Advances in Brief

Genetic Interactions between *atm* and *p53* Influence Cellular Proliferation and Irradiation-induced Cell Cycle Checkpoints

Christoph Heiner Westphal, Cornelius Schmalz, Sheldon Rowan, Ari Elson, David Erich Fisher, and Philip Leder

Department of Genetics and Howard Hughes Medical Institute, Harvard Medical School (C. H. W., P. L.), and Department of Pediatric Oncology, Dana-Farber Cancer Institute and Harvard Medical School (C. S. R., D. E. F.) and Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot 76100 Israel (A. E.)

Abstract

Ataxia-telangiectasia and Li-Fraumeni syndrome, pleiotropic disorders caused by mutations in the genes *atm* and *p53*, share a marked increase in cancer rates. A number of studies have argued for an interaction between these two genes (for comprehensive reviews, see M. S. Meyn, Cancer Res., 55: 5991–6001, 1995, and M. F. Lavin and Y. Shilo, Annu. Rev. Immunol., 15: 177–202, 1996). Specifically, *atm* is placed upstream of *p53* in mediating G1-S cell cycle checkpoint control, and both *atm* and *p53* are believed to influence cellular proliferation. To analyze the genetic interactions of *atm* and *p53*, mouse embryonic fibroblasts (MEFs) homozygously deficient for both *atm* and *p53* were used to assess cell cycle and growth control. These double-null fibroblasts proliferate rapidly and fail to exhibit the premature growth arrest seen with *atm-null* MEFs. MEFs null for both *atm* and *p53* do not express any p21wfaf1 or p14arf, showing that *p53* is required for p21wfaf1 expression in an *atm-null* background. By contrast, homozygous loss of either *atm*, *p53*, or both results in similar abnormalities of the irradiation-induced G1-S cell cycle checkpoint. Our results suggest two separate pathways of interaction between *atm* and *p53*, one linear, involving G1-S cell cycle control, and another more complex, involving aspects of growth regulation.

Introduction

Ataxia-telangiectasia is a multifaceted disorder marked by progressive neurological degeneration, specific immunodeficiencies, telangiectasias, lymphoreticular malignancies, infertility, growth retardation, and premature signs of aging. Recently, three laboratories have reported the generation of mice null for *atm* (1—3). These mice are small and prone to thymic lymphomas and yield MEFs (4, 5) that undergo premature growth arrest rapidly. Mice deficient in *p53* are similarly prone to cell cycle checkpoints and apoptosis, and both genes have been implicated in regulating cellular proliferation (6, 7). To analyze genetic interactions between *atm* and *p53*, MEFs were derived from mice bearing homozygous null mutations in *atm*, in *p53*, or in both genes. The predisposition to cell growth arrest and the fidelity of the irradiation-induced G1-S cell cycle checkpoint were analyzed in cell lines null for both genes. We find that the additional loss of *p53* rescues the growth arrest phenotype seen in *atm-null* MEFs. In addition, homozygous loss of *atm*, *p53*, or both *atm* and *p53* abrogates the ionizing radiation-induced G1-S checkpoint. These results argue for a role for *p53* in mediating the growth arrest observed in the *atm-null* background and provide a description of the genetic interactions between the *p53* and *atm* genes in a linear pathway regulating cell cycle control.

Materials and Methods

Derivation of MEFs. MEFs were derived using standard procedures (8). Day 11.5 to 16.5 post coitum embryos were dissociated, treated with DNase and trypsin, and plated in DMEM containing 15% FCS. DNA for Southern analysis was extracted from embryo heads.

Growth Assays and Passaging Experiments. MEF growth was assessed as described previously (1). Four × 10⁴ cells (all at first passage) obtained from tissue culture dishes of equivalent density were plated in duplicate wells on six-well tissue culture plates. Cells were trypsinized and counted in duplicate using a hemocytometer at days 2, 4, 6, and 8 after plating. Each data point represents the average of duplicate wells.

Cell lines homozygously deficient in *atm*, *p53*, or both were passaged continuously using 1:5 dilutions in tissue culture until growth arrest was observed. The *atm-null* cell lines underwent growth arrest between passages 3 and 4, whereas the *p53* and *atm/p53* doubly deficient cell lines continued at a doubling rate of 18–20 h past passage 20.

Irradiation and Fluorescence-activated Cell Sorting Analysis. Unstained early-passage (p2–p4) MEFs, growing on tissue culture dishes, were irradiated from a ¹³⁷Cs-source (Gammatron 40, Atomic Energy of Canada, Ltd.) at a dose rate of 1 Gy/min. Cells were harvested 16 h after irradiation, fixed in 50% ethanol, and stained for 30 min in a solution of 2.5 μg/ml propidium iodide and 50 μg/ml RNase A. Flow cytometry was carried out on a FACSscan (Becton Dickinson) using CellQuest Software. The data were analyzed subsequently with ModFit LT V1.01 for cell cycle determination.

Protein Analysis. Early-passage wild-type, *atm-null*, *p53-null*, and double-null cells were harvested, and 500,000 cells were lysed to make extracts. Entire extracts were separated by 13% PAGE and transferred onto a nitrocellulose filter (Schleicher & Schuell). Protein was detected using a polyclonal antibody (C-19; Santa Cruz Biotechnology) or a α-3-tubulin-specific antibody (TUB 2.1, Sigma Chemical Co.) Filters were incubated with horse-radish peroxidase-coupled secondary antibody (Cappel) and visualized with enhanced chemiluminescence.

Results

Generation of *atm*(*−/−*) *p53*(*−/−*) MEFs. To produce MEFs null for both the *atm* and *p53* genes, compound heterozygotic (*atm*(*+/−*) *p53*(*+/−*)) mice were mated, and pregnant females were sacrificed to derive MEFs between 11.5 and 16.5 days post coitum. A total of 79 primary cell lines were derived in this manner, yielding all possible genotypic combinations, including two deficient for both *atm* and *p53*. These cell lines were used for subsequent growth experiments and cell cycle analysis.

Control of Cellular Proliferation Is Mediated by *atm* and *p53*. The effects of *atm* and *p53* on cellular proliferation were studied via cell growth experiments on early-passage MEFs. As shown in Fig. 1A, and consistent with earlier observations, *atm-null* MEFs undergo fewer than four doublings before undergoing growth arrest (1—3). By contrast, homozygous deletion of *p53* in an *atm-null* background...
ATM and P53 MODULATE PROLIFERATION AND G1-S CHECKPOINTS

Fig. 1. A, MEFs deficient in both atm and p53 proliferate more rapidly than MEFs deficient in atm alone. Both double-null lines grow significantly faster than any atm-deficient cell line described to date. B, Southern blot analysis of representative MEFs derived in the present cross. The atm knockout allele is 7 kb, and the wild-type allele is 5 kb (2). The p53 knockout allele is 8 kb, and the wild-type allele is 16 kb (5). MEF primary cell line ap38 (bold), the growth properties of which are described in A, is derived in the present cross. The atm knockout allele is 7 kb, and the wild-type allele is 5 kb. MEF deficient in atm alone. Both double-null lines grow significantly faster than any atm-deficient cell line described to date. B, Southern blot analysis of representative MEFs derived in the present cross. The atm knockout allele is 7 kb, and the wild-type allele is 5 kb (2). The p53 knockout allele is 8 kb, and the wild-type allele is 16 kb (5). MEF primary cell line ap38 (bold), the growth properties of which are described in A, is atm(—/—) pS3(—/—). MEFs ap39 to ap43 represent a range of other genotypic combinations. ko, knockout allele; wt, wild-type allele.

-overcomes this growth arrest (two independently derived MEFs, ap38 and ap24, are depicted in Fig. 1A). The signal for growth arrest seen in atm-null MEFs is hence dependent on p53 function. Fig. 1B shows a representative Southern blot of MEFs derived in the present cross, including ap38, one of the double-null MEFs used for the growth experiments shown in Fig. 1A. atm(—/—) pS3(—/—) cells have been monitored for more than 20 passages without signs of the slowed growth indicative of crisis (data not shown). atm(+/+) pS3(—/—) cells were monitored in parallel and proliferated at the same rate over 20 passages.

To address the role of p21WAF1/CIP1 expression in the control of cellular proliferation, protein expression was analyzed in wild-type, single-null, and double-null cells. p21WAF1/CIP1 protein is present at equivalent levels in both proliferating and nonproliferating atm-null MEFs and is expressed at lower levels in wild-type cells (Fig. 2, Lanes 1, 2, and 5; see also Ref. 3). Interestingly, neither atm(—/—) pS3(—/—) nor atm(+/+) pS3(—/—) cells express p21WAF1/CIP1, as measured by Western blot (Fig. 2, Lanes 3 and 4, respectively). These observations support the view that p53 is the dominant modulator of p21WAF1/CIP1 expression, whereas loss of atm leads to up-regulation of p21WAF1/CIP1 protein levels via direct or indirect mechanisms (see also Fig. 4A).

atm(—/—) pS3(—/—) MEFs Have an Impaired Irradiation-induced G1-S Cell Cycle Checkpoint. Patients with ataxia-telangiectasia and Li-Fraumeni syndrome are known to develop cancers at an increased rate. In both cases, the pathogenic role of increased genomic fragility has been implicated (9, 10). Deficiencies in either atm or p53 lead to profound defects in γ-irradiation-induced cell cycle checkpoints, which likely play a role in the predisposition to cancer seen in both diseases (11). p53 activation of p21WAF1/CIP1 plays a central role in the G1-S checkpoint after certain types of DNA damage, such as that caused by ionizing radiation (8, 12). The G2-M damage-activated checkpoint, on the other hand, appears to be partially p53 independent (13). Loss of atm has a complex effect on the G2-M cell cycle checkpoint (14–17), which is felt to be independent of p53. A more detailed analysis of the G2-M checkpoint is hence not presented here.

To understand the epistatic relationship between atm and p53 in cell cycle control, atm/p53 double-null cells were compared to littermate control MEFs with respect to the function of their G1-S cell cycle checkpoint in response to γ irradiation. The primary data are presented in Fig. 3, A–D. Because atm homozygously deficient cell lines undergo growth arrest quite rapidly, Southern analysis of embryos was completed within 2.5 days after harvesting, and atm-null cells were subjected immediately to cell cycle analysis. The cell cycle analysis involved γ-irradiating cells at the indicated doses and harvesting cells 16 h later for fixation, staining, and analysis by flow cytometry.

As shown in Fig. 3A, wild-type cells show a functional checkpoint at G1-S. By contrast, the G1-S checkpoint is impaired significantly in p53-null cells (Fig. 3B). Similarly, in the case of the atm-null MEFs (Fig. 3C), there is a clear deficiency in the G1-S checkpoint, which is likely underestimated because a significant proportion of these cells are already growth arrested and residing in G0. This premature growth arrest has been documented extensively (1–3). Cells null in both atm and p53 again show loss of the G1-S checkpoint (Fig. 3D).

Fig. 3E represents the percentage change seen in G1-G0 after 5 or 10 Gy of irradiation. These data are consistent at 5, 10, and 20 Gy, showing that wild-type MEFs manifest an increasing percentage of cells in G1-G0 after irradiation, whereas single- and double-null cells show a decreasing percentage over the range of doses analyzed. Note that both double-null MEFs derived in this cross are shown here, and that the percentage decrease in G1-G0 is similar in these two primary lines. The results presented in Fig. 3A–E support a previously suggested model in which atm and p53 assume equivalent roles in mediating irradiation-induced G1-S cell cycle arrest, consistent with their participation in a linear regulatory pathway (Fig. 4B; Refs. 14 and 15).

Discussion

Loss of p53 Suppresses the Growth Arrest Seen in atm-null MEFs. All previously described human and mouse atm-deficient fibroblast cell lines show significant growth defects. In the mouse, the growth...
Fig. 3. MEFs deficient in both atm and p53 show lack of the G1-S checkpoint. A, intact G1-S checkpoint in an atm(+/+) p53(+/-) control cell line. B and C show lack of the G1-S checkpoint in atm(+/-) p53(-/-) and atm(-/-) p53(+/-) control cell lines, respectively. D, a representation of the cell cycle profile of atm(-/-) p53(-/-) cells, again with deficiency in the G1-S cell cycle checkpoint. E represents a direct comparison of the percentage change in G1-G0 after 5 and 10 Gy of irradiation (compared to unirradiated) in two double-null cell lines, one wild-type cell line and each single-null cell line. These data are based on the primary data presented in A–D. Note that the percentage decrease in G1-G0 phase seen in atm(-/-) p53(+/+) is less pronounced than that seen in atm(+/-) p53(+/+) and atm(-/-) p53(-/-) cells. This is likely due to an increase in G0 phase of these cells, because these cells are about to undergo growth arrest, as described here and previously (1–3). A qualitative difference can be seen between wild-type (with increased G1-G0 phase after irradiation), and single- and double-null cells (with decreased G1-G0 phase after irradiation). This difference was reproducible over the range of doses shown in A–D, with each of these observations representing an independent experimental observation. Finally, both double-null cell lines represented in E showed virtually identical percentage decreases in G1-G0 at all irradiation doses. Phases: •, G1-G0; @, S; and 0, G2.

arrest is especially severe, leading to a senescent appearance at the third passage in tissue culture (1–3). In contrast, deletion of either p21^{cip1/waf1} or p53 increases MEF proliferative rates (8). Here we have shown that the homozygous genetic deletion of p53 in an atm-deficient background overcomes the growth arrest seen in atm-null MEFs.

These results argue for a dominant role for p53 in mediating MEF growth arrest, because MEFs null for atm and p53 proliferate much more rapidly than MEFs deficient in atm alone. These double-null cells grow as rapidly as cells null for p53. Although the exact role, if any, of p21^{cip1/waf1} is presently not clear, it is possible that a portion of the growth arrest seen in atm-null cells is mediated by a p21^{cip1/waf1}-dependent mechanism, because p21^{cip1/waf1} is overexpressed in atm-null cells. These elements can be brought together in the simplified model outlined in Fig. 4A. It is important to note that suppression of
Homozygous loss of either gene clearly impairs the irradiation-induced G1-S checkpoint, whereas deletion of both genes in tandem does not appear to significantly exacerbate this phenotype. These and previous observations argue for an equivalent role for atm and p53 in a common pathway mediating the irradiation-induced G1-S checkpoint (Fig. 4B; Refs. 14 and 15).

Interestingly, loss of p21^{cip1/waf1} similarly perturbs the G1-S checkpoint (8, 12). Moreover, p21^{cip1/waf1}-null mice do not appear to develop cancer at increased frequencies (8). Inasmuch as loss of either atm or p53 predisposes to malignancy in both mouse and human, this phenotype seems less likely to involve a p21^{cip1/waf1}-mediated signal transduction pathway. The studies reported here highlight the paradox that atm function may be either growth-inhibiting (at the G1-S radiation-induced cell cycle checkpoint) or growth-promoting (in MEFs grown in vitro), and that p53 may modulate both of these effects.

References

Genetic Interactions between \( atm \) and \( p53 \) Influence Cellular Proliferation and Irradiation-induced Cell Cycle Checkpoints

Christoph Heiner Westphal, Cornelius Schmaltz, Sheldon Rowan, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/9/1664

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.