The Ataxia telangiectasia Gene Product Is Required for Oxidative Stress-induced G1 and G2 Checkpoint Function in Human Fibroblasts*

Received for publication, December 14, 2000, and in revised form, March 19, 2001
Published, JBC Papers in Press, April 4, 2001, DOI 10.1074/jbc.M011303200

Rodney E. Shackelford, Cynthia L. Innes, Stella O. Sieber, Alexandra N. Heinloth, Steven A. Leadon§, and Richard S. Paules‡

From the Growth Control and Cancer Group, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709 and the §Department of Radiation Oncology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Ataxia telangiectasia (AT) is an autosomal recessive disorder characterized by neuronal degeneration accompanied by ataxia, telangiectasias, acute cancer predisposition, and sensitivity to ionizing radiation (IR). Cells from individuals with AT show unusual sensitivity to IR, severely attenuated cell cycle checkpoint functions, and poor p53 induction in response to IR compared with normal human fibroblasts (NHFs). The gene mutated in AT (ATM) has been cloned, and its product, pATM, has IR-inducible kinase activity. The AT phenotype has been suggested to be a consequence, at least in part, of an inability to respond appropriately to oxidative damage. To test this hypothesis, we examined the ability of NHFs and AT dermal fibroblasts to respond to t-butyl hydroperoxide and IR treatment. AT fibroblasts exhibit, in comparison to NHFs, increased sensitivity to the toxicity of t-butyl hydroperoxide, as measured by colony-forming efficiency assays. Unlike NHFs, AT fibroblasts fail to show G1 and G2 phase checkpoint functions or to induce p53 in response to t-butyl hydroperoxide. Treatment of NHFs with t-butyl hydroperoxide activates pATM-associated kinase activity. Our results indicate that pATM is involved in responding to certain aspects of oxidative damage and in signaling this information to downstream effectors of the cell cycle checkpoint functions. Our data further suggest that some of the pathologies seen in AT could arise as a consequence of an inability to respond normally to oxidative damage.

EXPERIMENTAL PROCEDURES

Cell Cultures and Culture Conditions—NHFs are normal human fibroblast cultures derived from foreskins of apparently healthy neonates and were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) and at passages 13–19 (27). GM03349, a normal dermal fibroblast strain from a 10-year-old male, was obtained from NIGMS (National Institutes of Health) Human Genetic Mutant Cell Repository (Camden, NJ) and at passages 15–19. ATM-deficient fibroblasts were obtained from the NIGMS Human Genetic Mutant Cell Repository (strain designations GM02052 and GM03395 (Camden, NJ)) and the NIA Aging Cell Repository (strain AG03658 (Camden, NJ)). The donor for the cells of strain GM02052 was a 15-year-old Moroccan female. Cells of this strain contain a mutation at nucleotide 103 that carry one defective copy of the AT gene has been estimated to be around 0.5–1% of the general population (2). Those AT heterozygotes have been reported to exhibit elevated cancer risk, particularly for breast cancer and lymphoproliferative disease (3–5). Cultured AT dermal fibroblasts show increased chromosomal instability and acute sensitivity to IR in comparison to age-matched normal human fibroblasts (NHFs) (6–10). Cells from individuals with AT exhibit poor p53 induction and severely impaired G1, S, and G2 phase checkpoint functions in response to IR (11–14). The gene mutated in AT, ATM, has been identified (15), and the gene product, pATM, has been shown to have IR-inducible protein kinase activity (16, 17). ATM shares homology with the family of phosphatidylinositol 3’-kinases (15), which include DNA-PK, ATR, MEC1, TEL1, TOB, and FRAP among others (18). Members of this family of proteins have been reported to be involved in various aspects of the detection of DNA damage and control of cell cycle progression (18, 19).

pATM has been suggested to function, at least in part, in the cellular response to oxidative damage (for review see Ref. 20). Support for this hypothesis comes from observations that pATM-deficient cells are unusually sensitive to the toxic effects of hydrogen peroxide, nitric oxide, and superoxide as determined by colony-forming efficiency assays. Additionally, they resynthesize glutathione unusually slowly after depletion with diethyl maleate (21–25). Furthermore, Barlow and colleagues (26) have shown that ATM-deficient mice have elevated markers of oxidative stress, particularly in organs such as the cerebellum, which are consistently affected in individuals with AT. Therefore, we hypothesized that pATM-deficient fibroblasts would lack normal cell cycle checkpoint function in response to oxidative stress. To test this hypothesis, we compared the effect of IR, and reactive oxygen species produced by treatment with t-butyl hydroperoxide, on normal and ATM-deficient human fibroblast strains.

This paper is available online at http://www.jbc.org

This is an Open Access article under the CC BY license.
the cells of strain AG03058 are dermal fibroblasts cultured from a skin biopsy from a 14-year-old black female. The exact mutations of ATM in strains GM03395 and AG03058 are not known. However, no pATM could be detected by immunoblotting in protein extracts from any of these strains, and the donors expressed the typical phenotype of the AT disease. Therefore, cells were used at passages 14–20. To establish an independent protein kinase-inhibitory peptide (pATM) and Oxidative Stress-induced Checkpoints

pATM and Oxidative Stress-induced Checkpoints

in vitro

Affinity Purification of pATM Antibody, aATM 7 a.p.—In order to isolate the highest affinity, highest specificity aATM antibodies from the rabbit polyclonal antiserum, antibody was affinity-purified using the original peptide immunogen. Peptide corresponding to pATM residues 826–840 (pATM peptide) was immobilized to generate a peptide affinity column and tumbled overnight at 4 °C.

Affinity Purification of pATM Antibody, aATM 7 a.p.—In order to isolate the highest affinity, highest specificity aATM antibodies from the rabbit polyclonal antiserum, antibody was affinity-purified using the original peptide immunogen. Peptide corresponding to pATM residues 826–840 (pATM peptide) was immobilized to generate a peptide affinity column and tumbled overnight at 4 °C.

Affinity Purification of pATM Antibody, aATM 7 a.p.—In order to isolate the highest affinity, highest specificity aATM antibodies from the rabbit polyclonal antiserum, antibody was affinity-purified using the original peptide immunogen. Peptide corresponding to pATM residues 826–840 (pATM peptide) was immobilized to generate a peptide affinity column and tumbled overnight at 4 °C.

Affinity Purification of pATM Antibody, aATM 7 a.p.—In order to isolate the highest affinity, highest specificity aATM antibodies from the rabbit polyclonal antiserum, antibody was affinity-purified using the original peptide immunogen. Peptide corresponding to pATM residues 826–840 (pATM peptide) was immobilized to generate a peptide affinity column and tumbled overnight at 4 °C.

Affinity Purification of pATM Antibody, aATM 7 a.p.—In order to isolate the highest affinity, highest specificity aATM antibodies from the rabbit polyclonal antiserum, antibody was affinity-purified using the original peptide immunogen. Peptide corresponding to pATM residues 826–840 (pATM peptide) was immobilized to generate a peptide affinity column and tumbled overnight at 4 °C.
1× PBS overnight at 4 °C. The peak fractions were verified by Western blotting of total protein extracts from normal and ATM-deficient fibroblasts that had been resolved by 6% SDS-PAGE (acylamide/bisacylamide ratio of 100:1). Affinity-purified αATM 7 (αATM 7 a.p.) antibody was incubated for 30 min prior to its addition to 1.5-ml microcentrifuge tubes, and quick-frozen in dry ice/ethanol until assayed. Determination of thymine glycol formation was performed as described previously (29). Each experimental point was performed in triplicate.

RESULTS

ATM Fibroblast Strains Are Hypersensitive to the Toxic Effects of t-Butyl Hydroperoxide—To examine the relative toxicity of reactive oxygen species exposure between normal and pATM-deficient fibroblasts, we treated different fibroblast strains with t-butyl hydroperoxide. We employed t-butyl hydroperoxide as a source of oxidative stress as it is poorly hydrolyzed by catalase (30). We reasoned that this is important since catalase activity has been reported to be low in ATM-deficient cells (31, 32) and thus, using a peroxide that can be hydrolyzed by catalase, would introduce experimental variation due to differences in cellular catalase activities.

To initiate these studies, two normal and two ATM-deficient fibroblast strains were treated with increasing levels of IR, and toxicity was assayed by colony-forming efficiency. As reported previously (7), exposure to increasing amounts of IR inhibited colony formation in ATM-deficient fibroblast strains more effectively than in NHFs (Fig. 1A). To compare the effects of t-butyl hydroperoxide on normal and ATM-deficient fibroblasts, normal NHF1 and GM03349 cells and ATM-deficient

![Image](pATM and Oxidative Stress-induced Checkpoints 21953)
GM02052 and GM03395 cells were treated with increasing concentrations of t-butyl hydroperoxide. As shown in Fig. 1B, the ATM-deficient fibroblasts were more sensitive to the colony-forming inhibiting effects of t-butyl hydroperoxide than the normal fibroblasts. LC50 (lethal concentration for 50% of population) for both normal fibroblast strains was in the 40–50 μM t-butyl hydroperoxide range, whereas the LC50 for the ATM-deficient dermal fibroblast strains was in the 6–8 μM range (Fig. 1B). The colony-inhibiting effect of t-butyl hydroperoxide in both normal and ATM-deficient fibroblast strains was biphasic, with an initial high sensitivity to low concentrations of t-butyl hydroperoxide (1–10 μM), followed by less sensitivity at higher concentrations (>10 μM). This apparent biphasic response is pATM-independent and suggests to us that higher concentrations of t-butyl hydroperoxide induce a pATM-independent resistance or adaptive response to the effects of t-butyl hydroperoxide in both cell types (Fig. 1B, compare 1–10 μM to 10–300 μM t-butyl hydroperoxide).

Peroxides are thought to exert some of their damaging effects through the production of reactive oxygen intermediates via events such as the Fenton reaction (for review see Ref. 33). We pretreated fibroblasts with mannitol for 1 h prior to t-butyl hydroperoxide treatment in order to ascertain if the colony-inhibiting effects of t-butyl hydroperoxide could be reduced by co-treatment with an antioxidant. Mannitol, which is effective at scavenging hydroxyl radicals (34), was used as an antioxidant. As shown in Fig. 1C, pretreatment with mannitol partially inhibited the killing effect of t-butyl hydroperoxide on both normal and ATM-deficient fibroblast strains. For most concentrations examined, the differences between treatment with and without mannitol are significant. However, pretreatment with mannitol of the ATM-deficient cells showed less reduction of killing following treatment with the highest concentration of t-butyl hydroperoxide (100 μM) (Fig. 1C). This is likely to be due to the very low number of ATM-deficient cells that can still form colonies at this concentration of t-butyl hydroperoxide. Nevertheless, these data show that the toxic effect of t-butyl hydroperoxide can be partially reversed by pretreatment with an antioxidant.

**TABLE I**

| Treatment/concentration | Relative percentage of cells in early S phase* | ATM-normal NHF1 | ATM-deficient AG03058 |
|-------------------------|----------------------------------------------|----------------|-----------------------|
| **A. t-Butyl-OOH**      |                                              |                |                       |
| 0 μM                    | 100 (±8)                                     | 100 (±8)       |                       |
| 10 μM                   | 100 (±7)                                     | 104 (±10)      |                       |
| 30 μM                   | 70 (±11)                                     | 117 (±4)       |                       |
| 100 μM                  | 54 (±5)                                      | 93 (±5)        |                       |
| **B. γ-IR**             |                                              |                |                       |
| 0 Gy                    | 100 (±7)                                     | 100 (±7)       |                       |
| 3.0 Gy                  | 19 (±9)                                      | 96 (±6)        |                       |

* Percentage of treated cells in early S phase relative to the percentage of mock-treated control cells in early S phase.

______________________________________________________________________________________________

**Fig. 2. Flow cytometric analysis of G1 checkpoint delay in response to t-butyl hydroperoxide exposure and IR treatment in ATM-normal NHF1 and ATM-deficient AG03058 fibroblasts.** Exponentially growing cells were treated as indicated. Four hours after treatment, BrdUrd (BrdU) was added for the last 2 h of incubation. Cells were fixed and stained with α-BrdUrd-fluorescein isothiocyanate and propidium iodide. Dot plots show incorporation of BrdUrd into DNA as an indication of DNA synthesis and propidium iodide fluorescence as an indication of DNA content. The regions drawn represent areas from which data were taken for analysis.

The same concentrations of t-butyl hydroperoxide (Table I and Fig. 2). As demonstrated previously (11, 35), normal fibroblasts exhibit G1 checkpoint arrest in response to IR, whereas ATM-deficient fibroblasts did not (Table I and Fig. 2). p53 protein stabilization and activation, which is necessary for the elicitation of full G1 checkpoint response to cellular damage from IR exposure for more than a transient period (36–38), is severely attenuated in ATM-deficient cells (35–37). Since ATM-deficient cells exhibit poor p53 induction in response to IR and fail to show a G1 checkpoint function in response to both IR and t-butyl hydroperoxide treatment, we hypothesized that p53 induction in response to t-butyl hydroperoxide treatment would similarly be poor in AT fibroblasts.
blasts compared with NHFs. When p53 immunoprecipitations were performed, followed by Western blotting with a second anti-p53 antibody, we found that p53 was induced in NHF1 cells in response to both t-butyl hydroperoxide and IR (Fig. 3). p53 induction in response to t-butyl hydroperoxide was slower than induction in response to IR, with significant induction following IR exposure by 1 h and induction by t-butyl hydroperoxide peaking later, ~1–2 h (Fig. 3). At the time points examined here, neither IR nor t-butyl hydroperoxide treatment significantly induced p53 protein levels in the ATM-deficient fibroblast strain GM02052 (Fig. 3).

p33<sup>CDK2</sup>/cyclin E-associated kinase activity has been shown to be required for progression through late G<sub>1</sub> and into S phase (39). To test the effect of t-butyl hydroperoxide on p33<sup>CDK2</sup>/cyclin E-associated kinase activity, we treated the NHF1 fibroblast strain with either 300 μM t-butyl hydroperoxide or 3.0 Gy IR, followed by incubation for 1–10 h, and we measured p33<sup>CDK2</sup>/cyclin E histone H1 <em>in vitro</em> kinase activity. As shown in Fig. 4, both treatments resulted in p33<sup>CDK2</sup>/cyclin E histone H1 <em>in vitro</em> kinase activity suppression. Maximal inhibition occurred at 6 h, followed by partial recovery at 10 h. The inhibition observed following treatment of NHF1 cells with 300 μM t-butyl hydroperoxide was less than that with exposure to 3.0 Gy IR and occurred with delayed kinetics compared with IR treatment (compare Fig. 4, A and B). When NHF1 cells were treated with t-butyl hydroperoxide over a 10 μM to 1 μM range and incubated for 6 h, p33<sup>CDK2</sup>/cyclin E histone H1 <em>in vitro</em> kinase activity was found to fall in a concentration-dependent manner, with kinase activity reduced ~70% at concentrations of 100 μM t-butyl hydroperoxide and above (data not shown).

When the ATM-deficient dermal fibroblast strain GM02052 was treated with 300 μM t-butyl hydroperoxide or 3.0 Gy IR, followed by incubation for 1–10 h, the ATM-deficient fibroblasts failed to exhibit significant inhibition of kinase activity by either agent (Fig. 4, A and B).

<em>T-Butyl Hydroperoxide Exposure Induces a G<sub>2</sub> Checkpoint Response in Normal Fibroblasts That Is Defective in Fibroblasts Lacking pATM Function</em>—Exposure of logarithmically growing cells from the normal fibroblast strain NHF1 to 1.5 Gy IR resulted in a rapid delay of entry into mitosis 2 h post-treatment, with a reduction in the mitotic index to only 3% (~±10%) of that of the mock-treated cells. ATM-deficient fibroblast cells (GM02052) showed no significant reduction in the mitotic index relative to mock-treated controls (94 ± 2%), in agreement with previous findings (40). Similarly, exposure of NHF1 cells to 10–300 μM t-butyl hydroperoxide generated a strong G<sub>2</sub> checkpoint response (Fig. 5). Under the same conditions over the same concentration range of t-butyl hydroperoxide treatment, no significant inhibition of entry into mitosis was observed with the ATM-deficient fibroblast strain cells (GM02052) 2 h post-treatment (Fig. 5).

To explore further the G<sub>2</sub> checkpoint response to t-butyl hydroperoxide treatment, we performed p34<sup>CDC2</sup>/cyclin B histone H1 <em>in vitro</em> kinase activity assays on protein extracts from two normal and two ATM-deficient fibroblast strains. As shown in Fig. 6A, p34<sup>CDC2</sup>/cyclin B histone H1 <em>in vitro</em> kinase activity was suppressed in a concentration-dependent manner 2 h post-treatment with 1–300 μM t-butyl hydroperoxide in both normal human fibroblast strains, NHF1 and GM03349. Similarly, exposure to 1.5 Gy IR inhibited p34<sup>CDC2</sup>/cyclin B histone H1 <em>in vitro</em> kinase activity from normal human fibroblasts, as we have previously reported (40, 41) (Fig. 6B). Neither treatment with t-butyl hydroperoxide at any concentration nor exposure to 1.5 Gy IR caused the two ATM-deficient fibroblast strains, GM02052 and GM03349, to display a significant suppression of p34<sup>CDC2</sup>/cyclin B histone H1 <em>in vitro</em> kinase activity from normal human fibroblasts, as we have previously reported (40, 41) (Fig. 6B). A representative p34<sup>CDC2</sup>/cyclin B histone H1 <em>in vitro</em> kinase assay for NHF1 and GM02052 fibroblasts is shown in Fig. 6C.

To investigate the possibility that the ATM-deficient fibroblast strains were capable of exhibiting a G<sub>2</sub> checkpoint function but with delayed kinetics in response to exposures of t-butyl hydroperoxide, a time course analysis of p34<sup>CDC2</sup>/cyclin B histone H1 <em>in vitro</em> kinase activity following treatment was performed. The results indicate that treatment with 300 μM...
t-butyl hydroperoxide and exposure to 1.5 Gy IR were both effective at suppressing p34\(^{CDCl}_2\)/cyclin B histone H1 kinase activity in NHF1 cells at 1 h post-treatment, with maximal suppression of kinase activity occurring at 2 h. Activity recovered to roughly untreated levels by 6 h (Figs. 7, A and B). These kinetics closely resemble those reported previously for the suppression of mitotic entry by 1.5 Gy IR (40). Neither ATM-deficient fibroblast strain (GM02052 and GM03395) showed significant suppression of p34\(^{CDCl}_2\)/cyclin B histone H1 in vitro kinase activity in response to either treatment with 300 \(\mu M\) t-butyl hydroperoxide or exposure to 1.5 Gy IR at any time points examined (Fig. 7, A and B). Mannitol pretreatment reduced the p34\(^{CDCl}_2\)/cyclin B histone H1 in vitro kinase activity inhibition initiated by 100 \(\mu M\) t-butyl hydroperoxide treatment (data not shown). Thus an antioxidant may partially reverse the depressive effects of t-butyl hydroperoxide treatment on the G2 checkpoint function as indicated by the inhibition of p34\(^{CDCl}_2\)/cyclin B kinase activity.

**t-Butyl Hydroperoxide Treatment Activates pATM-associated Kinase Activity**—IR treatment of melanoma and lymphoblast cell lines was found to increase pATM-associated kinase activity toward the PHAS-1 and p53 proteins in *in vitro* kinase activity assays (16, 17). IR-inducible kinase activity was found in pATM normal cell lines but not in cell lines derived from AT patients. In these studies, pATM kinase activity was found in pATM normal cell lines but not in cells derived from AT patients. These studies, pATM kinase activity was reported to be induced by roughly 2-fold following treatment with IR (16, 17). Based on the data above, we hypothesized that oxidative damage generated by treatment with t-butyl hydroperoxide should induce pATM-associated kinase activity.

A rabbit polyclonal antiserum to a peptide corresponding to pATM residues 826–840 was raised and affinity-purified to the cognate peptide. Based on Western blot analysis of whole cell and/or nuclear protein extracts from NHF1 and HeLa cells, it was determined that the anti-ATM 7 affinity-purified antibody (αATM 7 a.p.) recognizes a protein of ~350 kDa, the predicted size of pATM. This band was not detected in dermal fibroblasts derived from individuals with AT (Fig. 8).

NHF1 cells and the ATM-deficient dermal fibroblast strain AG03058 treated with either 6.0 Gy IR or 15 min with 300 \(\mu M\) t-butyl hydroperoxide were examined for pATM kinase activity. As shown in Fig. 9A, low levels of pATM-associated *in vitro* kinase activity were found to be associated with immunoprecipitated protein complexes from extracts from untreated NHFs using the αATM 7 a.p. antibody. This activity was roughly 50% higher than the background level of kinase activity associated with protein G-agarose bead mock immunoprecipitations (using no antibody) with extracts from IR-treated NHFs. When protein extracts from NHF1 cells treated with either IR or t-butyl hydroperoxide were assayed for pATM-associated *in vitro* kinase activity toward PHAS-1 protein, the level of activity in the immunocomplexes was significantly increased. t-Butyl hydroperoxide treatment induced pATM-associated kinase activity an average of 2.2-fold over the level found associated with pATM immunocomplexes from untreated NHF1 cells in 3 independent experiments. IR treatment increased pATM-associated kinase activity an average of 2.1-fold (average of 6 independent experiments) (Fig. 9A). A representative pATM kinase assay is shown in Fig. 9B. To examine the pATM-dependent specificity of these *in vitro* kinase assays, αATM 7 a.p. antibody was preincubated with either the peptide that it was raised against (ATM-N826) or an irrelevant peptide of the same length (data not shown). As shown in Fig. 9A, the ATM-N826 peptide largely blocked IR-
induced pATM-associated kinase activity to PHAS-1. Finally, pretreatment with 1 mM mannitol significantly reduced t-butyl hydroperoxide-induced pATM-associated kinase activity toward the PHAS-1 protein (data not shown), indicating that an antioxidant could reverse the pATM-activating effects of t-butyl hydroperoxide in this assay.

Further confirmation that the in vitro kinase activity toward PHAS-1 was associated with pATM was found in that in vitro kinase activity assayed from protein extracts from fibroblasts lacking pATM showed no significant difference whether anti-pATM antibody was present in the assays or not. Furthermore, with extracts from IR- or t-butyl hydroperoxide-treated ATM-deficient fibroblasts, the addition of ATM-N826 peptide to the assay did not suppress the background level of in vitro kinase activity (Fig. 9B).

**Heme Oxygenase-1 Induction Is Normal in AT Fibroblasts—** Heme oxygenase-1 (HO-1) is induced in human skin fibroblasts by peroxides (42). To determine whether AT fibroblasts were deficient in this response to oxidative stress, we treated four fibroblast strains (1 normal and 3 from individuals with AT) with 0, 1, 3, 10, 30, and 100 μM t-butyl hydroperoxide for 4 h and performed Western blot analysis for HO-1 protein. As shown in Fig. 10, t-butyl hydroperoxide treatment resulted in an induction of HO-1 protein at 10 μM concentrations and above in all four fibroblast strains. There was no significant difference in the response of any of the fibroblasts to HO-1 induction. Western blot analyses demonstrated that t-butyl hydroperoxide maximally induced HO-1 protein at 4–5 h in each cell type (data not shown). Pretreatment of NHF1 fibroblasts with mannitol for 1 h followed by treatment with 10 μM t-butyl hydroperoxide for 4 h in the continued presence of mannitol resulted in a significant inhibition of HO-1 protein induction, demonstrating that an antioxidant could partially reverse the effects of t-butyl hydroperoxide (data not shown).

**Thymine Glycol Formation Induced by t-Butyl Hydroperoxide Treatment Is the Same in pATM-normal and pATM-deficient Fibroblasts—** NHF1 cells and ATM-deficient fibroblasts (AG03058) were treated with t-butyl hydroperoxide or IR and examined for thymine glycol formation. As shown in Table II, treatment of either fibroblast type with 100 or 300 μM t-butyl hydroperoxide (A) or 6.0 Gy γ-IR (B) resulted in essentially equal thymine glycol formation between the fibroblast types. Thus, the initial damage produced by t-butyl hydroperoxide and IR treatment in pATM normal and deficient fibroblasts is not significantly different, as measured by thymine glycol formation.

**DISCUSSION**

We have examined the role of pATM in cellular responses to oxidative damage generated by t-butyl hydroperoxide treatment of normal human fibroblasts and ATM-deficient dermal fibroblasts. We undertook this study for several reasons as follows. 1) ATM-deficient cells have been reported to be unusually sensitive to oxidants such as nitric oxide, superoxide, and hydrogen peroxide in colony-forming efficiency assays compared with normal cells (21, 23–25). 2) ATM-deficient cells re-synthesize glutathione unusually slowly after depletion with diethyl maleate (22). 3) Cellular damage by IR exposure has been suggested to involve damage due to reactive oxygen species (43). 4) The RAD9 gene product, which has similar functions to pATM, has been found to be necessary for checkpoint arrest in response to peroxide exposure (44). 5) BRCA1, which has been demonstrated to play a role in protecting cells from damage by hydrogen peroxide (45), has recently been found to...
be phosphorylated by activated pATM (46). 6) ATM-deficient mice have been found to have elevated markers of oxidative damage, such as nitrotyrosine and hemeoxygenase activity, in organs known to be affected by the AT phenotype. Interestingly, cerebellar HO levels were found to be 600% greater in ATM-deficient mice than in ATM-normal mice (26). This last observation is particularly interesting as cerebellar pathologies are a hallmark of AT (47–49). Finally, pATM has been suggested to function, in part, as a sensor of oxidative stress (20). This last observation is particularly interesting as cerebellar pathologies are a hallmark of AT (47–49). Finally, pATM has been suggested to function, in part, as a sensor of oxidative stress (20).

We report that compared with pATM normal fibroblasts, ATM-deficient fibroblasts exhibit the following characteristics after oxidative damage: 1) greater sensitivity to t-butyl hydroperoxide that is both pATM-independent and similar to that observed in normal fibroblasts. The biphasic response in both normal and AT cells suggests that higher concentrations of t-butyl hydroperoxide (1–10 μM), followed by lesser sensitivity at higher concentrations (>10 μM). Whereas ATM-deficient fibroblasts were clearly more sensitive to the colony-forming inhibitory effects of t-butyl hydroperoxide, the appearance of the biphasic response in both normal and AT cells suggests that higher concentrations of t-butyl hydroperoxide (>10 μM) induce a resistance to the effects of t-butyl hydroperoxide that is both pATM-independent and similar to that seen in normal cells. The biphasic colony-inhibitory response we found here is similar to one reported with mouse embryonic stem cells exposed to hydrogen peroxide (45), suggesting that this biphasic response may be a common adaptive event following peroxide exposure.

One possible interpretation of our findings could be that ATM-deficient cells may respond normally to oxidative damage generated by t-butyl hydroperoxide treatment but not exhibit normal checkpoint responses or p53 induction due to their receiving less damage than normal fibroblasts after exposure to the same concentration of t-butyl hydroperoxide. To address

![Western blot analysis of heme oxygenase induction in response to t-butyl hydroperoxide treatment.](image)

**Fig. 10.** Western blot analysis of heme oxygenase induction in response to t-butyl hydroperoxide treatment. One normal and three ATM-deficient fibroblast strains were treated as indicated; whole cell lysates were prepared, and protein extracts were subjected to SDS-PAGE. The resolved proteins were then transferred onto nitrocellulose and probed using an anti-heme oxygenase antibody.

**Table II**

| Treatment/concentration | Thymine glycol/10^7 bases^a |
|-------------------------|-----------------------------|
| 0 μM                    | ND^b                        |
| 100 μM                  | 19 ± 3.0                   |
| 300 μM                  | 60 ± 4.1                   |
| 6.0 Gy                  | 55 ± 0.3                   |

^a Cells were harvested immediately after treatment and quick-frozen until analysis. Thymine glycol quantification was performed as reported previously (29).

^b ND, not detectable.
this issue we quantified DNA damage induced by IR and t-butyldihydroperoxide exposure by quantifying thymine glycol formation in normal and ATM-deficient fibroblasts. We found that thymine glycol formation was not significantly different between the fibroblast types directly following exposure to either agent. Based on these data, we conclude that the initial damage received by normal and ATM-deficient fibroblasts following IR and t-butyldihydroperoxide exposure is approximately the same and that, in fact, it is the absence of pATM function that accounts for the differences seen between the cell types.

A major hallmark of cells from individuals with AT is the ablation of the G1 and G2 checkpoint functions in response to IR, as well as other IR response-related events, such as p53 and p21 induction (11, 12, 36, 37, 50). Here we have shown that after exposure to t-butyldihydroperoxide, ATM-deficient fibroblasts lack G1 and G2 checkpoint responses, lack p53 induction, and show enhanced toxicity, reminiscent of the cellular responses seen with ATM-deficient fibroblasts exposed to IR. Thus our data support the hypothesis that pATM plays a role in resistance to oxidative stress (20).

Cell cycle checkpoints are thought to function, in part, to allow cells time to repair damage, particularly damage to DNA, before re-entering the cell cycle and completing subsequent cellular events such as DNA replication or mitosis. Ablation of cellular checkpoint functions, such as occurs in ATM-deficient cells or in cells treated with methylxanthines, can result in greater lethality following exposure to toxic agents (for review see Ref. 33). One question we have not addressed is whether or not pATM responds to oxidative damage to DNA or responds directly to a change in intracellular redox state independent of DNA damage. Although our data do not address this question specifically, we do demonstrate that ATM-deficient fibroblasts fail to exhibit normal G1 and G2 checkpoint responses following exposure to oxidative stress. Thus the inability of ATM-deficient cells to exhibit G1 and G2 checkpoint responses following oxidative damage is likely to be one mechanism that predisposes these cells to enhanced toxicity in response to oxidative stress. Our results suggest that there is an inability to respond to oxidative stress in cells from individuals with AT, as a consequence of which may be relevant to the mechanisms of Purkinje cell death and other pathological changes observed in AT patients.

Acknowledgments—We thank B. Alex Merrick for the generous provision of p53 antibody, and Bradley Sturgeon, Chia Chiao, and Cynthia Ashfari for their many helpful discussions and suggestions.