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X-ray-induced telomeric instability in Atm-deficient mouse cells☆

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Abstract

The gene responsible for ataxia telangiectasia (AT) encodes ATM protein, which plays a major role in the network of a signal transduction initiated by double strand DNA breaks. To determine how radiation-induced genomic instability is modulated by the dysfunction of ATM protein, we examined radiation-induced delayed chromosomal instability in individual cell lines established from wild-type $Atm^{+/+}$, heterozygote $Atm^{+/-}$, and knock-out $Atm^{-/-}$ mouse embryos. The results indicate that $Atm^{-/-}$ mouse cells are highly susceptible to the delayed induction of telomeric instability and end-to-end chromosome fusions by radiation in addition to the elevated spontaneous telomeric instability detected by telomere fluorescence in situ hybridization (FISH). The telomeric instability was characterized by abnormal telomere FISH signals, including loss of the signals and the extra-chromosomal signals that were associated and/or not associated with chromosome ends, suggesting that Atm deficiency makes telomeres vulnerable to breakage. Thus, the present study shows that Atm protein plays an essential role in maintaining telomere integrity and prevents chromosomes from end-to-end fusions, indicating that telomeres are a target for the induction of genomic instability by radiation.

Keywords: Atm; Radiation; Genomic instability; Chromosome aberration; Telomeric instability

Ataxia telangiectasia (AT) is an autosomal recessive disorder characterized by progressive cerebellar degeneration, premature aging, growth retardation, gonadal atrophy, immunodeficiency, high sensitivity to ionizing radiation, and cancer predisposition [1,2]. The gene responsible for AT, *ATM* (ataxia telangiectasia mutated), encodes ATM protein, which plays a crucial role in the signal transduction network that modulates cell-cycle checkpoints, DNA repair, and other cellular responses to DNA damage [3–5]. When this gene is mutated, those signaling networks are disrupted, resulting in impaired response to DNA damage [6].

Atm, the mouse homolog of ATM, shows 84% amino acid identity and 91% similarity to ATM [7]. Atm-deficient mice exhibit many of the same symptoms found in AT patients such as growth retardation, neurologic dysfunction, infertility, severe defects in T-cell maturation, and extreme sensitivity to ionizing radiation, implying that this mouse model provides a powerful tool for dissecting the pleiotropic phenotypes of AT [8,9]. Cells from Atm-deficient mice show slow growth and premature senescence in culture [8,9]. They also show chromosome abnormalities in the form of end-to-end fusions [10], as seen in AT [11,12], indicating the importance of Atm function in maintaining telomere integrity.

Telomeres contain both DNA and proteins that together appear to stabilize the ends of eukaryotic DNA [13]. In contrast to the unstable ends induced by chromosome breakage, telomeres stabilize chromosome ends by protecting chromosomes from degradation by exonucleolytic attack and end-to-end fusions [14]. There are several lines of evidence to indicate that telomere dysfunction is one of the characteristics of Atm-deficient mouse cells. For example, more than the

☆ *Abbreviations:* Atm, ataxia telangiectasia mutated; FISH, fluorescence in situ hybridization.

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expected number of telomere FISH signals is seen in interphase nuclei and extra-chromosomal telomere signals that are not associated with chromosomes are also observed in mitosis in $Atm^{-/-}$ mouse fibroblasts [10]. Interestingly, evidence indicating the presence of telomeric instability has also been seen in fibroblasts from AT patients [10,11].

Radiation induces genomic instability that is manifested in several endpoints observed in descendants of survivors exposed to radiation [15]. Delayed chromosomal aberrations are a typical consequence of radiation-induced genomic instability [16], of which mechanism for the induction remains unclear. There has been no clear correlation reported thus far between radiation-induced chromosomal instability and a well-defined gene or chromosome. However, recent works demonstrate that one structure crucial to maintain chromosomal integrity is the telomere [17]. It is therefore intriguing to determine whether telomeres are a target for induction of delayed chromosomal instability by radiation.

In the present study, we investigated radiation-induced delayed chromosomal instability in Atm-deficient mouse cell lines to elucidate the relationship between Atm function and genomic instability caused by radiation. Because the recognition process for DNA strand breaks (DSBs) and the subsequent network of signal transduction are impaired in $Atm^{-/-}$ mouse cells, residual damage leading to genomic instability is expected to accumulate more in $Atm^{-/-}$ mouse cells than in wild-type $Atm^{+/+}$ mouse cells. Our results demonstrate that $Atm^{-/-}$ mouse cells are susceptible to induction of delayed chromosomal instability by radiation via telomere dysfunction, indicating a significant function of Atm protein in the maintenance of chromosome stability.

Materials and methods

Cells and cell culture. The three cell lines used were established from whole embryos of C3H/He wild-type $Atm^{+/+}$, heterozygote $Atm^{+/-}$, and knock-out $Atm^{-/-}$ mice. These cell lines were spontaneously immortalized, of which chromosome numbers were 61.7 ± 16.8, 70.8 ± 13.6, and 68.9 ± 14.0 in wild-type $Atm^{+/+}$, heterozygote $Atm^{+/-}$, and knock-out $Atm^{-/-}$ mouse cells, respectively. The cells were cultured in α-modified minimum essential medium (α-MEM; Gibco-BRL) supplemented with 10% fetal calf serum (FCS), 20 mM Hepes, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere with 5% CO_2.

X-irradiation. For X-irradiation, 5×10^5 cells were inoculated into a T-25 cm^2 tissue-culture flask and cultured until they became confluent. The confluent culture was irradiated with X-rays using an X-ray generator (M-150WE; Softex, Osaka) operating at 150 kVp and 5 mA with a 0.1-mm Cu filter at a dose rate of 0.43 Gy/min.

Assay for cell survival. The killing effect of X-rays was determined by a colony formation assay. The cells were irradiated with X-rays, seeded into 100-mm dishes, and then incubated for 10 days for wild-type $Atm^{+/+}$ mouse cells and 3 weeks for heterozygote $Atm^{+/-}$ and knock-out $Atm^{-/-}$ mouse cells.

In an assay for delayed lethal damage, the cells were exposed to the equivalent 10% survival dose, 6 Gy for wild-type $Atm^{+/+}$, 4 Gy for heterozygote $Atm^{+/-}$, and 3 Gy for knock-out $Atm^{-/-}$ mouse cells. Primary surviving colonies were then harvested, replated again, and incubated for another 10 days or 3 weeks for colony formation as secondary colonies. The resultant colonies were fixed, stained with 5% Giemsa, and scored under a dissecting microscope. Colonies consisting of more than 50 cells were scored as survivors.

Analysis of delayed chromosome aberrations. Delayed chromosome aberrations were investigated in secondary surviving colonies as described above. The secondary colonies were harvested and replated into a 100-mm dish, and exponentially growing cells were treated with Colcemid (80 ng/ml) for 4–8 h to harvest mitotic cells. Chromosome samples were prepared as described previously [18]. They were then subjected to the telomere FISH procedure.

Telomere-FISH analysis. The chromosome slides were immersed in PBS(−) at 37 °C for 30 min, fixed in 4% formaldehyde in PBS(−) for 2 min, and washed in PBS(−) for 5 min three times. The slides were then treated with 1 mg/ml pepsin solution, pH 2.0, at 37 °C for 1 min. After washing with PBS(−) for 10 s, the slides were fixed in 4% formaldehyde in PBS(−), washed with PBS(−) three times, and applied with 10 μl hybridization mixture containing 70% formamide, 1% (w/v) blocking reagent (Roche Diagnostics, Tokyo) in maleic acid buffer, pH 7.0, and 3 ng of fluorescence-labeled telomeric PNA probe (FITC-$(CCCTAA)_3$; PerSeptive Biosystems). Then, the slides were heat-denatured at 80 °C for 4 min and hybridized with the PNA probe for 5 h in a humidified dark box. After hybridization, the slides were washed twice in 70% formamide/10 mM Tris, pH 7.2, for 15 min, followed by washing with 50 mM Tris/150 mM NaCl, pH 7.5/0.05% Tween 20 for 5 min three times. Finally, DNA was counterstained with 250 ng/ml DAPI in antifade solution. The chromosome samples were observed using a fluorescence microscope (Olympus, Tokyo) and digital images were recorded using a CCD camera (Photometrics).

Results

Radiosensitivity

We examined the radiosensitivity of three mouse cell lines by colony formation assay. As shown in Fig. 1,

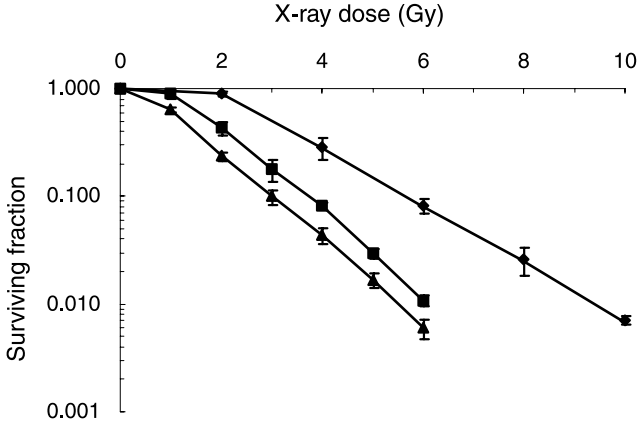

Fig. 1. Radiosensitivity in $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells. Cellular sensitivity to X-rays is determined by clonogenic assay. Diamonds, wild-type $Atm^{+/+}$; squares, heterozygote $Atm^{+/-}$; triangles, knock-out $Atm^{-/-}$. The data represent means ± SE calculated from three independent experiments.

$Atm^{-/-}$ and $Atm^{+/-}$ cells were found to be more radiosensitive than $Atm^{+/+}$ cells. The D_0 values of $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells were 1.65, 1.08, and 1.06 Gy, respectively. Although the D_0 value of $Atm^{+/-}$ mouse cells was close to that of $Atm^{-/-}$ mouse cells, the difference in radiosensitivity between them was distinguishable, with $Atm^{-/-}$ being the most radiosensitive.

Delayed cell death

To determine the effects of Atm deficiency on the induction of genomic instability, we first examined delayed cell death by radiation. Radiation-induced delayed cell death was determined by the cloning efficiency of the secondary colonies, where the cells were exposed to an equivalent 10% survival dose. The cloning efficiencies of $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ cells were 0.76 ± 0.06, 0.38 ± 0.09, and 0.17 ± 0.02, respectively (Fig. 2). Consistent with the result of acute lethality, $Atm^{-/-}$ cells were most sensitive to the induction of delayed cell death by radiation among the three cell lines, indicating that the dysfunction of Atm protein enhances delayed cell death induced by radiation.

Delayed chromosome aberrations

To further elucidate the effects of Atm dysfunction on radiation-induced genomic instability, we investigated the delayed chromosome aberrations in the three cell lines derived from the secondary colonies, which were exposed to an equivalent 10% survival dose. Among the chromosome aberrations, we focused on fragments and end-to-end fusions, as the majority of aberrations observed consisted of these types of aberrant chromosomes, and also those aberrations have been reported to be one of the characteristics of Atm-deficient mouse cells [10]. The end-to-end fusions were categorized into four types: fusions between the ends of q arms (dicentrics; dic); fusions between the ends of p arms (Robertsonian fusions: Rob); fusions between the ends of p and q arms (translocation-type; tra); and complex fusions. Because the frequency of complex fusions was negligible, we compared the remaining three types of aberrations, dic, Rob, and tra, among the three mouse cell lines.

Telomere-FISH analysis revealed that the three fusion-type aberrations can be divided into two groups: those with FISH signals at a fused site (tel+) and those without signals (tel−) as shown in Fig. 3. In Fig. 4A, total chromosome aberrations including fragments and all fusions are presented. As seen in Figs. 4B and C, it is obvious that the response to radiation differs remarkably between the two groups. Spontaneous levels of tel+ fusions were quite similar in the three cell lines, and enhanced induction by radiation was only observed in $Atm^{+/+}$ cells. In contrast, spontaneous levels of tel− fusions were altered depending on the status of the *Atm* gene, being the highest in $Atm^{-/-}$ cells (6.4 ± 1.1/chromosome), the lowest in $Atm^{+/+}$ cells (0.7 ± 0.4/chromosome), and intermediate in $Atm^{+/-}$ (2.1 ± 0.9/chromosome). In contrast to tel+ fusions, radiation enhanced the tel−fusions in the three cell lines depending on the status of the *Atm* gene, being the highest in $Atm^{-/-}$ cells (22.2 ± 2.3/chromosome), the lowest in $Atm^{+/+}$ cells (4.0 ± 1.2/chromosome), and intermediate in $Atm^{+/-}$ (7.4 ± 1.4/chromosome) (Fig. 4C). This result suggests that Atm dysfunction increases the susceptibility to telomere fusions via a loss of telomeres.

In the tel− fusion-type aberrations, the induction level of dicentrics by radiation was not distinctly different among the three cell lines (Fig. 5). In contrast, the induction levels of Robertsonian and translocation-type

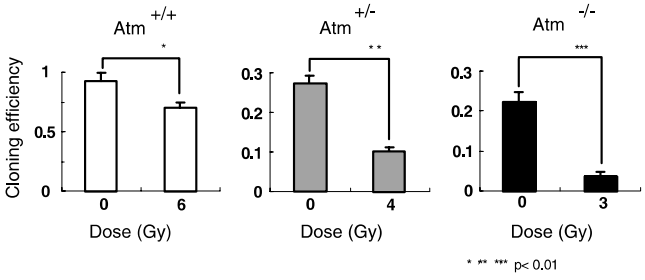

Fig. 2. Radiation-induced delayed cell death in $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells. Delayed cell death by X-irradiation is determined by the cloning efficiencies of secondary colonies of wild-type $Atm^{+/+}$, heterozygote $Atm^{+/-}$, and knock-out $Atm^{-/-}$ mouse cells, where they are exposed to an equivalent 10% survival dose. The value is represented as the cloning efficiency (CE). The data represent means ± SE calculated from three independent experiments. Statistical analysis was performed using Student's *t* test.

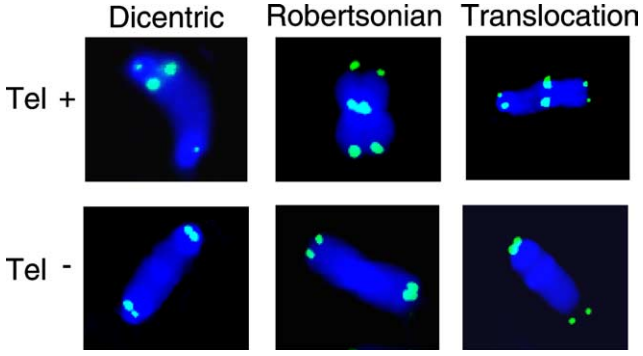

Fig. 3. Fusion-type aberrations with and without telomere FISH signals. Metaphase spreads prepared from three mouse cell lines are visualized using the telomere FISH procedure, as described in Materials and methods. Fusions between the ends of q arms (dicentrics, A,D), p arms (Robertsonian fusions, B,E), and p and q arms (translocation-type, C,F) are presented. Each type of fusion is categorized into two groups, those with FISH signals at a fused site (tel+, A–C) and those without signals (tel−, D–F).

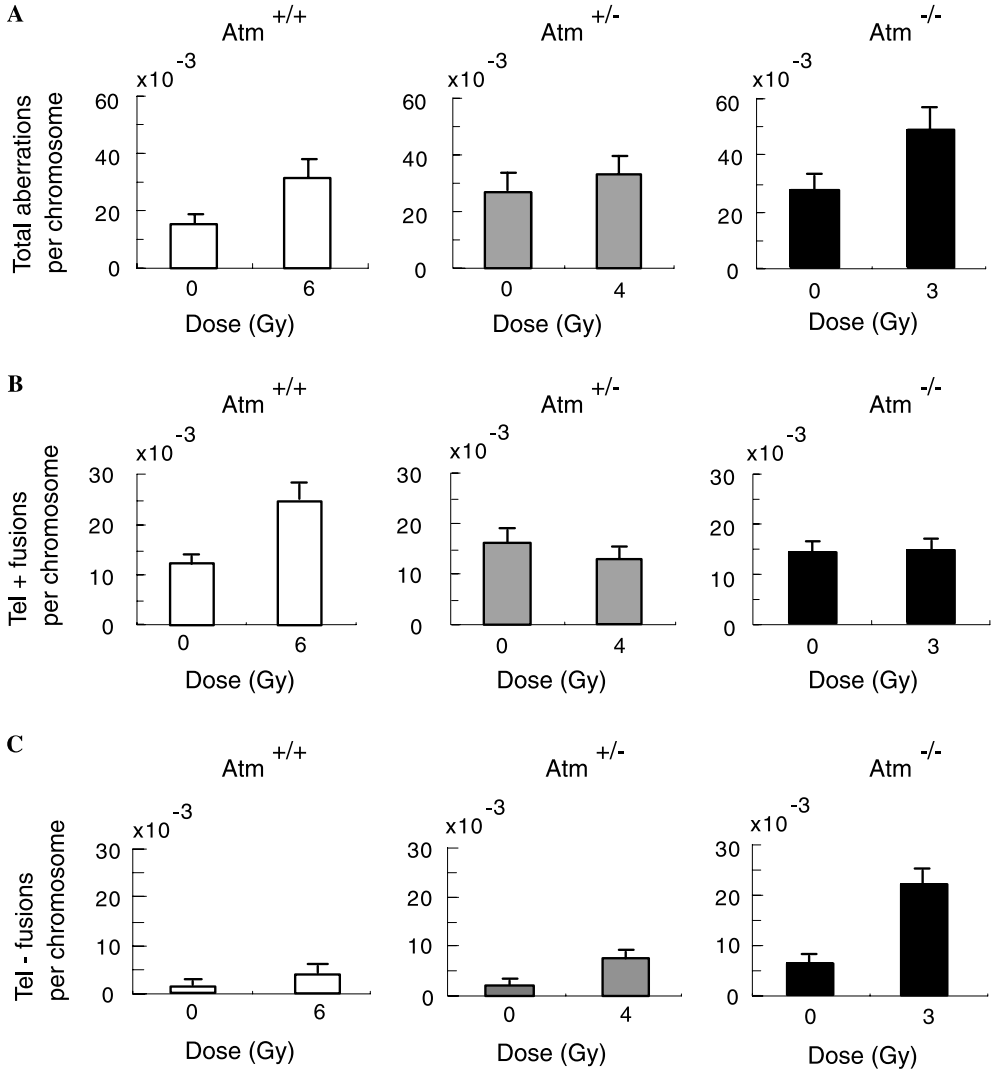

Fig. 4. Radiation-induced delayed chromosome aberrations in $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells. Frequencies of total chromosome aberrations, including fragments and all fusions (A), tel+ fusions (B), and tel− fusions (C), are presented. The modes of chromosome numbers of three mouse cell lines are within a range of 55–72. The data were obtained by analyses of 40–80 metaphases. The number of aberrations per chromosome ± SE is calculated as $x/N \pm \sqrt{x/N}$, where x and N represent the numbers of aberrations and chromosomes analyzed, respectively, based on the Poisson distribution.

fusions by radiation were highest in $Atm^{-/-}$ cells among the three cell lines (Fig. 5).

Telomere dysfunction

Because the analysis for delayed chromosome aberrations suggested a loss of telomere function in Atm-deficient mouse cells, we scored the abnormal telomere FISH signals, including a complete loss of signals (null signal) and extra-chromosomal signals in the three cell lines. The latter signals were divided into two groups: the extra signals associated with chromosome ends and those existing outside chromosomes, as shown in Fig. 6. Spontaneous levels of the loss of telomere FISH signals and the extra-chromosomal telomere FISH signals in $Atm^{-/-}$ cells were higher than those in $Atm^{+/-}$ and $Atm^{+/+}$ cells, indicating that the abrogation of Atm function accelerates the loss of telomere DNA sequences and leads to telomere dysfunction (Fig. 7). Of particular interest was that radiation enhanced this telomere dysfunction, especially in $Atm^{-/-}$ cells, as shown in Fig. 7. This result, conversely, suggests that radiation reduces proportions of a chromosome possessing intact four telomere signals. Indeed, the chromosome analysis revealed that the proportions of a chromosome possessing intact four telomere signals were 80%, 69%, and 64% in unirradiated $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells, respectively, and decreased to 73%, 61%, and 55% in irradiated $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells, respectively. This means that exposure to radiation

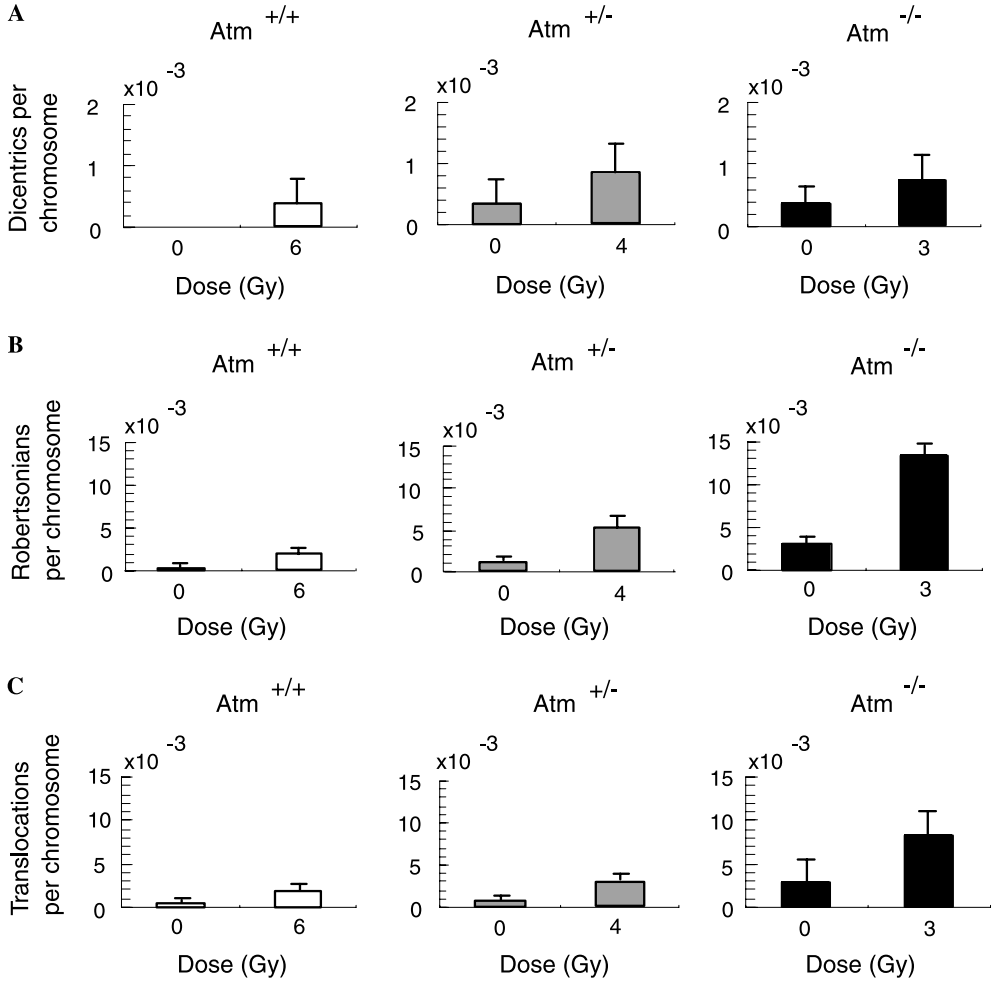

Fig. 5. Telomeric fusions in $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells. The frequencies of tel– fusions including dicentrics (A), Robertsonian fusions (B), and translocation-type fusions (C) are presented. The data are obtained by analyses of 40–80 metaphases. The number of aberrations per chromosome ± SE is calculated as $x/N \pm \sqrt{x}/N$.

decreases the percentage of chromosomes with the intact signals of $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells at 8.8%, 11.6%, and 14.1%, respectively, confirming the acceleration of telomere loss by radiation.

Discussion

In the present study, we demonstrated that Atm protein plays an essential role in telomere stabilization, protecting chromosomes from end-to-end fusions. Of particular interest is that $Atm^{-/-}$ cells show elevated levels of the loss of telomere signals and extra telomere signals associated and/or not associated with chromosome ends. This result showing spontaneously elevated levels of this type of abnormal telomere signals in Atm-deficient mouse cells is consistent with that reported by Hande et al. [10]. The presence of such abnormal telomere signals implies that a part of telomere DNA is fragmented, possibly because of Atm dysfunction. The similar telomere dysfunction associated with Atm deficiency as presented here has been also recently demonstrated in mice doubly null for *Atm* and the telomerase RNA component (*Terc*) [19].

In addition, so far as we know, this is the first study to demonstrate that ionizing radiation enhances delayed telomeric instability, resulting in elevated levels of end-to-end fusions by impaired telomere structure over many (>20) cell divisions post-irradiation.

However, the levels of radiation-induced delayed dicentric chromosomes were not found to be dramatically increased in $Atm^{-/-}$ cells compared with $Atm^{+/+}$ and $Atm^{+/-}$ cells (Fig. 5A). This result is in contrast to the elevated levels of Robertsonian and translocation-type aberrations (Figs. 5B and C). Although the reason for this discrepancy remains unknown, one possible interpretation is that dicentrics, a typical unstable chromosome aberration, are closely associated with cell death via impaired cell divisions. In fact, $Atm^{-/-}$ cells are highly susceptible to the induction of delayed cell death

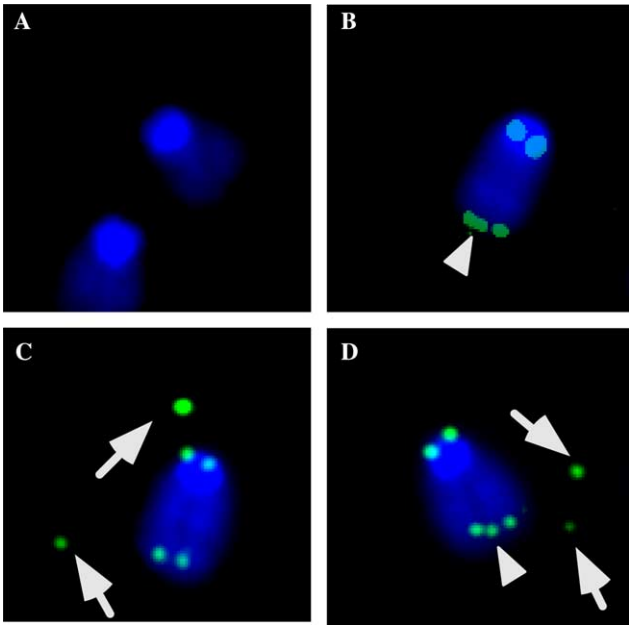

Fig. 6. Examples of chromosomes with abnormal telomere FISH signals. (A) Loss of all telomere FISH signals; (B) an extra telomere FISH signal at the telomere (an arrowhead); (C,D) an extra telomere FISH signal (an arrowhead) and extra-chromosomal FISH signals (arrows).

by radiation (Fig. 2). This high incidence of delayed cell death may eliminate the accumulation of cells containing dicentrics. In contrast to dicentrics, Robertsonian and translocation-type aberrations might be stably transmitted to progeny cells and be accumulated in growing cell populations because some of them were apparently found to expand clonally. We speculate that one of the centromeres of those two aberrant chromosomes might be inactivated and thus nonfunctional.

There is accumulated evidence to indicate that ionizing radiation induces delayed chromosomal instability in the progeny of survivors over many cell divisions post-irradiation [15,16]. While the mechanism for this induction remains unclear, the present and former studies of telomeric instability [10] indicate that one of the crucial targets in generating delayed chromosomal instability is telomeres. It has been reported that the cells from Atm-deficient mice showed extensive telomere shortening corresponding to a 35–40% loss of telomeric DNA when the length was measured by quantitative FISH (Q-FISH) in metaphase chromosomes [10]. Because one of the functions of telomeres is to prevent end-fusion of chromosomes, severe telomere erosion may cause chromosomal instability [17]. Indeed, high levels of spontaneous and radiation-induced end-to-end fusions were observed in $Atm^{-/-}$ cells compared with $Atm^{+/+}$ cells (Fig. 4A). Intriguingly, the majority of end-fusions in $Atm^{+/+}$ cells were found to consist of tel+ type fusions, whereas such a tendency was not seen in $Atm^{-/-}$ cells (Figs. 4B and C). Radiation clearly enhanced the formation of tel+ fusions in $Atm^{+/+}$ cells (Fig. 4B), suggesting that the telomere function to protect chromosome ends is attenuated by radiation

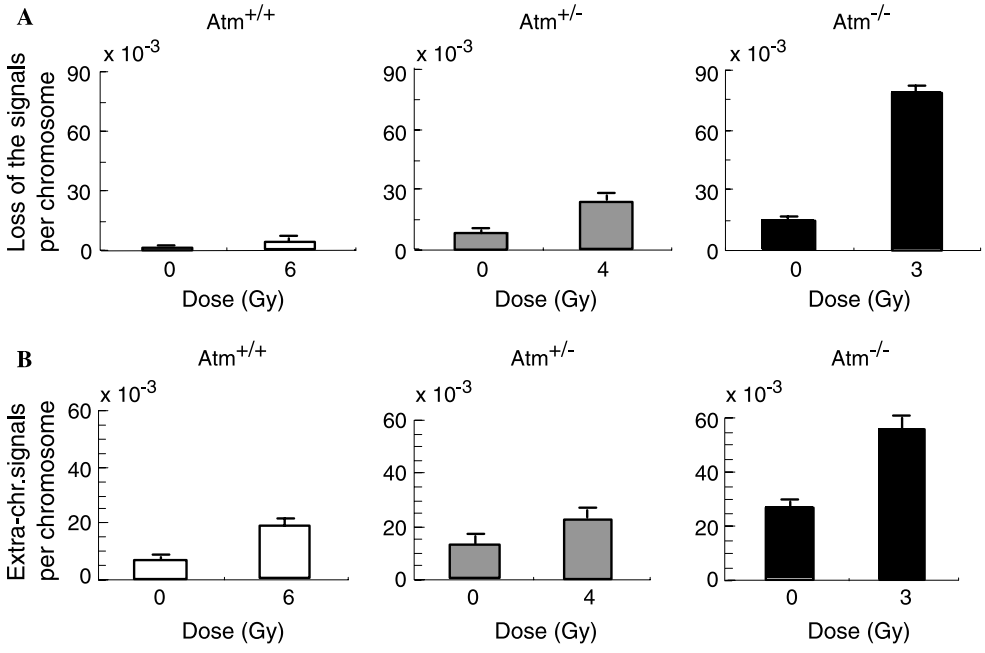

Fig. 7. Abnormal telomere FISH signals in $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ mouse cells. Frequencies of abnormal telomere FISH signals, including loss of the signals (A) and the extra-chromosomal signals (B), are presented. The data were obtained by analyses of 40–80 metaphases. The number of aberrations per chromosome ± SE is calculated as $x/N \pm \sqrt{x}/N$.

exposure, despite the presence of telomere DNA sequences detected by telomere FISH. In contrast, such enhanced induction of tel+ fusions by radiation was not seen in $Atm^{+/-}$ and $Atm^{-/-}$ cells, indicating that radiation-induced attenuation of telomere function by a similar mechanism to that observed in $Atm^{+/+}$ cells did not occur in $Atm^{+/-}$ and $Atm^{-/-}$ cells. In contrast, the induction level of tel−fusions by radiation was remarkably elevated in $Atm^{-/-}$ cells (Fig. 4C), suggesting that the extensive telomere loss caused by Atm deficiency, rather than by the attenuation of telomere function, is accelerated by radiation, resulting in the promotion of end-to-end fusions in $Atm^{-/-}$ cells.

The acceleration of telomere loss by Atm deficiency implies that Atm function maintains telomere integrity. However, the molecular mechanism responsible for the loss of telomere FISH signals and extra-chromosomal telomere FISH signals in Atm-deficient cells remains unknown. Hande et al. [10] suggest that Atm dysfunction leads to defective replication of telomeric DNA, which results in the breakage of telomeric DNA. They have raised the possibility that telomere DNA is vulnerable to breakage because DNA-strand breaks (DSBs) in telomeres might not be sensed and /or repaired in the absence of Atm. There is evidence indicating that DSBs are preferentially accumulated in telomeres [20–22] for which the repair efficiency is defective in the telomere DNA [23]. As a result, telomere DNA might be susceptible to fragmentation in Atm-deficient mouse cells.

At the ends of telomeres, the G-rich strand is longer than the C-rich strand, forming long 3′ G-rich overhangs [24]. Recently, Griffith et al. [25] proposed the new model of telomere structure of mammalian cells, where telomeres end in large duplex loops, designated as T-loops. The T-loops are formed by invasion of the 3′ telomeric overhang into the duplex telomeric repeat array. It is very like that the extensive telomere erosion results in destroying the T-loops and that this impaired chromosome ends are recognized as DSBs that are targets for DSB repair activities. Our present results demonstrate that Atm protein is critically involved in maintaining telomere integrity probably protecting telomere DNA from breakage. Elevated telomere fusions, as a result of Atm dysfunction, may contribute to genomic instability early in tumorigenesis in Atm-deficient mice.

We demonstrated the clear difference in the radiation sensitivities between $Atm^{+/+}$ and $Atm^{+/-}$ mouse cells as shown in Figs. 1 and 2. This is in contrast to the radiosensitivity of human AT heterozygotes, where sensitivity to ionizing radiation is increased only in some AT heterozygotes but indistinguishable from normal radiosensitivity in the other AT heterozygotes when radiosensitivity is examined by cell survival [26,27]. This difference of radiation response between human $ATM^{+/-}$ cells and mouse $Atm^{+/-}$ cells remains unknown. Barlow et al. [28] reported that $Atm^{+/-}$ mice displayed premature greying and increased sensitivity to sublethal dose (4 Gy) of γ-irradiation in regard to radiation effects on longevity of the mice although they showed no clear evidence of increased acute radiosensitivity. Furthermore, cell lines from $Atm^{+/-}$ mice and $Atm^{+/-}$ thymocytes indicated intermediate levels of checkpoint function after radiation exposure compared with cell lines from $Atm^{+/+}$ or $Atm^{-/-}$ mice [28]. Therefore, we speculate that *Atm* heterozygosity of mice could result in phenotypic changes of radiation response including cell lethality possibly caused by dosage reduction.

In summary, the present study indicates that radiation accelerates the fragmentation of telomeric DNA, detected as the extra-chromosomal signals by telomere FISH in Atm-deficient mouse cells over many (>20) cell divisions post-irradiation. This telomere erosion enhances end-to-end chromosome fusions, which may result in the promotion of genomic instability in Atm-deficient mouse cells. Thus, we propose that telomeres are a critical target for induction of delayed chromosome instability induced by ionizing radiation.

Acknowledgments

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