Atm Knock-in Mice Harboring an In-frame Deletion Corresponding to the Human ATM 7636del9 Common Mutation Exhibit a Variant Phenotype

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ABSTRACT

ATM, the gene mutated in the human immunodeficiency disorder ataxia-telangiectasia (A-T), plays a central role in recognizing ionizing radiation damage in DNA and in controlling several cell cycle checkpoints. We describe here a murine model in which a nine-nucleotide in-frame deletion has been introduced into the Atm gene by homologous recombination followed by removal of the selectable marker cassette by Cre-loxP site-specific, recombination-mediated excision. This mouse, Atm-SARI, was designed as a model of one of the most common deletion mutations (7636del9) found in A-T patients. The murine Atm deletion results in the loss of three amino acid residues (SRI; 2556–2558) but produces near full-length detectable Atm protein that lacks protein kinase activity. Radiosensitivity was observed in Atm-SARI mice, whereas the immunological profile of these showed greater heterogeneity of T-cell subsets than observed in Atm−/− mice. The life span of Atm-SARI mice was significantly longer than that of Atm−/− mice when maintained under nonspecific pathogen-free conditions. This can be accounted for by a lower incidence of thymic lymphomas in Atm-SARI mice up to 40 weeks, after which time the animals died of other causes. The thymic lymphomas in Atm-SARI mice were characterized by extensive apoptosis, which appears to be attributable to an increased number of cells expressing Fas ligand. A variety of other tumors including B-cell lymphomas, sarcomas, and carcinomas not seen in Atm−/− mice were observed in older Atm-SARI animals. Thus, expression of mutant protein in Atm-SARI knock-in mice gives rise to a discernibly different phenotype to Atm−/− mice, which may account for the heterogeneity seen in A-T patients with different mutations.

INTRODUCTION

The human genetic disorder A-T3 is characterized by immunodeficiency, neurodegeneration, sensitivity to ionizing radiation, and cancer predisposition (1, 2). Chromosomal instability in this disease is characterized by abnormal rearrangements involving chromosomes 7 and 14 in the vicinity of the TCR and immunoglobulin genes (3, 4). It has been suggested that a reduced capability in processing double-strand breaks in DNA is responsible for the radiosensitivity, immunodeficiency, infertility, and cancer predisposition (5, 6). Evidence in support of this has been provided by disruption of the Atm gene in mice. These animals developed malignant thymic lymphomas by 4–5 months of age (7–9), and these lymphomas had translocations in chromosome 14 occurring in both alleles of the TCR α/β locus (10).

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The abbreviations used are: A-T, ataxia-telangiectasia; TCR, T-cell receptor; mAb, monoclonal antibody; SIEL, in situ end-labeling; ES, embryonic stem; SF, single positive; DP, double positive; SPF, specific pathogen-free; FoxL, Fox ligand; IL, interleukin.

These data suggest that breaks occurring within the TCR locus during T-cell development undergo inappropriate end-joining, giving rise to genome instability and cancer predisposition. Consistent with this, Liao and Van Dyke (11) failed to observe the development of thymic lymphomas in Atm−/− Rag1−/− mice in which V(D)J recombination had been disrupted. On the other hand, Petiniot et al. (10) reported a lower frequency and longer latency period for thymic lymphomas in Atm−/− Rag2−/− mice compared with Atm−/−mice. Overall, the results point to an important but nonessential role for V(D)J recombination in tumorigenesis in Atm-deficient thymocytes (10).

The phenotype observed in Atm−/− mice generally reflects that seen in A-T patients with the exception of neurodegeneration, where the majority of studies failed to reveal such abnormalities (7–9, 12). In two cases, some evidence for neuronal degeneration and abnormal development of Purkinje cells was provided in Atm−/− mice (13, 14). In all of the Atm−/− mice generated to date, the disruption of the gene was achieved by means of gene inactivation leading to complete loss of Atm protein expression (7–9, 12, 14). Mice on either inbred or mixed genetic backgrounds did not show any phenotypic variation (7). Disruption of Atm led to a smaller size at birth, body weights of these mice were reduced during the early growth period, and fibroblasts from these animals grew poorly (7–9). Although the architecture of various lymphoid tissues was normal in Atm−/− mice, these organs were generally smaller in size (7–9).

Although there are clear hallmarks that characterize the A-T phenotype, some variability exists in these features (1), which might be explained by the nature of the mutation or genetic background. In addition to a phosphatidylinositol 3-kinase domain, the ATM protein contains several regions that interact with other proteins or are predicted to be functionally important (15, 16). Furthermore, there is evidence for a dominant-negative effect of a region of the ATM protein containing a putative leucine zipper motif (17). Because up to 20% of A-T patients express mutant protein and none of the Atm−/− mice produced to date express Atm protein, it was important to generate a mouse expressing mutant ATM protein and to determine whether these mice display any variation in the characteristics associated with the A-T phenotype. Accordingly, we produced mice homozygous for a nine-nucleotide in-frame deletion (7666del9) in Atm, which was predicted to give rise to a protein with three amino acids deleted (SRI; 2556–2558). This mutant corresponds to one of the most common A-T mutations found to date (15, 18). We report here that the phenotype of Atm-SARI mice is significantly different to that described for Atm−/− mice.

MATERIALS AND METHODS

Gene Targeting and Generation of Atm-SARI Mice. To generate a mutant mouse model of the human 7636del9 mutation found in A-T patient AT1ABB (15, 18), we used homologous recombination and the Cre-loxP system to introduce a nine-nucleotide in-frame deletion into exon 54 of mouse Atm (19). The gene targeting vector was constructed using a 5.5-kb EcoRV-
PstI genomic fragment isolated from a AZAP2 clone containing a mouse Atm insert derived from 129/SvJ genomic DNA (ADA1.1). On the basis of the mouse Atm cDNA sequence, removal of nucleotides 7666–7674 resulted in deletion of three amino acid residues (SRI; 2556–2558) at a position corresponding to the human 7636del9 mutation from patient AT1ABR. Site-directed mutagenesis was used to introduce this mutation into exon 54.

A 36-bp mutagenesis primer (5′-CTGATAGCTAATCTCACTTGATCAC-3′) was used to delete nucleotide 7666–7674 and also to create a novel BclI restriction site neutral polymorphism at nucleotides 7680. This restriction site was included as a marker to track the deletion mutation. A second 29-bp mutagenesis primer (5′-GTAACTGCAAGGGCCCTTTTGT-3′) was also used to introduce a unique Apal site within intron 54, into which a loxP-neo/gpt-loxP selection cassette was inserted (Fig. 1). After transfection into 129SvJ ES cells (C1368) and G418 selection, two independently targeted clones were injected into C57Bl/6J blastocysts, and 10 high-grade chimeric males were produced. These were, in turn, backcrossed with C57Bl/6J, and of these, 8 transmitted the mutation through the germ line. The floxed neo/gpt selection marker was removed subsequently by crossing homozygous cDNA sequence, removal of nucleotides 7666–7674 resulted in deletion of three amino acid residues (SRI; 2556–2558) at a position corresponding to the human 7636del9 mutation from patient AT1ABR. Site-directed mutagenesis was used to introduce this mutation into exon 54.

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a percentage of the total number of cells. Thymic lymphomas were disaggregated and analyzed in the same way.

**Production of mAbs.** The mAb MAT3-4G10/8 was raised against a peptide spanning positions 1967–1988 of murine Atm, to which a cysteine residue was added at the NH₂ terminus for coupling to keyhole limpet hemocyanin. Mice were immunized with 50 μg of antigen (total of six injections over 15 weeks), and the appearance of antigen-specific antibodies was monitored by ELISA based on the antigen peptide and then by immunoblotting of mouse tissue extracts and immunoprecipitation of Atm from these tissues. Hybridomas were established using standard methods, and antibody production was monitored as described above. Seventeen clones were finally stocked, and clone 4G10/8, which produces heavy chain IgG1, was used in this study. The antibody was purified using fast protein liquid chromatography over a HiTrap protein G column (Pharmacia Biotech) and dialyzed against PBS.

**Western Blotting.** Protein extracts were prepared from spleens by homogenization in TGN buffer [50 mM Tris (pH 7.5), 50 mM β-glycerophosphate, 150 mM NaCl, 10% glycerol, 1% Tween 20, 1 mM NaF, 1 mM Na₂VO₄, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM DTT] and centrifuged at 13,000 × g for 15 min. Protein concentrations were determined using the Bio-Rad DC protein assay kit according to the manufacturer’s recommendations. Protein samples (100 μg) were separated on 5 or 10% denaturing gels and blotted onto Hybond-C nitrocellulose membranes. After blocking in 4% milk powder, 0.1% Tween 20, and PBS for at least 1 h, blots were incubated for 1 h at room temperature or overnight at 4°C with the relevant primary antibody. Immunoreactive bands were visualized with Renaissance chemiluminescence substrate and captured and recorded using an Amersham autoradiography cassette and a phosphorimager. The primary antibodies used in this study were mouse mAb anti-Atm MAT3-4G10/8 and rabbit antiantiactin (Sigma Chemical Co.).

**In Vitro Atm-Kinase Assay.** Atm-kinase activity was determined using the method described by Canman et al. (21). For whole cell lysate isolation, cells were lysed on ice in TGN buffer. After centrifugation at 13,000 × g for 15 min, 1 mg of extract was pre-cleared with protein A-Sepharose beads. Atm was immunoprecipitated with Atm antibody (MAT3), and the kinase activity was determined.

**Cell Survival.** Thymi were dissected from mice, and thymocytes were disaggregated in RPMI 1640 (10/rib/ml) as described above. Cells were incubated with or without IL-2 (60 μg/ml) at 37°C in an atmosphere of 5% CO₂ for 24 h. Cells were exposed to ionizing radiation (0–4 Gy), and cell viability was determined by adding 0.1 ml of 0.4% trypan blue to a 0.5-ml suspension (22). Thymic lymphomas were disaggregated in RPMI 1640 (10/rib/ml) as described above. Cells were incubated with or without IL-2 (60 μg/ml) at 37°C in an atmosphere of 5% CO₂ for 24 h. Cells were exposed to ionizing radiation (0–4 Gy), and cell viability was determined by adding 0.1 ml of 0.4% trypan blue to a 0.5-ml suspension (22). The number of viable cells was determined at 48 and 72 h after irradiation.

**Induced Chromosome Aberrations.** Cells were irradiated with 1 Gy of γ-rays. For G₂-phase cells, Colcemid (final concentration, 0.1 μg/ml) was added immediately after irradiation, 1–2 h before harvesting. The cells were treated for 15 min in 0.075 m KCl, fixed in methanol:glacial acetic acid, 3:1 (vol/vol), and spread on glass slides. The cells were then stained with Giemsa, and 50 metaphases were analyzed for each sample (22).

**ISEL of Tumor Sections.** Paraffin sections of thymoma from Atm⁻/⁻ and Atm-ΔSRI mice were dewaxed, digested with pepsin, and subjected to ISEL, essentially as described by Ansari et al. (23) using Biotin-16-dUTP (Enzo Diagnostics) in the labeling mix. Incorporated biotin-dUTP was detected with horseradish peroxidase coupled to streptavidin (DAKO) and 3,3'-diaminobenzenidine staining. The sections were counterstained with hematoxylin, mounted, and photographed using an Olympus BH2 microscope.

**RESULTS**

**Generation of Atm-ΔSRI Mutant Mice Expressing Mutant Atm Protein.** Because the Atm protein is a high molecular weight molecule containing several potential domains in addition to the kinase domain, it is important to establish whether the presence of mutant Atm protein influences the A-T phenotype. To date, all of the Atm mouse models produced result in Atm gene inactivation and a complete abrogation of Atm protein expression. We therefore generated mice homozygous for a nine-nucleotide in-frame deletion (7666del9) in Atm resulting in the loss of three amino acids (SRI: 2556–2558), hereafter referred to as Atm-ΔSRI. The strategy used to introduce the ΔSRI mutation into Atm included the insertion of a loxP-flanked selectable marker cassette into the intron downstream of exon 54 (Fig. 1a; Ref. 24), which contains the nine-nucleotide deletion. Chimeric mice generated from two independently targeted 129SVJ ES cell clones (D11A and C9B) were crossed with C57BL/6J mice to produce Atm-ΔSRI heterozygotes. The loxP-selectable marker cassette was removed by crossing Atm-ΔSRI heterozygous mice with an Elia-Cre deleter mouse strain that constitutively expresses Cre recombinase (20), Atm-mutant mice were identified by Southern blot analysis of tail tip DNA (Fig. 1b) or PCR analysis of a region containing the deletion mutation and BclI site (Fig. 1c, top panel). Using this analysis, it is possible to distinguish Atm-ΔSRI homozygotes (714 bp) from heterozygotes and wild type (663 bp). The increased size of the Atm-ΔSRI homozygous band is attributable to the presence of a single loxP site in intron 54. Cleavage with BclI confirmed the presence of the mutation (Fig. 1c, bottom panel). Deletion of the nine-nucleotide sequence was verified by genomic DNA sequencing (Fig. 1d). These ΔSRI mutant mice expressed near full-length Atm protein, whereas no Atm protein was detected in Atm⁻/⁻ mice, as determined by immunoblotting of spleen cell extracts with a mAb (MAT 3) derived against the mouse Atm sequence (Fig. 2a). Because we have shown previously that the corresponding human mutant protein typified in the cell line AT1ABR (15, 18) is kinase-dead and thereby incapable of phosphorylation of p53 on serine 15 (25), we measured the kinase activity of Atm-ΔSRI splenocytes using p53₁₋₄₀ as a substrate as described previously (21, 26). A marked increase in Atm-kinase activity was observed in wild-type spleen cell extracts in response to radiation exposure, whereas only a low level of residual kinase activity, which did not respond to radiation, was observed in Atm-ΔSRI extracts (Fig. 2b). As expected, extracts from Atm⁻/⁻ mice displayed no Atm-kinase activity.
expression and abnormally differentiated Purkinje cells were apparent (14). As with Atm<sup>−</sup> mice, Atm-DSR1 mice, 2–16 months of age, failed to show any evidence of gross neuronal degeneration, specific loss, or abnormal migration of Purkinje cells (results not shown). Similar to Atm<sup>−</sup> mice, there was also evidence of testicular abnormalities (poorly developed testes) and disruption of spermatogenesis in Atm-DSR1 mice (n = 2). In contrast to seminiferous tubules of testes from wild-type mice where abnormalities were not observed, tubules from Atm-DSR1 mice were disrupted with evidence of degeneration of spermatocytes and loss of spermatids (Fig. 4, a and b). Similarly, oogenesis in Atm-DSR1 mice (n = 2) was disrupted with an observed lack of maturing follicles and oocytes (results not shown).

**Immunological Abnormalities in Atm-DSR1 Mice.** A variable but common characteristic in A-T is immunodeficiency and immune defects, which are reproduced in Atm<sup>−</sup> mice. To characterize the T-cell development/phenotype in Atm-DSR1 mice, cell samples of thymus and inguinal lymph node were analyzed by flow cytometry and compared with those from age-matched (7–12 weeks) wild-type and Atm<sup>−</sup> mice. Atm-DSR1 mice had smaller thymi, spleens, and lymph nodes than wild-type mice and 10 times fewer thymocytes than wild-type mice, in agreement with data for Atm<sup>−</sup> mice. A reduction of 30–50% in the total number of thymocytes expressing α/βTCR and CD3 was observed in the thymi of Atm<sup>−</sup> and Atm-DSR1 mice, which is consistent with previous results for Atm<sup>−</sup> mice (9). More detailed analysis of eight 12-week-old Atm-DSR1 mice revealed that three of these mice showed significant increases in cell size, as reflected in scatter analysis and apparently random accumulation of either CD4<sup>−</sup>CD8<sup>−</sup>, CD8<sup>−</sup>CD8<sup>−</sup>low, CD8<sup>+</sup> (39%), or CD4<sup>+</sup> (64%) SP populations. This heterogeneity in three Atm-DSR1 mice is illustrated in Fig. 5a. These changes in T-cell subtypes were also evident in two younger (8-week) Atm-DSR1 mice. On the other hand, Atm<sup>−</sup> mice more closely resembled the wild type in the proportions of DP and SP T cells.

The lymph nodes of Atm<sup>−</sup> mice contain only 50% of lymphocytes expressing either CD3, CD4<sup>+</sup>CD8<sup>+</sup>, and CD4<sup>+</sup>CD8<sup>+</sup> compared with Atm-DSR1 and wild-type mice (results not shown). On the basis of the combination of low CD3, low CD62L, and low CD44 expression, the Atm<sup>−</sup> lymph nodes appear to consist primarily of T cells at an early stage of development (29). Both CD62L and CD44 are putative markers for memory T cells in mice.

**Life Span, Thymic Lymphomas, Apoptosis, and Up-Regulation of FasL in Atm-DSR1 Mice.** Patients with A-T have been demonstrated to develop a spectrum of tumors, primarily leukemias and lymphomas (6). On the other hand, Atm<sup>−</sup> mice develop exclusively malignant thymic lymphomas and die between 2 and 5 months of age when held in non-SPF facilities (7–9, 28). In this study (under non-SPF conditions), the life span of Atm-DSR1 mice (n = 43) was increased dramatically compared with Atm<sup>−</sup> mice (n = 26). When 100% of Atm<sup>−</sup> mice were dead, only 50% of Atm-DSR1 animals had died (43) was increased dramatically compared with Atm<sup>−</sup> mice (n = 26). When 100% of Atm<sup>−</sup> mice were dead, only 50% of Atm-DSR1 animals had died.

**Fig. 4.** Effect of Atm disruption on reproductive capability. a and b. H&E-stained sections through seminiferous tubules from wild-type (a) and Atm-DSR1 mice (b). ×40.
Morphological examination of thymic lymphomas from Atm-ΔSRI mice (>3 months of age) revealed that these tumors were more spongy and the cells less tightly aggregated than in the Atm<sup>−/−</sup>-tumors. Histological examination of tumors showed that there was a significantly increased proportion of cells undergoing what appeared to be spontaneous apoptosis based on nuclear fragmentation in Atm-ΔSRI tumors (Fig. 7b) compared with Atm<sup>−/−</sup>-tumors (Fig. 7a). These cells were confirmed as apoptotic by ISEL staining (Fig. 7c and d), and ~50% of Atm-ΔSRI thymic lymphoma cells were Annexin V positive compared with ~15% in Atm<sup>−/−</sup> thymic lymphomas (results not shown). To investigate these differences, we isolated disaggregated cells from the two types of tumors as well as thymocytes from wild-type and from the Atm-disrupted mice and performed flow cytometric analysis to compare surface markers for susceptibility to undergo apoptosis (32). Down-regulation of the Fas receptor (Fas/CD95) was evident in thymocytes in both Atm<sup>−/−</sup> and Atm-ΔSRI mice, with 30–40% fewer Fas-positive cells than in wild type (Table 1). This pattern of down-regulation is also seen in thymic lymphomas.

Died (Fig. 6). At this stage, autopsies revealed the presence of thymic lymphomas in all of these animals, indicating that death was a consequence of malignancy. Almost 30% of Atm-ΔSRI mice were alive after 16 months. The results obtained here for Atm<sup>−/−</sup> mice are consistent with previous reports on survival of these animals (9, 30).

Difference in survival appears not to be attributable to genetic background because both types of mice used here were of mixed genetic background: (a) C57BL/6J crossed with 129SvJ for Atm-ΔSRI; and (b) C57BL/6J crossed with 129SvEv for Atm<sup>−/−</sup>. Differences between 129Sv substrains do not significantly affect tumor incidence (31), and previous results have failed to demonstrate phenotype variation between mixed and inbred backgrounds for Atm<sup>−/−</sup> mice (7).

Phenotypic analysis of the thymic lymphomas revealed that the Atm<sup>−/−</sup>-phenotype was characterized predominately by CD4<sup>+</sup>CD8<sup>+</sup> DP cells and low CD3 (data not shown), whereas the Atm-ΔSRI lymphomas were more diverse, reflecting the greater heterogeneity of DP and SP populations of T cells seen in the thymi of these animals and may represent polyclonal tumors (Fig. 5b).

Fig. 5. FACS analysis of T-cell surface antigen expression in normal thymocytes and thymic lymphomas. a, forward scatter analysis (top panel) of thymocytes from wild-type, Atm<sup>−/−</sup>, and Atm-ΔSRI mice. Bottom panel, surface expression for CD4 and CD8 T-cell populations. Top right quadrants, double-positive cell populations. b, surface expression of CD4 and CD8 on thymic lymphomas from Atm<sup>−/−</sup> and Atm-ΔSRI mice.

Fig. 6. Kaplan-Meier survival plot of wild-type (WT), Atm<sup>−/−</sup>, and ΔSRI mice. P was calculated by comparing life spans of mice from each genotype using the SPSS statistical package. Atm<sup>−/−</sup> and Atm-ΔSRI mice of either 129Sv inbred or 129SvC57BL/6J mixed genetic background were used in this study. No differences in survival were observed with mice of different genetic backgrounds.

Fig. 7. Histological examination of thymic lymphomas. a and b, H&E staining of thymic lymphoma sections from Atm<sup>−/−</sup> and Atm-ΔSRI mice. ×40. The Atm<sup>−/−</sup>-thymic lymphoma (a) shows a reduced nuclear fragmentation indicative of apoptosis as compared with the Atm-ΔSRI thymic lymphoma (b), which shows extensive apoptosis. c and d, ISEL of paraffin sections of thymic lymphomas from Atm<sup>−/−</sup> and Atm-ΔSRI mice. Cells were labeled with Biotin-16-dUTP, and apoptosis was detected with horseradish peroxidase-coupled streptavidin and 3,3′-diaminobenzidine.

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from both forms of mutant mice. Expression of FasL was approximately the same in thymocytes from wild-type and mutant mice (Table 1). A small increase was evident in thymic lymphomas from Atm−/− mice, but in thymic lymphomas from Atm-ΔSRI mice there was an ~4-fold increase in the number of cells expressing FasL (Table 1). This corresponds well with the increased extent of apoptosis detected in thymic lymphomas from Atm-ΔSRI mice compared with Atm−/− mice. However, this extent of apoptosis was not observed, and FasL was not up-regulated in Atm-ΔSRI mice developing lymphomas at <2 months of age. To test the functionality of the FasL expression in Atm-ΔSRI tumors, we incubated thymic lymphoma cells with a target cell line AT1ABR, a lymphoblastoid cell line from a patient with A-T, containing the same mutation as in the Atm-ΔSRI mice (18). This cell line has a high expression of Fas. At a ratio of 3:1 lymphoma:target cells, viability of AT1ABR was reduced by 80% after 12-h incubation demonstrating that the up-regulated FasL was active in the apoptotic process observed in Atm-ΔSRI tumors (results not shown). This was substantiated additionally by attempts to establish the thymus lines in culture, where it was observed that those from Atm-ΔSRI were more difficult to grow.

**Development of a Spectrum of Tumors in Long-lived Atm-ΔSRI Mice.** It is evident from the data in Fig. 6 that the longevity of Atm-ΔSRI mice is markedly greater than that of most Atm−/− mice. As indicated above, all of the Atm−/− mice had died from thymic lymphomas by 40 weeks in this study. The 44% (19 of 43) of Atm-ΔSRI mice that had died by the same time also died with the same tumors. Very few Atm-ΔSRI mice died after this time from thymic lymphomas, but a significant number of deaths (7) were associated with a variety of other tumors. Upon autopsy, the mice usually displayed marked splenomegaly but with minimal involvement of the thymus. Immunochemistry showed that three of these animals had B-cell leukemia. The B-cell tumors grew aggressively with no evidence of FasL up-regulation. Furthermore, histopathological examination of various tissues/organisms revealed the presence of multiple tumor types including ovarian granulosa cell tumors, epithelial carcinomas, histiocytic/reticulum cell tumors, ovarian sex cord, and stromal cell tumors. Mutant Atm protein was expressed in both lymphoid and solid tumors (results not shown).

**DISCUSSION**

The Atm-ΔSRI mouse represents a dysfunctional Atm model that expresses mutant protein unlike all of the other Atm−/− mice described to date, which do not express Atm protein because of the nature of the gene disruption (7–9, 12, 14). In one case, a neo<sup>+</sup> gene replaced a portion of the Rad-3 homology domain of Atm, but neither full-length Atm transcript nor protein were detected (14). The mutant mouse described here corresponds to a commonly observed mutation in A-T patients (763delG; Refs. 15 and 18). As with the human mutant, Atm-ΔSRI protein is less stable as judged by the amount of Atm determined by immunoblotting, and no Atm-kinase activity was detected, similar to that for the human mutant (33). However, some variability in the amount of Atm-ΔSRI protein was observed in different experiments. Because this short deletion is upstream from the kinase domain, it is likely that it causes an inactivating, conformational change in the Atm protein or prevents binding of a protein critical for activity. Since the SRI sequence is in a putative domain, FAT (Ref. 34; FRAP, ATM, and TRAPP), present in a subfamily of phosphatidylinositol 3-kinases, and because this domain is implicated in multimeric protein complex formation, its loss may interfere with protein-protein interactions. There is evidence that ATM is present in a large protein complex with BRC1A1, BLM, Mrell-Rad50-NBS1, and DNA repair proteins, called BRC1A1-associated genome surveillance complex (35), but none of these proteins have been shown to interact with this region of ATM.

In this study, thymocytes and splenocytes from both Atm−/− and Atm-ΔSRI mice were more susceptible to radiation-induced killing than wild-type cells. This was confirmed by radiation-induced chromosome aberrations, which were 3-fold higher in both types of mutant mouse thymocytes than in wild-type cells. This is in agreement with two previous reports that showed that ES cells from Atm−/− mice were hypersensitive to radiation at low doses (7). Although other studies suggest that Atm−/− cells are equally sensitive as wild-type cells in mitogen-stimulated T cell proliferation (36), in some instances these cells are only equally sensitive to differentiating factors (37). In other studies, exposure conditions were different in that whole animal irradiation occurred prior to cell isolation, higher doses were used (5–20 Gy), and apoptosis was the end point (12, 28, 30). However, at least in the case of embryonic fibroblasts, extremely poor growth makes it difficult to differentiate between the two cell types (7). Although Elson et al. (9) have shown that the relative extent of the increase in chromosome damage by bleomycin is similar in Atm-deficient and wild-type murine fibroblasts, the absolute value, 9.71 breaks/metaphase, is considerably greater in Atm−/− cells than in wild type (1.70 breaks/metaphase). This might well translate into increased cell killing. Indeed, Westphal et al. (36) have shown that loss of Atm radiosensitizes multiple cell types including fibroblasts, bone marrow, and gastrointestinal cells as well as p53-null bone marrow cells. Furthermore, whereas p53−/− cells are considered generally to be radioreistant, mitogenic stimulation of p53−/− lymphocytes renders them susceptible to radiation-induced killing (37). In other studies, exposure conditions were different in that whole animal irradiation occurred prior to cell isolation, higher doses were used (5–20 Gy), and apoptosis was the end point (12, 28, 30, 36, 38). All of these studies point to reduced apoptosis in irradiated Atm−/− cells. However, because death by apoptosis may only account for 30% of radiation-induced killing in A-T cells (39), the data obtained may not reflect the actual extent of cell killing. In addition, Brown and Wouters (40) have reported that when clonogenic survival is used as an end point of cell killing, ability to undergo apoptosis does not contribute significantly to the sensitivity of tumor cells to radiation and chemotherapeutic drugs.

Although a number of the Atm−/− phenotypic characteristics have been reproduced in the Atm-ΔSRI mice, the most dramatic difference is the significantly greater life span of these animals. In the experiments conducted here, Atm-ΔSRI and Atm−/− mice were bred in a non-SPF facility. In previous reports, Atm−/− mice were also housed under non-SPF conditions, and all of the animals had died with thymic lymphomas between 2 and 5 months of age (7–9, 30). The Atm−/− mice used here died at rates comparable with those observed previously for these animals (9). Longer life spans in Atm−/− mice appear to be attributable to breeding in pathogen-free conditions. This is particularly evident with the Atm−/− mice produced by Barlow et al. (7), where the mice did not survive beyond 4.5 months, but when the same animals were maintained in SPF housing, 40% of animals were still alive after 18 months (10). Breeding was also carried out in

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</tr>
<tr>
<td>Wild-type</td>
<td>90.6 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>Atm</td>
<td>62.7 ± 2.4</td>
<td>2.8 ± 1.2</td>
</tr>
<tr>
<td>Atm-ΔSRI</td>
<td>54.8 ± 4.5</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Thymic lymphoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atm−/−</td>
<td>60.8 ± 7.7</td>
<td>7.5 ± 1.5</td>
</tr>
<tr>
<td>Atm-ΔSRI</td>
<td>57.8 ± 10.8</td>
<td>30.7 ± 0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> n = 5 mice in each group.

<sup>b</sup> Percentage of total thymocytes ± SE.

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4566
pathogen-free facilities by Borghesani et al. (14), where 50% of Atm mutant mice were alive after 10 months. Clearly, SPF conditions were not required for the prolonged life span described here in Atm-ΔSRI mice.

The extent of apoptosis occurring in thymic lymphomas from Atm-ΔSRI mice may contribute to the longevity in Atm-ΔSRI mice. Previous studies have failed to observe apoptotic cells in tumors from the Atm-/- mice used in the present investigation (41). However, thymic lymphomas derived from Atm/p21 double-null mice show high levels of apoptotic cells. It appears likely that loss of p21 potentiates and it provides a model to investigate additionally the molecular basis described here provides additional evidence for heterogeneity in A-T, the apoptotic response presumably because of a lack of G2 arrest (41).

Whereas the Atm-ΔSRI thymic lymphomas and consequent apoptosis may explain why only 50% of Atm-ΔSRI mice succumb to thymic lymphomas after 40 weeks, at which time all of the Atm/-/- mice (n = 26) in this study had died from thymic lymphomas (Fig. 6). The “take rate” of these tumors may be reduced by Fas/FasL-induced apoptosis. In all, 19 of 43 (44%) of Atm-ΔSRI mice died of thymic lymphomas up to 10 months of age. Some of the other animals died from splenetic, ovarian, and B-cell tumors with evidence of metastatic spread. To date, no B-cell tumors have been recorded in Atm-/-/- mice, and there has been no evidence of tumor metastases in these mice. Whereas the Atm-ΔSRI mice have significantly increased longevity over the Atm/-/- mice, it appears that there may be two periods of tumor susceptibility: (a) the initial period up to 10 months where animals develop thymic lymphomas; and (b) for the survivors, a later onset of tumorsogenesis with a distinct set of tumors. This second period of tumor susceptibility can be explained by the development of age-related tumors. No tumors were observed in wild-type controls during the same time frame. Thus, the phenotype of the mutant described here provides additional evidence for heterogeneity in A-T, and it provides a model to investigate additionally the molecular basis of tumor development in A-T.

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REFERENCES


**Atm Knock-in Mice Harboring an In-frame Deletion Corresponding to the Human ATM 7636del9 Common Mutation Exhibit a Variant Phenotype**

Kevin Spring, Simone Cross, Chung Li, et al.

*Cancer Res* 2001;61:4561-4568.

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