Nuclear ceramide is associated with ATM activation in the neocarzinostatin-induced apoptosis of lymphoblastoid cells

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Abbreviation list:
aSMase, acid sphingomyelinase; A-T, ataxia telangiectasia; ATM, ataxia telangiectasia mutated; CerS, ceramide synthase, DDR, DNA damage response; DSB, DNA double-strand break; GBA,
glucosylcerebrosidase; MRE11, meiotic recombination 11; MRN, MRE11/Rad50/NBS1; NBS1, Nijmegen breakage syndrome 1; NCS, neocarzinostatin; nSMase, neutral sphingomyelinase; SM, sphingomyelin; SMase, sphingomyelinase; SMS, sphingomyelin synthase.

**Keywords:** Ceramide, sphingomyelin, neutral SMase, sphingomyelin synthase, ATM, MRN complex, DSB, apoptosis, NCS
Abstract

Ceramide is a bioactive sphingolipid that mediates ionizing radiation- and chemotherapy-induced apoptosis. Neocarzinostatin (NCS) is a genotoxic anti-cancer drug that induces apoptosis in response to DNA double-strand breaks (DSBs) through ataxia telangiectasia mutated (ATM) activation. However, the involvement of ceramide in NCS-evoked nuclear events such as DSB-activated ATM has not been clarified. Here, we found that nuclear ceramide increased by NCS mediated apoptosis through the enhanced assembly of ATM and the Mre11/Rad50/NBS1 (MRN) complex proteins in human lymphoblastoid L-39 cells. NCS induced an increase of ceramide production through activation of neutral sphingomyelinase (nSMase) and suppression of sphingomyelin synthase (SMS) upstream of DSB-mediated ATM activation. In ATM-deficient lymphoblastoid AT-59 cells compared with L-39 cells, NCS treatment showed a decrease of apoptosis even though ceramide increase and DSBs were observed. Expression of wild-type ATM, but not the kinase-dead mutant ATM, in AT-59 cells increased NCS-induced apoptosis despite similar ceramide accumulation. Interestingly NCS increased ceramide content in the nucleus through nSMase activation and SMS suppression and promoted colocalization of ceramide with phosphorylated ATM and foci of MRN complex. Inhibition of ceramide generation by the overexpression of SMS suppressed NCS-induced apoptosis through the inhibition of ATM activation and assembly of the MRN complex. In addition, inhibition of ceramide increase by the nSMase inhibitor GW4869 prevented NCS-mediated activation of the ATM. Therefore,
our findings suggest the involvement of the nuclear ceramide with ATM activation in NCS-mediated apoptosis.

**Significance Statement**

We demonstrate that regulation of ceramide with neutral sphingomyelinase (nSMase) and sphingomyelin synthase in the nucleus in double-strand break–mimetic agent neocarzinostatin (NCS)-induced apoptosis. We also showed that ceramide increase in the nucleus plays a role in NCS-induced apoptosis through activation of the ataxia telangiectasia mutated/Mre11/Rad50/Nbs1 complex in human lymphoblastoid cells.
Introduction

Excessive exposure of cancer cells to DNA-damaging stress such as ionizing radiation (IR) and enediyne antibiotics induces the formation of DNA double strand breaks (DSBs) and subsequent induction of DNA damage response (DDR) pathways, including apoptotic cell death (Bhattacharya et al., 2018; Maier et al., 2016; Woods and Turchi, 2013). After cell stress, DSBs are rapidly recognized by the meiotic recombination 11 (Mre11)/double-strand break repair (Rad50)/Nijmegen breakage syndrome 1 (NBS1) (MRN) complex (Lavin et al., 2015). The MRN complex accumulates at DSBs and then recruits and activates the ataxia telangiectasia mutated (ATM) kinase from the cytosol to the nucleus to repair DSBs (Lavin et al., 2015; Stracker and Petrini, 2011). ATM is a member of the phosphatidylinositol 3-kinase (PI3K) family and activates a diverse range of downstream signals including apoptosis-inducing molecules such as p53 and caspases (Enoch and Norbury, 1995; Kuang et al., 2009; Tripathi et al., 2013). DSBs are then repaired by cellular recovery pathways such as non-homologous end junction or homologous recombination (Zhao et al., 2020). However, in tumor cells, DSBs result in activation of the ATM/p53-mediated apoptotic pathway, leading to prevention of cancer cell progression (Hsu et al., 2009; Wang et al., 2021). Thus, DSB-mediated apoptosis has been targeted for the prevention of cancer progression by the use of IR and anti-cancer drugs.

Ceramide is a sphingolipid that functions as an essential component of lipid bilayer in the cellular membrane and plays a central role in sphingolipid metabolism, regulating sphingomyelin (SM), sphingosine 1-phosphate (S1P), and glycosphingolipids (GSLs) (Hannun and Obeid, 2018). Ceramide
has also been shown to function as a bioactive mediator involved in diverse cellular functions such as cell death, senescence, differentiation, and autophagy (Hannun and Obeid, 2018; Ogretmen, 2018; Taniguchi and Okazaki, 2014; Taniguchi and Okazaki, 2020). Numerous cellular stresses including IR, oxidative stress, death ligands, and anti-cancer drugs were reported to enhance ceramide generation to induce apoptosis (Carroll et al., 2015). Ceramide generation is regulated through three enzymatic pathways: i) de novo ceramide synthesis, which involves serine and palmitoyl-CoA condensation by serine-palmitoyl transferase (SPT), N-acylation of sphinganine by ceramide synthase (CerS), and desaturation of dihyroceramide by sphingolipid delta-4 desaturase (DES); ii) the salvage pathway, which involves recycling ceramide from sphingosine catabolized from GSLs or S1P by CerS, and iii) the SM/ceramide cycle, which involves SM synthase (SMS) synthesizing SM from ceramide and sphingomyelinase (SMase) hydrolyzing SM to ceramide (Hannun and Obeid, 2018). We previously showed that ceramide and SM are closely related to cell death and proliferation/survival, respectively, and SM/ceramide balance through the SM/ceramide cycle seems to plays a critical role in stress-induced apoptotic cell death (Taniguchi and Okazaki, 2014; Taniguchi and Okazaki, 2020). Early studies showed that DDR induces apoptosis with ceramide generation through the hydrolysis of SM by neutral SMase (nSMase) (Haimovitz-Friedman et al., 1994). Other reports showed that acid SMase (aSMase)-knockout cells derived from a patient with Nieman–Pick disease were resistant to IR-induced cell death, which suggests that the SM/ceramide balance is involved in DSB-mediated cell death (Chmura et al., 1997; Santana et al., 1996). However, the precise relation between the
mechanistic pathway underlying DSB-induced DDR, such as ATM activation and MRN complex formation, and ceramide has not been clarified.

Neocarzinostatine (NCS) is an enediyne anti-cancer drug that induces DSBs (Dedon and Goldberg, 1990; Hensens et al., 1994). NCS treatment rapidly induces DSB-mediated ATM activation, resulting in apoptosis through the activation of downstream molecules including p53 and caspases (Bar-Shira et al., 2002; Yuan et al., 2002; Yuan et al., 2003). Therefore, NCS has been used for the prevention of cancer progression in various types of tumors, including neuroblastoma, estrogen-responsive breast cancer, cervical cancer, and thyroid cancer (Cortazzo and Schor, 1996; Klubo-Gwiazdzinska et al., 2017; Liang et al., 1995).

In the present study, we investigated the mechanism of ceramide generation in response to NCS and the relation of nuclear ceramide and DSB-mediated DDR. In particular, the role of nuclear ceramide regulated by nSMase and SMS in NCS-mediated nuclear events, including MRN complex proteins and ATM activation, was elucidated.

**Materials and methods**

**Materials**

Expression vectors encoding human ATM were kindly gifted from Dr. Yosef Shiloh and Dr. Yael Ziv (Tel Aviv University, Ramat Aviv, Israel). NCS, 4′,6-diamino-2-phenylindole (DAPI) and GW4869 were obtained from Sigma-Aldrich (St Louis, MO, USA). N-acetylphosphosine (C2-ceramide) was
obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti-phospho ATM (S1981) (ab81292 and ab36810), anti-Golgi matrix protein 130 (GM130) (G7295) and anti-Mre11 (ab214), and anti-Rad50 (ab89) antibodies were from Abcam (Cambridge, MA, USA). Anti-NBS-1 (GTX70224) antibodies were purchased from GeneTex (San Antonio, TX, USA). Anti-ATM (2873S), anti-lamin A and C (2032S), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (2118S), and anti-cytochrome c oxidase subunit 4 (COX IV) (4850P) antibodies were from Cell Signaling (Danvers, MA, USA). Anti-transferrin receptor antibody was obtained from Zymed Laboratory Inc. (San Francisco, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Promega (Madison, WI, USA). Alexa Fluor-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA). Anti-ceramide monoclonal IgM antibody (NHCER-2) was obtained previously (Taniguchi et al., 2015). Acetyl-Asp-Met-Gln-Asp-aldehyde (DMQD-CHO) and acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7 amide (DEVD-MCA) were purchased from the Peptide Institute (Osaka, Japan).

Cell culture

Normal human lymphoblastoid L-39 cells and ataxia-telangiectasia (A-T)-derived AT-59 cells were kindly gifted from Dr. Y. Shiloh and Dr. Y. Ziv (Tel Aviv University, Ramat Aviv, Israel) (Matsuoka et al., 2000) and cultured in RPMI 1640 (Wako, Tokyo, Japan) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin-streptomycin. Cell viability was greater than 95% in all experiments as
assayed by 0.025% (w/v) trypan blue dye exclusion method.

**Detection of apoptotic cells by DAPI staining**

Apoptotic cells were determined by staining with DAPI described previously (Taniguchi et al., 2012). Treated cells were harvested, fixed with 4% (w/v) paraformaldehyde for 20 min at 4°C, and then stained with DAPI for 20 min at room temperature. At least 200 cells in one experiment were counted under fluorescent microscopy, and cells with nuclear condensation and fragmentation were judged as apoptotic cells.

**Detection of apoptotic cells by flow cytometry**

Apoptotic cells exposing phosphatidylserine were detected by staining with FITC-conjugated Annexin V (FITC-Annexin V) (BD Biosciences, San Jose, CA, USA) and flow cytometry analysis was performed as described previously (Taniguchi et al., 2015). Briefly, cells were suspended in Annexin V binding buffer (BD Biosciences) and stained with FITC-Annexin V for 15 min at room temperature. Stained cells were then analyzed with Gallios (Beckman Coulter, Miami, FL, USA). For each group, at least 20,000 cells were analyzed.

**Measurement of caspase-3 activity**

The DEVD-MCA cleavage assay was conducted as described previously (Iwai et al., 2003). Briefly,
cells were lysed in buffer containing 10 mM HEPES-KOH (pH 7.4), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (w/v) 3-(3-cholamidopropyl-dimethylammonio) propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 U/ml aprotinin, and 50 μg/ml leupeptin. After centrifugation at 10,000 g for 10 min, the supernatant was collected and added to reaction buffer containing 10% (w/v) sucrose, 10 mM HEPES-KOH (pH 7.4), 5 mM DTT, 0.1% (w/v) CHAPS, and 10 μM DEVD-MCA, and the mixture was incubated at 25°C for 60 min. Fluorescence of 7-amino-4-methyl-coumarin was measured by a fluorescent microplate reader Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA, USA) using 360 nm excitation and 450 nm emission filters. For the DEVD-pNA cleavage assay, the treated cells were analyzed with APOPCYTO™ Caspase-3 Colorimetric Assay Kit (MBL, Tokyo, Japan), following the manufacturer’s protocol.

**Liquid chromatography tandem mass spectrometry (LC-MS/MS)**

For whole cells, lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959) after the addition of 1 nmol of internal standards for LC-MS/MS, C_{17}-SM, C_{17}-ceramide, C_{17}-lactosylceramide, C_{17}-glucosylceramide, and C_{17}-sphingosine (Avanti Polar Lipids, Alabaster, AL, USA). The extract was saponified with 0.4 M KOH in methanol at 37°C for 2 h to remove glycolipids. The alkali-stable fraction was recovered with chloroform and then washed with water. The chloroform phase was evaporated under a stream of nitrogen and then dissolved in 150 μl of acetonitrile/methanol.
An LC-MS system consisting of Prominence UFLC system (Shimadzu, Kyoto, Japan) coupled to a 3200 QTRAP System (SCIEX, Foster City, CA, USA) was used for the analysis of lipids. Extracted lipids were injected onto an InertSustain column (5 μm particle size, 2.1 × 150 mm, GL Science, Tokyo, Japan). MS analysis was run in the positive ion mode with following instrument parameters: curtain gas of 10 psi, ion spray voltage of 5500 V, temperature of 300°C, nebulizer gas of 30 psi, auxiliary gas of 50 psi and collision cell exit potential of 10 V. The contents of individual lipids were calculated by relating the peak area of analyte to the peak area of the corresponding internal standard. Data acquisition and analysis were performed using Analyst Software version 1.6 (SCIEX).

For nuclear fractions, the analysis of ceramide and SM species in lipid extracts was performed using a high-performance Surveyor/TSQ 7000 LC-MS system (Thermo Fisher Scientific) as described previously (Bielawski et al., 2006). The amounts of ceramide were expressed as the total of species with various carbon chains (C_{14}, C_{16}, C_{18}, C_{18:1}, C_{20}, C_{20:1}, C_{20:4}, C_{22}, C_{22:1}, C_{24}, C_{24:1}, C_{26}, and C_{26:1}). The amounts of SM were similarly expressed as the total of species with various carbon-chains (C_{14}, C_{16}, C_{18}, C_{18:1}, C_{20}, C_{20:1}, C_{22}, C_{22:1}, C_{24}, C_{24:1}, C_{26}, and C_{26:1}).

**Ceramide measurement by diacylglycerol kinase (DGK) assay**

Ceramide amounts were measured by DGK assay as described previously (Taniguchi et al., 2012). After extracting lipids from cells, ceramide was labeled by *E. coli* DGK and [γ-^{32}P] ATP as radio-labeled ceramide-1 phosphate and separated in solvent containing chloroform/acetone/methanol/acetic
acid/H_2O (10:4:3:2:1, vol/vol/vol/vol/vol) on a thin layer chromatography plate. Radioactivity within spots of radio-labeled C1P was calculated with a BAS2000 Image Analyzer (Fujifilm, Tokyo, Japan).

**Measurement of SMS, glucosylceramide synthase (GCS), and sphingomyelinase activities**

The activities of sphingolipid enzymes including SMS, GCS, nSMase, and aSMase were assessed with the previously described methods (Shakor et al., 2011; Taniguchi et al., 2015). For SMS and GCS activities, cells were homogenized in buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 10 mM EGTA, and inhibitor cocktail (Roche, Basel, Switzerland). Homogenates (100 μg of protein) were incubated in reaction solution (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 20 μM C_6-NBD-ceramide, 0.1 mM UDP-glucose, 120 μM phosphatidylcholine). For nSMase and aSMase activities, cells were sonicated in lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% (w/v) Triton X-100, and inhibitor cocktail. Lysate was added to the reaction solution for nSMase (100 mM Tris-HCl [pH 7.5], 10 μM C_6-NBD-SM, 10 mM MgCl_2, 0.1% [w/v] Triton X-100, and 5 mM DTT) or aSMase (100 mM sodium acetate [pH 5.0], 10 μM C_6-NBD-SM, and 0.1% [w/v] Triton X-100). The reaction solutions were incubated for 60 min at 37°C. Lipids were extracted using the Bligh–Dyer method, applied onto thin layer chromatography plates, and developed with a solvent consisting of chloroform–methanol–12 mM MgCl_2 (65:25:4, vol/vol/vol). The fluorescent lipids were detected using LAS-4000 (Fujifilm) and quantified using Multi Gauge 3.1 (Fujifilm).
Western blot analysis

Cells were lysed in RIPA buffer (Wako) for 20 min on ice. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After incubation with Blocking One solution (Nacalai Tesque, Kyoto, Japan) or Blocking One-P (Nakalai Tesque) for 15 min at room temperature, the membranes were incubated overnight at 4°C with primary antibodies. The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies for 45 min at room temperature. Immunoreactive protein bands were detected with the LAS-4000 system.

Plasmid constructs, transfection and stable cell lines

The plasmids encoding FLAG-tagged wild-type ATM (containing the full-length cDNA for ATM) and kinase-dead ATM (kd ATM; containing the full-length cDNA with point mutations at Asp 2870 to Ala 2870 and Asn 2875 to Lys 2875) were previously described (Li et al., 2000). AT-59 cells were transiently transfected by electroporation with Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA). Plasmids (50 μg) were mixed with 5 x 10⁷ of cells and the mixture was exposed to a high voltage pulse (300V, 960 μF). The transfected cells were cultured in RPMI-1640 medium supplemented with 20% (v/v) FCS and 80 ng/ml of G418. The media were replaced with 10% (v/v) FCS at 48 h before experiments.

L-39 cells over-expressing SMS1 and SMS2 were established by retroviral transfection using pDON-
hSMS1 (L-39-SMS1) and pDON-hSMS2 (L-39-SMS2), respectively, as described previously (Shakor et al., 2011). Control cells were established using the empty vector pDON-AI (L-39-Vector). The infected cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FCS and 400 μg/ml of G418.

**Immunocytochemistry**

Cells were cytospun onto coated slide glasses at 800 g for 5 min at room temperature (Matsunami Glass Ind., Ltd., Osaka, Japan) and fixed with 0.2% (w/v) glytalardehyde/3% (w/v) paraformaldehyde containing 0.1% (w/v) Triton X-100 for 20 min at 4°C. The cells were permeabilized with PBS containing 0.2% (w/v) Triton X-100 for 20 min and blocked with PBS containing 3% (w/v) BSA, 5% (v/v) normal goat serum, and 0.1% (w/v) Tween 20 for 1 h. Cells were treated with primary antibodies against ceramide, phospho-ATM, Mre11, Rad50, and NBS-1 (1:200 dilution) at 4°C for overnight and then incubated with goat anti-rabbit antibody conjugated with Alexa Fluor 488 and goat anti-mouse antibody conjugated with Alexa Fluor 594 (1:500 dilution) for 1 h at room temperature. Nuclei were counter-stained with DAPI for 20 minutes at room temperature. Images were obtained using a Carl Zeiss LSM710 confocal microscope (Carl Zeiss Co., Ltd., Jena, Germany) or a BZ-X700 fluorescent microscope (Keyence, Osaka, Japan). Fluorescent intensity of nuclear protein was measured with Image J 1.43u software (NIH, Bethesda, MD, USA).
**Isolation of nuclear fractions**

Cells were suspended in Buffer A (10 mM HEPES-KOH [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 2.5 μg/ml leupeptin, and 2.5 μg/ml aprotinin) and incubated for 15 min on ice. Then, 0.625% (w/v) Nonidet-P40 was added, mixed by vortex for 15 sec, and centrifuged at 15,000 g for 30 sec at room temperature. Pellets were collected as nuclear fractions.

**Statistical analysis**

Statistical analyses were performed using the GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA) and the results are represented as the mean ± standard deviation (S.D.). Statistically significances were determined using paired t test or one-way ANOVA test with a follow-up Tukey’s test. A P-value < 0.05 was considered to indicate statistical significance.

**Results**

**NCS treatment induces caspase-dependent apoptosis and ceramide accumulation in non-malignant lymphoblastoid cells**

NCS is a complex of a single chain protein (113 amino acids, 11 kD) and enediyne chromophore molecule, and well-known radiometric drug that induces DSBs and subsequent apoptosis in various cancer cell types. We first confirmed the apoptotic effect of NCS in L-39 non-malignant lymphoblastoid cells. NCS treatment increased the numbers of apoptotic cells, as defined by nuclear
fragmentation and Annexin V-positive cells, in a dose-dependent manner (Figs. 1A and B). In parallel with the increase in apoptotic cells, NCS also augmented caspase-3 activity (Fig. 1C). Treatment with DMQD-CHO, a specific inhibitor of caspase-3, blocked NCS-induced apoptosis in L-39 cells (Fig. 1D), which indicates that NCS induced caspase-dependent apoptosis in L-39 cells.

Previously, some reports showed that DSBs induced ceramide increase and its-mediated apoptosis (Ch'ang et al., 2005; Mesicek et al., 2010). Therefore, we confirmed ceramide generation by NCS treatments in L-39 cells. In fact, NCS increased ceramide amounts at dose-dependent manner (Fig. 1E). In addition, LC-MS/MS analysis revealed that NCS treatment increased intracellular C16:0 ceramide (Fig. 1F). Ceramide mediates apoptosis in response to numerous cellular stresses through the SM/ceramide cycle (Taniguchi and Okazaki, 2020). Therefore, we next examined whether SM/ceramide cycle-related enzymes including SMS and SMase were changed by NCS treatment in L-39 cells. SMS activity was suppressed in cells treated with NCS compared with controls (Fig. 1G). In addition, NCS treatment enhanced nSMase activity (Fig. 1H). However, aSMase activity was not altered in NCS-treated L-39 cells (Fig. 1I). Moreover, NCS had no change of GCS activity, which transfers glucose to ceramide and mediates ceramide metabolism (Fig. 1J). These results suggest that NCS may have induced upregulation of nSMase and suppression of SMS in L-39 cells, leading to ceramide accumulation.

**NCS induces ATM-independent ceramide accumulation and ATM-dependent apoptosis.**
Previous reports showed that NCS causes DBS-mediated apoptosis through the ATM pathway. We confirmed that NCS treatment of L-39 cells increased the phosphorylation of ATM at serine 1981, which is a sign of its activation (Supplemental Fig. S1A) (Bakkenist and Kastan, 2003). *In vitro* kinase assays also showed that NCS increased the kinase activity of activated ATM (Supplemental Fig. S1B). We also observed phosphorylation of p53, which is a target of activated ATM and downstream factor of ATM-mediated apoptosis, in cells with NCS treatment (Supplemental Fig. S1C).

To determine whether ATM plays a role in NCS-mediated ceramide accumulation, we used ATM-deficient lymphoblastoid cells AT-59 derived from an A-T patient (Fig. 2A). As shown in Figs. 2B and 2C, NCS-induced apoptosis and caspase-3 activation were suppressed in AT-59 cells compared with L-39 cells. However, treatment of AT-59 cells with NCS resulted in ceramide accumulation (Fig. 2D). Similar to NCS-treated L-39 cells, NCS-treated AT-59 cells also showed decreased SMS activity and increased nSMase activity (Figs. 2E and 2F). Phosphorylation of H2A histone family member X (γH2AX) is marker of DSBs (Stucki et al., 2005). Immunocytochemistry showed that NCS treatment increased γH2AX in both L-39 cells and AT-59 cells, confirming that NCS induced DSBs regardless of the presence or absence of ATM (Supplemental Fig. S2).

Exogenous ceramide treatment was shown to induce caspase-3 activation and apoptosis (Iwai et al., 2003). Treatment of L-39 cells with N-acetyl sphingosine (C2-ceramide) increased apoptotic cells and caspase-3 activation (Figs. 2G and 2H). We also found that C2-ceramide induced ATM activation, as shown by increased ATM phosphorylation and kinase activity (Supplemental Figs. S1A and S1B).
Moreover, phospho-p53 was elevated in L-39 cells treated with C₂-ceramide (Supplemental Fig. S1C). However, AT-59 cells treated with C₂-ceramide showed no induction of apoptosis or caspase-3 activation (Figs. 2G and 2H). Therefore, these data while NCS induces ceramide accumulation in an ATM-independent manner, ATM is required for NCS-mediated apoptosis.

**Introduction of ATM recovers the sensitivity of AT-59 cells to ceramide and NCS-mediated apoptosis.**

To elucidate the involvement of ATM in NCS-ceramide-mediated apoptosis, we performed experiments with wild-type ATM (Wt ATM) and kinase-dead mutant ATM (Kd ATM), the inactivated form, in AT-59 cells (Fig. 3A). Over-expression of Wt ATM in NCS-treated AT-59 cells increased apoptotic cells and caspase-3 activity while these effects were not observed in cells expressing Kd ATM (Figs. 3B and 3C). Notably, NCS treatment caused ceramide accumulation with both Wt ATM and Kd ATM at the same level (Fig. 3D), which suggests that ATM and its kinase activity were essential for ceramide-mediated apoptosis in NCS treatment. Overexpression of Wt ATM also increased C₂-ceramide-mediated apoptosis (Fig. 3E).

**NCS induces nuclear ceramide accumulation and subsequent ATM activation.**

We next focused on the involvement of ceramide in ATM activation induced by NCS treatment. Following DSB formation, ATM is rapidly phosphorylated at serine 1981 and localizes to DNA
damage sites as detected by punctate structures in the nucleus (Kurose et al., 2005). We found that NCS treatment clearly induced the phosphorylation of ATM (p-ATM) and its nuclear foci formation in L-39 cells (Figs. 4A and 4B). We then assessed whether ceramide was involved in ATM activation or foci formation in the nucleus. As shown Fig. 4B, ceramide was increased after NCS treatment in L-39 cells and partially but clearly co-localized with p-ATM foci in the nuclei. To validate the accumulation of nuclear ceramide, we performed nuclear fractionation (Fig. 4C). Mass spectrometry of nuclear fractions revealed an increase of ceramide and decrease of SM in the nucleus after NCS treatment (Fig. 4D). Our results above showed that NCS suppressed SMS and increased nSMase activity in whole cell lysate of L-39 cells (Figs. 1G and 1H). We previously reported the presence of nSMase and SMS activities in nuclei (Watanabe et al., 2004). NCS treatment also induced the activation of nSMase and suppression of SMS in the nuclear fraction, similarly to its effects in whole cells (Fig. 4E). In addition, exogenous C2-ceramide treatment induced phosphorylation of ATM and foci formation in the nucleus (Fig. 4F).

In response to genotoxicity, MRE11, Rad50, and NBS1 form the MRN complex on the DSB sites as an early sensor system and recruit p-ATM from the cytosol to the nucleus (Lavin et al., 2015; Stracker and Petrini, 2011). We found that NCS treatment increased the colocalization of pATM with MRN complex proteins such as MRE11, Rad50, and NCS1 as the nuclear foci (Fig. 4F). Exogenous C2-ceramide also induced MRN complex proteins co-localizing with p-ATM. These data suggested that nuclear ceramide may be increased by NCS through regulation of the SM/ceramide cycle and
associates with the MRN complex following ATM activation.

**SMS overexpression suppresses activated ATM accumulation in the nucleus**

So far, there are two isoforms of SMS; SMS1 is localized in the Golgi apparatus, and SMS2 is in the both the Golgi apparatus and plasma membrane (Taniguchi and Okazaki, 2014). In the NCS treatment, suppression of nuclear SMS induced nuclear ceramide accumulation. Whereas SMS activity was clearly detected in the nucleus, its-responsive protein was largely unclear. We previously showed that mouse embryonic fibroblasts established from SMS1 and SMS2 double knockout mice showed complete absence of SMS activity (Asano et al., 2012; Taniguchi et al., 2016). To examine the possibility that SMS activity suppresses ATM signaling, we established L-39 cells overexpressing SMS1 or SMS2 and examined NCS-induced ATM activation and apoptosis in these cell lines. As shown in Fig. 5A, phosphorylation of ATM at Ser1981 induced by NCS treatment was suppressed in either SMS1 or SMS2 overexpression. In addition, p53 phosphorylation was also decreased in both SMS1 and SMS2-overexpressing cells treated with NCS (Supplemental Fig. S1E). While NCS treatment induced nuclear co-localization of p-ATM and ceramide in control L-39 cells, both SMS1- and SMS2-overexpressing cells suppressed ceramide increase and ATM activation in response to NCS compared with control cells (Fig. 5B). In addition, L-39-SMS1 and L-39-SMS2 cells showed inhibition of pATM foci formation after NCS treatment compared with L-39-vector cells (Fig. 5C). Consistent with these data, NCS-mediated apoptosis was also suppressed in L-39-SMS1 and -SMS2
cells compared with L-39-vector cells (Fig. 5D).

Inhibition of SM hydrolysis suppresses MRN complex accumulation in the nucleus

In NCS treatment, nSMase activity was enhanced to catabolize SM hydrolysis. Therefore, we assessed the effect of the nSMase inhibitor GW4869 on NCS-mediated ATM activation and MRN complex proteins. In fact, pre-treatment of cells with GW4869 suppressed NCS-induced increase of nSMase activity (Supplemental Fig. S3). In addition, GW4869 treatment decreased NCS-induced activation of ATM and p53 (Fig. 6A and Supplemental Fig. 1F). Moreover, pre-treatment of cells with GW4869 inhibited nuclear ceramide accumulation and ATM activation by NCS (Figs. 5B and 6A). GW4869 also suppressed NCS-induced increase of MRN complex protein at nuclei (Fig. 6B–D). Therefore, the production of nuclear ceramide by SM hydrolysis with nSMase may be important for the activation of ATM activation and the formation of MRN complexes in response to NCS treatment.

Discussion

Ceramide is a bioactive sphingolipid that is involved in numerous cell functions including apoptotic cell death. Previous studies have reported the molecular mechanisms of ceramide-mediated apoptosis in diverse types of cancer cells treated with anti-cancer agents (Ogretmen, 2018). In this study, we found that the genotoxic reagent NCS induced an increase of nuclear ceramide content with apoptotic
cell death in lymphoblastoid cells. Nuclear ceramide produced by the activation of nSMase and inhibition of SMS played a role in ATM activation and foci formation of the MRN complex during NCS-induced apoptotic cell death as a result of DDR, as shown by γH2AH dot formation (Supplemental Fig. S2). Interestingly, ATM-deficient AT-59 cells were resistant to NCS-induced apoptosis compared with ATM-positive L-39 cells, even though nuclear ceramide accumulation occurred in both cells. NCS induced DSBs and subsequent activation of ATM with nuclear foci formation of the MRN complex in L39 cells to higher levels than in AT-59 cells. Therefore, these results suggested that nuclear ceramide is upstream of ATM/MRN complex formation, resulting in the induction of DDR-mediated apoptotic cell death.

We previously showed that anti-cancer drug adriamycin induced accumulation of nuclear ceramide in the apoptosis of HL-60 leukemia cells but not in adriamycin-resistant cells (Kawase et al., 2002). Similarly, nuclear ceramide increased by up-regulation of nSMase and down-regulation of SMS and activated caspase-3 in Fas-induced apoptosis of Jurkat T cells (Watanabe et al., 2004). In addition, Albi and colleagues reported that UV-C irradiation of normal and differentiated rat follicular thyroid FRTL-5 cells induced apoptosis with an increase of nuclear ceramide/diacylglycerol balance through SMase activation (Albi et al., 2008; Albi et al., 2014). Gentamicin inhibited cell proliferation of SUP-T1 human lymphoma cells with nuclear SM elevation from the decrease of nSMase and increase of SMS (Codini et al., 2015). Furthermore, Jaffrézou et al. showed that IR treatment of human erythromyeloblastic TF-1 cells induced ceramide generation with activation of nuclear nSMase (Jaffrezou et
al., 2001). These reports supported our findings that the NCS increased-ceramide/balance regulated by nuclear nSMase and SMS in the nucleus plays a role in DSB/DDR-induced apoptosis.

SMS1 is localized at the Golgi apparatus whereas SMS2 is located at both Golgi apparatus and the plasma membrane (Taniguchi and Okazaki, 2020). Our results showed that over-expression of SMS1 or SMS2 inhibited both NCS-induced apoptosis and increase of nuclear ceramide in L-39 cells (Fig. 5), which suggests a nuclear function for SMS. The localization of SMS in the nucleus has not been confirmed because of the lack of efficient antibodies (Kitatani et al., 2015). Because SMS proteins undergo various modification such as palmitoylation (Tani and Kuge, 2009) and caspase-mediated cleavage (Lafont et al., 2010), SMSs may undergo protein-processing to enable nuclear localization after protein modification. In addition, SMS1 has several alternative splicing forms, and nuclear-specific SMS may be transcribed from the SMS1 (SGMS1) or SMS2 (SGMS2) genes at low levels (Filippenkov et al., 2018; Yang et al., 2005). We also used GW4869, a specific inhibitor of nSMase2 (Luberto et al., 2002), to suppress ceramide accumulation induced by NCS. Our results showed that GW4869 inhibited NCS-induced activation of the ATM/MRN complex and apoptosis with a decrease of nuclear ceramide (Figs. 5 and 6). Four isoforms of nSMase have been reported: nSMase1, nSMase2, nSMase3, and mitochondria-associated nSMase (Kitatani et al., 2015; Xiang et al., 2021). Among them, only nSMase1 has been reported to localize in the nucleus (in rat hepatoma cells) (Mizutani et al., 2001). Other studies reported the localization of human and mouse nSMase1 at the endoplasmic reticulum (Fensome et al., 2000; Rodrigues-Lima et al., 2000; Tomiuk et al., 2000). In
addition, over-expression or knockout of nSMase1 had no effects on ceramide metabolism in cells and
mice (Tepper et al., 2001; Tomiuk et al., 1998; Zumbansen and Stoffel, 2002). Here, we showed the
inhibitory effect of GW4869 on both nuclear nSMase activity and nuclear ceramide, which suggests
the role of nSMase2 in nuclear ceramide generation. Therefore, further investigation is required to
elucidate how nSMase2 is contributing to the regulation of nuclear ceramide content.

Previous studies reported that IR treatment of HeLa cells induced the increases of C16:0, C24:0, and
C24:1 ceramide, which were blocked by addition of fumonisin B1, an inhibitor of CerS (Mesicek et
al., 2010). There are six isoforms of CerS (CerS1–6), which are involved in the regulation of carbon
chain lengths in ceramides at the endoplasmic reticulum (Levy and Futerman, 2010). We showed that
C16:0 ceramide increased after treatment with NCS, but we did not detect significant changes of
C24:0 and C24:1 ceramide (Fig. 1). C16:0 ceramide is mainly produced by CerS5 and CerS6, whereas
long-chain C24:0 and C24:1 ceramides are produced by CerS2 (Grosch et al., 2012). In addition, X-
ray irradiation was reported to increase ceramide generation through the activation of CerS in A-T
patient–derived cells and enhance the radiosensitivity in crypt stem cells of ATM-deficient mice,
suggesting that ATM inhibited CerS activity at the post-transcriptional level (Ch'ang et al., 2005).
These findings suggest that CerS-dependent ceramide generation is regulated downstream of ATM.
However, ceramide generation by NCS was not inhibited in Kd ATM cells or AT-59 cells (Figs. 1–3).
Therefore, NCS-mediated generation of nuclear ceramide may be independent of the CerS-mediated
de novo and salvage pathways.
ATM is a member of the PI3K family along with ATR, DNA-PK and mTOR (Menolfi and Zha, 2020), and its downstream signal pathways involving p53 and caspases have been vigorously investigated (Paull, 2015). In fact, caspase-3 specific inhibitor DMQD-CHO suppressed NCS-induced apoptosis (Fig. 1D). However, the inhibitory effect of DMQD-CHO on NCS-induced apoptosis was about 50% in L-39 cells. Previously, we have shown that the same concentration of DMQD-CHO completely inhibits caspase-3 activity in heat shock treatment of HL-60 cells, but suppresses apoptosis by only about 50% (Kondo et al., 2000). Sahu et al. showed that the tetracyclic condensed quinolone compound induced not only caspase-dependent but also caspase-independent apoptosis by caspase-inducing factors through ATM and p53 activation (Sahu et al., 2013). Therefore, DMQD-CHO may not completely suppress cell death because NCS-induced apoptosis also involves a caspase-independent pathway.

Here we showed that NCS-increased ceramide induced ATM-dependent activation of p53 (Supplemental Fig. S1). However, how the upstream regulatory pathways of ATM are mostly unknown. The MRN complex is a critical molecule that recruits ATM to DSBs region. The MRN complex rapidly recognizes DSBs and associates with ATM, leading to the induction of survival or apoptosis according to the extent of DDR. In this study, we found that ceramide accumulation induced by NCS was upstream of ATM activation and nuclear accumulation of MRN complex proteins. Inhibition of ceramide accumulation by GW4869 or overexpression of SMSs suppressed ATM activation, resulting in rescue from apoptotic cell death (Figs. 5 and 6). However, the precise
mechanism of ceramide in MRN complex–mediated ATM activation in DDR is unknown. In vitro reconstitution experiments using recombinant ATM protein, MRN proteins, and DNA showed induction of p53 phosphorylation through ATM activation (Lee and Paull, 2005). The addition of C2-ceramide to in vitro kinase assays using immunoprecipitated ATM from L-39 cells had no effect on the activation of ATM (data not shown). However, NCS-increased nuclear ceramide colocalized with ATM/MRN complex foci, which suggests the possibility that nuclear ceramide was involved in MRN complex formation (Fig. 4). We previously showed that ceramide inhibited the PI3K family member mTOR through ceramide-activated protein phosphatases (CAPPs) (Taniguchi et al., 2012). Thus, it is likely that ceramide indirectly is related to the function of ATM/MRN complex. Recently, the modulators of the MRN complex have been found: UFMylation of histone H4 by UFM1 specific ligase 1 (UFL1) recruiting to MRE11 for ATM activation and inhibited MRE11 exonuclease activity by complement component 1, q subcomponent binding protein (C1QBP) (Bai et al., 2019; Qin et al., 2019). In addition, SPO11 was reported to work as a regulator of MRE in meiotic DSBs (Paiano et al., 2020), further investigation is required to address the relation of MRN regulators such as C1QBP and SOP11 with nuclear ceramide to activate ATM.

Chemotherapeutic enediyne antibiotics including NCS have been applied clinically to various malignancies because their toxicity is much higher than that of anthracycline antibiotics such as adriamycin. NCS is useful for the treatment of tumors such as neuroblastomas, estrogen-responsive breast cancers, and cervical cancer (Cortazzo and Schor, 1996; Liang et al., 1995). In the clinical
setting, cancers sensitive to NCS become quickly resistant to NCS-induced apoptosis. Our previous study showed a lower level of ceramide in chemoresistant leukemia patients than in chemosensitive leukemia patients (Itoh et al., 2003). Therefore, the lower content of nuclear ceramide through the ceramide/SM balance may be a cause of resistance to NCS treatment. Therefore, it may be important to develop novel agents to increase nuclear ceramide to activate apoptotic ATM signals such as p53 and caspases and enhance the anti-tumor effect of NCS.

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**Authorship Contributions**

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Footnotes

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The authors declare that they have no conflict of interest.
Figure Legends

Figure 1 NCS treatment induces apoptosis and ceramide accumulation in L-39 cells.

(A–C) L-39 cells were treated with the indicated concentration of NCS for 12 h. (A) Apoptotic cells, identified as cells with nuclear fragmentation, were detected by DAPI staining. (B) Cells were stained with FITC-Annexin V. Apoptotic cells, indicated by FITC-Annexin V-positive cells, were measured by flow cytometry. (C) Caspase-3 activity was measured by DEVD-pNA cleavage assay. (D) L-39 cells were pretreated with caspase-3 inhibitor DMQD-CHO for 1 h before treatment with the indicated concentrations of NCS for 12 h. Apoptotic cells were detected by DAPI staining. (E and F) Ceramide contents were measured by DGK assay (E) and LC-MS/MS (F). L-39 cells were treated with NCS at 200 ng/ml for 4 h. (G-J) Activities of sphingomyelin synthase (SMS) (G), neutral sphingomyelinase (nSMase) (H), acid sphingomyelinase (aSMase) (I) and glucosylceramide synthase (GCS) (J) in L-39 cells were examined under the indicated conditions. The values are shown as mean ± S.D from three independent experiments (n=3). *P < 0.05, **P<0.001.

Figure 2 NCS and C12-ceramide treatments induce apoptosis in ATM-expressing lymphoblast cells.

(A) Western blot analysis for ATM expression in L-39 and AT-59 lymphoblast cell lines. (B and C) L-39 and AT-59 cells were treated with NCS. After 12 h treatment, apoptotic cells were detected by DAPI staining (B). After 6 h treatment of NCS, caspase-3 activity was measured by the DEVD-MCA
cleavage assay (C). (D) After treatment of AT-59 cells with 200 ng/ml NCS, cells were harvested at the indicated times. Intracellular ceramide was measured by the DGK assay. (E and F) Activities of sphingomyelin synthase (SMS) (E) and neutral sphingomyelinase (nSMase) (F) were examined in AT-59 cells treated with the indicated concentrations of NCS. (G and H) L-39 and AT-59 cells were treated with C₂-ceramide. After 12 h, apoptotic cells were detected by DAPI staining (G). After 6 h treatment of C₂-ceramide, caspase-3 activity was measured by the DEVD-MCA cleavage assay (n=3) (H). The values are shown as mean ± S.D from three independent experiments (n=3). *P < 0.05, **P<0.001.

**Figure 3 Introduction of ATM recovers the sensitivity to ceramide and NCS-mediated apoptosis.**

(A) Immunoblot analysis for FLAG-tagged ATM in AT-59 cells transfected with pcDNA3 expression vectors encoding FLAG-tagged wild-type ATM (Wt ATM) or kinase-dead ATM (Kd ATM). (B) At 48 h after transfection, cells were treated with NCS and C₂-ceramide for 12 h. Apoptotic cells were detected by DAPI staining. (C) Caspase-3 activity was measured by DEVD-MCA cleavage assay. The cells were treated with NCS for 6 h and then assayed (n=3). (D) Ceramide content was determined by the DGK assay. (E) After treatment with C₂-ceramide at indicated concentrations for 12 h, apoptotic cells were detected by DAPI staining (n=3). The values are shown as mean ± S.D from three independent experiments (n=3). *P < 0.05, **P<0.001. NS, no significance.
Figure 4 NCS induces ceramide production and subsequent activation of ATM in the nucleus.

(A) L-39 cells were treated with or without 200 ng/ml NCS for 1 h. Western blot analysis was performed for ATM phosphorylated at Ser1981 (p-ATM). (B) NCS-treated cells were immunostained with anti-phosphorylated ATM (p-ATM) (Ser1981) and anti-ceramide antibodies. Images were obtained by confocal microscopy, and the numbers of nuclear p-ATM foci were counted in ten cells obtained from three independent experiments (n=10). Scale bars, 10 μm. (C) Nuclear fractions were isolated from L-39 cells and examined by western blot analysis. Organelle markers were as follows: Lamin A and C (Lamin A/C), nuclear; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cytosol; transferrin receptor, membrane and endosomes; calnexin, endoplasmic reticulum; cytochrome c oxidase subunit 4 (COX IV), mitochondria; Golgi matrix protein 130 (GM130), Golgi apparatus. (D and E) L-39 cells were treated with or without 200 ng/ml NCS for 1 h. (D) Nuclear fractions were extracted and ceramide (left graph) and SM (right graph) were measured by LC-MS/MS. (E) Sphingomyelin synthase (SMS) and neutral sphingomyelinase (nSMase) enzymatic activities in nuclear fractions were analyzed and are represented as the percentage of non-treated cells. Data are shown as mean ± S.D from three independent experiments (n=3). *P < 0.05, **P<0.001. (F) L-39 cells were treated with 200 ng/ml NCS or 20 μM C₂-ceramide (C₂-Cer) for 1 h and then immunostained with anti-Rad50, anti-p-ATM, anti-NBS, or anti-Mre1 antibodies. Nuclear staining was performed by DAPI. Cells were examined by confocal microscopy. Scale bars, 10 μm. NT, no treatment.
Figure 5 SMS overexpression suppresses NCS-mediated activation of ATM and MRN complex formation in the nucleus.

L-39 cells over-expressing SMS1 or SMS2 were established by retroviral transfection of hSMS1 (L-39-SMS1) and hSMS2 (L-39-SMS2). (A) Cells were treated with 200 ng/ml NCS for 1 h. Then, western blot analysis was performed for ATM phosphorylated at Ser1981 (p-ATM). (B) Cells were pre-treated with 5 μM of GW4869 (GW) for 1 h, followed by treatment with NCS for 20 min. The fixed cells were immunostained with anti-p-ATM and anti-ceramide antibodies and examined by confocal microscopy. Nuclei were counter-stained with DAPI. Scale bars, 10 μm. (C) Nuclear p-ATM foci were counted from three independent experiments (n=10). (D) Cells were treated with the indicated concentrations of NCS for 12 h. Apoptosis cells were detected by DAPI staining. The values are shown as mean ± S.D from four independent experiments (n=4). *P < 0.05, **P<0.001. NT, no treatment.

Figure 6 Inhibition of SM hydrolysis suppresses MRN complex accumulation in the nucleus.

L-39 cells were pre-treated with 5 μM GW4869 (GW) for 10 min and then treated with 200 ng/ml NCS for 1 h. (A) ATM protein was detected with immunoblotting. (B-E) The fixed cells were immunostained with anti-ceramide (B), anti-Rad50 (C), anti-NBS1 (D) and anti-MRE11 (E) and observed with fluorescent microscopy. Nuclear staining was performed with DAPI. Scale bars, 10 μm.
Fluorescent intensity in nuclear were measured in ten cells from three independent experiments with Image J software (n=10). The values are shown as mean ± S.D. **P<0.001. NT, no treatment.
Figure 1
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