Defective Control of Apoptosis and Mitotic Spindle Checkpoint in Heterozygous Carriers of ATM Mutations

Teruko Shigeta, Masatoshi Takagi, Domenico Delia, Luciana Chessa, Satoshi Iwata, Yusuke Kanke, Minoru Asada, Mariko Eguchi, and Shuki Mizutani

ABSTRACT

Ataxia telangiectasia (AT) carrier-derived lymphoblastoid cell lines (AT-LCLs/hetero) with suboptimal ATM protein expression were examined for the regulation of radiosensitivity, apoptosis, and mitotic spindle checkpoint in response to DNA-damaging agents. Although AT-LCLs/hetero showed intermediate radiation sensitivity, as determined by clonogenic assay, they were resistant to early-onset apoptosis, as much as AT patient-derived LCLs (AT-LCLs/homo). Furthermore, two of three AT-LCLs/hetero showed defective mitotic spindle checkpoint control in response to X-ray irradiation, which is a recently characterized biological feature in AT-LCLs/homo. Our findings indicate that carriers of ATM mutation have biological abnormalities due to haploinsufficiency of ATM protein or dominant-negative effect of mutant ATM protein. Thus, although it is still controversial whether ATM mutation carriers are at higher risk for cancer during adulthood, our findings based on in vitro biological indicators support the notion that at least some of such carriers are at a higher risk for cancer development than those without ATM mutation. Our findings may help to reevaluate epidemiological studies on cancer susceptibility in AT carriers.

INTRODUCTION

AT is an autosomal recessive, multisystemic disorder with progressive cerebellar ataxia, bulbar telangiectasia, and immunological deficiencies. Patients with AT are also at a high risk for the development of malignancies, such as malignant lymphoma and leukemia (1, 2), and an increased tendency for the development of cancer, particularly breast cancer, has been reported in AT carriers (3, 4). The responsible gene, ATM, was recently identified and characterized (5, 6). It encodes a 350,000 nuclear phosphoprotein that contains, at its COOH terminus, a phosphatidylinositol 3-kinase catalytic domain, and at its central part, a RAD3-homologous domain. ATM protein is involved in intracellular signaling, cell cycle control, and DNA repair and recombination in response to DNA damage. The role of ATM in DNA damage signal transduction is primarily supported by data showing that the oncosuppressor p53-dependent G1 cell cycle checkpoint pathway is defective in AT (7–10) and that ATM phosphorylates p53 at Ser 15 (11, 12). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2 and mediates radiation-induced transactivation of cyclin-dependent kinase inhibitor p21Cip1/WAF1 through accumulation of p53 protein. The important role of ATM in G1-S and S-phase control in response to genotoxic stimulation has also been suggested by the physical interaction between ATM and c-Abl tyrosine kinase (13, 14) and a possible hyperphosphorylation of the replication protein A (RPA) by ATM (15, 16). Furthermore, the involvement of ATM in the cell cycle control at S-G2 and G2-M was recently indicated by the finding that chk2, the mammalian homologue of Rad53 (Saccharomyces cerevisiae), and Cds1 (Saccharomyces pombe) protein kinase undergo phosphorylation after radiation in an ATM-dependent manner (17).

Thus, these findings suggest that ATM protein plays multiple key roles in intracellular signaling, cell cycle control, and DNA repair and recombination. We have recently reported the biological features of LCLs from AT patients and showed perturbation of the DNA damage-associated early-onset apoptosis and cell cycle regulations at multiple cell cycle checkpoints including mitotic spindle checkpoint in AT patients (18).

AT carrier-derived LCLs from heterozygous AT mutation (AT-LCLs/hetero) are reportedly more or less radiosensitive (2), and it is still controversial whether these individuals have an increased risk for the development of cancer in adulthood (3, 4, 19, 20). Although AT-LCLs/hetero were demonstrated to have ~50% of ATM protein of normal control LCL (Wt-LCL; Ref. 9), the biological features of somatic cells from heterozygous carriers of AT mutations have been poorly characterized. Because they are clinically indistinguishable from normal subjects and it is estimated that up to 1% of the general population are heterozygous carriers of AT mutations (3), characterization of the biological features of AT-LCLs/hetero may help extend our understanding of the genetic basis of cancer patients in the general population.

MATERIALS AND METHODS

Cell Lines and Their Characteristics. EBV-immortalized LCLs were established by infecting lymphocytes with the EB virus strain B95-8 as described previously (21). Immortalized LCLs were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) with 10% FCS (Life Technologies, Inc., Gaithersburg, MD) at approximately 5 × 105 cells/ml at 37°C in 5% CO2. These samples were obtained after informed consent and approval of the study by the local ethics committee. Cell lines AT43RM and AT52RM, showing homozygous ATM mutation 7517 del 4 and compound heterozygous mutations 7626 C → T, 8283 del, and 7820 del, respectively, have been described (22) and will be referred to as AT-LCLs/homo. Cell lines 155RM, 227RM, and 373RM, showing one wild-type and a second mutated allele of 7792 C-T, 7517 del 4, and 8283 del, respectively, have been described (22) and will be referred to as AT-LCLs/hetero (Table 1).

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4 D. Delia, unpublished data.
Table 1 Cell lines and ATM gene mutations

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>ATM gene</th>
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<tbody>
<tr>
<td>Wt-LCL</td>
<td>N/N*</td>
</tr>
<tr>
<td>AT43RM</td>
<td>7517 del 4</td>
</tr>
<tr>
<td>AT52RM</td>
<td>7626 C → T/8365 del A</td>
</tr>
<tr>
<td>155RM</td>
<td>7792 C → T/N</td>
</tr>
<tr>
<td>227RM</td>
<td>7517 del 4/N</td>
</tr>
<tr>
<td>373RM</td>
<td>8283 del TCN</td>
</tr>
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</table>

* N, wild-type allele.

Molecular Analysis of ATM Gene Mutations. Mutations in AT patients involved in this study were identified by restriction endonuclease fingerprint or protein truncation test. Briefly, the ATM coding region of 9186 bp was subdivided into six contiguous fragments of approximately 1600–1800 bp each, with a partial overlap of 150–300 bp. Each fragment was independently amplified with specific primers on cDNA derived from total mRNA, isolated from LCLs or peripheral blood lymphocytes. All primers on 5' contained a T7 promoter region, spacer of 3–6 bp, Kozak consensus sequence, and in-frame amplified ATM sequence. After in vitro transcription/translation incorporating [35S]methionine, the product was electrophoresed on 14% SDS-polyacrylamide gel. The presence of a putative “truncation mutation” allowed us to identify by sequencing the gene fragment where the mutation belongs.

Anti-ATM Antibody and Western Blotting for Protein Analysis. The cDNA sequence of ATM corresponding to amino acids 1–150 was amplified from reverse-transcribed mRNA from normal lymphoblastoid cells, inserted in-frame with a 6×Hist-tag sequence in the expression vector pTrcHis-B (Invitrogen, San Diego, CA), cloned and checked by automatic sequencing. Escherichia coli strain BL21 was transformed with pTrcHis-ATM-N; 6 h after induction with 100 μM isopropyl-β-D-galactoside, BL21 was lysed in 6 M urea, sonicated, and centrifuged. The supernatant was loaded on a Talon column (Clontech, Palo Alto, CA), and after elution with 20–100 mM imidazole, the Hist-tag protein was recovered and checked for purity by SDS-PAGE. Mice were immunized with the recombinant ATM-N protein, and sera were collected, purified against ATM-N protein, and used for Western blotting. A two-gradient SDS-PAGE (bottom 4 cm consisted of a 10% gel with an acrylamide:bis-acrylamide ratio of 100:1) was used to separate and retain on a N/A, wild-type allele.

Clonogenic Assay. Clonogenic assay was conducted as described previously (24). Briefly, cells were resuspended at a concentrations of 1000 cells/ml in 10% FCS RPMI containing 50% of culture supernatant of Wt-LCL, and 100 μl of cell suspension were plated to each well of a 96-well microplate. Cells received X-IR with 0, 1, 2, or 5 Gy at a dose rate of 1 Gy/min. After 2 weeks of culture, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added at a concentration of 5 mg/ml, and the well that had a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-positive cluster comprised of more than 37 cells was defined as positive (18, 24).

RESULTS

ATM Protein Expression in AT-LCLs/hetero. ATM protein expression, as determined by Western blot analysis using anti-ATM N protein antibody, demonstrated normal expression in Wt-LCL. AT-LCLs/homo (AT43RM and AT52RM) expressed no ATM protein, whereas 155RM, 227RM, and 373RM derived from heterozygous carrier of ATM mutation (AT-LCLs/hetero) showed 45%, 40%, and 59% of the amount of ATM protein expressed in Wt-LCL, as demonstrated in a representative example shown in Fig. 1. The lack or barely detectable expression of ATM protein in AT-LCLs/hetero is in agreement with the previous observation in LCLs with ATM mutation on both alleles (9).

AT-LCLs/hetero Are Resistant to Acute Onset Apoptosis. Apoptosis sensitivity has been a controversial issue in AT-derived somatic cells, and recently we have shown that AT-LCLs/homo are resistant to radiation-induced acute but not late-onset apoptosis (18). These findings led us to study the sensitivity to acute-onset apoptosis against 5 Gy X-IR in AT-LCLs/hetero. When dead cells were determined by their PI uptake in unfixed condition, a significant population of cells was identified as dead only in Wt-LCL. This proportion was much smaller in AT-LCLs/hetero and was comparable with AT-LCLs/homo (Fig. 2a). When apoptosis was determined by fractions

![Fig. 1](image-url)
with subdiploid DNA content after PI staining and analyzed on a linear scale by FACS analysis of PI-positive (dead) and PI-negative (viable) cells, 0 h (□), 24 h (●), 48 h (▲), and 72 h (●) after 5 Gy X-IR. Data represent the result of three experiments with similar features. g, percentage of apoptotic cells, 0 h (□), 24 h (●), and 48 h (▲) after 5 Gy X-IR as determined by subdiploid DNA contents. Values are means (n = 3 for each LCL); bars, SD. h, percentage of apoptotic cells among cells treated with H2O2 for 24 h as determined by subdiploid DNA contents. Values are means (n = 3 for each LCL); bars, SD. i, flow cytometric analysis of loss of ΔΨm after 5 Gy X-IR. Loss of ΔΨm was determined as described in “Materials and Methods.” Data are representative results of three experiments at 0 h (□) and 24 h (●) after X-IR with similar features. In all panels, columns 1–6 represent Wt-LCL, AT43RM, AT52RM, 155RM, 227RM, and 373RM, respectively.

Table 2. G0/G1 versus G2/M ratio 24 h after irradiation

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>G0/G1 versus G2/M ratio</th>
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<tbody>
<tr>
<td>Wt-LCL</td>
<td>2.18 ± 0.53</td>
</tr>
<tr>
<td>AT43RM</td>
<td>0.77 ± 0.41</td>
</tr>
<tr>
<td>AT52RM</td>
<td>0.93 ± 0.23</td>
</tr>
<tr>
<td>155RM</td>
<td>1.67 ± 0.17</td>
</tr>
<tr>
<td>227RM</td>
<td>1.12 ± 0.17</td>
</tr>
<tr>
<td>373RM</td>
<td>2.1 ± 0.24</td>
</tr>
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</table>

Apoptosis induced by C2-ceramide was also tested by treatment with 100 μM C2-ceramide for 24 h. Similar to H2O2 stimulation, 155RM, 227RM, and 373RM showed resistance to C2-ceramide-induced apoptosis (apoptosis %: 16.6 ± 2.7, 14.5 ± 2.1, and 21.1 ± 4.0, respectively), of which the difference is comparable or significant when compared with AT-LCLs/homo [14.0 ± 2.2% (AT43RM) and
13.6 ± 8.2% (AT52RM) or Wt-LCL (32.6 ± 4.5%), respectively (Fig. 2d).

To avoid possible failure in identification of apoptotic population using subdiploid DNA as an indicator of apoptosis, additional indicators were used. The reduction of mitochondrial transmembrane potential (ΔΨm) is one of the cellular hallmarks for apoptotic cells and can be determined using a mitochondrial transmembrane potential-sensitive dye DiOC₆ (3). Thus, an assay for DiOC₆ (3) staining was used after X-IR. In Wt-LCL, a marked increase in the population with reduced ΔΨm was noted. This increase in loss-of-ΔΨm population in Wt-LCL was in contrast with the results in AT-LCLs/hetero and AT-LCLs/homo. Both of the two AT-LCLs/homo and all of the three AT-LCLs/hetero showed less increase in loss-of-ΔΨm population (Fig. 2e). H₂O₂ treatment yielded identical results to those of X-IR (data not shown). An apoptosis-resistant phenotype in AT-LCLs/hetero was further characterized by an assay for transmitting proapoptotic signal following application of apoptotic stimuli. Although DEVD-sensitive caspase 3-like protease activity as studied 72 and 144 h after X-IR was examined by FACS. In addition to the characteristic features such as decreased synthesis without cytokinesis 72 and 144 h after X-IR was examined by FACS. The proportion of cells that developed a second round of DNA synthesis without cytokinesis 72 and 144 h after X-IR was examined by FACS. In addition to the characteristic features such as decreased apoptosis (Fig. 3) and G₁ delay, accumulation of DNA contents greater than 4C was noted in AT-LCLs/hetero (3.2% in 155RM (Fig. 3c), 9.6% in 227RM (Fig. 3d), and 10.6% in 373RM (Fig. 3e). The finding in 227RM and 373RM was significant compared with Wt-LCL and was comparable with AT43RM (11.3%; Fig. 3b). Hyperploidy was confirmed in 227RM by FISH using centromere probes for chromosomes 3 (Fig. 3f) and 18 (data not shown), demonstrating more than four signals in at least 6% of cells analyzed (data not shown). These results indicate that at least some of AT-LCLs/hetero show significant abnormality of X-IR-associated mitotic spindle
checkpoint, and the defect in mitotic spindle checkpoint is not entirely correlated to the failure in induction of G1 arrest.

**AT-LCLs/hetero Show Intermediate Radiosensitivity.** It is well established that AT cells are hypersensitive to ionizing irradiation. We have also shown increased radiosensitivity in our AT-LCLs/homo (Ref. 18; Fig. 4). AT-LCLs/hetero are, on the other hand, reported to be more or less radiosensitive compared with normal cells (2). Because AT-LCLs/hetero showed a significant apoptosis-resistant phenotype, the radiosensitivity of these AT-LCLs/hetero was studied by clonogenic assay after exposure to variable doses of X-IR. All AT-LCLs/hetero demonstrated intermediate radiosensitivity (Fig. 4). Thus, the radiosensitivity of AT-LCLs/hetero was not significantly increased but rather intermediate between AT-LCLs/homo and Wt-LCL.

**Late-Onset Apoptosis in AT-LCLs/homo and AT-LCLs/hetero.** The findings in AT-LCLs/hetero of intermediate radiosensitivity, resistance to acute onset apoptosis, and defect in cell cycle control were studied further. A simultaneous analysis of DNA ploidy and TUNEL assay was used in 227RM and compared with AT52RM and Wt-LCL cultivated for 144 h after X-IR. TUNEL-positive cells with diploidy or more than diploidy may represent those in early-stage apoptosis, whereas cells at advanced-stage apoptosis are defined by subdiploid DNA content and are briefly indicated for Wt-LCL, AT52RM, and 227RM in Fig. 3. When the fraction with subdiploid DNA content was excluded, 42, 38, and 14% of cells were TUNEL positive in AT52RM, 227RM, and Wt-LCL, respectively (Fig. 5), thus indicating that significantly more cells are actively dying in AT-LCLs/hetero and AT-LCLs/homo than Wt-LCL in longer cultures. It was also noted that although cells with diploidy constituted the major fraction of TUNEL-positive cells in Wt-LCL, cells with hyperploidy constituted the major fraction of TUNEL-positive cells in AT52RM and 227RM, although to a lesser amount in the latter.

**DISCUSSION**

AT is an autosomal recessive multisystemic disorder with a progressive cerebellar ataxia, bulbar telangiectasia, and immunological deficiencies. AT patients also manifest an increased incidence of malignancies, such as malignant lymphoma and leukemia. Corresponding to the pleiotropic functions of ATM protein, cells with homozygous or compound heterozygous ATM mutation show elevated radiosensitivity, abnormal regulation of cell cycle control, and defect in DNA repair in response to ionizing radiation and other environmental stresses. The biological features of cells from heterozygous carriers of AT mutation, on the other hand, have not been fully characterized, and it is still controversial whether they are predisposed to cancer in adulthood (3, 4, 19, 20). Thus, LCLs from heterozygous carriers of AT mutation await further investigation with regard to radiosensitivity and recently characterized apoptosis resistance and mitotic spindle checkpoint abnormalities.

We selected AT-LCLs/homo (AT52 and AT43) and AT-LCLs/hetero (155RM, 227RM, and 373RM) from archives of LCLs, which have been shown to express no or <50% of normal ATM protein, respectively, by quantitative Western blotting. Hyperradiosensitivity is the hallmark of AT-LCLs/homo. We have shown recently that radiosensitivity is not obligatorily associated with apoptosis sensitivity in AT-LCLs/homo, which is in accord with several recent reports (26, 27). Similar to AT-LCLs/homo, all three AT-LCLs/hetero were resistant to acute-onset apoptosis in response to various apoptogenic stimuli, as verified by various indicators such as subdiploid DNA contents, reduction of ΔΨm, and DEVD-sensitive caspase 3-like protease activity. These findings indicate that heterozygous ATM mutation has a dominant effect in induction of apoptosis-resistant phenotype in AT-LCLs/hetero. Because p53 plays a central role for DNA damage-induced apoptosis and AT-LCLs/hetero are more or less defective in p53 transactivation (8), it would be a subject for additional studies whether the apoptosis-resistant phenotype is due to a direct effect of ATM mutation or a consequence of defective p53 transactivation.

In contrast to the less profound dysregulation in G1 checkpoint control, mitotic checkpoint after X-IR was significantly defective in two of the three AT-LCLs/hetero. Mitotic checkpoint abnormality in AT-LCLs/hetero suggest that heterozygous ATM mutation has a dominant influence on chromosomal instability in response to DNA-damaging agents, supporting previous notions based on radiation-induced chromosomal damages in AT carriers (28–30). Furthermore, our results support the notion that AT-LCLs/hetero, although resistant to early-onset apoptosis, are nevertheless sensitive to late-onset apoptosis due to accumulation of cell cycle checkpoint abnormalities. This would consequently lead to a reduction in clonogenic activity, although less significantly compared with AT-LCLs/homo. These biological features of AT-LCLs/hetero could be due to ATM haplosufficiency or dominant-negative effect of mutant ATM protein. Although apoptosis resistance was a common feature in AT-LCLs/hetero, 227RM and 373RM with 7517del4 mutation and 8283 delTC mutation, respectively, showed more prominent abnormalities in X-IR-associated mitotic checkpoint regulation than 155RM with 7792C to T mutation. Because the amount of wt-ATM protein as determined by Western blot analysis in each AT-LCL/hetero was not different (45 ± 18, 40 ± 14, and 59 ± 40% of the level in Wt-LCL for 155RM, 227RM, and 373RM, respectively; Fig. 1), it is less likely that these defects are due to haplosufficiency of ATM protein. All these mutant proteins are associated with truncating mutation either due to a frameshift subsequent to four and two nucleotides deletion at nucleotides 7517 and 8283 (227RM and 373RM) or missense mutation from C to T at nucleotide 7792 [codon 2598 (CGA (R) to TGA (X); 155RM]. All these mutant proteins contain leucine zipper motif, along...
with the entire 5' coding region. Thus, it is less likely that the heterogeneity of the negative effect of mutant ATM proteins depends on whether they include leucine zipper (31). Although mutant ATM protein is presumably very unstable, its dominant-negative effect is more likely to explain these features in AT-LCLs/hetero and would be a subject for a further characterization.

On the basis of our finding that heterozygous ATM mutations have more or less dominant-negative effects on the function of wild-type ATM, it is indicated that AT carriers are at a higher risk of cancer development than noncarriers. It has been estimated that up to 1% of the general population are heterozygous carriers of ATM mutations who are clinically indistinguishable from normal individuals (3). Although it is epidemiologically still controversial that AT carriers are predisposed to cancer, the results presented here may influence the interpretation of epidemiological data based on the associated risk in AT carriers. Additional molecular/epidemiological studies are warranted, based on the in vitro biological abnormalities of AT-hetero LCLs.

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REFERENCES

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