Dual roles of ATM in the cellular response to radiation and in cell growth control

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The gene mutated in ataxia-telangiectasia (AT) patients, denoted ATM, encodes a putative protein or lipid kinase. To elucidate the functions of ATM, we disrupted the mouse ATM gene through homologous recombination in mice. Consistent with cellular defects of AT patients, the ATM⁻/⁻ cells are hypersensitive to γ-irradiation and defective in cell-cycle arrest following radiation, correlating with a defective up-regulation of p53. In addition, ATM⁻/⁻ mouse thymocytes are more resistant to apoptosis induced by γ-irradiation than normal thymocytes. ATM⁻/⁻ fibroblasts are inefficient in G1 to S-phase progression following serum stimulation and senesce after only a few passages in culture. They have an increased constitutive level of p21CIP/WAF1. The ATM protein is therefore critical both for cellular responses to ionizing radiation and for normal cell-cycle progression. ATM⁺/⁺ fibroblasts and thymocytes showed intermediately defective responses to irradiation but no growth defect, suggesting that the increased cancer risk of AT heterozygotes could be attributable to poor checkpoint function.

[Key Words: DNA repair; cell-cycle arrest; p53; p21; cellular proliferation]

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Ataxia-telangiectasia (AT) is an autosomal recessive human hereditary disorder characterized by multisystem defects. These include progressive cerebellar degeneration leading to ataxia, dilated blood vessels (telangiectasia) in the eye and facial area, sensitivity to γ-irradiation, high incidence of tumorgenesis in the lymphoid system, degeneration of thymus, and deficiency in both humoral and cellular immune responses [Lehmann 1982; Swift et al. 1991; Thacker 1994]. Whereas AT patients are relatively rare (about 4×10⁻⁴), about 0.5% of the general population harbors a single mutant allele [Swift et al. 1987, 1991]. Significantly, cells derived from people heterozygous for an AT mutation have an intermediate sensitivity to γ-irradiation, and it has been suggested that heterozygous people have a cancer predisposition about fivefold that of the normal population [Swift et al. 1987, 1991]. Therefore, understanding the molecular basis of the AT mutation is of great importance.

AT cells are hypersensitive to DNA damaging agents, such as γ-irradiation and restriction enzyme digestion, that can induce double-stranded DNA breaks [Thacker 1994]. Furthermore, the ability of AT cells to arrest in G1 following DNA damage is deficient, indicating defects in the cell-cycle checkpoint control [Meyn 1995]. Because the induction of the p53 protein after γ-irradiation is responsible for the arrest at G1 checkpoint [Kuerbitz et al. 1992], studies have been carried out to evaluate the p53 response to γ-irradiation in human AT cells. The initial study suggested that the p53 response to γ-irradiation is defective in AT cells [Kastan et al. 1992]. However, later studies found either no substantial defect in the p53 response to γ-irradiation in AT cells or that the p53 response is reduced with a delayed kinetics [Khanna and Lavin 1993; Lu and Lane 1993]. Genetic heterogeneity among mutant and control cells may have masked effects.

A gene mutated in all tested AT patients, denoted ATM, has been identified recently through positional cloning [Savitsky et al. 1995]. The ATM gene encodes a large protein that is similar to a number of large proteins that define a subfamily of kinases including the PI-3 (lipid) kinase [Savitsky et al. 1995]. Members of this family contain a highly conserved carboxy-terminal kinase domain [Zakian 1995]. Several members of this family, including yeast proteins Rad3, Mec1, and Tel1, are involved in DNA repair and cell-cycle checkpoint control [Weinert and Lydall 1993; Greenwell et al. 1995; Hari et al. 1995; Morrow et al. 1995; Paulovich and Hartwell 1995]. Other members of the family, such as yeast proteins Tor1p and Tor2p and their mammalian counterpart FRAP, control cell-cycle progression from the G1 phase into the S phase [Brown et al. 1995]. A direct link between this kinase family and DNA repair was provided by the discovery that the DNA-dependent protein kinase [DNA-PK] is a member of this family [Hartley et al. 1995].
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1995]. It is involved in double-stranded DNA break repair and in lymphocyte V(D)J recombination—a defective DNA-PK leads to severe immunodeficiency in mice [Blunt et al. 1995]. If structural homology implies functional similarity, ATM could be involved in a cell-cycle checkpoint responding to DNA damage.

The PI-3 kinase family members, including p110α, β, and γ, phosphorylate inositol phospholipids that are involved in signal transduction pathways [Hunter 1995]. Members of the ATM kinase family have so far failed to show any intrinsic lipid kinase activity [Hunter 1995]. However, Tor proteins and DNA-PK, as well as PI-3 kinase, display protein kinase activity [Carpenter et al. 1993; Dhand et al. 1994; Brown et al. 1995; Hartley et al. 1995], suggesting that ATM may function as a protein kinase.

To elucidate the mechanisms of defective cell-cycle checkpoint control, neurodegeneration, and immunodeficiency in AT patients, we created mutant mice in which the ATM gene is disrupted through homologous recombination. The ATM−/− mice are viable and have developed immune and other defects similar to certain symptoms of AT patients [Xu et al., this issue]. Our study of mouse embryo fibroblasts [MEFs] and thymocytes from mice indicates a dual role of ATM relating to cell-cycle checkpoint control. Following γ-irradiation, the ATM−/− cells arrest cell cycle poorly and have a defective p53 response. These characteristics support the idea that ATM is responsible for signaling the p53-mediated checkpoint control, neurodegeneration, and immunodeficiency in AT patients. To generate ATM−/− ES cells, the ATM+/− ES cells were selected with higher concentrations of G418 as described previously [Xu et al. 1996b]. ES colonies surviving the selection of 4.8 mg/ml G418 were expanded and analyzed by Southern blotting. The ATM−/− ES cells were subcloned and confirmed (data not shown).

Six out of seven ATM−/+ ES clones injected into blastocysts gave rise to chimeric mice that transmitted the ATM mutation into germ-line. Mice heterozygous for the ATM mutation [ATM−/+] were intercrossed to generate mice homozygous for the ATM mutation [ATM−/−]. Tail DNA derived from ATM−/+ and ATM−/− MEFs. The amplified products from the synthesized cDNA are correlated with the primers indicated with arrowheads.

**Results**

**Targeted disruption of the ATM gene in mice**

Because the kinase domain of ATM is frequently mutated in AT patients—alteration of one serine residue in the domain can cause the AT phenotype [Savitsky et al. 1995]—we designed a targeting construct to disrupt conserved sequence in the ATM kinase domain [Fig. 1A,B]. To screen for homologous recombination events, genomic DNA isolated from transfected embryonic stem (ES) clones was digested with BamHI and probed with probe A that was outside the transfected targeting construct, giving a 18-kb germ-line band and a 10-kb mutant band [Fig. 1A–C]. Positive ES clones were further confirmed by BamHI digestion and hybridization to probe B, giving a 18-kb germ-line band and a 4.3-kb mutant band [Fig. 1A–C].

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**Figure 1.** Targeted disruption of the ATM gene in mice. (A) Genomic configuration of the germ-line ATM locus. Exons are represented with open boxes except that the exonal region encoding the conserved kinase sequence is represented with a filled box. The length of the BamHI diagnostic restriction fragment and the locations of the probes are shown. The transcriptional direction of the ATM gene and the locations of the primers are indicated with arrowheads. (B) Targeting construct to insert the PGK-neoG gene into one ATM exon and simultaneously to replace the second exon. The transcriptional direction of the PGK-neoG gene is indicated with an arrowhead and is opposite that of the ATM gene. (C) Targeted locus following homologous recombination between the targeting construct and the endogenous ATM locus. The lengths of the diagnostic restriction fragments are shown. (D) Southern blot analysis of the tail DNA from ATM−/−, ATM−/+ and ATM+/+ mice. Tail DNA [10 μg] was digested with BamHI and hybridized to probe A. Because of the polymorphic nature of the BamHI restriction at this locus, the size of the germ-line band is either 18 kb if the allele is derived from ES cells or 20 kb if the allele is derived from C57BL/6. The locations of the DNA size marker are indicated. (E) RT–PCR analysis of the ATM mRNA in ATM−/−, ATM−/+ and ATM+/+ MEFs. The amplified products from the synthesized cDNA are correlated with the primers indicated with arrowheads.
and ATM−/− mice were analyzed by Southern blot [Fig. 1D].

Because the ATM gene is expressed ubiquitously [Savitsky et al. 1995], we employed RT–PCR to check the expression of ATM in the MEFs derived from day-14 ATM+/+, ATM+/−, and ATM−/− embryos. In the PCR assays, primers 1 and 2 were used to detect the coding region that was disrupted directly by the PGK-neo gene; primers 3 and 4 were outside the targeting construct and used to amplify a larger coding region containing the disrupted exons [Fig. 1A]. Using both sets of primers, no expression of the ATM mRNA could be detected in the ATM−/− MEFs [Fig. 1E], indicating that the expression of the ATM kinase domain had been disrupted. Because the ATM kinase domain appears to be essential for the function of ATM, this targeted mutation should destroy the production of a functional ATM.

ATM−/− ES cells are hypersensitive to γ-irradiation

Taking advantage of the capability of ES cells to grow clonally, we established a clonal survival assay to examine the sensitivity of ATM−/− ES cells to γ-irradiation. Consistent with cellular hypersensitivity of AT cells, the ATM−/− ES cells are also much more sensitive to the γ-irradiation than ATM+/+ and ATM+/− ES cells [Fig. 2].

Cell-cycle arrest following irradiation

In response to DNA damage caused by agents such as γ- or UV irradiation, mammalian cells undergo cell-cycle G1 arrest [Kastan et al. 1992; Lu and Lane 1993]. However, inhibition of cell-cycle progression by γ-irradiation is reduced in cells derived from AT patients [Meyn 1995]. Therefore, we examined the ability of ATM+/+, ATM+/−, and ATM−/− MEFs to arrest cell cycle at G1 following γ-irradiation or UV irradiation using a protocol originally developed by others [Kastan et al. 1991, 1992; Kuerbitz et al. 1992]. The cells were asynchronously growing at the time of irradiation and their cell-cycle position was assayed using the fluorescence-activated cell analyzer to measure DNA content and bromodeoxyuridine (BrdU) incorporation during a pulse label at 14 hr after irradiation. In the absence of irradiation, the ATM−/− and ATM+/− cells had somewhat smaller S-phase fractions than the wild-type cells [Fig. 3A; not shown]. In normal cells, there was a 75% loss of S-phase cells following irradiation of 10 Gy and an accumulation of cells in G2/M and G1/G0 [Fig. 3A]. The ATM−/− MEFs had only about a 40% loss of S-phase at this dose of irradiation. G2/M phase cells accumulated extensively but there were relatively fewer G1/G0 cells [Fig. 3A,B]. The effects on S-phase cells were dose-dependent [Fig. 3B]. The ATM−/− MEFs exhibited an intermediate defect in cell-cycle arrest following higher dosages [≥5 Gy, Fig. 3A,B], but apparently behaved normally if exposed only to a lower dose [2 Gy; data not shown]. In contrast, ATM+/+, ATM+/−, and ATM−/− MEFs showed equivalent ability to reduce their S-phase fraction following UV-irradiation [Fig. 3B].

To measure the optimal defect of ATM−/− MEFs in G1, cell-cycle arrest following γ-irradiation, we examined the ability of ATM+/+, ATM+/−, and ATM−/− MEFs to arrest their cell cycle in G1 phase at different time points following 10-Gy γ-irradiation. Whereas maximal defects in G1 were observed 10 hr following γ-irradiation, ATM−/− MEFs exhibited defective G1 cell-cycle arrest and ATM+/− MEFs showed an intermediate defect at all tested time points following γ-irradiation [Fig. 3C].

The p53 response to DNA damage

The p53 protein level is up-regulated transiently after DNA damage and cell-cycle arrest following DNA damage requires an intact p53 gene [Kastan et al. 1992]. The role of p53 may be to activate the expression of a number of genes including p21CIP1/WAF1 [El-Diery et al. 1993; Xiong et al. 1993; Macleod et al. 1995]. To understand the basis of the defective cell-cycle G1 arrest following γ-irradiation but not UV-irradiation in ATM−/− cells, the protein levels of p53 at different time points following γ- or UV irradiation were evaluated by immunoblot analysis of whole-cell extracts. It was confirmed that equivalent amounts of protein were transferred in each lane by staining the nitrocellulose membrane after transfer. One hour following 10 Gy γ-irradiation, a significant increase of p53 protein was seen in both ATM+/+ and ATM+/− MEFs [Fig. 4A], although the magnitude of the up-regulation in ATM−/− MEFs appeared to be less than that in ATM+/+ MEFs. The p53 protein levels decreased at later time points [2, 3, 4, 5, 8 hr] following γ-irradiation in both ATM+/+ and ATM+/− MEFs [Fig. 4A, data not shown]. In contrast, no increase of p53 protein level

Figure 2. Clonogenic survival of ATM+/+ [●], ATM+/− [◆], and ATM−/− [□] ES cells following exposure to graded doses of γ-irradiation. For each irradiation dosage, colonies from duplicated wells were counted. The genotypes are indicated in the figure. Consistent results were obtained from two sets of independently produced ATM+/+ and ATM−/− ES cells.

Functions of ATM in cell-cycle regulation

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Figure 3. Defective ability of the ATM−/− MEFs to arrest in G2/M phase following γ-irradiation. (A) A FACScan analysis of the untreated and irradiated ATM+/+, ATM+/−, and ATM−/− MEFs following 10 Gy γ-irradiation. DNA synthesis is revealed by FITC-conjugated anti-BrdU antibody and the DNA content is revealed by propidium iodide (PI) staining. Boxes representing cells in G1, S, and G2/M phases as well as the percentage of total cells are indicated. We have not observed consistent difference between the number of S-phase cells of ATM+/+ and ATM+/− MEFs. (B) Quantitative analysis of the number of cells in S phase following γ-irradiation versus the number of cells in S phase in an untreated control. Bars as in B. Three independent experiments were performed for γ-irradiation at each dosage and two for UV-irradiation at a dosage of 60 J/m². The mean value is presented with error bars showing standard deviation. Consistent results were obtained from independently derived MEFs.

Figure 4. The p53 response to radiation and p21 protein levels in ATM+/+, ATM+/−, and ATM−/− MEFs. (A) The protein levels of p53 at 0, 1, 4, 8 hr following 10-Gy γ-irradiation. The lanes are labeled on the top. p53 is indicated with arrowheads. (B) The protein levels of p53 at 0 and 14 hr following 60 J/m² UV-irradiation. The lanes are labeled at the top and protein indicated with arrowheads. (C) The p21 protein level in proliferating and resting MEFs. Proliferating MEFs of various passage number were harvested and MEFs resting at G0 were obtained by serum starvation for 96 hr. The genotypes and passage numbers are shown on the top and p21 is indicated with arrowheads. (D) Protein levels of p21 at different time points after serum stimulation of G0 MEFs. The time points and genotypes are shown on the top and p21 is indicated with arrowheads.

was observed in ATM−/− MEFs 1 hr after γ-irradiation and only a small increase of p53 protein level was detected at 3 or 4 hr but not at 2, 5, 8 hr following irradiation (Fig. 4A; data not shown). Therefore, the p53 response to γ-irradiation was defective in ATM−/− cells.

Based on the reported kinetics of the p53 response to UV-irradiation in mouse cells (Lu and Lane 1993), we analyzed the p53 response in MEFs 14 hr following UV irradiation (Fig. 4B; data not shown). A similar up-regulation of p53 protein level was observed in MEFs of all genotypes (Fig. 4B), indicating that the p53 response to UV irradiation is normal in ATM−/− MEFs.

p21 expression in MEFs

We also analyzed the p21 protein levels in ATM+/+, ATM+/−, and ATM−/− MEFs. The specificity of the polyclonal anti-p21 antibody we used has been verified previously (Deng et al. 1995). A considerably higher level of p21 protein was detected in both proliferating and resting ATM−/− MEFs (Fig. 4C). In addition, analysis of the p21 protein level at different time points following serum stimulation of synchronously growth-arrested cells also indicated higher levels of p21 protein at all time points in ATM−/− MEFs (Fig. 4D). p21 protein lev-
els in cells derived from AT patients have been examined [Canman et al. 1994]. However, no difference was detected between AT cells and cells derived from normal individuals. This could be attributable to a difference between human and mouse cells or the fact that the AT cells used in these studies were immortalized and EBV-immortalization may mask this p21 effect.

\[ \gamma\text{-irradiation-induced apoptosis of thymocytes in vivo} \]

Because \( \gamma\text{-irradiation-induced apoptosis of immature thymocytes is mediated by p53 [Clarke et al. 1993; Lowe et al. 1993] and the p53 response to } \gamma\text{-irradiation is defective in } ATM^{-/-}\text{ MEFs [Fig. 4A], we examined the apoptotic response of } ATM^{+/+}\text{ and } ATM^{-/-}\text{ thymocytes to in vivo } \gamma\text{-irradiation. T-cell development in the thymus of both } ATM^{+/+}\text{ and } ATM^{-/-}\text{ mice is defective and will be described in detail elsewhere [Fig. 5A; Xu et al., this issue]. As expected, the immature CD4^+CD8^+ thymocytes, which are most susceptible to apoptosis, essentially disappear in the thymus of normal mice 48 hr after exposure to 5-Gy } \gamma\text{-irradiation [Smith et al. 1989; Clarke et al. 1993; Lowe et al. 1993; Fig. 5A–C]. Similar to } ATM^{-/-}\text{ mice, total thymocyte number is also reduced significantly in } ATM^{-/-}\text{ mice 48 hr after exposure to } \gamma\text{-irradiation, indicating that } ATM^{-/-}\text{ thymocytes are also sensitive to } \gamma\text{-irradiation [Fig. 5C]. However, compared with that of } ATM^{+/+}\text{ mice, the much higher percentage of immature thymocytes surviving the effect of } \gamma\text{-irradiation in } ATM^{-/-}\text{ mice indicates that } ATM^{-/-}\text{-immature thymocytes are more resistant to the effects of } \gamma\text{-irradiation than normal immature thymocytes, implying that p53-mediated apoptosis following } \gamma\text{-irradiation is defective in } ATM^{-/-}\text{ thymocytes [Fig. 5A–C]. } ATM^{+/+}\text{ thymocytes displayed an intermediate resistance to } \gamma\text{-irradiation [Fig. 5A,B].} \]

\[ \textbf{Impaired proliferation of } ATM^{-/-}\text{ MEFs in cultures} \]

Fibroblasts derived from AT patients display a premature senescence [Shiloh et al. 1985]. Therefore, we examined the growth properties of \( ATM^{+/+}\text{, } ATM^{+/-}\text{, and } ATM^{-/-}\text{ MEFs. Irrespective of their passage numbers, } ATM^{-/-}\text{ MEFs proliferated more slowly than } ATM^{+/+}\text{ and } ATM^{+/-}\text{ MEFs [Fig. 6A,B]. In addition, and in contrast to p53^{-/-}\text{ MEFs, the saturation density of } ATM^{-/-}\text{ MEFs is only about half that of } ATM^{+/+}\text{ and } ATM^{+/-}\text{ MEFs [Fig. 6A,B; Deng et al. 1995]. After passage 6, the } ATM^{-/-}\text{ MEFs essentially became senescent, whereas the } ATM^{+/-}\text{ and } ATM^{+/+}\text{ MEFs continued to proliferate [Fig. 6C].} \]

\[ \textbf{G}_{1}/\text{S progression in the cell cycle of } ATM^{-/-}\text{ MEFs} \]

To examine the defects in cellular growth involved in the slow proliferation and ultimate senescence of the mutant fibroblasts, we asked how efficiently the cells came into S phase after serum stimulation. \( ATM^{+/+}\text{, } ATM^{+/-}\text{, and } ATM^{-/-}\text{ MEFs of passage 3 synchronized at } G_{0}\text{ by serum starvation were released into medium containing 10% fetal calf serum and 24 or 72 hr later the percentage of cells in S phase was determined. Compared with } ATM^{+/+}\text{ and } ATM^{+/-}\text{ MEFs, a considerably lower percentage of } ATM^{-/-}\text{ cells had entered the S phase 24 hr after serum stimulation [Fig. 7A,B]. However, } >90\%\text{ of } ATM^{+/+}\text{ and } ATM^{-/-}\text{ MEFs had entered the S phase by 72 hr [data not shown]. Therefore, } ATM^{-/-}\text{ MEFs have a defective ability to enter S phase, probably accounting for their slower growth.} \]

\[ \textbf{Discussion} \]

Previous work had demonstrated a number of defects in
ATM−/− human cells derived from patients, but there was disagreement in the literature about their nature, probably as a consequence of the genetic heterogeneity inherent in human studies. In particular, it is not clear that all human ATM alleles give the same phenotype. Using ATM−/− cells derived from mice with a disrupted ATM gene, we could confirm certain previous studies and provide a clear picture of two types of defects in the cells, one for the response to ionizing radiation and the other in normal growth control. As significantly, we could demonstrate a series of defects in ATM+−/− cells that might explain an increased tendency to carcinogenesis in heterozygous individuals.

Studying the response of cells to γ-irradiation, we found that—consistent with previous studies of fibroblasts derived from AT patients (Meyn 1995)—the ATM−/− cells do not arrest as effectively as wild-type cells. They maintain more S-phase cells after irradiation, indicating a lack of an effective block at the G1/S border. They were not defective in their response to UV irradiation. Thus, ATM behaves as a checkpoint controller for cells that have been exposed to ionizing radiation, perhaps indicating that the protein is involved in sensing the DNA strand breaks caused by the irradiation. An important conclusion from these studies is that in the absence of ATM function, γ-irradiation does not induce p53 effectively. This indicates that p53 is downstream of ATM.

Consistent with studies of human AT cells, ES cells that are ATM−/− are more sensitive to γ-irradiation than normal ES cells. A defective ability of AT cells to cell-cycle arrest following DNA damage has been suggested previously to contribute to the hypersensitivity of AT cells to ionizing radiation and the high frequency of tumorigenesis in AT patients (Lehmann 1982; Shiloh et al. 1985).

Paradoxically, ATM−/− CD4+CD8+ thymocytes that have been irradiated in situ are more resistant to γ-irradiation than their wild-type counterparts but this probably indicates that ATM is involved in signaling an apoptotic response in some cells and a growth arrest re-
response in others, just as seen for p53 signaling [Clarke et al. 1993; Lowe et al. 1993].

ATM appears to play a key role in normal control of fibroblast growth. MEFs from ATM−/− mice showed three defects in in vitro growth: a lower saturation density, slow passage through the G1/S border after serum stimulation, and a more rapid senescence. Fibroblasts derived from AT patients [Shiloh et al. 1985] display an apparent premature senescence. In addition, ATM−/− mouse cells also showed a higher than normal constitutive level of p21 at all times. What causes the increased p21 is not clear, and whether it is the cause of the various effects on cell growth must be tested, but the correlation of increased p21 and senescence has been noted before [Halevy et al. 1995; Nakanishi et al. 1995; Parker et al. 1995].

ATM+/- cells showed partial defects in certain parameters we measured but not others. In particular, they were unaltered in their sensitivity to γ-irradiation, but that was only measured in ES cells at a low radiation dose (≤5 Gy); they showed a partial defect in cell-cycle arrest at high doses of ionizing radiation; their radiation-induced p53 response was lower than wild-type; they showed some resistance to thymocyte death, but their in vitro growth parameters were unaffected. The defects are probably not caused by the generation of a dominant negative gene product from the mutant ATM allele, because humans heterozygous for different mutations of the ATM gene, including a deletion of the whole ATM locus or missense mutations in the kinase domain, share similar intermediate cellular defects [Lehmann 1982; Shiloh et al. 1985]. Heterozygous humans have a somewhat increased cancer rate but none of the other symptoms of AT [Swift et al. 1987, 1991]. The defects in radiation response of the heterozygous cells suggest that the increased cancer risk could be attributable to the incomplete cell-cycle arrest and a decreased apoptotic response following DNA damage. The lack of changed cell-cycle parameters in ATM+/- cells suggests that normal cell growth may be less sensitive to the level of ATM than the checkpoint functions and the multigene defects such as growth retardation, premature aging, and thymus degeneration in AT patients [Lehman et al. 1982] could be more a consequence of the cell growth defect in ATM−/− cells than the lack of radiation response. The cancer risk in AT heterozygotes, however, could be a direct reflection of the poor radiation response in the heterozygous cells.

ATM regulates the p53 response to ionizing radiation

It has been shown that the cell-cycle arrest in G1 following DNA damage is mediated by p53 and that DNA damage leads to a rapid and transient increase of p53 protein level [Kastan et al. 1992; Kuerbitz et al. 1992; Lu and Lane 1993]. Previous studies to evaluate the p53 response to γ-irradiation in AT cells have yielded conflicting results [Kastan et al. 1992; Lu and Lane 1993]. In general agreement with previous reports by Kastan et al. [1992] and Khanna et al. [1993], we found that the p53 response to γ-irradiation in ATM−/− MEFs is substantially defective, indicating that ATM plays a significant role in signaling the p53 response following ionizing irradiation. The delayed kinetics of the p53 response to γ-irradiation we observed in ATM−/− MEFs has also been reported by Lu and Lane [1993] in human AT fibroblasts. However, the greatly reduced p53 response in ATM−/− cells was not observed by Lu and Lane.

γ-irradiation-induced apoptosis of thymocytes is mediated by p53 [Clarke et al. 1993; Lowe et al. 1993]. Therefore, the finding that ATM−/− thymocytes, although still sensitive to γ-irradiation, are more resistant to apoptosis induced by γ-irradiation than normal immature thymocytes suggests that the p53 response to DNA damage also may be defective in ATM−/− thymocytes, supporting further the notion that ATM is responsible for signaling the p53 response to DNA-strand-break damage. The resistance of these ATM mutant thymocytes to p53-mediated apoptosis following DNA damage would allow thymocytes harboring damaged DNA to survive and undergo selection for the mutated cells that are neoplastic. This might explain why the predominant tumors found in AT patients are of lymphoid origin (Lehmann 1982). In fact, a greatly increased frequency of thymic lymphomas is now appearing in our ATM−/− mice and these tumors are of CD4+CD8+ origin [Xu et al., this issue].

Synthesis of p53 protein is regulated negatively by wild-type p53 that binds to the 5′ untranslated region and inhibits the translation of its own mRNA [Mosner et al. 1995]. The rapid and transient up-regulation of p53 following DNA damage is mediated through a post-transcriptional mechanism perhaps involving protein stabilization [Kastan et al. 1992; Mosner et al. 1995]. In this context, ATM may function by sensing DNA damage and signaling events like relief of the translational inhibition or stabilization of p53. In addition, ATM may carry out its function by directly phosphorylating p53, as suggested by the observation that DNA-PK, a relative of ATM, can phosphorylate p53 in vitro [Hartley et al. 1995].

After this paper was submitted, Barlow et al. [1996] published their description of independently generated ATM mutant mice. Fibroblasts derived from their ATM mutant mice are also defective in both cell-cycle checkpoint control following γ-irradiation and cellular proliferation.

Materials and methods

Cloning of the human ATM cDNA fragment by PCR

A 516-bp cDNA fragment encoding the conserved kinase sequence was amplified from a human B cell cDNA library (gift from S. Elledge, Baylor College of Medicine, Houston, TX) by PCR using primers specific for the human ATM gene [Hunter 1995; Savitsky et al. 1995]. The PCR reaction was done in a final volume of 100 μl containing 2 μg of cDNA, 150 ng of each primer, 1× PCR buffer (Boehringer Mannheim), 0.2 μM dNTP, and 5 units of Taq polymerase (Boehringer Mannheim). PCR was run for 35 cycles, each consisting of 1 min at 94°C, 1.5 min
at 62°C and 1 min at 72°C. The final reaction step was followed by an extension at 72°C for 10 min. Half of the reaction was analyzed on a 1.2% agarose gel. The sequences of the primers used were as follows: primer 1: 5'-GCTGTTATCAAGATAATCTCTGTT-3'; primer 2: 5'-AAGTCTTTTGAGAGAAATTGTATGA-3'.
Cells were recovered from mice of all genotypes 48 hr after exposure to γ-irradiation (5 Gy) and stained with PE-conjugated anti-CD4 antibody and FITC-conjugated anti-CD8 antibody (both from Pharmingen). As controls, thymocytes from mice without any treatment were also analyzed. Stained cells were analyzed with a FACScan (Becton-Dickinson) using a CellQuest program.

Proliferation assay and analysis of G_s/S transition during the cell-cycle progression

Cell proliferation and saturation density assays were performed essentially as described [Deng et al. 1995], except that three plates were counted at each time point. To synchronize cells at G_s, 70–80% confluent asynchronous culture was washed with PBS and then placed in DMEM containing 0.1% FBS for 96 hr. Cells were harvested and released into DMEM containing 10% FBS and 10 μM EdU for 24 or 72 hr and harvested. Cells in the S phase were analyzed with flow cytometry as described above.

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