mutual exclusion between the apoptotic ring and phosphorylation of H2AX on tyrosine 142.

MDC1 and 53BP1 are not recruited to the apoptotic ring

Our finding that P^{Y142} -H2AX decreases during apoptosis questions the hypothesis of Cook *et al.* (18) that sustained phosphorylation on tyrosine 142 is the main mechanism for preventing the binding of MDC1 to γ -H2AX during apoptosis. We found that MDC1 staining was significantly reduced in the γ -H2AX-positive apoptotic cells (Fig. 2A, B). Thus, our experiments demonstrate that during apoptosis, and contrary to ionizing radiation (Fig. 2A), MDC1 tends to decrease and is not recruited to γ -H2AX, which in turn probably explains why 53BP1 is not recruited to the apoptotic γ -H2AX ring (9). Notably, after UV damage, which like apoptosis produces a diffuse pan-staining γ -H2AX response, 53BP1 does not colocalize with γ -H2AX (21,22).

MDC-1 cleavage and inactivation by caspase-3

Next, we used Western blotting to analyze MDC1 in apoptotic cells. Figure 3 (panels A and B) shows that full-length MDC1 disappears rapidly during TRAIL-induced apoptosis. Concomitantly, TRAIL-treatment induced the appearance of a 70-kDa polypeptide that reacted with the MDC1 antibody (Fig. 3A-B, asterisk). The 70-kDa band was decreased by down-regulation of MDC1 with siRNA, indicating it was derived from MDC1 (Fig. 3A). These experiments suggested cleavage of MDC1 in cells undergoing apoptosis. Apoptotic MDC1 cleavage appears a general process as we also found it following treatment with Fas antibody (Supplemental Fig. 1A), as well as in response to the anticancer agents, camptothecin and cisplatin (Supplemental Fig. 1B). Moreover, apoptotic MDC1 cleavage was observed both in cancer and normal (prostate epithelial) cells (Supplemental Fig. 1C).

To confirm that MDC1 cleavage at the early phase of apoptosis was related to caspases, HCT116 cells were treated with the broad-spectrum caspase inhibitor Z-VAD-fmk (23) together with TRAIL. The apoptotic MDC1 cleavage (disappearance of full length MDC1 and appearance of the 70-kDa MDC1 band) were both suppressed by the broad-spectrum Z-VAD-fmk (23) but not by the proteasome inhibitor MG132 (Fig. 3B). Because caspase-3 is the main effector caspase, which migrates to the nucleus during apoptosis (24,25) whereas

caspase-2 is primarily nuclear like MDC1 (3), we tested which of those two caspases was responsible for the cleavage of MDC1. Expression of MDC1 in rabbit reticulocyte lysates enabled us to perform *in vitro* cleavage assays with recombinant caspases. Figure 3C shows that caspase-3 but not caspase-2 cleaves MDC1 at multiple sites. Next we searched caspase-3 consensus sequences in MDC1 (3,26) and mutated 19 potential caspase-3 target sites at aspartate residues (Supplemental Fig. 2). Mutation of aspartate 173 to alanine partially abolished caspase-3-mediated MDC1 cleavage (Fig. 3D). This caspase-3 disjoins the N-terminus of MDC1 (see Fig. 5).

To determine the impact of MDC1 inactivation on TRAIL-induced apoptosis, we tested the effects of TRAIL in HCT116 cells with MDC1 downregulation by siRNA. Figure 4A, B, C demonstrates that downregulation of MDC1 increases TRAIL-induced DNA fragmentation and caspase-2 activation. These data indicate an active participation of MDC1 in promoting apoptosis. It is not excluded that the effect of MDC1 on apoptosis could be indirect. Inactivation of MDC1 could impair DNA repair leading to excessive DNA damage and consequently enhance apoptosis.

Discussion

MDC1 is a large protein (2090 amino acids; 226 kDa) and the identification of the caspase-3 cleavage site at position 173 is sufficient by itself to cleave MDC1 between its FHA (forkhead-associated) and BRCT (breast cancer C-terminal) domains (Fig. 5). The integrity of MDC1 is likely critical for DNA repair and DDR as γ -H2AX binds the BRCT domain (12) and ATM the FHA domain (17,27) of MDC1. Under normal DNA repair and checkpoint conditions, the recruitment of ATM by MDC1, bound to chromatin *via* γ -H2AX, leads to additional H2AX phosphorylation, which in turn recruits more MDC1 and engages a full DDR activation (Fig. 5). Our results show that during apoptosis, cleavage of MDC1 by caspase-3 prohibits these interactions and therefore aborts the DDR activation (Fig. 5). Nijmegen breakage syndrome (NBS) constitutes another example where disruption of BRCT and FHA domains disturbs the assembly of DDR complexes; the most frequent NBS1 mutations cause the scission of Nbs1 in two distinct segments, one corresponding to the FHA domain and the other to the ATM and Mre11 binding sites (28). The fact that MDC1 is cleaved by caspase-3 during apoptosis is consistent with other studies demonstrating the cleavage of ATM and DNA-PK, two other critical DNA damage responsive proteins, after apoptotic stimuli (29-31). The cleavage of ATM inactivates its kinase function, which prevents DNA repair and DNA damage signaling (30). Similarly, the cleavage of DNA-PK in late apoptosis abrogates its activity (31).

In conclusion, our study reveals a novel mechanism by which caspase-mediated cleavage inactivates DNA repair and checkpoint responses. Moreover, our finding that MDC1 inactivation tends to reduce apoptosis warrants further studies. Indeed, MDC1 inactivation has recently been found in a significant number of breast and lung carcinomas (32,33), which could impact therefore on tumor cell survival and tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Decreased H2AX phosphorylation at tyrosine 142 during apoptosis. **A**, Kinetics of the effects of TRAIL on γ -H2AX, pY142-H2AX and total H2AX in HCT116 cells. Cells were treated with 0.1 μ g/mL TRAIL for the indicated times. GAPDH was used as loading control. **B**, Differential staining patterns for pT2609-DNA-PK and pY142-H2AX in response to TRAIL. pT2609-DNA-PK and pY142-H2AX confocal immunofluorescence staining in HCT116 cells treated with TRAIL (0.1 μ g/mL, 2 h). pT2609-DNA-PK was labeled in red and pY142-H2AX in green.

Figure 2.

Mutual exclusion of MDC1 and γ -H2AX during apoptosis. **A**, Differential staining patterns for γ -H2AX and MDC1 in response to TRAIL. γ -H2AX and MDC1 confocal immunofluorescence staining in HCT116 cells treated with TRAIL (0.1 μ g/mL, 2 h) or irradiated (3 Gy). γ -H2AX was labeled in red and MDC1 in green. **B**, Relationships between γ -H2AX and MDC1 intensities. One hundred untreated (γ -H2AX negative) (lower panel) and two hundreds TRAIL-treated (0.1 μ g/mL, 2 h) HCT116 cells were analyzed by confocal microscopy [γ -H2AX positive cells (upper panel) represented 50% of the TRAIL-treated cells]. MDC1 intensity was quantified using Adobe Photoshop 7.0. The x axis indicates the MDC1 intensity and the y axis indicates the number of cells.

Figure 3.

In vivo (A, B) and *in vitro* (C, D) cleavage of MDC1 by caspase-3.

A, HCT116 cells were treated for the indicated times with TRAIL (0.1 µg/mL) 96 h after transfection with siRNA against MDC1 or a negative control siRNA. MDC1 was analyzed by Western blotting. Tubulin was used as loading control. The gray star corresponds to the main MDC1 cleavage fragment. **B**, HCT116 cells were treated with MG132 (MG, 30 µM) or Z-VAD-fmk (ZV, 100 µM) for 1 h prior to the addition of TRAIL (0.1 µg/mL for 3 h). MDC1 was analyzed by Western blotting. Tubulin was used as loading control. The gray star corresponds to the main MDC1 cleavage fragment. **C**, Expression of wild-type GFP-MDC1 cDNA in rabbit reticulocyte lysates and study of its cleavage by recombinant caspases-2 or -3 in the presence of Z-VAD-fmk (ZV). The stars correspond to cleavage fragments. **D**, Expression of wild-type or mutated D173A GFP-MDC1 cDNA in rabbit reticulocyte lysates and study of its cleavage by recombinant caspase-3 in the presence of Z-VAD-fmk (ZV). The stars correspond to cleavage fragments.

Electrophoretic migration of molecular mass markers (kDa) is indicated to the right of each panel. The epitope recognized by the MDC1 antibody maps to a region between residues 475 and 525 of MDC1 (panels A and B). Differently, the use of a radiolabeled MDC1 protein (panels C and D) allows the full visualization of MDC1 fragments.

Figure 4.

Anti-apoptotic function of MDC1. **A**, Western blotting of a representative experiment showing the efficiency of MDC1 downregulation by siRNA. HCT116 cells were transfected with siRNA against MDC1 or negative control siRNA. MDC1 was analyzed by Western blotting. Tubulin was used as a loading control. **B**, Effect of MDC1 on sub-G1. HCT116 cells were treated with TRAIL (0.1 µg/mL) 96 h after transfection with siRNA against MDC1 or a negative control siRNA. The y axis represents the percentage of cells with sub-G1 DNA. The cells transfected with negative control siRNA are in black, and those transfected with siRNA against MDC1 are in gray. **C**, Effect of MDC1 on procaspase-2 level. HCT116 cells were treated with TRAIL (0.1 µg/mL) 96 h after transfection with siRNA against MDC1 or a negative control siRNA. Procaspase-2 was analyzed by Western blotting. Tubulin was used as loading control.

Figure 5. Schematic representation of MDC1 interactions during DNA damage response (DDR) and apoptosis.