ATM Induces Cell Death with Autophagy in Response to H₂O₂ Specifically in Caenorhabditis elegans Nondiving Cells

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1. Introduction

Ataxia-telangiectasia-mutated (ATM) kinase plays a critical role in the DNA damage response and DNA repair [1]. In response to DNA double-stranded breaks (DSBs), ATM is activated by autophosphorylation of serine 1981 and induces DNA repair, cell cycle arrest, and cell death together with the MRE11-RAD50-NBS1 (MRN) complex [2]. Dysfunction of ATM results in ataxia-telangiectasia (AT) in humans [3]. AT is an autosomal recessive inherited disorder with characteristic symptoms such as cerebellar ataxia, oculocutaneous telangiectasia, immunodeficiency, and cancer predisposition [3]. Nijmegen breakage syndrome (NBS), which is induced by the dysfunction of NBS1, is also an autosomal recessive inherited disorder with characteristic symptoms, such as immunodeficiency and cancer predisposition similar with AT [3]. Although NBS1 and ATM function in the same pathway, the cerebellar ataxia is not observed in NBS patients [3]. Therefore, ATM is considered to have additional roles to DNA damage response (DDR).

Recently, it was reported that the oxidation of cysteine 2991 of ATM results in the formation of disulfide bond between coupled cysteine 2991 of dimeric ATM followed by autophosphorylation of serine 1981 [4], which phosphorylate p53 or Chk2 in vitro. This suggests that ATM can be directly activated by reactive oxygen species (ROSs) without DSBs [4]. ROSs, such as superoxide anion radical (•O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (•OH), are generated by normal cell metabolism, drug treatments, and radiation [5].

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As lots of ROSs are generated in the cerebellum, the response of ATM to ROSs is expected to be related to the cerebellar ataxia in AT syndrome [6]. However, the physiological function of the oxidized active dimer of ATM in nondividing cells has not been determined.

In order to elucidate the physiological function of the response of ATM to ROSs in nondividing cells, we analyzed the function of ATM using Caenorhabditis elegans (C. elegans). C. elegans has been used as a model organism of aging and apoptosis [7]. In C. elegans, the cell fates have been completely determined, and adult hermaphrodites contain 959 nondividing somatic cells [8, 9]. Therefore, C. elegans is a good model animal for the analysis of the stress response in nondividing cells. Thus far, we have reported the function of DNA mismatch repair (MMR) unique to nondividing cells using C. elegans [10].

In previous studies, C. elegans ATM-1 (CeATM-1) was found to protect germ cells from γ-rays irradiation, suggesting that CeATM-1 functions in DSB repair as in mammals [11]. In addition, we previously reported that atm-1(tm5027) worms exhibited sensitivity to methyl methanesulfonate (MMS) at both larval and adult worm stages [10]. This suggests that ATM-1 is also required for DSB repair in both dividing and nondividing somatic cells.

Thus, C. elegans was expected as good animal model to investigate the response to ROSs of ATM in the neuron. In this study, we determined the function of ATM in nondividing cells and speculated the cause of the cerebellar ataxia.

2. Materials and Methods

2.1. C. elegans Strains and Culture Conditions. The wild-type strain (Bristol N2) [7], JK2739[lin-6(e1466) dpy-5(e61) I/hT2 [bli-4(e937) let-5(q782) qIs48] (IIIII)] [12], and MAH236; [lgg-1p::GFP::lgg-1+ odr-1p::RFP]) [13] were supplied by the Caenorhabditis Genetics Center (Minneapolis, USA). The atm-1(tm5027) mutant was supplied by the National BioResource Project (Tokyo, Japan) [10]. A deletion in the atm-1 gene was verified by PCR using two primer pairs listed in the Supplementary Table (available here). The atm-1(tm5027) mutant worms were backcrossed with Bristol N2 twice and maintained with the GFP balancer hT2 to avoid the accumulation of mutations [12]. The lgg-1 reporter strain (tm5027, [lgg-1p::GFP::lgg-1+ odr-1p::RFP]) was generated by crossing each strain. Worms were cultured on 50 mm NGM plates containing 0.3% (w/v) NaCl, 0.25% (w/v) poly-L-lysine, 0.005% (w/v) cholesterol, 1 mM CaCl2, 1 mM MgSO4, 25 mM potassium phosphate (pH 6.0), and 0.17% (w/v) agar with a lawn of Escherichia coli (E. coli) OP50 at 20°C [14].

2.2. Establishment of a Stable atm-1(tm5027) Worm Line. However, its contribution to somatic cells was not clarified. In order to analyze the function of CeATM-1 in somatic cells, we first established a stable maintenance system for atm-1(tm5027) worms, because it was previously reported that CeATM-1 contributes to genome integrity in C. elegans germ cells [11]. We backcrossed the atm-1(tm5027) worms with wild-type N2 worms twice, and then we crossed backcrossed atm-1(tm5027) worms with JK2739 (hT2) worms to keep the worms heterozygous [12]. We maintained atm-1(tm5027/hT2) heterozygous worms by picking GFP-positive worms until use and isolated atm-1(tm5027) homozygous worms for experiments by picking GFP-negative worms. Previous atm-1 knockdown worms exhibited normal growth [15, 16]. Backcrossed atm-1(tm5027) worms had the same percent growth (L1 to adult) as N2 worms, suggesting that background mutation was sufficiently restored (Figure S1).

2.3. Synchronizations of Worms. Starved L1 larvae were prepared in order to obtain synchronized worms as previously described [10]. In brief, worms on NGM plates were harvested and incubated in alkaline hypochlorite [500 mM NaOH and 1.2% (v/v) hypochlorite] until their bodies were completely dissolved (5–10 minutes). Eggs were then washed 3 times with S basal [50 mM potassium phosphate (pH 6.0) and 100 mM NaCl]. Eggs were hatched and synchronized by incubation at 20°C overnight without food.

2.4. L1 Growth Assay. The time-course drug treatments were performed using synchronized L1 worms as previously described [10]. The synchronized L1 larvae were treated with several drugs in M9 buffer at 20°C. Then, the worms were transferred to NGM plates and incubated for 4 days. Synchronized L1 worms were irradiated with UV and UVC on NGM plates and then incubated for 4 days. After 4 days, the percentage of worms that grew from L1 to adults was calculated. At least 150 animals were counted for each condition.

2.5. Adult Worm Drug Resistance Assay. Drug resistance assays using adult worms were performed as previously described [10]. Briefly, synchronized L1 larvae were cultured on NGM plates until they completely developed to the adult stage (4 days). They were harvested and then treated with drugs for 1 hour at 20°C in M9 buffer. They were transferred to NGM plates and incubated for 1 day. The percent survival was then calculated. At least 150 animals were counted for each condition.

2.6. AO Staining. AO staining was performed as previously described [10]. Briefly, synchronized L1 larvae were cultured on NGM plates until they developed completely to the adult stage (4 days). Adult worms were harvested and treated with drugs in M9 buffer for 1 hour at 20°C. The worms were then washed with M9 buffer twice and stained with 5 mg/ml acridine orange (AO) in M9 buffer for 5 minutes. The worms were destained twice with 1 ml of M9 buffer for 10 minutes and fixed with phosphate-buffered saline (pH 7.4) (PBS) containing 4% paraformaldehyde (PFA). After washing twice with 1 ml of M9 buffer, they were observed by fluorescence microscopy with excitation by a 488 nm argon laser. At least 150 animals were counted for each condition. The microscopy was performed with a Carl Zeiss LSM510 microscope (Carl Zeiss, Germany).
2.7. Reporter Assay. The Lgg-1 reporter assay was performed as previously described [10]. Briefly, Lgg-1 reporter (tm5027 [lgg-1p::GFP::lgg-1 + odr-1p::RFP]) adult worms were harvested with M9 buffer and treated with drugs for 7 hours at 20°C in M9 buffer. The worms were fixed with PBS containing 4% PFA for 10 minutes at 20°C. After washing twice with M9 buffer, the worms were observed by fluorescence microscopy with excitation by a 488 nm argon laser. At least 150 animals were counted for each condition. The microscopy was performed with a Carl Zeiss LSM510 microscope (Carl Zeiss, Germany).

2.8. Statistics. Qualitative data were representative data of at least three experiments. Unless otherwise noted, quantitative data were expressed as the mean ± S.D. The significance of differences was examined by Student’s t-test. p < 0.05 was considered significant.

3. Results

3.1. atm-1(tm5027) Worms Were Sensitive to DNA-Damaging Agents. Many studies using cultured mammalian cells have demonstrated that dysfunction of ATM results in sensitivity to ROSs and several DNA-damaging agents, including γ-rays and UVC [17, 18]. Previous studies using C. elegans reported that atm-1(tm5027) germ cells are sensitive to γ-rays and UVC [11, 19]. We also found that atm-1(tm5027) L1 larvae are sensitive to the SN1-type alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and SN2-type alkylating agent MMS [10]. However, the sensitivity to MNNG of atm-1(tm5027) larvae was dependent on mismatch repair (MMR), whereas sensitivity to MMS was not [10]. These differences in sensitivities to both types of alkylating agents suggest that the mechanism of DSB generation is largely dependent on other DNA repair pathways in C. elegans somatic cells. Therefore, a comprehensive analysis using different DNA-damaging agents is needed to understand which kinds of DNA damage generate DSBs. Thus, we first performed drug treatment assays using L1 larvae with several DNA-damaging agents.

We treated L1 larvae with γ-rays, UVC, and a crosslinking agent (mitomycin C; MMC). γ-rays induce DSBs in genomic DNA by two ways: direct breaking or indirect breaking via generation of ROSs [20]. UVC generates pyrimidine dimers in genomic DNA [21]. As pyrimidine dimers strongly block transcription, they result in DSBs [22, 23]. MMC is an antitumor drug that alkylates genomic DNA and forms interstrand crosslinking (ICL) [24]. Due to the high ability of ICL to block the progression of replication and transcription, the accumulation of ICLs leads to generation of DSBs during replication and transcription [25]. Different types of DNA-damaging agents all generate DSBs in different ways.

Using these different DSB sources, we assessed whether they induce DSBs in C. elegans dividing somatic cells. As shown in Figures 1(a)–1(c), atm-1(tm5027) larvae exhibited sensitivity to almost all of the DNA-damaging agents.

Interestingly, atm-1(tm5027) adult worms were sensitive to NaHSO₃, a deaminating agent that induces DSBs via formation of uracil in genomic DNA, but atm-1(tm5027) larvae were not (Figures S2A and B).

3.2. atm-1(tm5027) Adult Worms Exhibited Resistance to H₂O₂. Next, we evaluated the sensitivity of atm-1(tm5027) worms to ROSs using H₂O₂ and MV. H₂O₂ is a typical ROS generated in vivo [26] and is used in some signaling pathways [27, 28]. MV generates O₂⁻ in vivo [29, 30]. First, we tested the sensitivity of larvae, and atm-1(tm5027) larvae exhibited sensitivity to both H₂O₂ and MV (Figures 2(a) and 2(b)).

In general, ROSs also generate DSBs and activate cell cycle checkpoints in dividing cells [31, 32]. Therefore, because of the lack of cell cycle checkpoints, adult worms were expected to have different responses to ROSs from those of larvae. In order to analyze the response to ROSs of CeATM-1, we treated fully matured worms with H₂O₂ and MV.

Drug resistance assays using adult worms demonstrated that atm-1(tm5027) adult worms had significant resistance to H₂O₂, but similar sensitivity to MV as wild-type (Figures 2(c) and 2(d)).

3.3. CeATM-1 Induces Intestinal Cell Death in Response to H₂O₂ Treatment. In order to address the mechanisms of resistance of atm-1(tm5027) adult worms to H₂O₂, we performed dead cell imaging using acridine orange AO [33]. AO is a nonfluorescent dye that fluoresces only when it stably binds to nucleic acids [33]. As AO is actively exported out of the living cells, fluorescence is observed only in dead cells after sufficient destaining [33]. In nontreated worms, fluorescence was not detected in both wild-type and atm-1(tm5027) worms (Figure 3(a)). The head, which contains the pharynx and neurons, was severely injured by the MMS treatment in atm-1(tm5027) worms, whereas that of wild-type worms was not (Figures 3(a) and 3(b)). On the other hand, the intestines of wild-type worms were severely injured by the H₂O₂ treatment, whereas those of atm-1(tm5027) worms were not (Figures 3(a) and 3(b)).

For further confirmation, we next observed AO fluorescence under caffeine treatment. Caffeine is often used as an inhibitor of ATM and ATR proteins [34]. In a previous study, 1 mM caffeine decreased ATM kinase activity to approximately 20% [34]. We pretreated adult worms with 2.5 mM caffeine for 2 hours before the H₂O₂ treatment and then treated them with 88 mM H₂O₂ for 1 hour followed by AO staining. Caffeine at 2.5 mM did not affect wild-type or atm-1(tm5027) worms without H₂O₂ treatment (Figures 4(a) and 4(b)). In contrast, caffeine significantly suppressed the intestinal cell death in wild-type worms, but it did not in atm-1(tm5027) worms (Figures 4(a) and 4(b)). These results indicate that CeATM-1 induced intestinal cell death in response to H₂O₂.

3.4. CeATM-1 Induces Cell Death with Autophagy in Response to H₂O₂ Treatment. Next, we tried to identify the type of cell death induced by CeATM-1. In mammalian dividing cells, ATM generally induces apoptosis via phosphorylation of p53 [35]. However, in C. elegans, CEP-1
(p53 homologue in *C. elegans*) is abundantly expressed in dividing cells like germ cells, but is hardly expressed in nondividing somatic cells [36]. Therefore, we examined p53-independent cell death pathway. Previously, we reported that MMR induces cell death with autophagy in *C. elegans* nondividing somatic cells [10]. In addition, emerging evidence suggests that ATM plays key roles in autophagy, mitophagy, and pexophagy [37, 38]. Thus, we examined whether ATM induces cell death with autophagy in response to H2O2.

In *C. elegans*, the increase of LGG-1 (Atg8/LC3 homologue in *C. elegans*), a member of the autophagosome, is a marker of autophagy [39]. We made an *lgg-1* reporter strain (tm5027 [lgg-1p::GFP::lgg-1 + odr-1::RFP]) and performed reporter assays. The expression of *lgg-1* was elevated by H2O2 treatment in wild-type intestinal cells (Figures 5(a) and 5(b)). In contrast, the expression level of *lgg-1* was not increased in *atm-1(tm5027)* somatic cells, but increased expression of *lgg-1* was observed in the embryos held in *atm-1(tm5027)* adult worms (Figure 5(a)). In addition, we observed expression of *lgg-1* after the MMS treatment. The expression of *lgg-1* was elevated by MMS treatment in both the somatic cells and embryos of *atm-1(tm5027)* worms, but its induction was not observed in wild-type worms (Figures 5(a) and 5(b)). These results suggest that H2O2 and MMS induced cell death with autophagy.

### 4. Discussion

In the present study, we obtained interesting finding that *atm-1(tm5027)* adult worms were resistant to H2O2.

A recent study revealed that ATM is directly activated by H2O2 and becomes an active dimer [4]. Thus, ATM is considered to be a sensor of ROSs. In addition, ATM is known as a regulator of ROSs [37, 38]. Previous studies reported that ATM downregulates cellular ROS levels via phosphorylation of p53 [40]. Dysfunction of ATM is known to result in diabetes by increasing ROSs followed by abnormal activation of the ASK1/JNK pathway [41]. Therefore, ATM is considered to function as a sensor and direct regulator of ROSs.

As a further role, we found that ATM can induce cell death in response to H2O2 (Figure 3). H2O2 ATM dependently induced intestinal cell death. The lack of ATM in adult worms resulted in resistance to H2O2 (Figure 2(d)). On the other hand, *atm-1(tm5027)* larvae exhibited sensitivity to H2O2 (Figure 2(b)). This difference is considered to be due to the cell cycle checkpoints. ATM plays a central role in DDR, especially in DSB repair [2]. Therefore,
dividing cells that have dysfunctional ATM are sensitive to ROSs because ROSs induce DSBs in genomic DNA and activate cell cycle checkpoints [31, 32]. On the other hand, nondividing cells do not have cell cycle checkpoints. This demonstrates the advantage of *C. elegans* for analyzing the function of ATM.

Interestingly, *atm-1(tm5027)* adult worms did not exhibit sensitivity to O$_2^-$ (Figure 2(c)). Previously, it was reported that pretreatment of pyocyanin, one of the O$_2^-$-inducing chemicals, inhibits ATM activation induced by γ-rays irradiation [42]. As H$_2$O$_2$ and O$_2^-$ have different oxidation potentials [43], this difference implies that O$_2^-$ cannot induce ATM-1 signaling in response to ROSs due to too strong oxidation potentials, which may result in excessive oxidation of ATM.

Recent studies highlighted the regulation of autophagy by ATM. Alexandera et al. revealed that ATM induces autophagy via the LKB/AMPK/mTOR pathway in human cultured cells [44]. In addition, Qi et al. reported that Parkin accumulates in response to spermidine treatment followed by ATM activation, and mitophagy is activated [45]. In our study, *C. elegans* adult worms exhibited ATM-dependent cell death with autophagy (Figure 5). Our results suggest a new significance of induction of autophagy by ATM. However, cell death with autophagy may be unique to *C. elegans*. Because *C. elegans* adult somatic cells have the unique condition of little caspase or CEP-1, and ATM usually induces apoptosis via p53 in mammalian cells [1, 36], and abnormality of ATM induces ataxia-telangiectasia in humans (3), *atm-1(tm5027)* did not have obvious abnormalities in behavior.

Previously, we reported that MMR induces intestinal cell death with autophagy by MV treatment [10]. MMR also induced cell death with autophagy by MNNG treatment, but in the pharynx and neurons [10]. This type of tissue-specific response was also observed in CeATM-1 (Figures 3 and 5). As discussed in the previous report, the *C. elegans* intestine may be more sensitive to ROSs than other tissues, and how those differences are established needs to be elucidated.

In summary, we found that ATM induces cell death in response to H$_2$O$_2$. In general, cells lacking ATM are thought...
Figure 3: CeATM-1 induces intestinal cell death in response to H$_2$O$_2$. (a) Adult N2 and atm-1 (tm5027) worms were treated with 0.5% MMS or 88 mM H$_2$O$_2$ for 1 hour at 20°C. After the treatment, the worms were stained with AO for 5 minutes at 20°C and observed microscopically after destaining twice with M9 buffer and fixing with PFA. (b) The fraction of animals with somatic AO fluorescence. At least 150 animals were counted for each condition. All data are mean ± SD and * means significantly different by Student’s t-test (p < 0.05). The photographs of a worm and a microscope were obtained from TogoTV (© 2016 DBCLS TogoTV).

Figure 4: Caffeine prevents H$_2$O$_2$-dependent intestinal cell death. (a) Adult N2 and atm-1 (tm5027) worms were pretreated with caffeine (20 mM caffeine for at least 6 hours). Then, the worms were treated with 88 mM H$_2$O$_2$ for 1 hour at 20°C. After the treatment, the worms were stained with AO for 5 minutes at 20°C and observed microscopically after destaining twice with M9 buffer and fixing with PFA. (b) The fraction of animals with somatic AO fluorescence. At least 150 animals were counted for each condition. All data are mean ± SD and * means significantly different by a multiple-comparison one-way ANOVA (Tukey’s test) (p < 0.05).
to be sensitive to ROSs, but our results demonstrated that lack of ATM results in resistance to H\textsubscript{2}O\textsubscript{2} in nondividing conditions (Figure 2(d)). Thus, the lack of ATM-dependent cell death may be one reason why the cerebellar ataxia is observed in AT patients but not in NBS patients [3]. We are considering that the lack of these ATM-dependent cell deaths might be the one of the reasons of the cerebellar ataxia. However, why do the resistant AT patient’s cerebellar cells are dysfunctional is unknown. We hypothesize that ATM prevents necrosis, which injures other cells via release of lysosomal enzymes, by inducing cell deaths with autophagy in response to H\textsubscript{2}O\textsubscript{2}, protecting the tissue integrity.

5. Conclusion

In this study, we found that deficiency of ATM results in tolerance to H\textsubscript{2}O\textsubscript{2} in nondividing cells. We demonstrated that ATM can induce cell death in response to H\textsubscript{2}O\textsubscript{2}, but not O\textsubscript{2}\textsuperscript{−}, and this cell death is not apoptosis but cell death with autophagy.

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

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**Supplementary Materials**

Supplementary Table: the primers used in this study. These primers were used for the verification of the atm-1(tm5027) deletion allele. Supplementary Figure S1: percent growth of atm-1(tm5027) worms. The percent growth (L1 to L4). Data represent the mean ± S.D. from five independent experiments. N.S. means not significantly different (p value ≥ 0.05 by Student’s t-test). Supplementary Figure S2: drug resistance assay for NaHSO3. (A) Synchronized L1 larvae of N2 ( ) and atm-1(tm5027) ( ) were treated with 50 mM NaHSO3 at 20°C. After treatments, the worms were cultured for 4 days and we calculated the ratio of adult worms/transferred L1 worms. (B) Synchronized adult N2 (white bars) and atm-1(tm5027) (gray bars) worms were treated with 105 mM NaHSO3 for 1 hour at 20°C. 24 hours later, the percent survival was calculated. All data are mean ± S.D and * means significantly different by Student’s t-test (p < 0.05). (Supplementary Materials)

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