Checkpoint Abrogation in G_2 Compromises Repair of Chromosomal Breaks in Ataxia Telangiectasia Cells

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Abstract

Checkpoint abrogation in G_2 compromises repair of DNA double-strand breaks (DSB) and confers enhanced G_2 chromosomal radiosensitivity in ataxia telangiectasia (AT) cells. To directly test this hypothesis, we combined calyculin A–induced premature chromosome condensation with conventional cytogenetics to evaluate chromosome damage before and after the G_2 checkpoint in irradiated primary AT and normal human lymphocytes and their lymphoblastoid derivatives. Direct analysis of radiation damage in G_2 by premature chromosome condensation reveals practically indistinguishable levels of chromosomal breaks in AT and normal cells. Yet a 4-fold increase in metaphase chromosome damage is observed in AT cells as compared with normal cells which, in contrast to AT cells, exhibit a strong G_2 arrest manifest as an abrupt reduction in the mitotic index. Thus, an active checkpoint facilitates repair of chromosomal breaks in normal cells. Treatment with caffeine that abrogates the G_2 checkpoint without significantly affecting DSB rejoining increases metaphase chromosome damage of normal cells to the AT level but leaves unchanged interphase chromosome damage in G_2. Caffeine has no effect on any of these end points in AT cells. These observations represent the first direct evidence that the G_2 checkpoint facilitates repair of chromosome damage, presumably by supporting repair of DNA DSBs. Failure to arrest will lead to chromatin condensation and conversion of unrepaired DNA DSBs to chromosomal breaks during G_2-to-M phase transition. (Cancer Res 2005; 65(24): 11292-6)

Introduction

Ataxia telangiectasia (AT) is a rare, autosomal recessive syndrome characterized by progressive cerebellar degeneration, immunodeficiency, genomic instability, defective cell cycle checkpoints, increased sensitivity to ionizing radiation, and cancer predisposition (1–3). The AT mutated (*ATM*) gene is located on chromosome 11q22.23 and the protein product belongs to the family of phosphatidylinositol 3 kinase–like family of protein kinases. The hypersensitivity of AT cells to ionizing radiation and radiomimetic agents, but not to UV or alkylating agents, suggests the rather specific involvement of ATM in the processing of DNA double-strand breaks (DSB), a particularly deleterious lesion generated by these agents.

As a component in cell signaling, ATM phosphorylates p53, Chk2, Brac1, Nbs1, Smc1, and other down stream effectors and seems to serve as a central hub in the development of the early responses to DNA DSBs (1–3). As a result, AT cells exhibit strongly diminished checkpoints when irradiated in G_1 or S and a practically complete defect in checkpoint activation when irradiated in G_2 (4). Cells irradiated in G_1 or S display a normal or even enhanced checkpoint response on arrival in G_2, suggesting the operation of distinct, ATM-independent mechanisms of activation (4). The complete abrogation of checkpoint response in G_2-irradiated AT cells is particularly interesting as it allows a "clean" examination of the consequences of checkpoint activation (or abrogation) on the response to ionizing radiation.

In the present article, we describe experiments designed to investigate the interplay between DNA DSB repair defect and checkpoint abrogation and its contribution to the enhanced G_2 chromosomal radiosensitivity observed in AT cells. The developed experimental design allows us to focus on the G_2 phase of the cell cycle and the G_2-to-M phase transition to take advantage of the clear and practically sole dependence of the G_2 checkpoint on ATM (4). Specifically, we combine calyculin A–induced premature chromosome condensation (5) with conventional cytogenetics to evaluate radiation-induced chromosome damage in interphase, before the G_2 checkpoint, and compare this damage to that scored at metaphase. This protocol allows us to examine directly the role of an active G_2 checkpoint in the repair of chromosomal breaks. Furthermore, we use caffeine to abrogate the G_2 checkpoint, without significantly affecting DSB rejoining, and to force cells with DNA damage into mitosis, which converts DNA DSBs into chromosomal breaks via the associated maturation-promoting factor activation and chromosome condensation (6–9).

Materials and Methods

Three EBV-transformed lymphoblastoid cell lines, GM 15786, GM03188A, and GM09899, derived from an AT patient, an obligatory *ATM* heterozygote, and a normal individual, respectively, were used. Cells were maintained in RPMI (Life Technologies, Invitrogen, Paisley, Scotland) supplemented with HEPES and sodium bicarbonate, 15% fetal bovine serum (FBS), 1% L-glutamine (2 mmol/L), and antibiotics (penicillin, 100 units/mL; streptomycin, 100 μg/mL). All incubations were at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. To study peripheral blood lymphocytes (PBL), 5-mL blood was drawn, after obtaining consent, from four clinically characterized AT homozygotes, three obligate AT heterozygotes, and four healthy controls. None of these individuals were under treatment at the time of blood sampling. For PBL culture, 0.5 mL of whole blood was added to 5 mL of McCoy's 5A medium supplemented with 10% FBS, 1% phytohemagglutinin (PHA), 1% glutamine, and antibiotics. Cultures were incubated at 37°C for 72 hours before use in experiments.

Irradiation was carried out in a GammaCell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada) at room temperature and at a dose rate of 1 Gy/min. Calyculin A was prepared as a 1 mmol/L solution in ethanol. PHA was dissolved in water at a concentration of 0.24 mg/mL.

Caffeine was prepared as a 100 mmol/L stock solution in PBS. All chemicals were from Sigma Chemical, Co., (St. Louis, MO) unless stated otherwise.

Proliferating cells (lymphoblasts or lymphocytes) were exposed to radiation (1 Gy) and incubated for 30 minutes at 37°C to allow division of cells irradiated at mitosis. Subsequently, the culture was divided and one half was treated with colcemid for 1 hour to arrest dividing cells at metaphase whereas the remaining half was incubated at 37°C for 30 minutes in the presence of colcemid, and then 50 nmol/L calyculin A was added and allowed to act for an additional 30 minutes to induce premature chromosome condensation. At 90 minutes postirradiation, cells from all cultures were collected by centrifugation, treated in 75 mmol/L KCl for 10 minutes, fixed in methanol/glacial acetic acid (3:1, v/v), and processed for cytogenetics analysis. Standard procedures were used for chromosome preparation and staining (9). For each experimental point, ~ 100 cells were scored for chromatid damage based on standard criteria. For scoring, we considered chromatid breaks and gaps, the latter only when longer than a chromatid width. Light microscopy was coupled to an image analysis system (MetaSystems, Altlussheim, Germany) to facilitate scoring.

Results and Discussion

Stimulated PBLs and actively growing lymphoblastoid cell lines were exposed to 0 or 1 Gy and returned to 37°C for 30 minutes to allow division of cells irradiated past the G$_2$ checkpoint but before the colcemid metaphase arrest point (see Fig. 1*A*). Subsequently, colcemid was added for 1 hour to arrest at metaphase cells irradiated in G$_2$ to analyze radiation-induced chromosome damage and to measure the mitotic index. To quantitatively evaluate the role of the G$_2$ checkpoint in the repair of chromosome damage, we employed calyculin A to directly visualize and score damage in G$_2$ phase, and thus presumably before the G$_2$ checkpoint, in ATM-proficient or ATM-deficient cells. Calyculin A, a specific inhibitor of protein phosphatases type 1 and 2A (10), initiates premature chromosome condensation in all phases of the cell cycle and causes complete chromosome condensation in G$_2$ phase, allowing analysis of chromosomal damage in G$_2$-phase cells. Prematurely condensed chromosomes in G$_2$ are morphologically similar to metaphase chromosomes but lack visible constriction at the centromeric region (Fig. 1*B, left*). This enables an easy and clear distinction in the same sample between cells at metaphase (Fig. 1*B, right*) and cells in G$_2$ and allows parallel scoring of chromosome damage before and after the G$_2$ checkpoint.

Figure 2*A* shows a 5- to 10-fold reduction in mitotic index in irradiated wild-type cells, indicative of a strong arrest in G$_2$. *ATM*-deficient cells show a marginal reduction in mitotic index, in agreement with an abrogation of the G$_2$ checkpoint. Stimulated lymphocytes from *ATM* homozygotes or normal individuals give results similar to those obtained with lymphoblastoid cell lines. PBLs of *ATM* heterozygotes show a response similar to that of normal controls (Fig. 2*A*). An auxiliary to the above discrimination in the same sample between mitotic and G$_2$-phase cells is the ability to evaluate G$_2$ arrest after ionizing radiation. This is because the ratio of cells in G$_2$ to cells in G$_2$ + M is expected to increase in cells sustaining checkpoint activation, but to remain unchanged in cells unable to develop this response, such as AT cells. This is indeed observed in the results shown in Fig. 2*B*. An increase in this ratio is seen in normal and AT heterozygote cells but no significant change is detected in AT cells.

To evaluate the consequences of G$_2$ checkpoint defect in AT cells, we scored chromatid aberrations at metaphase (see example in Fig. 1*B, right*). Normal controls show low levels of chromatid aberrations at metaphase, either in the lymphoblastoid cell line or the PBL cultures, whereas AT cells show an increased frequency of chromatid aberrations, in agreement with the increased radiosen-

sitivity to killing (Fig. 3*A*). This is in agreement with the hypothesis that an intact, ATM-dependent G$_2$ checkpoint prevents G$_2$ cells from entering mitosis before repair reduces damage load (11). Figure 1*B* (*left*) shows an example of ionizing radiation–induced chromatid damage in G$_2$ phase and Fig. 3*B* shows the results obtained from such analysis. Notably, chromatid damage in G$_2$
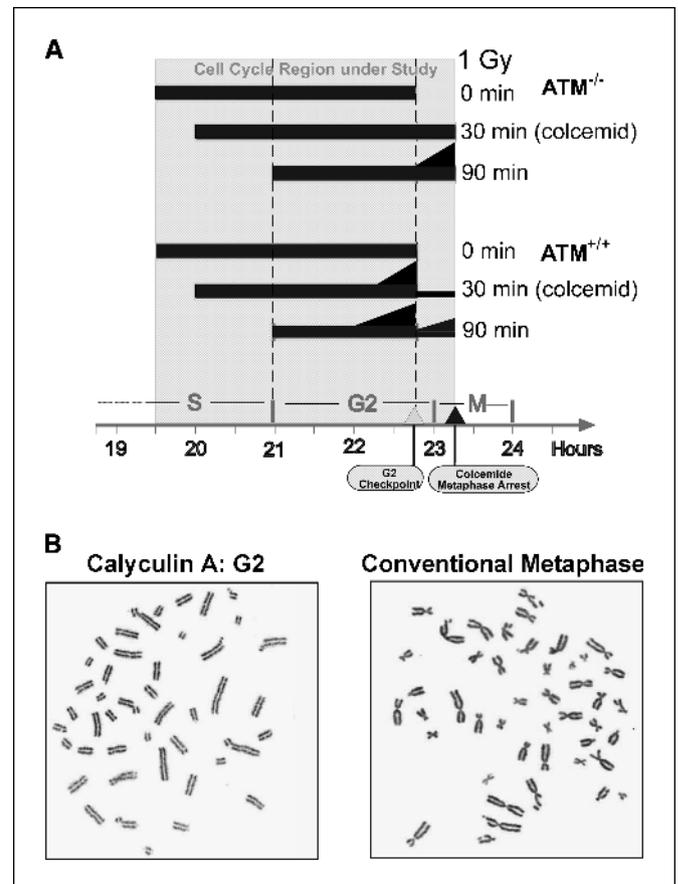

Figure 1. Outline of the experimental design and visualization of chromosome breaks at metaphase or in G$_2$ phase. *A,* characteristics of the experimental design. The adopted experimental design allows analysis of chromosome damage in a well-defined window of the cell cycle that covers G$_2$ and the end of S phase (*gray area*). For illustration purposes, a 24-hour cell cycle is assumed with 14-, 7-, 2-, and 1-hour duration for G$_1$, S, G$_2$, and M phase, respectively. Only the part of the cell cycle relevant for the experiments presented here is shown. *X-axis, white* and *black triangles,* approximate locations of the G$_2$ checkpoint and the colcemid arrest point at metaphase. *Horizontal bars,* distribution of cells through the part of the cell cycle under investigation at the moment of ionizing radiation exposure, as well their anticipated distribution at time points relevant for the experimental design. Due to differences in response, progression through the cycle after irradiation is depicted separately for ATM$^{+/+}$ and ATM$^{-/-}$ cells. *Widening of the bars,* accumulation in G$_2$ phase as a result of checkpoint activation or accumulation in M phase as a result of colcemid treatment. Irradiation is at 0 minutes. During the first 30 minutes after irradiation, before addition of colcemid, cells past the G$_2$ checkpoint traverse metaphase and are thus excluded from analysis. AT cells experience no delay in G$_2$ during this period of time whereas normal cells begin arresting in G$_2$ and display a reduction in mitotic index (*narrowing of the bars*). After an additional 60 minutes, AT cells accumulate at metaphase due to the action of colcemid but show no accumulation at the G$_2$ checkpoint; normal cells accumulate at both blocks although accumulation at metaphase is reduced compared with AT cells. See text for details and justification of assumptions made in the drawing of this schematic. *B, left,* example of an irradiated cell treated with 50 nmol/L calyculin A during the G$_2$ phase of the cell cycle. Note the complete condensation of chromosomes that allows visualization and analysis of chromosome damage. Note also the lack of pericentric constriction that allows distinction between G$_2$ phase and metaphase cells. *Right,* metaphase appearance of a cell irradiated in G$_2$ phase. Note the chromatid-type damage present as well as the clearly outlined pericentric constriction.
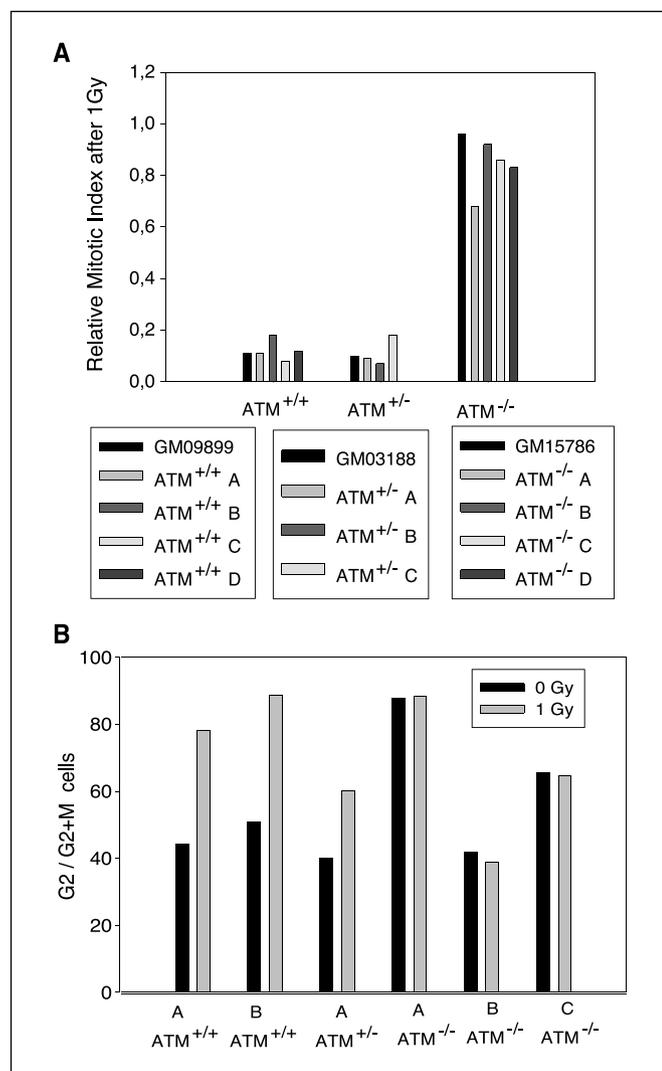
Figure 2. Effect of 1-Gy γ-irradiation during G_2 on cell cycle progression and transition into M phase. *A,* relative mitotic indices of lymphoblastoid cell lines and PBLs of different *ATM* statuses measured 90 minutes after exposure either to 0- or 1-Gy γ-radiation. Mitotic index is defined as the percent of cells at mitosis. The plotted values were calculated as the ratio of mitotic indices of irradiated and nonirradiated cells. Due to the timing of collection, cells analyzed have been exposed to radiation in the G_2 phase of the cell cycle (see Fig. 1*A*). *B,* G_2 to G_2 + M ratio calculated by estimating the percent of cells in G_2 and M using differences in pericentric morphology after treatment with calyculin A.

under these experimental conditions is similar in AT and normal cells. Comparison between Fig. 3*A* and *B* shows that the level of damage measured in all types of cells in G_2 is similar to the level of damage measured in AT metaphase cells. The 2- to 3-fold reduction in the level of chromosome damage in normal metaphase cells suggests a modulation of scorable damage at the checkpoint transition. Lymphoblastoid cell lines and lymphocytes give very similar results, suggesting that the underlying G_2 checkpoint mechanism is not altered by immortalization and culturing.

To test the hypothesis that in normal cells checkpoint function reduces chromosome damage at metaphase, we employed caffeine to abolish G_2 checkpoint by inhibiting ATM (12, 13). Caffeine was administered 30 minutes before ionizing radiation to three lymphoblastoid cell lines or to lymphocytes obtained from two healthy donors, and chromosome damage was scored at metaphase. Figure 4*A* shows the results obtained. Treatment with caffeine of

normal or AT heterozygotes increases ionizing radiation–induced chromosome damage at metaphase to the levels scored in AT cells. Notably, caffeine did not further increase chromosome damage measured in AT cells. This result is compatible with an abrogation by caffeine of the G_2 checkpoint through inhibition of ATM. Because increase in chromosome damage at metaphase after caffeine treatment can, in principle, also be caused by inhibition of DNA repair (14), we used calyculin A to score chromosome damage in G_2. We anticipated that if caffeine inhibited DNA repair during the 90 minutes allowed by the experimental protocol, it would increase damage scored in irradiated, treated G_2-phase cells as compared
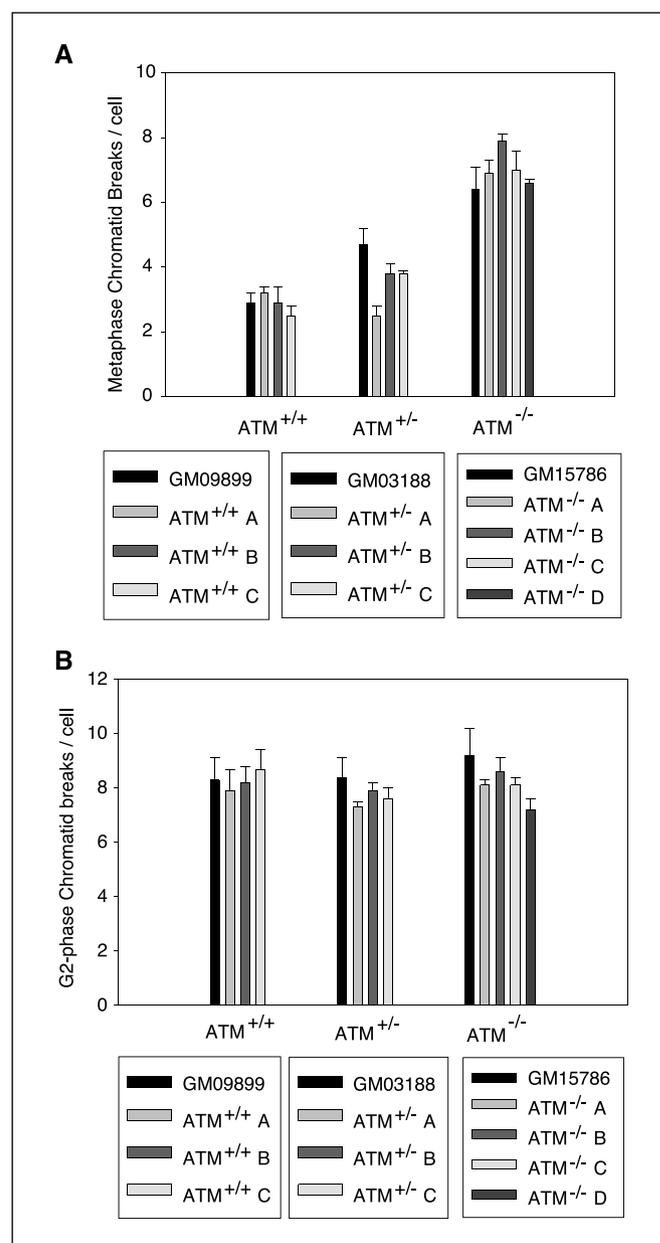

Figure 3. Analysis of chromosomal breaks at metaphase or directly in G_2 phase using premature chromosome condensation in cells of different *ATM* statuses. *A,* chromatid breaks scored at metaphase in normal cells, AT heterozygotes, and AT cells after 1-Gy γ-irradiation and 90 minutes postirradiation incubation as indicated in Fig. 1*A*. *B,* chromatid breaks per cell after 1-Gy irradiation of lymphoblastoid cell lines or PBLs obtained from AT patients, carriers, and control donors, scored directly in G_2 phase in prematurely condensed chromosomes visualized after treatment with calyculin A.
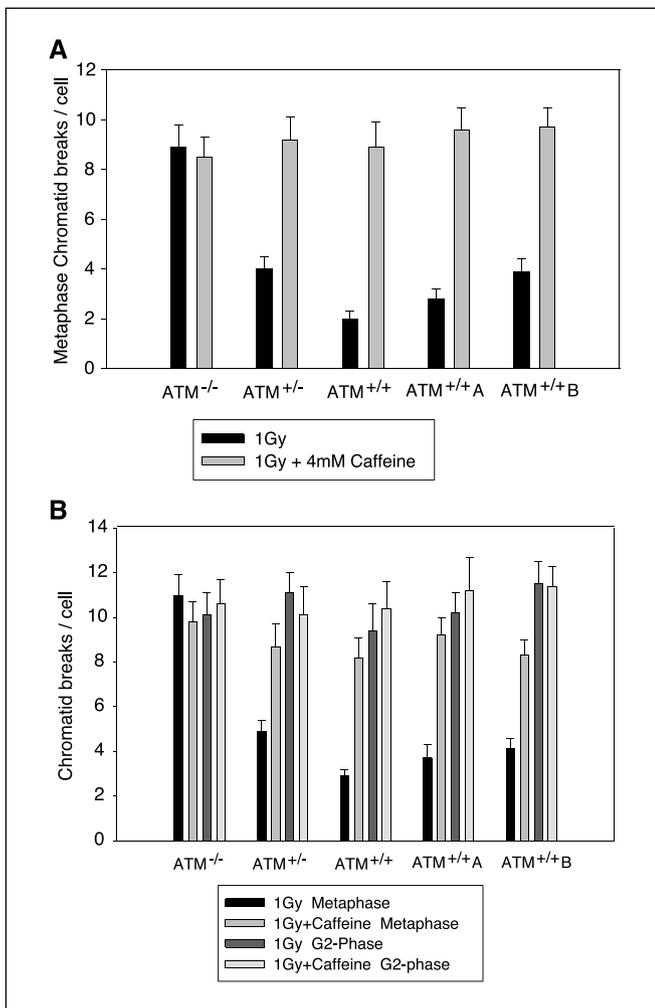
Figure 4. Effect of caffeine on the yield of chromatid breaks in G_2 after calyculin A–induced premature chromosome condensation, as well as by conventional metaphase analysis, in lymphoblastoid cell lines of different *ATM* statuses and PBLs of two normal donors. *A,* results obtained by conventional analysis at metaphase of cells exposed to 1-Gy and treated for 90 minutes in the presence or absence of 4 mmol/L caffeine. *B,* results obtained from calyculin A–treated samples in which G_2 prematurely condensed chromosomes were analyzed together with metaphase chromosomes. The latter was achieved by taking advantage of the lack of centromeric constriction in the G_2 chromosomes of calyculin A–treated samples.

with untreated G_2-phase cells. The results in Fig. 4*B* indicate that chromosome damage scored in G_2 phase in normal cells, heterozygotes, or AT cells is not affected by caffeine, confirming that the effect of caffeine in this phase of the cell cycle derives predominantly from abrogation of the G_2 checkpoint.

Figure 4*B* also includes the analysis of chromosome damage at metaphase in the calyculin A–treated samples, carried out by exploiting the above indicated difference in centromeric morphology to differentiate mitotic from G_2-phase cells. The results are similar to those obtained by conventional metaphase analysis carried out without calyculin A, confirming that centromeric morphology is a powerful discriminator between mitotic and G_2-phase cells. This observation validates the use of this cytogenetic approach for visualizing and scoring chromosome damage before and after the G_2 checkpoint.

The above presented results show the unique features of our cytogenetic approach that allow simultaneous analysis on the same

preparation of chromosome repair, checkpoint function, and chromosome aberration (lethal lesion) production, and contribute to our understanding of the mechanism of ATM function in the following key end points. First, they provide strong evidence that the ATM-dependent checkpoint activated in cells irradiated in G_2 contributes significantly to the reduction of chromosome damage. Second, because both AT and normal cells have the same time to carry out DNA repair before premature chromosome condensation, the similarity in the number of chromatid breaks scored in G_2 suggests that the underlying lesion (i.e., the DNA DSB) is repaired with similar kinetics in both types of cells. This is in agreement with reports that repair of DNA DSBs in AT cells is compromised only in a small ($\sim 10\%$) subset of DNA DSBs that is hypothesized to represent complex lesions that are difficult to repair (15). Lastly, checkpoint abrogation and failure of cells to arrest in G_2 phase will allow chromatin condensation, which can convert unrepaired DNA DSBs to chromosomal breaks during G_2-to-M phase transition (6–9).

Because chromosome damage correlates with cell radiosensitivity to killing, we infer that the checkpoint will also contribute to cell radiosensitivity to killing. This observation is in line with observations by others indicating that G_2 checkpoint activation in G_2 relies exclusively on ATM (4). At this point, it is important to emphasize that the above conclusion is only valid for cells in G_2 and seems to be in line with the low-dose hypersensitivity to killing (16) but cannot explain observations with AT cells exposed to radiation in other phases of the cell cycle (15, 17). The same holds true for the lack of caffeine effect in G_2-irradiated AT cells as it is known that caffeine radiosensitizes exponentially growing AT cells to killing (14). This may be mediated either through an effect on the ATM-independent component of the G_2 checkpoint evident in cells irradiated before G_2 (4) or by an effect on DNA DSB repair by homologous recombination (18).

Recent reports implicate phosphorylation of Smc1 by ATM at Ser[957] and Ser[966] in the repair of ionizing radiation–induced chromosome breaks in the G_2 phase of the cell cycle. Thus, knock-in mouse cells in which these phosphorylation sites are mutated to alanine show defects in the S-phase checkpoint with a magnitude equivalent to that of AT cells, reduced chromosome repair, and a marginal increase in radiosensitivity to killing (19). Smc1 is a component of cohesin and the recombination complex RC-1 and is phosphorylated after ionizing radiation at the sites of DNA DSBs by ATM in an Nbs1/Brca1–dependent manner (19). It will be informative to examine the effect of these knock-in mutations on chromosome repair and checkpoint response using the approaches described here.

Finally, there is evidence that the effects of ATM on checkpoint response and radiosensitivity to killing can be genetically separated (20). The results presented above indicate that the G_2 phase may be unique in the sense that the checkpoint and survival functions of ATM coincide and emphasize the importance of a careful evaluation of radiation effects in the different phases of the cell cycle.

Acknowledgments

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Cancer Res 2005;65:11292-11296.

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