Protective roles for ATM in cellular response to oxidative stress

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Abstract ATM (ataxia telangiectasia mutated), the gene mutated in ataxia telangiectasia, is related to a family of large phosphatidylinositol 3-kinase domain-containing proteins involved in cell cycle control and DNA repair. We found that $ATM^{-/-}$ DT40 cells were more susceptible than wild-type cells to apoptosis induced not only by ionizing radiation and bleomycin but also by non-DNA-damaging apoptotic stimuli such as C_2-ceramide. Furthermore, the apoptosis induced by C_2-ceramide and H_2O_2 was blocked by anti-oxidants, indicating that the $ATM^{-/-}$ DT40 cells had a heightened susceptibility to apoptosis induced by reactive oxygen intermediates (ROI), presumably due to defective ROI-detoxification activities. In support of this hypothesis, we found that more ROI were generated in $ATM^{-/-}$ DT40 cells than in wild-type cells, following treatment with the above apoptotic stimuli. These results indicate that ATM plays important roles in the maintenance of the cell homeostasis in response to oxidative damage.

Key words: Reactive oxygen intermediate; Apoptosis; Ataxia telangiectasia mutated; Gene targeting

1. Introduction

Ataxia telangiectasia (A-T) is an autosomal recessive disorder with complex clinical features including progressive cerebellar ataxia, oculocutaneous telangiectasias, growth retardation, cellular and humoral immunodeficiency, increased predisposition to lymphoma and leukemias and gonadal abnormalities. Cells derived from A-T patients are characterized by a high level of chromosomal abnormalities, a defect that is greatly potentiated by ionizing radiation (IR) and by hypersensitivity to IR. In A-T cells, DNA damage fails to induce an arrest in DNA synthesis (leading to the phenomenon of radioresistant DNA synthesis) or an appropriate arrest at the G1/S or G2/M cell cycle checkpoints, suggesting anomalous cell cycle regulation as a major underlying cause of the disease [1,2]. The gene mutated in A-T (designated ATM, ataxia telangiectasia mutated) was identified recently and is a member of a family of large proteins that have a PI3 kinase domain at the C-terminus [3]. Several members of this family, including the yeast proteins Rad3, Mec1 and Tel1, are involved in DNA repair and cell cycle checkpoint control, further indicating an important role for ATM in chromosomal maintenance [4,5]. However, the roles for ATM in another important cellular response to DNA damage, apoptosis, are less clear and conflicting results from experiments using cells derived from A-T patients and ATM-knockout mice have been reported [6–11]. Furthermore, the molecular mechanisms underlying the apoptosis of postmitotic cerebellar Purkinje cells in A-T patients in the absence of DNA damage have remained elusive. Because DNA-damaging agents such as IR generate reactive oxygen intermediates (ROI) which damage macromolecules and induce cell death [12], ATM abnormalities may lead to apoptotic cell death due to oxidative damage. Consistent with this hypothesis, decreased levels of catalase and increased levels of lipid hydroperoxides were observed in several A-T cell lines [13] and increased levels of oxidant-modified proteins and lipids were detected in tissues, including brain, of ATM-knockout mice [14].

To further study the roles played by ATM in the cellular reaction to oxidative damage, we used an isogenic set of stable cell lines differing only in their ATM status, which we created previously from the chicken B cell line DT40. Although these stable DT40 cell lines, as with most transformed chicken cell lines, do not express p53, they display many of the characteristics of A-T cells, most notably a hypersensitivity to IR [15]. We found that $ATM^{-/-}$ DT40 cells are more susceptible to apoptotic cell death induced by oxidative stress and that $ATM^{-/-}$ DT40 cells show an increased intracellular ROI generation following oxidative stress. These results indicate that ATM plays important roles in the maintenance of cell homeostasis in response to oxidative damage.

2. Materials and methods

2.1. Cell culture and analysis of apoptosis

The generation of $ATM^{-/-}$ mutant clones was described previously [15]. Wild-type and $ATM^{-/-}$ DT40 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (Equitech-Bio.), 1% chicken serum (Sigma), 2 mM L-glutamine, 10^{-5} M β-mercaptoethanol and penicillin/streptomycin at 39.5°C in a humidified 95% air/5% CO_2 atmosphere. X-ray irradiation was carried out at room temperature using an X-ray irradiator (Hitachi) at 150 kV with 0.5 mm Cu+0.5 mm Al filtration at a dose of 1.00 Gy/min. Four hours after treatment with either 4 Gy X-ray irradiation, 100 μM H_2O_2, 100 μM C_2-ceramide (Sigma) or 100 μg/ml bleomycin (Wako), the proportion of apoptotic cells was determined, using an ApoAlert Annexin V Apoptosis kit (Clontech). Apoptotic morphologic changes were analyzed by acridine orange staining as described previously [16].

2.2. Analysis of intracellular oxidant production and anti-oxidative enzyme activities

Intracellular oxidant production was analyzed by flow cytometry using a DCFH-DA dye [17]. In brief, 1×10^6 cells were washed with HBSS and treated with either 4 Gy X-ray irradiation, 100 μg/ml bleomycin or 100 μM C_2-ceramide. After 2 h, the cells were stained with 5 μM DCFH-DA for 30 min at 39.5°C, then analyzed by flow cytometry (FACScan, Becton-Dickinson). The specific activities of cellular catalase and glutathione peroxidase were measured as described previously [18].

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Abbreviations: A-T, ataxia telangiectasia; ATM, ataxia telangiectasia mutated; DSB, double-stranded break; IR, ionizing radiation; PI, phosphatidylinositol; ROI, reactive oxygen intermediates

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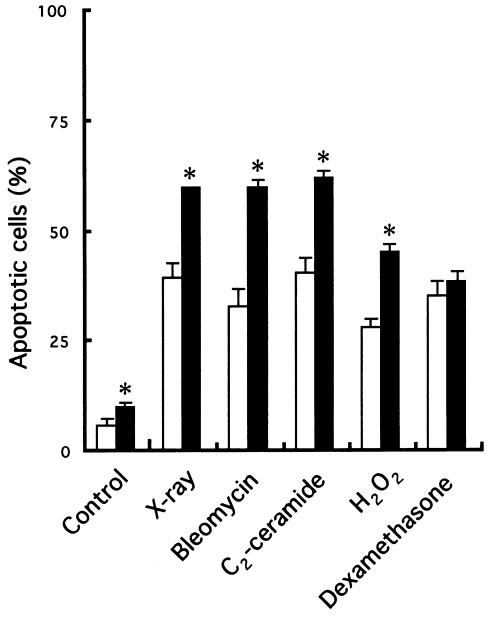

Fig. 1. Flow cytometric analysis of apoptosis by Annexin V staining. Wild-type (□) and ATM$^{-/-}$ (■) DT40 cells were treated with either IR (4 Gy), bleomycin (100 μg/ml), C_2-ceramide (100 μM), H_2O_2 (100 μM) or dexamethasone (10^{-8} M). After 4 h, the cells were stained with Annexin V for 15 min, then analyzed using flow cytometry. Data are expressed as mean ± S.D. ($n = 3$). *$P < 0.05$, treated ATM$^{-/-}$ cells compared with wild-type DT40 cells. Similar results were obtained in two consecutive experiments with two different ATM$^{-/-}$ DT40 clones.

2.3. Karyotypic analysis

For karyotype analysis, colcemid (Gibco-BRL) was added to the culture medium to a concentration of 0.1 μg/ml for 3 h, after which metaphase spreads were placed on glass slides and Giemsa-stained as described previously [19].

3. Results and discussion

Since markedly decreased clonogenic survival following IR is a hallmark of cells from A-T patients and was reproduced in ATM$^{-/-}$ DT40 cells, we first analyzed the effects of ATM disruption on IR-induced apoptosis. To eliminate possible effects due to decreased clonogenic survival following IR [15], we monitored the initial phase of apoptosis, using Annexin V staining at 4 h following IR and subsequent flow cytometric analysis. As shown in Fig. 1, the frequencies of both spontaneous and IR-induced apoptosis were significantly higher in ATM$^{-/-}$ than in wild-type DT40 cells. In addition, ATM$^{-/-}$ DT40 cells were also more susceptible to apoptosis induced by another double-stranded break (DSB)-inducing agent, bleomycin. We then studied the susceptibility of ATM$^{-/-}$ DT40 cells to apoptosis induced by C_2-ceramide or H_2O_2, since IR generates ceramides and ROI, which are possible mediators of apoptosis [12,20–22]. As shown in Fig. 1, ATM$^{-/-}$ DT40 cells were also more susceptible to apoptosis induced by these agents than were wild-type cells. In addition, when cells were exposed continuously to low levels of H_2O_2 using a glucose oxidase system (20 mUnits/ml glucose oxidase and 1 M glucose), the proportions of apoptotic cells were significantly higher in ATM$^{-/-}$ DT40 cells (57.8 ± 2.20%) than in wild-type DT40 cells (34.9 ± 2.00%). However, there was no significant difference between ATM$^{-/-}$ and wild-type DT40 cells in the frequency of apoptosis following dexamethasone (Fig. 1) or UV irradiation (data not shown), indicating that ATM$^{-/-}$ DT40 cells had increased susceptibility only to some specific types of apoptotic stimuli. Essentially similar results were obtained in the analysis of apoptotic morphologic changes with acridine orange staining (data not shown).

We were interested in whether the apoptosis that followed C_2-ceramide and H_2O_2 treatment induced DNA damage to trigger apoptosis. As shown in Fig. 2, IR and bleomycin induced chromosomal breaks, the frequencies of which were highly elevated in ATM$^{-/-}$ DT40 cells, as reported previously [15]. However, C_2-ceramide did not induce significant chromosomal breaks. (We could not determine the extent of chromosome breaks with H_2O_2 for technical reasons.) It is therefore unlikely that the apoptosis induced by C_2-ceramide was due to DNA damage.

ATM$^{-/-}$ DT40 cells were more susceptible to H_2O_2-induced apoptosis than wild-type cells (Fig. 1), and ROI is implicated in ceramide-induced apoptosis [23,24]. The results of a recent study further suggest that ATM-deficient cells are more susceptible to oxidative damage than wild-type cells [14]. Taken together, these results raise the interesting possibility that more ROI are generated in ATM-deficient cells due to a defective ROI-detoxification mechanism. We therefore determined the amount of ROI generation in ATM$^{-/-}$ and wild-type DT40 cells, following apoptotic stimuli (IR, bleomycin, and C_2-ceramide): 2 h after treatment with these agents, intracellular oxidant products were measured using DCFH-DA dye staining. As shown in Fig. 3, intracellular oxidant products were significantly increased in ATM$^{-/-}$ DT40 cells compared with wild-type DT40 cells. Furthermore, the addition of catalase (1 mg/ml) or *N*-acetyl cysteine (5 mM) to cell cultures stimulated with C_2-ceramide and H_2O_2 inhibited not only

Table 1
Effect of anti-oxidants on apoptosis in wild-type and ATM$^{-/-}$ DT40 cells

Treatment	Wild-type			ATM$^{-/-}$		
	None	+Catalase	+NAC	None	+Catalase	+NAC
Control	6.6 ± 0.46	6.5 ± 1.78	5.7 ± 0.77	9.5 ± 1.11	9.5 ± 0.35	8.7 ± 1.15
X-ray	36.4 ± 3.21	13.3 ± 0.68	19.0 ± 0.58	58.3 ± 2.45	27.3 ± 2.58	38.5 ± 4.11
Bleomycin	29.2 ± 3.28	12.1 ± 0.93	17.1 ± 1.53	59.2 ± 1.86	28.7 ± 1.95	41.2 ± 2.25
C_2-ceramide	35.2 ± 2.41	7.1 ± 0.90	15.3 ± 0.96	49.4 ± 3.56	8.4 ± 0.76	25.2 ± 1.17
H_2O_2	28.1 ± 0.91	7.3 ± 0.92	7.2 ± 0.87	44.9 ± 0.92	8.6 ± 0.65	8.5 ± 0.64

Wild-type and ATM$^{-/-}$ DT40 cells had received X-ray irradiation (4 Gy) or were treated with bleomycin (100 μg/ml), C_2-ceramide (100 μM) or H_2O_2 (100 μM) with or without anti-oxidants (catalase: 1 mg/ml, NAC: 5 mM). After 4 h, the cells were stained with Annexin V for 15 min, and then analyzed with flow cytometry. Data are expressed as mean ± S.D. ($n = 3$). Similar results were obtained in two consecutive experiments.

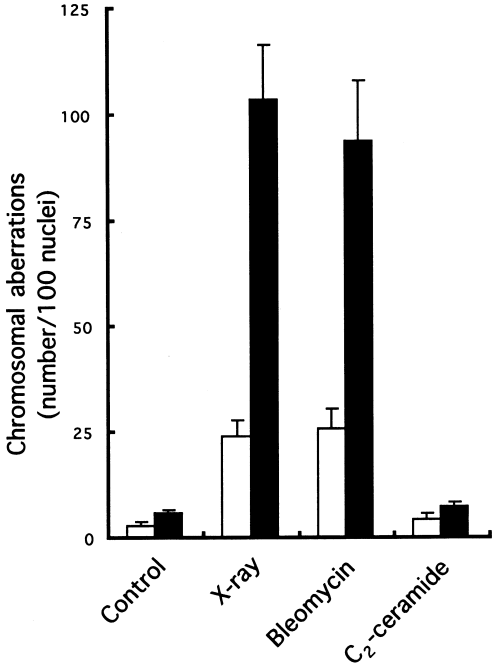

Fig. 2. Frequencies of chromosomal aberrations following apoptotic stimuli. Wild-type (□) and ATM$^{-/-}$ (■) DT40 cells were treated with colcemid for 3 h following treatment with either IR (4 Gy), bleomycin (100 μg/ml), or C_2-ceramide (100 μM). Data are presented as macrochromosomal (1–5 and Z) aberrations per 100 metaphase spreads, and expressed as mean ± S.D. ($n = 3$).

intracellular oxidant production (data not shown) but also apoptosis (Table 1), indicating that ROI are important mediators for the apoptosis induced by these agents. However, while the addition of these anti-oxidants to cell cultures stimulated with IR and bleomycin inhibited intracellular oxidant production (data not shown), these anti-oxidants were only partially effective in inhibiting the apoptosis induced by these agents (Table 1). To study further possible mechanisms for the increased susceptibility of ATM$^{-/-}$ DT40 cells to oxidative stress, we measured catalase and glutathione peroxidase activities in wild-type and ATM$^{-/-}$ DT40 cells. As shown in Table 2, reduced levels of catalase activity were observed in ATM$^{-/-}$ DT40 cells in agreement with the study on cells derived from A-T patients [13], though there was no significant difference in glutathione peroxidase activity between wild-type and ATM$^{-/-}$ DT40 cells.

Although markedly decreased clonogenic survival following IR is consistently seen in cells from A-T patients, conflicting results on IR-induced apoptosis have been reported with cells derived from A-T patients and ATM-knockout mice [6–11]. Recently, A-T cells have been shown to be more susceptible to IR-induced apoptosis due to enhanced ceramide generation [25]. In the present study, we have shown that ATM$^{-/-}$ DT40 cells are more susceptible than wild-type cells to apoptosis induced not only by IR and bleomycin but also by C_2-ceramide (Fig. 1). We further showed that, unlike IR and bleomycin, which induce DSB, C_2-ceramide does not induce significant DSB (Fig. 2), indicating that ATM$^{-/-}$ DT40 cells are also more sensitive to the apoptosis induced by non-DNA-damaging apoptotic stimuli. Although the precise mechanism underlying ceramide-induced apoptosis is still poorly defined [26,27], ROI are candidate mediators [23,24]. In support of this hypothesis, we found that ATM$^{-/-}$ DT40 cells also are more susceptible to H_2O_2-induced apoptosis (Fig. 1), and that the apoptosis induced by C_2-ceramide and H_2O_2 is completely blocked by anti-oxidants such as catalase or *N*-acetyl cysteine (Table 1). Several groups of investigators recently reported evidence that ATM-deficient cells are more susceptible than wild-type cells to oxidative damage [13,14,28]. These results raise the interesting possibility that ATM is involved in ROI-detoxification. In support of this hypothesis, we found that more ROI are generated in ATM$^{-/-}$ DT40 cells than in wild-type DT40 cells, following treatment with several apoptotic stimuli (Fig. 3), and that a reduced level of catalase activity

Table 2
Anti-oxidative enzyme activities in wild-type and ATM$^{-/-}$ DT40 cells

	Wild-type	ATM$^{-/-}$
Catalase (Units/mg protein)	1.6 ± 0.92	0.19 ± 0.15*
Glutathione peroxidase (mUnits/mg protein)	16.7 ± 0.42	16.7 ± 0.07

Wild-type and ATM$^{-/-}$ DT40 cells were washed with PBS(−) twice and suspended in PIPA buffer (0.01% digitonin, 0.25% sodium cholate). Catalase and glutathione peroxidase activities in cells were determined as described in Section 2. Data are expressed as mean ± S.D. ($n = 3$). *$P < 0.05$, treated compared to wild-type DT40 cells. Similar results were obtained in two consecutive experiments.

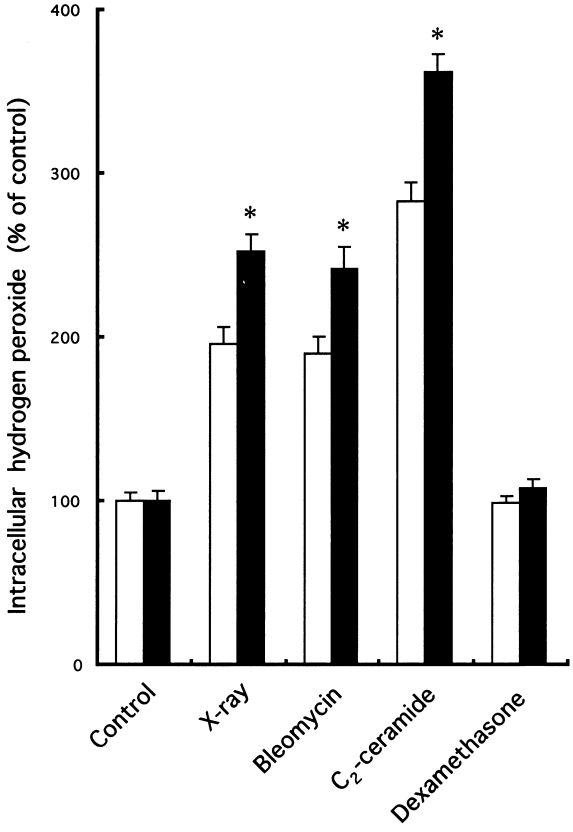

Fig. 3. Production of intracellular oxidants in response to apoptotic stimuli. Wild-type (□) and ATM$^{-/-}$ (■) DT40 cells were treated with either IR (4 Gy), bleomycin (100 μg/ml), C_2-ceramide (100 μM) or dexamethasone (10^{-8} M). After 2 h, the cells were stained with DCFH-DA (5 μM) for 30 min at 39.5°C, then analyzed using flow cytometry. Data are expressed as mean ± S.D. ($n = 3$). *$P < 0.05$, treated ATM$^{-/-}$ cells compared with wild-type DT40 cells. Similar results were obtained in two consecutive experiments with two different ATM$^{-/-}$ DT40 clones.

was observed in ATM$^{-/-}$ DT40 cells (Table 2). These results are also consistent with those of a recent study that showed increased levels of oxidant-modified products, decreased levels of catalase and enhanced heme oxygenase activity in ATM-deficient cells and tissues [13,14]. However, at the present time, we cannot exclude the possibility that ATM also functions in anti-apoptotic pathways. Further investigation will be required to define how ATM functions in the cellular response to oxidative stress.

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