Loss of the ARF Tumor Suppressor Reverses Premature Replicative Arrest but not Radiation Hypersensitivity Arising from Disabled Atm Function

Takehiko Kamijo, Esther van de Kamp, Miriam J. Chong, Frederique Zindy, J. Alan Diehl, Charles J. Sherr, and Peter J. McKinnon

Howard Hughes Medical Institute [T. K., J. A. D., C. J. S.], and Departments of Tumor Cell Biology [T. K., E. v. d. K., F. Z., J. A. D., C. J. S.] and Genetics [M. J. C., P. J. M.], St. Jude Children’s Research Hospital, Memphis, Tennessee 38105

ABSTRACT

The alternative reading frame product (p19ARF) of the mouse INK4a/ARF locus is induced by oncoproteins such as Myc and E1A as part of a checkpoint response that limits cell cycle progression in response to hyperproliferative signals. ARF binds directly to Mdm2 to prevent down-regulation of p53 and thereby promotes p53-dependent transcription and cell cycle arrest. However, ARF is not required for p53 induction in response to ionizing radiation or other forms of DNA damage. Animals lacking a functional ataxia telangiectasia (Atm) gene are exquisitely sensitive to ionizing radiation; Atm-null mouse embryo fibroblasts (MEFs) undergo premature replicative arrest, which is relieved by the loss of p53. Here we show that the loss of ARF expands the life expectancy of Atm-null MEFs, but alters neither the sensitivity of Atm-null mice to ionizing radiation nor their propensity to develop lymphomas early in life. Therefore, whereas ARF and Atm signal to p53 through distinct pathways, the loss of ARF can modify p53-dependent features of the Atm-null phenotype.

INTRODUCTION

DNA damage triggers a coordinated series of cellular responses that ultimately lead to cell cycle arrest and DNA repair, or alternatively, to apoptosis. Cell cycle arrest is mediated by the p53 tumor suppressor, an ephemeral transcription factor that is stabilized in response to genotoxic stress and activates a series of genes, some of which inhibit cell proliferation (1, 2). Among the known p53 targets is the gene encoding p21Cip1 (3), an inhibitor of several classes of cyclin-dependent kinases whose functions are vital for cell cycle progression (4, 5).

In response to ionizing radiation, one of the proteins that lies within a pathway that signals to p53 is the AT1 gene product, ATM (6). AT is a pleiotropic genetic disorder characterized by neurodegeneration, telangectasias (dilated terminal blood vessels), thymic degeneration and immunodeficiency, gonadal dysgenesis, sensitivity to ionizing radiation, genome instability, and predisposition to lymphoreticular malignancies (reviewed in Ref. 7). The product of the human ATM locus is a large 370-kDa protein (3056 amino acid residues) that contains a COOH-terminal domain (~400 residues) homologous to the catalytic subunits of phosphatidylinositol 3-kinases and that encodes a protein kinase activity specific for serine and threonine residues (reviewed in Refs. 8, 9). Other segments of ATM exhibit more extensive NH2-terminal homology to a novel class of transcriptional coactivators of the TRRAP family (10–12). These regions are conserved throughout a family of proteins that are involved in cellular responses to DNA damage and maintenance of genome stability.

Current thinking suggests that activation of the ATM kinase by DNA damage leads in turn to p53 accumulation. ATM immunoprecipitates phosphorlylates p53 on serine-15 (13, 14), presumably inhibiting its interaction with its negative feedback regulator Mdm2 and thereby stabilizing the protein ([15, 16]; reviewed in Ref. 17).

Unlike normal MEFs, cells taken from mice lacking functional Atm proliferate slowly and, after only a few passages in culture, reach a growth-arrested state resembling senescence (18, 19). These cells exhibit constitutively high levels of p53 and p21Cip1, whereas MEFs explanted from Atmpl and Atmp21 double-null animals proliferate rapidly and do not arrest prematurely (20, 21). Therefore, the activation of p53 and its p21 target are determinants of the growth arrest observed in Atm-null MEFs. This may seem paradoxical in light of the fact that the loss of Atm compromises p53 activation in response to ionizing radiation (6, 22). However, cells lacking Atm accumulate unrepaired chromosome breaks (23), and it is likely that these are sensed by collateral pathways that act to stabilize p53. The loss of Atm compromises S-phase and G2 checkpoint functions that are less likely to depend directly on p53 function (8, 9). Additionally, ATM signals to as yet unidentified targets that modulate other responses to ionizing radiation, possibly including elements of the repair machinery itself. For example, although the gastrointestinal epithelium of Atm-deficient mice is highly sensitive to ionizing radiation, its sensitivity is not reversed in animals lacking both Atm and p53 (24), implying that ATM has other targets.

Activation of p53 in MEFs also occurs in response to hyperproliferative signals mediated by overexpressed oncoproteins such as Myc and E1A (25–28). Both Myc and E1A induce synthesis of the p19ARF tumor suppressor ([29, 30]; reviewed in Ref. 31). In turn, ARF binds directly to and inhibits Mdm2 function (32–35), stabilizing p53 and eliciting a p53 transcriptional response that leads to p21Cip1 induction and growth arrest or apoptosis, depending on culture conditions. The loss of either ARF or p53 function is sufficient to immortalize MEFs, converting them into established cell lines that can be transformed by oncogenic ras alleles alone (36). Indeed, the so-called immortalizing functions of Myc and E1A (37, 38) in part depend on their ability to select for cells that have disabled the ARF-p53 pathway and are, therefore, resistant to Myc- and E1A-induced apoptosis (29). Importantly, whereas the loss of ARF severely compromises the p53-dependent “oncogene checkpoint” response to Myc and E1A (29, 30), it is not required for the p53-mediated DNA damage response (35, 36). This suggests that ARF and ATM not only respond to different upstream signals, but that they reside within distinct biochemical pathways that independently activate p53. To explore possible interactions between these pathways, we asked whether the loss of ARF might alleviate the premature senescence observed in cells lacking Atm. The results indicate that the loss of ARF enables Atm-null MEFs to proliferate indefinitely, but does not affect the hypersensitivity of Atm-deficient animals to ionizing radiation.

MATERIALS AND METHODS

Interbreeding of Mice. ARF-null (36) and Atm-null (39) mice, each of 129/svJ × C57BL/6 background, were established in our laboratories and
interbred to yield animals lacking both genes. Initial crosses were performed using ARF+/− × Atm−/− heterozygotes to generate 8 double-null animals from 82 offspring (9.8% observed frequency versus 6.25% expected). Six of these animals were observed prospectively for tumor development. Atm-null mice are infertile, and subsequent crosses were carried out between ARF+/− and Atm−/− animals, yielding 15 double-null animals from 70 offspring (21% observed frequency versus 25% expected). Six animals were used for experiments involving radiation sensitivity, of which five were followed until death. Nine animals of this group were left untreated and followed for tumor development. Double-null animals from additional crosses were sacrificed for studies of p53 and p21 inductions in various organs in response to ionizing irradiation. All animal experiments were performed according to strict institutional guidelines and under an approved Animal Resources Center protocol.

Cells and Culture Conditions. Mouse embryos explanted at day 13.5–14.5 of gestation from animals of the genotypes indicated were maintained on a 3T9 protocol (9 × 104 cells transferred to 3-day intervals in 60-mm diameter dishes; Ref. 36) and propagated in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, and 10 μg/ml gentamicin (Life Technologies, Rockville, MD). Where indicated, MEF cell strains at the indicated passage number were diluted to 1 × 104 cells per 60-mm diameter plate in replicate cultures, and the kinetics of cell proliferation were determined by counting trypsinized cells from triplicate cultures at daily intervals.

Protein Analysis. Proteins were detected by direct immunoblotting. Frozen MEF cell pellets at indicated passage numbers and homogenized tissues from animals 3 h post irradiation (∼2 mg of protein) were disrupted at 4°C in Tween 20 lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 1 mM phenylmethylsulfonil fluoride, 0.4 U/ml aprotinin, 1 mM NaF, 10 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate], sonicated twice for 7 s (Virtis VirSonic 475, 14% power), and left on ice for 30 min. Debris was removed by sedimentation in a refrigerated microcentrifuge (5 min at 15,000 rpm), and protein was quantitated using a BCA kit (Pierce, Rockford, IL). Samples (200 μg of protein per lane) were separated by denaturing PAGE and transferred to nitrocellulose membranes (MSI, Westboro, MA). Filters were washed in TBS-Tween [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20] and blocked in the same solution containing 10% (w/v) nonfat dry milk. Filters were washed for 45 min in TBS-Tween and incubated with a 1/2000 dilution of donkey antibodies to rabbit IgG (Amersham, Arlington Heights, IL). Filters exposed to antibodies to p19ARF were washed for 45 min in TBS-Tween and incubated with a 1/500 dilution of rabbit antiserum to mouse Bax (PharMingen, La Jolla, CA), or to a 1/2500 dilution of monoclonal antibodies directed to p53 (Ab-6; Calbiochem, La Jolla, CA). Filters exposed to antibodies to p19ARF were washed for 45 min in TBS-Tween and incubated with a 1/2000 dilution of donkey antibodies to rabbit IgG (Amersham, Arlington Heights, IL) in TBS-Tween containing 5% milk. All filters were rewarshed as described above, and antibody binding sites were visualized by enhanced chemiluminescence, using appropriate second antibody conjugates or horseradish peroxidase-conjugated antibody (data not shown). By passage 4, ARF-null cells (Fig. 1B, □) were smaller than their wild-type counterparts (Fig. 1B, ○), whereas Atm-null cells were significantly larger, as is characteristic of senescing populations (Fig. 1B, △). However, cells null for both ARF and Atm were again smaller than wild-type MEFs (Fig. 1B, ●), although not as small as ARF-null cells containing functional Atm alleles.

MEFs of different genotypes were explanted into culture and maintained on a strict passage protocol. Wild-type MEFs (Fig. 2, ○) were irradiated with 8 Gy from a cesium source (delivered at a rate of 0.9 Gy/min) and allowed 72 h for recovery. Tissues were collected following fixation by transcardial perfusion with 4% paraformaldehyde, processed for paraffin embedding, and sectioned at 5 μm. PCNA immunohistochemistry was performed with anti-PCNA (IgM) from Calbiochem at a concentration of 5 μg/ml. The Vectastain Elite ABC kit/VIP substrate avidin/biotin immunoperoxidase system (Vector Laboratories, Burlingame, CA) was used for visualization of primary antibody binding per manufacturer’s instructions. Sections were counter-stained with methyl green.

RESULTS

Explanted Atm-null MEFs proliferate slowly and arrest completely after only a few population doublings, expressing constitutively high levels of both p53 and p21Cip1, and becoming large and flat (19). Other markers that are expected to accumulate in prematurely senescent cells include the two products of the INK4a/ARF locus, p16INK4a (41) and p19ARF (40), respectively (29, 36, 42–45). In general, the levels of p53, p21Cip1, p16INK4a, and p19ARF all rise progressively as MEFs are passaged in culture, but their induction is accelerated in Atm-null cells. Fig. 1A illustrates this for p53, p19ARF, and p16INK4a in early-passage wild-type MEFs (passages 2–7, see below) and shows that a more rapid induction of all three proteins occurs in cells that lack functional Atm. ARF-null cells exhibit a phenotype opposite to that of Atm-null cells; namely, neither p53 nor p21Cip1 accumulate as the MEFs are passaged. However, p16INK4a still accumulates to levels that are significantly higher than those in early passage cells (36); similar results were obtained in cells lacking both Atm and ARF function (data not shown). By passage 4, ARF-null cells (Fig. 1B, □) were smaller than their wild-type counterparts (Fig. 1B, ○), whereas Atm-null cells were significantly larger, as is characteristic of senescing populations (Fig. 1B, △). However, cells null for both ARF and Atm were again smaller than wild-type MEFs (Fig. 1B, ●), although not as small as ARF-null cells containing functional Atm alleles.

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Fig. 1. Premature induction of p53, p19ARF, and p16INK4a and increases in cell volume in Atm-null MEFs. A, MEFs grown on a 3T9 protocol were sampled at each passage for p19ARF, p16INK4a, and p53 protein expression by direct immunoblotting. Equal quantities of cell lysate protein (200 g) were loaded per lane. After electrophoresis and transfer of separated proteins to nitrocellulose, regions of antibody binding to the filters were detected using enhanced chemiluminescence. B, at passage 4, MEFs of different genotypes were trypsinized, and their mean corpuscular volumes were determined using a Coulter counter. Genotypes: wild type (○), ARF-null (□), Atm-null (△), and Atm/ARF double-null (●).
of the indicated genotypes (36). The SDs (2 minimum of three separate experiments, each performed with cells from different embryos number plated at the start of each passage. Each data point represents an average from a interval can be estimated by comparing the number of cells on the ordinate with the (Fig. 3). No induction of the p53-responsive gene extracts of both small intestine and spleen 3 h after exposure to X-rays as expected, the p53 and p21 responses were both attenuated in which was used as a loading control) was observed in small intestine. ARF-ATM INTERACTIONS WITH P53

![Image](https://cancerres.aacrjournals.org) Fig. 2. MEF proliferation kinetics. A, cells passaged at 3-day intervals (abscissa) were counted after trypsinization (ordinate), and the numbers of cells per dish were recorded; 9 × 10^5 cells were replated every 3 days (3T9 protocol). The doubling time in each 3-day interval can be estimated by comparing the number of cells on the ordinate with the number plated at the start of each passage. Each data point represents an average from a minimum of three separate experiments, each performed with cells from different embryos of the indicated genotypes (36). The SDs (2 not shown) were all <20% of the means. Genotypes: wild-type (○), ARF-null (□), Atm-null (△), and Atm/ARF double-null (●). B, growth rates were determined for cells taken at passages 4 and 15. Cells diluted to 1 × 10^6 per 60-mm dish were replica plated, and individual cultures harvested every day thereafter were counted. The values represent averages of triplicate cultures for each genotype. Symbols are identical to those in A.

initially underwent more than two population doublings per 3 days, but as they were propagated, their growth slowed, and by passage 17, the cells ceased proliferating (Fig. 2A). ARF-null cells (Fig. 2, □) grew faster than wild-type MEFs and continued to proliferate without any apparent slowdown in their population doubling time. Even at passage 15 when the growth of wild-type cells had significantly slowed, ARF-null cells proliferated as rapidly as cells at passage 4 (Fig. 2B). Atm-null cells initially grew very slowly and soon stopped dividing (Fig. 2, passage 4, △). MEFs from animals lacking both Atm and ARF proliferated somewhat more slowly than cells lacking ARF alone, but unlike wild-type MEFs, they no longer underwent arrest (Fig. 2, ●). In this respect, the loss of ARF is dominant over the loss of Atm, enabling cells lacking ATM function to proliferate continuously.

When wild-type or ARF-null animals were irradiated with 8 Gy (~LD_{50}), we observed significant induction of both p53 and p21 in extracts of both small intestine and spleen 3 h after exposure to X-rays (Fig. 3). No induction of the p53-responsive gene Bax (or β-tubulin, which was used as a loading control) was observed in small intestine. As expected, the p53 and p21 responses were both attenuated in organs from Atm-null mice and were not completely restored in double-null animals that also lacked ARF (Fig. 3). The p53 responses were similar in spleen and intestine from Atm-null and double-null animals, but the p21 responses showed greater variability. Because pairs of Atm-null and double-null animals were compared in three such experiments (one of which is shown), the latter differences may not be significant.

Cohorts of 4-week-old Atm-null or Atm/ARF double-null mice died soon after being exposed to 8 Gy of irradiation (~LD_{50} dose for wild-type mice; Fig. 4A), having suffered almost complete loss of their intestinal epithelium (Fig. 4B). ARF-null mice (n = 28) may be somewhat more resistant to ionizing radiation than their wild-type littermates (n = 14; Fig. 4A), but the small differences observed were not statistically significant, given the numbers of animals studied. Whereas intestinal epithelium from wild-type and ARF-null mice was capable of regeneration, as indicated by staining of S-phase cells with antibodies to PCNA (Fig. 4B, e and g), epithelium from Atm-null mice, regardless of their ARF status, failed to regenerate (Fig. 4B, f and h). As reported by others (24, 46), animals lacking p53 alone were relatively resistant and survived (Fig. 4A). Therefore, the loss of ARF did not ameliorate the sensitivity of Atm-null animals to ionizing radiation.

Atm-null mice die with a mean latency of ~3.5 months, generally succumbing to T-cell lymphomas (19, 21). Of 15 Atm/ARF double-null animals observed for tumor formation, 10 developed lymphomas, 3 developed fibrosarcomas, and 1 developed a salivary gland carcinoma. All but one died by 6 months of age, with a mean latency of 4 months. In contrast, fibrosarcoma is the predominant tumor type in

Fig. 3. Stabilization of p53 and induction of p21^{CIP1} in tissues from irradiated mice. Animals were γ-irradiated with 8 Gy (+) or left untreated (−) and sacrificed 3 h later. Genotypes of animals are indicated above the lanes. Levels of p53, p21^{CIP1}, and Bax (for small intestine) were determined by direct immunoblotting as in Fig. 1. The two (+) symbols for wild-type (WT) and ARF-null (ARF−/−) intestinal extracts represent samples taken from duplicate irradiated mice. Equal loading per lane in each blot was verified using antibodies to β-tubulin (not shown). Null X2 refers to tissues from Atm/ARF double-null mice.

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animals were exposed to 8 Gy of whole body irradiation (n = 28) may be slightly radioresistant, but the observed differences between them and their wild-type littermates (n = 14) were not significant, given the number of animals studied. B, jejunum of irradiated animals was taken 72 h after exposure, fixed, and stained with H&E (a–d) or for PCNA (e–h), which identifies proliferating populations (S phase) in regenerating crypts.

**DISCUSSION**

ATM appears to play a role as a selective sensor of DNA damage that responds to double-stranded DNA breaks induced by ionizing radiation and radiomimetic drugs, but not to UV radiation or to certain other stress signals (6–9). Human diploid fibroblasts from AT patients senesce prematurely in culture (47) and also appear to undergo accelerated telomere loss, raising the possibility that ATM may somehow be involved in regulating telomere length (48). In principle, the accumulation of unrepaired DNA breaks in AT cells (23) and/or the shortening of telomeres, which lead to dicentric chromosome formation and damage cycles during mitosis, might trigger a number of cell cycle checkpoint responses, including p53-dependent G1 arrest (49). Whereas the ATM protein likely helps to ensure that DNA breaks are recognized and repaired efficiently in normal human cells, accumulating DNA damage in the absence of ATM function could induce premature senescence (49). In mice, the loss of Atm also leads to the premature growth arrest of cultured MEFs, as manifested by their capability to undergo very few population doublings, their increased cell size, and the expression of elevated levels of p53, p21Cip1, p16Nk4a, and p19Arf, all of which can inhibit cell cycle progression. In contrast, double-null Atm/p53 and Atm/p21 MEFs continue to proliferate (20, 21); therefore, inactivation of the p53-p21Cip1 pathway effectively reverses these particular effects of Atm deficiency, at least in rodent fibroblasts, in which telomere shortening may play a less prominent role in determining the cellular life span than in human cells (50).

ATM kinase has been reported to phosphorylate p53 directly on serine-15 (13, 14), a process that may inhibit its interaction with Mdm2 (15). Mdm2, in turn, is a p53-responsive gene whose product acts through negative feedback to inhibit p53 transactivation (51, 52) and to accelerate p53 turnover (53–56). Hence, interference of Mdm2 binding by ATM-mediated p53 phosphorylation is thought to stabilize p53 and increase its transcriptional activity. Conversely, the loss of ATM function leads to an attenuated p53 response during the acute phase following ionizing radiation (6). Why then, does p53 accumulate in cells lacking ATM function? One interpretation is that in Atm-null cells, collateral sensors recognize DNA damage and signal via ATM-independent pathways to p53. This is consistent with observations that p53 is still induced, albeit with retarded kinetics, in AT cells following exposure to ionizing radiation (6), and with the identification of ATM-related gene products, such as ATR, that might phosphorylate ATM-related gene products, such as ATR, that might...
overall latency. The majority of ARF-null mice develop fibrosarcomas and lymphomas, but ~25% manifest carcinomas and tumors of the central and peripheral nervous systems, and virtually all animals die of cancers by 15 months of age (36).3 ARF-Atm double-null mice primarily succumb to thyrmomas early in life (4 months mean latency), indicating that the Atm-null pattern of susceptibility predominates.

That the loss of ARF counteracts certain p53-dependent effects of dysfunctional Atm connotes a functional interaction between the ARF-p53 and ATM-p53 pathways, the exact nature of which are unknown. The ability of ARF to antagonize Mdm2 function suggests one mechanism by which the loss of ARF might reset p53 levels. Synergy between these pathways has been documented in another setting. When E1A or Myc induces ARF, it sensitizes MEFs to apoptosis, an outcome augmented by depriving the cells of serum survival factors. On one hand, ATM/TRRAP family members might contribute by transcriptionally coactivating Myc and E2F-1 (10). In addition, Myc and E1A-sensitized cells are far more susceptible than their wild-type counterparts to the toxic effects of γ-irradiation or certain genotoxic drugs, an effect that is abrogated in cells lacking ARF function (30).

Hence, ARF and ATM each can likely reinforce the other’s activities in response to different forms of stress.

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