Ataxia Telangiectasia Mutated Deficiency Affects Astrocyte Growth but not Radiosensitivity

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ABSTRACT

The cancer-prone neurodegenerative disorder, ataxia telangiectasia (A-T), results from mutations of ATM (ataxia telangiectasia mutated). Individuals with A-T manifest a diverse array of symptoms including immune deficiencies, genomic instability, predisposition to cancer, progressive neurodegeneration, and hypersensitivity to IR including immune deficiencies, genomic instability, predisposition to cancer, progressive neurodegeneration, and hypersensitivity to IR. A-T 3 is an autosomal recessive disorder resulting from mutation of ATM (ataxia telangiectasia mutated). Cells derived from individuals with A-T are hypersensitive to IR and other agents that produce DNA double-strand damage (4, 5). Cells derived from individuals with A-T are hypersensitive to IR and other agents that produce DNA double-strand damage (4, 5). Cells derived from individuals with A-T are hypersensitive to IR and other agents that produce DNA double-strand damage (4, 5). Cultured cells from A-T individuals or Atm−/− mice have cell cycle and growth defects and are generally considered radiosensitive. However, it has been shown recently that cell populations in the Atm−/− central nervous system are radiosensitive. To define specific IR sensitivities of neural populations, we analyzed Atm−/− astrocytes. Here we show that Atm−/− astrocytes exhibit premature senescence, express constitutively high levels of p21, and have impaired p53 stabilization. However, in contrast to radiosensitive Atm−/− fibroblasts and radiosensitive Atm−/− neurons, survival of Atm−/− astrocytes after IR was similar to wild-type astrocytes. Additionally, p53-null astrocytes, but not fibroblasts, were moderately more radiosensitive than their wild-type counterparts, suggesting that the deficit in p53 stabilization observed in Atm-null cells is not a measure of radiation susceptibility. Thus, in astrocytes, the function of Atm in cellular growth and radiosensitivity is distinct. These data may have implications for ATM disruption strategies as a radiosensitizing treatment for brain tumors.

INTRODUCTION

A-T 3 is an autosomal recessive disorder resulting from mutation of ATM. Individuals with A-T manifest a diverse array of symptoms including immune deficiencies, genomic instability, predisposition to cancer, progressive neurodegeneration, and hypersensitivity to IR (reviewed in Ref. 1). ATM is a large protein (M 6 , 370,000) with sequence similarity at its COOH-terminal region to the phosphoinositol-3-kinase family of proteins function in the cellular response to DNA damage. For example, DNA-PKcs responds to DNA strand breaks, whereas ATR (Atm and Rad3 related) can activate p53 after DNA damage (4, 5). Cells derived from individuals with A-T are hypersensitive to IR and other agents that produce DNA double-strand breaks, but they respond normally to UV irradiation (3, 6). Ionizing radiation promotes p53 stabilization and causes a p53-dependent G 1 checkpoint that requires ATM. Cell lines with dysfunctional ATM either show a lack, or altered kinetics of, IR-induced p53 stabilization (7–10). This is thought to occur because ATM phosphorylates Ser 15 of p53, which in turn inhibits the interaction of p53 with Mdm2, leading to p53 stabilization (11–16). Additionally, phosphorylation at Ser 68 , as well as dephosphorylation at Ser 207 of p53, may also be involved in Atm-dependent activation of p53 (17–19). However, in some cases, phosphorylation may be dispensable for p53 stabilization (20). Downstream components of the p53-dependent DNA-damage pathway are also required for G 1 arrest, such as the cyclin-dependent kinase inhibitor p21 (3, 8). Consequently, the usual cell cycle checkpoint responses to DNA damage are subverted, thereby contributing to the hallmark features of A-T cells such as radiosensitive DNA synthesis (21). ATM dysfunction can also affect DNA damage-induced checkpoint points in other phases of the cell cycle, although the mechanism in these cases is less clear (22, 23). Although the G 2 -phase checkpoint may involve p53 and Chk1 (24–28), the S-phase checkpoint is not well characterized, although it does not appear to involve RPA phosphorylation (29).

Mice in which Atm has been inactivated are acutely sensitive to IR because of intestinal toxicity (30, 31). Fibroblasts derived from Atm-null mice also demonstrate IR hypersensitivity and premature senescence with associated defects in p53 regulation (31, 32). However, Atm function, at least in response to DNA damage, is tissue specific. Recently, we found an absence of IR-induced apoptosis in the CNS of Atm−/− mice (33). In the CNS, Atm-dependent apoptosis requires p53 because p53-null mice display a similar radiosensitive phenotype (33). In this report, we extend these studies to determine whether, in addition to Atm-null neurons, the radiosensitivity to IR observed in vivo could be recapitulated in other CNS-derived cells. Gial cells constitute the major cell type in the CNS and play a crucial role in the development and maintenance of neurons. CNS tumors are largely of glial origin, and the treatment of these disorders usually includes radiotherapy. Therefore, an understanding of the cellular response of nervous system populations to radiation is important for treatment of brain tumors. We report here that Atm−/− astrocytes, although showing a marked defect in growth and p53 induction, do not show hypersensitivity to IR, underscoring the importance of cellular context for Atm function. Moreover, the disparate response of Atm-null astrocytes compared with other Atm-null cell types may have clinical relevance because disruption of ATM function is a potential therapeutic approach for radiosensitizing brain tumors.

MATERIALS AND METHODS

Cell Culture. Fibroblasts were prepared from eviscerated, minced truncal tissue of E13.5–E15.5 embryos of the indicated genotypes by trypsinization and trituration through an 18-gauge needle. Astrocytes were obtained in a similar manner using forebrains of mice 6–10 days of age. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mm glutamine, 5 units/ml penicillin, 5 μg/ml streptomycin, 50 μg/ml β-mercaptoethanol, and nonessential amino acids in a humidified, 5% CO 2 incubator at 37°C. Cells were passaged by trypsinization upon reaching confluency, typically being seeded at 1/4 density once per week. Cells from passages 3 to 6 were used for post-mortem survival studies. Assays of p21 were performed on cells at passages 3 to 8. DNA was extracted from residual starting tissue overnight at 50°C in Laird buffer with 250 μg/ml proteinase K to genotype cell lines by PCR analysis. Genotypes were determined by PCR using primers that were specific for the Atm and p53 mutant alleles.

Growth and Survival Characterization. Cells were seeded into 24-well plates at a density of 10,000 cells/well, incubated overnight, and then exposed to a DNA-damaging agent. Bleomycin (50 μg/ml in complete media) was applied to cultures for 2 h. IR was delivered from a 137Cs source at 6.25 Gy/min. Ultraviolet irradiation (254 nm) was delivered directly to medium-free cells at a rate of 5 J/m 2 /s. Control cultures were sham washed and exposed in

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3 The abbreviations used are: A-T, ataxia telangiectasia; ATM, AT mutated; IR, ionizing radiation; CNS, central nervous system; HRP, horseradish peroxidase; WT, wild type; PCNA, proliferating cell nuclear antigen; GFAP, glial fibrillary acidic protein.
an identical manner to their experimental counterparts. Medium was changed at least twice weekly in the case of extended assay conditions. Quantitation of cell growth and survival was measured using crystal violet staining of cells (34). For crystal violet assays, plates were washed twice in PBS, fixed for 20 min in 2% paraformaldehyde/PBS, rinsed with H2O2, air-dried, and stored at −20°C until all plates in one run could be assayed simultaneously. Fixed cells were stained for 20 min with 0.1% crystal violet in 200 mM boric acid (pH 9) and washed extensively with H2O2, and the dye was solubilized in 10% glacial acetic acid. The Promega (Madison, WI) Cell Titer kit was also used according to the manufacturer’s instructions to assay cell numbers by monitoring the catalysis of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; a 200-μl reaction volume/well was used. Additionally, titers of cells grown in six-well plates (seeding density, 50,000 cells/well) were directly determined by counting trypanstained cells that excluded trypan blue. Identical results were obtained from each method of cell quantification. For clonogenic assays, cells were trypsinized and irradiated in solution (10 Gy at 6.25 Gy/min) and seeded into 15-cm plates at various densities to yield —50–250 colonies/plate. After 2 weeks growth, focal colonies of 32 or more cells were scored as a positive clone. Cells were fixed for 10 min in methanol:glacial acetic acid (3:1), air dried, and then stained with crystal violet as described above. All experiments were performed in triplicate, using at least three independent cell lines established from separate embryos. Results are expressed as the mean ± SD. All experiments used matched mutant and control fibroblasts and were always derived from the same pregnant female or, for astrocytes, the same litter.

**Protein Immunoblot Analysis.** Cells (1–5 million) were seeded into 15-cm dishes, grown until ~75% confluent, and irradiated with 10 Gy at 6.25 Gy/min in a 137 Cs-Gammacell irradiator. Two h after IR, cells were washed twice with ice-cold PBS and then scraped directly into 250 μl of lysis buffer containing 150 mM NaCl, 0.5% w/v SDS, 0.5% v/v NP40, 0.5% w/v sodium deoxycholate, 1 mM EGTA, 0.5 mM ZnCl2, 50 mM NaF, 50 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, 1% v/v β-mercaptoethanol, and a mixture of protease inhibitors (Boehringer Mannheim; Complete-Mini tablets). Lysed cells were heated at 100°C for 5 min, sonicated, and kept on ice for 30 min, prior to centrifugation to clear the extracts. Protein concentrations were determined using a Bradford reagent (BioRad) according to the manufacturer’s instructions. Proteins (100-μg aliquots) were separated by SDS-PAGE on a 12.5% gel, transferred to nitrocellulose membranes, and stained with Ponceau-S to confirm equal loading and transfer. The membranes were blocked in 5% skim milk in PBS-Tween 20 (0.1% v/v) and incubated with diluted primary antibody (see below) in blocking buffer. Primary antibody binding was identified using a HRP-conjugated secondary antibody visualized by ECL (Amersham). Membranes were routinely reprobed with different antibodies after stripping at 50°C for 30 min in 62.5 mM Tris (pH 6.8), 1 mM β-mercaptoethanol, and 2% SDS, extensive washing in PBS-Tween 20, and reblocking. Antibody dilutions used were: sheep anti-p53 (1/2500; Calbiochem), rabbit anti-p21 (1 μg/ml; Santa Cruz Biotechnology), mouse anti-GFAP (0.1 μg/ml; Boehringer Mannheim), mouse anti-PCNA (1 μg/ml; Calbiochem), goat anti-actin (1 μg/ml; Amersham), HRPCongjugated sheep anti-mouse (0.2 μg/ml; Amersham #NA931), HRP-conjugated sheep anti-mouse (0.2 μg/ml; Amersham), HRP-conjugated donkey anti-rabbit (0.2 μg/ml; Amersham), HRP-conjugated donkey anti-goat (2 μg/ml; Santa Cruz Biotechnology), and HRP-conjugated rabbit anti-sheep (0.2 μg/ml; Pierce).

**RESULTS**

Astrocyte cultures derived from WT and Atm−/− mice were established with similar efficiency and consisted of both protoplasmic type I and fibrous type II cells, although the smaller type II astrocytes diminished over successive passages (data not shown). Both WT and Atm−/− astrocytes maintained their stellate arborization over the course of the experiments, and unlike fibroblasts, they stained positive for the astrocyte-specific marker GFAP in both histological (Fig. 1A) and protein immunoblot analysis (Fig. 1B). Astrocyte cultures contained >95% of cells that expressed GFAP. Initially, cultures from both WT and Atm−/− were indistinguishable. However, from passage 4, although morphologically indistinguishable from WT, Atm−/− astrocytes exhibited a significant growth rate deficit as assessed by crystal violet staining (Fig. 2A). To confirm Atm−/− astrocytes were replication competent, we used PCNA. PCNA is an integral component of the DNA polymerase complex and is a marker for actively dividing cells. Protein immunoblotting with anti-PCNA antibodies confirmed that both WT and Atm−/− astrocytes had essentially equivalent levels of PCNA (Fig. 2B). In line with the growth deficits of Atm-null cells (Fig. 2A), basal levels of p21 were constitutively higher in Atm−/− cells and increased further with time in culture (Fig. 2C). The high p21 levels correlated with early replicative senescence, because the cells grew very slowly in culture and never attained a population density equivalent to WT cells (data not shown).

IR treatment promotes p53 stabilization, and this response is deficient in Atm−/− fibroblasts and tissues (32, 33, 35, 36). Stabilization of p53 in both Atm-null astrocytes and fibroblasts 2 h after a 10-Gy dose of IR was markedly reduced compared with controls (Fig. 3). Thus, Atm is required for p53 stabilization at this time point in both astrocytes and fibroblasts. Because the two cell types exhibit similar defects in p53 stabilization, we assessed radiosensitivity of each cell type. Atm−/− fibroblasts are hypersensitive to IR and the radiomimetic drug bleomycin but are not abnormally sensitive to UV irradiation (3, 6). However, radiosensitivity resulting from Atm dysfunction...
Fig. 2. Atm−/− astrocytes and fibroblasts show growth defects and high constitutive p21 levels. A, WT (○) or Atm-null (●) cells were fixed and stained at 2-day intervals. Results are from three separate experiments normalized to the staining intensity of the initial 2-day time point. Bars, SD. B, protein immunoblot analysis of astrocytes and fibroblasts indicate similar levels of PCNA (upper panel) in both WT and Atm-null cell cultures. PCNA levels were determined on WT and Atm−/− cultures at day 8 in culture. C, p21 levels are increased in Atm−/− astrocytes. Analysis of p21 (upper panels) in astrocytes and fibroblasts shows elevation in Atm−/− cells, which becomes more pronounced at later passages. Actin detection was used to normalize protein loading (lower panels).

Fig. 3. Atm is required for p53 stabilization after IR. Analysis of p53 levels from WT (+) or Atm-null (−) cell cultures 2 h after a 10-Gy IR exposure shows that p53 stabilization is decreased in both Atm-null astrocytes and fibroblasts (upper panels). Actin detection was used to normalize protein loading (lower panels).

is dependent upon cellular context, because select cell populations in the Atm−/− CNS are radioresistant (33). Therefore, we compared the relative survival of Atm−/− astrocytes and fibroblasts after IR, bleomycin, and UV. Because Atm−/− astrocytes exhibited a reduction in proliferative capacity at later passage in culture, all survival assays were conducted on early-passage cells prior to any overt changes in their population doubling times. Although Atm−/− fibroblasts showed a striking radiosensitivity, Atm−/− astrocytes showed no difference relative to WT astrocytes (Fig. 4). Cell survival 6 days after exposure to 10 Gy IR or 50 μg/ml bleomycin shows a pronounced hypersensitivity of Atm-null fibroblasts but not astrocytes when compared with controls (Fig. 4, A and B). Cell survival after UV irradiation was independent of Atm status (Fig. 4C), confirming that Atm is not directly involved in the signal pathways affected by UV damage. Cell survival at days 2 and 4 after treatment also yielded similar results (data not shown). Other measures of survival, including metabolic activity assessed by the catabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or counting of trypan blue-excluded live cells yielded identical data (data not shown). Additionally, we used clonogenic survival to assess the proliferative capacity of Atm−/− astrocytes after IR exposure. Only cells that had gone through at least five rounds of division (32 or more cells/colony) were scored as proliferating colonies. Again, although Atm−/− fibroblasts are hypersensitive to IR, the sensitivity of Atm−/− astrocytes is indistinguishable from WT astrocytes (Fig. 5). Because ATM signals to p53 after IR-induced damage, we also compared p53-null astrocytes and fibroblasts. Similar to previous reports (37), we found no appreciable differences in radiosensitivity of p53-null fibroblasts to
WT controls (Fig. 5). Although in the case of p53-null astrocytes, there is a significant differential sensitivity to IR compared with controls ($P < 0.001$; Fig. 5). Thus, in primary cell lines, a lack of p53 or a deficiency of the p53 response to IR is not a measure of radiosensitivity.

**DISCUSSION**

Individuals with A-T and Atm-null mice are extremely radiosensitive (1, 3). In the case of Atm-null mice, this radiation hypersensitivity is attributable to acute and severe intestinal epithelial damage (30, 31, 38). However, it is becoming increasingly apparent that hypersensitivity to IR associated with Atm dysfunction is cell-type specific. For example, although Atm$^{-/-}$ fibroblasts are radiosensitive, select Atm$^{-/-}$ neuronal populations in the developing nervous system are radioresistant (32, 33, 35). An interrelationship of ATM, p53, and IR-induced DNA damage clearly exists (3, 23, 39). However, this relationship is quite diverse, because IR-induced death in the thymus requires p53 but not Atm (33, 35), whereas in the developing CNS, both Atm and p53 can be required for neuronal death (33). Although Atm-null astrocytes have a defect in p53 stabilization after DNA damage, their radiosensitivity is independent of Atm status. In contrast, p53-null astrocytes showed a significant increase in survival after IR. Thus, these data indicate that IR targets influencing survival are cell and tissue specific, with only selective involvement of Atm and p53.

Atm$^{-/-}$ astrocytes also had a reduced growth rate. Although apparent at early passage, this occurred even though they were replication competent, as indicated by similar PCNA levels to WT cells. Growth deficits seem to be a general feature of primary cells derived from A-T individuals or Atm-null mice (3, 30, 32, 40). This growth deficit potentially involves p21, because the levels are higher in the Atm$^{-/-}$ cells than WT at the same passage. Elevated p21 levels and replicative senescence observed in the Atm-null astrocytes are similar to Atm-null fibroblasts, indicating that this pathway may be cell type independent. Although the precise nature of this growth deficit remains unknown, it is dependent upon p53, p21, and p19ARF because fibroblasts derived from Atm/p53, Atm/p21, or Atm/p19ARF double-null fibroblasts do not undergo replicative senescence and are immortal (31, 36, 41). This suggests a fundamental role for Atm in cell growth and replication that is distinct from radiosensitivity.

Previously, we found a prominent resistance to IR-induced apoptosis in the CNS of Atm$^{-/-}$ mice (33). In some cases, such as the external granule layer of the developing cerebellum, the resistant Atm-null cells were neurons. In other regions such as the dentate gyrus, the radioresistant Atm-null cells could have been either neurons or glia, or both. However, the data presented here suggest that Atm-null glia may not possess the same radioresistant properties as Atm-null neurons. Because of cell type specificity in Atm function and the potential clinical implications of a radiosensitizing phenotype conferred by ATM dysfunction, we were interested in establishing radiosensitivity in Atm-null astrocytes. The possibility of affecting the radiosensitivity status of a tissue has potential therapeutic implications for radiation treatment of cancer. Because many brain tumors are resistant to present chemotherapy treatments (42, 43), radiosensitization based on disruption of ATM function is an attractive possibility. However, our data suggest that disruption of ATM in cells of a glial lineage may not confer hypersensitivity to radiation and may not be useful as a radiosensitizing agent. Of course, the situation in a tumor may be different than that of normal glial cells, or the radiosensitivity of astrocytes may change under *in vitro* culture conditions. In contrast, because Atm dysfunction confers radioresistance to neurons, ATM disruption may be beneficial by limiting the neuronal damage of normal tissue often associated with radiotherapy (44).

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GROWTH AND RADIOSENSITIVITY OF ATM-NULL ASTROCYTES


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