Double-strand break repair by homologous recombination is regulated by cell cycle-independent signaling via ATM in human glioma cells

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SUMMARY

To investigate double-strand break (DSB) repair and signaling in human glioma cells we stably transfected human U87 (ATM\(^+\), p53\(^+\)) glioma cells with a plasmid having a single I-SceI site within an inactive GFP expression cassette allowing for the detection of homologous recombination repair (HRR) by GFP expression. HRR and non-homologous end-joining (NHEJ) were also determined by PCR. DSB repair was first detected at 12 h post-infection with an adenovirus expressing I-SceI with repair reaching plateau levels between 24 and 48 h. Within this time frame, NHEJ predominated over HRR in the range of 3 - 50-fold. To assess the involvement of ATM in DSB repair we first examined whether ATM was associated with the DSB. Chromatin-immunoprecipitation showed that ATM was present at the site of the DSB as early as 18 h post-infection. In cells treated with caffeine, an inhibitor of ATM, HRR was reduced, whereas NHEJ was not. In support of this finding, GFP flow cytometry demonstrated that caffeine reduced HRR by 90% under conditions when ATM kinase activity was inhibited. Dominant-negative ATM expressed from adenovirus inhibited HRR by 45%, also having little to no effect of NHEJ. Furthermore, HRR was inhibited by caffeine in serum-starved cells arrested in G0/G1, suggesting that ATM is also important for HRR outside of the S and G2 cell cycle phases. Altogether, these results demonstrate that HRR contributes substantially to DSB repair in human glioma cells, and, importantly, ATM plays a critical role in regulating HRR, but not NHEJ throughout the cell cycle.
INTRODUCTION

Chromosomal double-strand breaks (DSBs) are considered the most toxic of DNA lesions (1,2). DSBs can be induced by a variety of insults including ionizing radiation (IR)\(^1\) and exposure of cells to radiomimetic drugs. DSBs are one of the most acute threats to cellular homeostasis (3,4), and have been shown to lead to loss of chromosomes and rearrangement of genetic material, potentially resulting in malignancy (5-7). Under certain circumstances DSBs have been shown to be potent triggers of cell cycle arrest and apoptosis (2,4,5). In order to maintain genomic integrity eukaryotes have developed intricate systems to identify and repair these lesions (1,2,8).

The two major DSB repair pathways are non-homologous end-joining (NHEJ) and homologous recombination repair (HRR). Although NHEJ is the predominant type of repair in mammalian cells, it is usually error prone. In contrast, HRR uses sister chromatids or DNA repeats as homologous templates ensuring high fidelity repair. Mutations of genes key to HRR lead to predisposition to cancer (7,9), suggesting that HRR is vital to the repair of at least some DSBs and is key to maintaining genomic integrity (10).

ATM (ataxia telangiectasia mutated) is a large 350-kDa protein defective in cells from individuals with ataxia telangiectasia (AT) (3,11-13). AT is an autosomal recessive disorder characterized by progressive neuronal degeneration, immunodeficiency, sterility and cancer predisposition (3,7). AT cells demonstrate severe radiosensitivity, impaired cell cycle checkpoint control, and a profound defect in all cellular responses to DSBs (4,11), resulting in highly error prone DSB repair (14). The ATM protein is a serine-threonine kinase and a member of the

\(^1\)ABBREVIATIONS: Ad, adenovirus; AT, ataxia telangiectasia; ATM, AT mutated; ATR, AT and Rad3-related; DNA-PK, DNA protein kinase; DNA-PK\(^\ast\), DNA-PK catalytic subunit; DSB, double-strand break; GFP, green fluorescent protein; HRR, homologous recombination repair; IR, ionizing radiation; k/o, knock-out; NHEJ, non-homologous end-joining; PIKK, phosphoinositide 3' kinase-like kinase; SSA, single-strand annealing.
phosphoinositide 3-kinase-like kinase (PIKK) family, which also includes DNA-PK (DNA protein kinase) and ATR (AT and Rad3-related protein) (15). These proteins are associated with DNA damage surveillance, control of cell cycle checkpoints, and cell growth regulation (13,15,16). In response to DSBs, ATM in effect ‘raises the alarm’ to DNA damage, phosphorylating many downstream effector targets such as p53, H2AX, Mdm-2, Brca1, c-Abl, Chk-2, 53BP1, SMC-1 (13,17). This swift response acts to halt the cell cycle and stop DNA replication; ATM then facilitates DNA repair or triggers apoptosis based on the severity of the damage (15,18). It has been demonstrated that ATM binds to DNA in vitro (19), localizes to the nucleus in response to agents that cause DSBs (20), and rapidly phosphorylates H2AX (21), a process documented as one of the earliest and critical events in DSB repair (22-24). In cells of the central nervous system, ATM appears to play an atypical role in that ATM knock-out astrocytes are not radiosensitive and ATM is instead required for apoptosis (25).

Caffeine (1,3,7-trimethylxanthine), a xanthine derivative and radiosensitizer, leads to inhibition of the G1, intra-S, and G2 cell cycle checkpoint when cells are treated prior to irradiation (26-30). The cellular effects of caffeine resemble some of the defects observed in AT cells (31). In vitro, caffeine inhibits the catalytic activity of the ATM (and ATR) kinase at concentrations (0.2 and 1.1 mM IC50, respectively) that have previously been shown to be radiosensitizing (32,33). Studies in chicken DT40 cells have shown that ATM is involved in HRR (34), and Asaad et al (35) suggested a role for ATM in HRR based on caffeine inhibition studies in hamster cells. Very recently, it was demonstrated that caffeine mainly affects checkpoint control and DSB repair responses linked to HRR in chicken DT40 cells, and radiosensitizes hamster and human fibroblasts primarily by an NHEJ-independent process, possibly HRR (36).
Although ATM is clearly linked to the repair process directly or indirectly through cell cycle regulation, its exact role in DSB repair is unclear. Recent work has suggested a possible regulatory role for ATM in V(D)J recombination and the NHEJ pathway, and the binding of ATM at the site of recombination (37). However, direct evidence for the involvement of ATM in HRR independent of its effect on the cell cycle has not been described in human cells. In this report, we use a DSB repair assay based on the rare-cutting endonuclease I-SceI to analyze DSB repair events in human glioma cells. We show that HRR is an important DSB repair pathway in these cells and that ATM binds to the DSB, and using pharmacologic and genetic inhibitors we demonstrate that ATM specifically regulates HRR but not NHEJ. We also show that ATM acts in this capacity outside of the S and G2 phases of the cell cycle.
EXPERIMENTAL PROCEDURES

Reagents. Caffeine, propidium iodide and other chemicals were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO), endonuclease I-SceI and restriction enzyme Bcgl were obtained from New England Biolabs (Beverly, MA). Antibodies for western analysis were: β-actin antibody from Santa Cruz Biotechnology (Santa Cruz, CA), anti-HA from Roche Applied Science (Indianapolis, IN), anti-Flag from Sigma-Aldrich Chemical Co (St. Louis, MO), and anti-ATM (Ab-3) antibodies from Calbiochem (San Diego, CA). ³H-thymidine (1 mCi/ml), ¹⁴C-thymidine (100 μCi/ml), and [α-³²P] dCTP (7000 Ci/mmole) for random priming of GFP DNA hybridization probe were obtained from ICN (Costa Mesa, CA).

Cell culture and adenovirus treatment. Human malignant glioma U87 (p53⁺) cells, and the M059K (DNA-PKcs⁺), M059J (DNA-PKcs⁻) cell pair (38,39) were cultured in α-MEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 20% FBS (Irvine Scientific, Santa Ana, CA) and penicillin/streptomycin as described (40). Cells were transfected with the DR-GFP plasmid (41) using LipofectaminePlus (Invitrogen, Carlsbad, CA). Puromycin-resistant colonies were selected with 5 μg/ml of puromycin, and clonal cell lines were established. The Adβgal and Ad-SceI-NG adenoviruses have been described previously (42,43). AdFlag-ATM(LZ) adenovirus expresses the leucine-zipper domain (amino acids 812 to 1241) of ATM with an amino-terminal Flag tag and was generated as described (44) based on work by Morgan et al. (45). Unless otherwise stated, adenovirus was added to culture medium at an MOI of 100 and incubated while rocking for 2 h at 37°C. Virus was then removed and fresh medium applied. Unless otherwise stated, caffeine was added to the culture medium to a final concentration of 2 mM immediately after virus infection, and remained in the medium for the duration of the experiment.
**Western blot analysis.** Proteins were separated by 4-20% or 5% Tris-glycine SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 5% dry milk in Tris-buffered saline containing 0.02% Tween-20 and probed with antibodies at 1:500-2000 dilution. Blots were developed using the horseradish peroxidase (HRP) Western Lightning System (PerkinElmer Life Sciences Inc., Boston, MA) followed by exposure of membranes to x-ray film. Equal loading of protein in each lane was verified by probing the same blot with antibody to β-actin. When necessary, due to low protein expression, cells lysates were immuno-precipitated with antibody prior to western blot analysis.

**Cell cycle analysis.** Cells were fixed in 70% ethanol, treated with RNase, stained with 5 µg/ml of propidium iodide and analysed for DNA content by FACS on a Coulter Epics XL-MCL flow cytometer at the Massey Cancer Center Flow Cytometry Facility. Alternatively, to determine the fraction of cells entering S, cells were labeled with 10 nCi/ml 14C-thymidine for 48 h, and arrested in G0 by starvation with 0.05% serum for 48 h. Cells were then treated with 5 µCi/ml of 3H-thymidine for 4 h, fixed with acetic acid/methanol, washed with 10% TCA, and cellular uptake of 14C and 3H-thymidine determined on a Packard Topcount™ scintillation counter. Relative 3H-thymidine incorporation was determined by normalizing to 14C-thymidine counts.

**Fluorescence-based DSB repair assay.** In order to study the involvement of ATM in DSB repair we established a Green Fluorescent Protein (GFP) - based DSB repair system in human glioma cells as described previously for hamster cells (41). The system utilizes the I-SceI rare-cutting endonuclease from *Saccharomyces cerevisiae* expressed from an adenovirus (Ad-SceI-NG) (43). I-SceI recognizes an 18-bp DNA sequence not found in the mammalian genome (46).
The system employs an inactive GFP expression cassette plasmid (DR-GFP) containing a restriction site for I-SceI in the 5’ copy of two tandem and truncated GFP reporter genes (41). We transfected the DR-GFP plasmid into human U87 glioma cells and established a number of stably transfected cell clones by selection with puromycin (5 µg/ml). Upon infection with Ad-SceI-NG a single site-specific DSB is generated with a number of different scenarios possible; the break can simply re-ligate without any evidence of DNA cleavage, or the ends can be joined by NHEJ and at the same time destroy the I-SceI site, or HRR may use the 3’ internal copy of GFP DNA (iGFP) as a homologous template for repair resulting in GFP⁺ cells. The iGFP copy carries a unique restriction site, BclI, which replaces the I-SceI site when HRR has occurred. Single-strand annealing (SSA) repair could also occur, which would not result in GFP⁺ cells, but would transfer the BclI site to the 5’ copy, with a concomitant loss of DNA between the two GFP copies. NHEJ and HRR/SSA events can thus be detected by a PCR-based assay using GFP primers directed to sites flanking the I-SceI restriction site. PCR products are cleaved with BclI or in combination with I-SceI to eliminate re-ligation events. Products cleaved by BclI constitute HRR and SSA, and DNA remaining after cleavage with BclI and I-SceI represents NHEJ events.

**GFP analysis.** GFP flow cytometry was performed on live cells on a Coulter Epics XL-MCL flow cytometer (Ex: 488 nm, with 525 nm [+/-15 nm] bandpass filter) at the Massey Cancer Center Flow Cytometry Facility. Standard flow cytometry was carried out as described (47). GFP fluorescence was detected using a Zeiss SV11 stereomicroscope (Zeiss, Jena, Germany) with a Hamamatsu digital CCD camera C4742-95-12NRB (Hamamatsu Corp., Bridgewater, NJ) and AttoArc2/HB 100 Arc variable intensity microscopy illuminator system (Zeiss, Jena, Germany) using excitation/emission filters (470/525 nm). Images were captured using AxioVision 3.1 software and processed with Adobe PhotoShop 5.0.
DNA analysis. Genomic DNA was extracted using DNAeasy® Tissue Kit from Qiagen, Inc. (Valencia, CA). PCR reactions included 0.2 mM dNTPs (Amersham Biosciences, Piscataway, NJ.), 10 pmoles of repair primers (top strand, 5’- GAC TTG AAG TCG TGC TG -3’; bottom strand, 5’- TGT CGT GTT ATT GGT CGT GC -3’), synthesized by the Massey Cancer Center Nucleic Acid Core at VCU, and 1 unit/reaction of Taq DNA Polymerase (Stratagene, Cedar Creek, TX). PCR was performed under the following conditions; 95°C for 2 min, then 40 cycles of 92°C for 30 s, 50°C for 2 min, and 72°C for 1 min, with a final elongation step of 72°C for 5 min. PCR products were visualized on an 1% agarose gel and purified by Qiaquick® gel extraction kit (Qiagen Inc., Valencia, CA). PCR products were digested with BcgI and/or I-SceI restriction enzymes (2 units/reaction) for 4 h at 37°C. Products were separated on a 1.3% Tris-Borate, pH 8.3 agarose gel and detected by Southern blotting using a 780-bp hybridization probe isolated from pEGFP-1 (BD Biosciences ClonTech, Palo Alto, CA) that spans the EGFP gene. The probe was labeled with [α-32P]-dCTP using Prime-It® Kit II (Stratagene, Cedar Creek, TX), and unincorporated nucleoside triphosphates removed by QIAquick® Nucleotide Removal Kit (Qiagen, Inc., Valencia, CA). Membranes were exposed to BioRad Phospho-screen and quantified using QuantityOne® analysis software (Bio-Rad Laboratories, Hercules, CA).

Chromatin-immunoprecipitation (ChIP) assay. The ChIP assay was used to study protein-DNA interactions at the DSB after cleavage with I-SceI expressed from adenovirus (48). Briefly, cells were harvested at different times after I-SceI virus infection and proteins cross-linked to DNA by treating with 1% formaldehyde for 10 min followed by quenching with 2 mM glycine. Cell lysates were then immunoprecipitated with anti-ATM antibody and protein A - agarose. The cross-linking was reversed in 1% SDS at 65°C overnight. DNA was then isolated by standard
proteinase K digestion at 55°C and subsequent phenol/chloroform extraction and ethanol precipitation. PCR was carried out with ChIP primers directed to the 5’ DNA sequence flanking site of the I-SceI site. PCR products were detected by Southern blotting on 1.3% agarose gels. Primers used were (top strand), 5’- GAC TTG AAG TCG TGC TG -3’; (bottom strand), 5’- TGT CGT GTT ATT GGT CGT GC -3’ (synthesized by the Massey Cancer Center Nucleic Acid Core at VCU).
RESULTS

**Expression of HA-I-SceI from adenovirus.** First, to demonstrate expression of the I-SceI endonuclease from the Ad-SceI-NG virus (43), U87 glioma cells were infected and harvested at various times post-infection. I-SceI endonuclease expressed from this Ad has a haemagglutinin A (HA) tag on its amino-terminus permitting western blot detection of protein expression. In agreement with Anglana and Bacchetti (43), we identified a prominent band of HA-I-SceI on a western blot with an apparent molecular size of 30 – 35 kDa that appeared as early as 6 h after infection and remained elevated until 48 h post-infection (Fig. 1), demonstrating that HA-I-SceI is produced from the virus at high levels in U87 cells very soon after infection.

**ATM is expressed in human glioma cell lines.** To demonstrate that ATM is expressed in the U87 cells and other glioma cells, ATM was immuno-precipitated from U87 cells and transferred onto a membrane for western blot analysis. As controls for ATM expression we included extracts from M059J and M059K cells, a pair of human malignant glioma cell lines established from the same human tumor that differ in DNA-PKcs status and ATM expression (39). Expression of ATM was detected in both U87 and M059K cells, whereas ATM was not detected in the M059J cells (Fig. 2). Chan *et al* (39) demonstrated that ATM is expressed in M059J cells, albeit at very low levels.

**DSB repair in DR-GFP-transfected cells.** To facilitate the analysis of DSB repair events, U87 cells were transfected with the DR-GFP plasmid carrying the inactive dual GFP expression cassette with a single I-SceI site and stable cell clones were selected and established by selecting for cells resistant to puromycin (41). We obtained a number of clones that produced GFP⁺ cells
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upon infection with Ad-SceI-NG but not with Adβgal virus (not shown). The stable integrant, U87/DR-GFP appeared to have a single, or few copies of DR-GFP integrated in the genome as indicated by a simple banding pattern on a Southern blot (not shown).

In order to study HRR events in response to I-SceI cleavage, U87/DR-GFP cells were infected with either Adβgal or Ad-SceI-NG, harvested at 24, 48 and 72 h post-infection and subjected to GFP flow cytometry. In several experiments, we detected a time-dependent increase in GFP+ cells following Ad-SceI-NG infection, with 4.6% GFP+ cells by 48 h, and 5.9% by 72 h (Fig. 3). On the other hand, Adβgal-infected cells had very few GFP+ cells out of 30-50,000 analyzed cells (<0.16%). This result demonstrates that HRR (yielding GFP+ cells) is stimulated in human glioma cells by site-specific cleavage with I-SceI expressed from Ad-SceI-NG in agreement with results using hamster cells (41). Further study of DSB repair was then carried out by a PCR-based assay allowing for the parallel determination of NHEJ and HRR (41,49). DSB repair was detected as early as 12 h after infection and reached maximum levels between 24 h and 48 h (Fig. 4). As expected, NHEJ was more prominent than HRR with an average ratio of HRR and NHEJ of 1 to 10.

**ATM binds to the I-SceI-induced DSB in vivo.** ATM binds to DNA ends in vitro (19). In order to determine whether ATM directly associates with the DSB in vivo we used the ChIP assay (48). U87/DR-GFP cells were infected with I-SceI virus and cells harvested at the times indicated (Fig. 5). We found that ATM is associated with the DSB, first detected at 18 h with increased binding seen at 24 h, whereas no such association was detected in parallel cultures which were not infected with I-SceI virus. This result demonstrates that ATM is associated with the DSB as early as 18 h post-infection.
Caffeine inhibits ATM kinase activity in vivo and in vitro. Caffeine is an established radiosensitizer that inhibits the G1, intra-S, and G2 checkpoints and was recently shown to be an inhibitor of ATM (and at higher concentrations also ATR (31,50)). To determine the effects of caffeine on the ATM kinase activity in vivo we treated U87/DR-GFP cells with caffeine and processed cells for western blot analysis. p53 ser-15 has been shown to be phosphorylated by ATM after exposure to IR (51-53). Therefore we used an antibody that recognizes ser-15-phophorylated p53 to determine the effect of caffeine on ATM kinase activity. We found that 2 mM of caffeine inhibited p53 phosphorylation on ser-15 by ~50% in otherwise untreated cells (Fig. 6A). Similar observations were made in vitro when a GST-p53 fusion protein was used as a substrate for immunoprecipitated ATM kinase domain expressed from adenovirus (data not shown; unpublished observations) in line with the report by Sarkaria et al. (31). This finding is in agreement with previous reports using other types of human cells that caffeine inhibits ATM kinase activity in vivo (54).

Caffeine reduces HRR but not NHEJ. To determine the effects of inhibiting ATM on DSB repair, U87/DR-GFP cells were treated with caffeine after infection with Ad-SceI-NG. At 48 h the samples were harvested and analyzed by GFP flow cytometry. We found that caffeine reduced the number of GFP^+ cells, and thus HRR by ~90% (Fig. 6B). We then used the PCR-based repair assay to determine the effect of caffeine on DSB repair in more detail. At 48 h, the inhibitory effect of caffeine on HRR was 85% whereas 9% inhibition of NHEJ was observed (Table 1). At 24 h, a similar but less pronounced inhibition was seen. Two subsequent repeats of this experiment showed that HRR was inhibited ~60% by caffeine at 24 h, again with little or no effect on NHEJ (Table 1). These results show that caffeine specifically inhibits HRR, in line
with previous studies (36) and consistent with a role for ATM, and possibly ATR in the regulation of HRR but not NHEJ.

**Dominant negative ATM adenovirus inhibits HRR but not NHEJ.** Although ATM is believed to be the primary target of caffeine at the concentration used in these experiments, caffeine also inhibits other PIKKs such as ATR and mTOR at higher concentrations (31). Therefore, to confirm that the inhibition of HRR observed with caffeine is due to inhibition of ATM, we expressed a dominant negative (DN) allele of ATM. ATM(LZ) adenovirus expresses the ATM leucine-zipper. Expression of this domain results in DN inhibition of ATM perhaps via dimerization with endogenous ATM (45). Expression of Flag-ATM(LZ) in U87 cells was demonstrated by western blotting using anti-Flag antibody (**Fig. 7A**). A prominent band at ~50 kDa, the expected size of Flag-ATM(LZ), was detected in extracts from cells infected with AdFlag-ATM(LZ) but not in controls. We then infected the U87/DR-GFP cells with AdFlag-ATM(LZ) 24 h prior to infection with I-SceI virus and determined the effect on repair 24 h later. We found that DN ATM(LZ) reduced the number of GFP+ cells compared to controls (**Fig. 7B**). When repair was determined by PCR/Southern blot assay we found that DN ATM(LZ) inhibited HRR by 45 and 42% at 24 and 48 h, respectively, and similar to the effect of caffeine, DN ATM(LZ) had little to no effect on NHEJ (**Table 2**). These data demonstrate that ATM is an important regulator of HRR, and suggest that the effect of caffeine on HRR is primarily attributable to the inhibition of ATM, rather than ATR or any other PIKK.

**Caffeine specifically inhibits HRR in serum-starved cells.** The effect of caffeine on ATM and cellular radiosensitivity is linked to the inhibition of the G2 checkpoint (50). To dissociate a potential direct effect of caffeine on DSB repair from the G2 checkpoint or any other cell cycle
effect, we next determined whether inhibition of ATM and its possible impact on DSB repair could be seen outside of the S and G2 phases of the cell cycle. Because the DR-GFP plasmid consists of two inactive direct repeats of GFP, DSB repair at the I-SceI site could in theory occur by HRR in any phase of the cell cycle, including G0/G1. Therefore, we serum-starved the U87/DR-GFP cells for 48 h prior to applying caffeine and then carried out the repair assay as before. We found that the U87/DR-GFP cells arrested almost completely in G0/G1, in agreement with a previous report (55). Flow cytometry demonstrated that after 48 h of serum starvation only <2% of the cells traversed into S (Table 3). This finding was substantiated by ³H-thymidine labeling experiments in which the S population was reduced to ~4% (data not shown).

As assessed by GFP flow cytometry, we found that HRR occurred even in starved cells. In cycling cells, treatment with caffeine reduced the number of GFP⁺ cells by 90% (average of three independent experiments; 3.8% to 0.4%). In starved cell cultures, the frequency of GFP⁺ cells was 60% lower compared to that in cycling cells, but was still reduced 73% by caffeine (Fig. 8A and B). These results suggest that even though the level of HRR is reduced in G0/G1, it is still dependent on ATM. That inhibition was less complete in starved G0/G1 than in cycling cells could perhaps be explained by an ATM-independent component of HRR, or by a less complete inhibition of ATM itself by caffeine in G0/G1 cells.
DISCUSSION

In this study we found that both HRR and NHEJ are operational in U87 human glioma cells. As demonstrated here these cells express ATM, which is inhibited by caffeine and DN ATM. A previous study showed that ATM is expressed in U87 and M059J cells, albeit at much reduced levels in the latter (39). In our hands, we found that the relative levels of HRR and NHEJ using our repair system span 1:50 to 1:3; results accumulated from many experiments. This variation might result from slight differences in cell density or growth between separate experiments. In cycling cells one expects HRR to contribute more to the overall DSB repair levels, whereas in growth-inhibited populations, primarily residing in G0 and G1, HRR is expected to be reduced (56). The relative levels of HRR and NHEJ in our system fall within previously reported levels of HRR and NHEJ, ranging from several percent to 50% of HRR, in rodent and human cells (46,49,57-59). However, it is likely that NHEJ is underestimated in this type of system since not all events are captured.

ATM has been identified as the primary sensor of certain types of DNA damage, particularly DSBs produced by IR (2-5,8,10). The importance of ATM to genomic integrity is highlighted in individuals with AT who display increased cancer susceptibility and a marked increase in sensitivity to ionizing radiation (60). Caffeine is an established radiosensitizer; it is now thought that its radiosensitizing property is due to its ability to inhibit ATM and at higher concentrations ATR kinase activity, abrogating cell cycle checkpoints and perhaps affecting DSB repair. In this report, we have shown that caffeine inhibits p53 ser-15 phosphorylation by ~50% at a concentration which reduces HRR between 60 - 90%. The p53 ser-15 site is a major substrate for ATM (51-53,61). These findings are further strengthened by results obtained with DN ATM(LZ)
adenovirus, which inhibited HRR by 40 - 45%. DN ATM(LZ) expressed from retrovirus or plasmid was previously shown to radiosensitize human colorectal carcinoma and SV40-transformed fibroblasts, and abrogate the S-phase checkpoint perhaps by interfering with DNA repair (45,62). Using our adenovirus we were able to document expression of ATM(LZ) by western blotting, which was not possible when ATM(LZ) was expressed from retrovirus (45). The ATM(LZ) adenovirus radiosensitizes U87 (unpublished observations), suggesting that interfering with HRR and ATM cell cycle checkpoint regulation affect radiosurvival. Altogether, our results suggest that ATM is of critical importance for successful HRR in human cells. Importantly, we also show that caffeine and DN ATM(LZ) have little to no effect on NHEJ, suggesting that ATM plays no significant role in the regulation of the NHEJ pathway, or, at least, we were not able to detect any such effect using our experimental conditions.

It was suggested recently that caffeine does not inhibit ATM or ATR in vivo (63). In fact, whereas cell cycle checkpoints were efficiently inhibited by caffeine, surprisingly, high concentrations (≥4 mM) of caffeine were stimulatory in combination with agents that inhibited DNA synthesis. However, the reason for this obscure in vivo effect is unclear. Another study concluded that the target for caffeine (when applied at 4 mM) is the ATR/Chk1-regulated G2/M checkpoint, and this treatment radiosensitizes normal as well as AT cells to similar extent (64). In contrast, we used 2 mM of caffeine, a concentration previously used to inhibit ATM in vitro and in vivo (33,50). This concentration does not appreciably affect growth and is not toxic to U87 cells or any other cells we have tested (not shown). Furthermore, our experiments did not use any genotoxic agent (except for I-SceI) in addition to caffeine, which seems necessary to obtain stimulation of ATM and ATR by high concentrations of caffeine (63). Most importantly,
our results with caffeine are fully supported by the results obtained with DN ATM(LZ), suggesting that under our experimental conditions caffeine most likely targets ATM.

Previously, it was demonstrated that ATM binds to DNA during V(D)J recombination, which uses many of the same repair factors as NHEJ, but it was not determined whether the presence of ATM influenced V(D)J recombination or not (37). Using our glioma cell repair system and ChIP analysis we have shown that ATM associates with the DNA directly at the site of DSB. Although the literature strongly suggested such binding (19,20), we believe our result is the first direct evidence of binding occurring when both HRR and NHEJ are assessed at sub-toxic DSB levels. The time when this association was first detected at 18 h is somewhat later than expected. However, keeping in mind that after infection HA-I-SceI first needs to get expressed and then allowing time for the enzyme to digest the DNA, our finding fits with a model where ATM plays an important role at the DSB during repair. We do not know at which point DSBs begin to accumulate in our system, but in an earlier study using the Ad-SceI-NG virus, DSBs were first detected 16 h after infection (43). Since we first detected repair at 12 h with maximum HRR levels seen between 24 – 48 h, the binding of ATM to the DSB would occur relatively early within this time window.

When ATM was inhibited either pharmacologically or genetically, we did not observe any influence (either positive or negative) on NHEJ. Other studies have shown that inhibiting NHEJ positively influences HRR (65-67), whereas our results suggest that NHEJ levels do not increase when HRR is inhibited. Our findings are supported by several studies on the role of ATM in DSB repair (34,35), but to our knowledge, our study is the first to show that in human cells when both HRR and NHEJ are examined in parallel, ATM only affects HRR. However, we did not see
complete inhibition of HRR with either caffeine or DN ATM(LZ), perhaps suggesting incomplete inhibition or that another PIKK, possibly ATR is also important for HRR.

It is important to note that the repair events scored by PCR/Southern blotting are based on transfer of the $BcgI$ cleavage site to the site of the DSB, and thus include products of both HRR (GFP$^+$ population) and SSA, (which does not produce GFP$^+$ cells). Because we observed similar inhibitory effects of caffeine and DN ATM(LZ) on repair using GFP flow cytometry and PCR/Southern blotting, two tentative conclusions can be made. First, most likely, the effect of caffeine is primarily through ATM and not ATR since with caffeine we observed ~60% inhibition at 24 h and with DN ATM(LZ) we observed 45% inhibition. The difference between inhibition levels at 48 h (caffeine; 85%, and DN ATM(LZ); 42%) could be due to induction of compensatory mechanisms perhaps via ATR at later times. Second, and more speculative, a similar 85-90% inhibition of HRR with caffeine was seen with both GFP flow cytometry and PCR/Southern blotting, suggesting that HRR and not SSA was primarily affected. Thus, ATM might control HRR but not SSA. SSA, is a relatively ill-defined repair pathway involving either homology-directed repair or micro-homology-directed NHEJ (1). Whether ATM and/or ATR are important for SSA, is also not known. Future studies are aimed at clarifying the role of ATM in SSA.

ATM is believed to regulate the G1, intra-S, and the G2 checkpoints. To dissociate the effects of ATM on DSB repair from those directly associated with cell cycle regulation, we used serum-starved G0/G1-arrested cells to examine repair. Similar to results using cycling U87/DR-GFP cells, we found that caffeine treatment of serum-starved cells inhibited HRR but not NHEJ. This result suggests that ATM also regulates HRR outside of the S and G2 phases of the cell cycle.
ATM Regulates Homologous Recombination in Human Cells

HRR can operate in G0/G1 cells in our system because the repair substrate has two direct and incomplete copies of the GFP gene, and thus, HRR should be able to occur throughout the cell cycle. Our finding that ATM functions throughout the cell cycle agrees with a recent report that demonstrated similar cell cycle-dependent radiosensitization of AT and normal cells, suggesting that ATM’s role in DSB repair is independent of the cell cycle (68).

Previous studies using the I-SceI-based repair assay have been carried out primarily in rodent cell systems (41,49,59,66) except for a few studies using the human breast carcinoma HCC1937 cell line expressing mutant Brca1 (69), and a human lymphoblastoid cell line (70), neither of which examined both HRR and NHEJ. Although it is very important to study DSB repair in human cells, the efficiency of transfection is in general much lower than for rodent cells. Thus, in experiments involving transient transfection of human cells with a I-SceI expression plasmid, it is very difficult to obtain sufficient yield of I-SceI expressing cells to allow for efficient measurement of DSB repair. By using adenovirus-expressed I-SceI we ensure much better yield of DSB repair events, and thus a more reliable assessment of both NHEJ and HRR. We also demonstrate here that adenovirus efficiently introduce genes expressing proteins important for DSB repair or interfering mutants. This approach will permit us to assess the impact of mutant and DN alleles on DSB repair in human cells more swiftly than using stably transfected cells.

In summary, we have demonstrated that HRR is an important type of DSB repair in human glioma cells with an overall contribution to repair between 2 – 30% with the remaining repair processed primarily through NHEJ. We have also shown unequivocally that ATM binds to the DSB and specifically regulates HRR whereas NHEJ is not influenced by ATM manipulation. Manipulating ATM function with subsequent effects on HRR can also be seen in serum-starved
cells suggesting that regulation of HRR by this important factor may occur throughout the cell cycle.
ACKNOWLEDGEMENTS

This work was supported by NIH grant P01CA72955. The Massey Cancer Center Flow Cytometry Facility is supported in part by NIH grant P30 CA16059. The DR-GFP plasmid was kindly supplied by Dr. M. Jasin, Memorial Sloan-Kettering Cancer Center, New York, NY, and the Ad-SceI-NG adenovirus was donated by Dr. Silvia Bacchetti, McMaster University, Hamilton, Ontario, Canada. M059J and M059K cells were from Dr. Joan Allalunis - Turner, University of Alberta, Edmonton, Alberta, Canada.
TABLE LEGENDS

Table 1. Caffeine specifically inhibits HRR in U87/DR-GFP cells. U87/DR-GFP cells were infected with Ad-SceI-NG and 2 mM caffeine was added 2 h post-infection. Caffeine remained in the medium throughout the experiment. Cells were harvested by trypsinization at 24 and 48 h, and genomic DNA extracted and used for PCR using repair primers. PCR products were digested with I-SceI and/or BcgI restriction enzymes, separated by agarose gel electrophoresis, and products visualized by Southern blot analysis as described in the legend to Fig. 4. In Exp.1, both 24 and 48 h time points were examined. Caffeine specifically inhibited HRR 59% at 24 h and 85% at 48 h. NHEJ was affected 16% and 9% at 24 and 48 h respectively. In Exp. 2 and 3, DSB repair was examined at 24 h. Again, HRR was inhibited ~60% whereas NHEJ was only affected <2%. The relative levels of HRR and NHEJ in these experiments varied between 1:4 – 1:50.

Table 2. Expression of DN ATM(LZ) specifically inhibits HRR in U87/DR-GFP cells. At 24 and 48 h genomic DNA was extracted and PCR performed using repair primers. PCR products were digested with restriction enzymes, separated by agarose gel electrophoresis, and products visualized by Southern blot analysis as described in the legend to Fig. 4. HRR was specifically inhibited 42- 45%, whereas NHEJ was not affected. The relative levels of HRR and NHEJ in these experiments were 1:8 – 1:10.

Table 3. Growth arrest of serum-starved U87/DR-GFP cells. Cells were starved for 24 or 48 h, then fixed and stained with propidium iodide. Flow cytometry was carried out as described in the Experimental Procedures.
FIGURE LEGENDS

Fig. 1. **Expression of HA-SceI in U87 glioma cells from adenovirus.** Adenovirus-based DNA delivery was used to achieve maximum HA-SceI expression yield. U87 glioma cells were infected with the replication-incompetent Ad-SceI-NG adenovirus expressing the *Saccharomyces cerevisiae* I-SceI endonuclease (43), and harvested 6 - 48 h post-infection. The endonuclease expressed by this virus has a hemagglutinin A (HA) tag on its amino-terminus permitting western analysis using anti-HA antibody to monitor expression.

Fig. 2. **ATM is expressed in human glioma cells.** ATM was immunoprecipitated from U87, M059K and M059J cells. The M059J cells are defective in NHEJ and also reported to produce little to no ATM (39,71). Cells were lysed, immunoprecipitated and western blot analysis performed with an anti-ATM antibody as described in Experimental Procedures.

Fig. 3. **Homologous recombination repair in U87 glioma cells.** U87/DR-GFP were infected with Ad-SceI-NG and harvested at various times as indicated in the figure. GFP⁺ cells and HRR events were determined by flow cytometry at 24, 48, and 72 h. (A) Fluorescence microscopy of cells transduced with either Ad-SceI-NG (top) or Adβgal (control virus; bottom) at 48 h post-infection. (B) Flow cytometry at 24, 48, and 72 h of U87/DR-GFP cells transduced with either Ad-SceI-NG or Ad-βgal. The large peak in the flow diagram represents cell auto-fluorescence, and the small peak represents GFP⁺ cells. As shown in the insert, a gating function (enclosed area) was applied to a 2-D plot of GFP-specific fluorescence vs. light scatter to identify GFP⁺ cells, which were then expressed as a percentage of the total cell number.
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**Fig. 4.** **U87 glioma cells carry out HRR and NHEJ.** U87/DR-GFP cells were infected with Ad-I-SceI-NG and harvested at the times indicated. Genomic DNA was extracted and PCR was performed using repair primers. PCR products were digested with I-SceI and BcgI restriction enzymes in vitro and fragments separated by 1.3% agarose gel electrophoresis. Products were visualized by Southern blot analysis using a $^{32}$P-labeled GFP probe, and quantified by Quantity One® analysis software (Bio-Rad, Richmond, CA). **(A)** HRR and NHEJ repair levels plotted as a function of time. Repair is quantified as the fraction of total PCR DNA that is digested with BcgI (HRR), and DNA remaining after BcgI + I-SceI digestion (NHEJ). **(B)** PCR/Southern blot data of results shown in A. Repair was detected as early as 12 h after transduction and reached maximum steady-state levels between 24 - 48 h. In this experiment the average relative ratio of HRR and NHEJ (24 – 48 h) was 1:10.

**Fig. 5.** **ATM binds to the I-SceI induced DSB in vivo.** U87/DR-GFP cells were infected with Ad-SceI-NG. At the indicated times, proteins were cross-linked to DNA by formaldehyde treatment and cells harvested for ChIP assay. Anti-ATM antibody and protein A-agarose was used in immunoprecipitations as described in Experimental Procedures. Input DNA isolated from cell lysates prior to immunoprecipitation was used as a control. The material equivalent to 60,000 cells were loaded on the agarose gel. Input DNA corresponded to 1000 and 500 cells, respectively.

**Fig. 6.** **Caffeine inhibits ATM kinase activity and HRR in vivo.** U87/DR-GFP cells were treated with 2 mM caffeine and harvested after 2 h followed by western blot analysis with phospho-ser15 p53 antibody. **(A)** Caffeine inhibits basal p53 phosphorylation on ser-15. **(B)**
Flow cytometry 48 h post-transduction with or without Ad-SceI-NG and treatment with caffeine (2 mM). Data was analyzed as described in the legend to Fig. 3B.

**Fig. 7.** Expression of DN ATM(LZ) specifically inhibits HRR in U87/DR-GFP cells. U87/DR-GFP cells were infected with AdFlag-ATM(LZ) 24 h prior to Ad-SceI-NG infection. Cells were harvested at 24 and 48 h post-infection with Ad-SceI-NG. (A) Expression of Flag-ATM(LZ) in U87 cells as shown by western blotting with anti-Flag antibody. Adβgal was used as control virus. The viruses were applied at an MOI of 30 and cells were harvested 3 days post-infection. Equal loading of samples was shown by blotting with anti-β-actin antibody. (B) At 48 h cells were analyzed by fluorescence microscopy to determine the levels of GFP⁺ cells (HRR). A dramatic reduction in the number of GFP⁺ cells was seen after transduction with AdFlag-ATM(LZ), expressing the LZ domain fused to a Flag-tag, compared to cells transduced with Adβgal.

**Fig. 8.** Caffeine inhibits HRR in serum-starved arrested cells. (A) Serum-starved (48 h) U87/DR-GFP cells were infected with Ad-SceI-NG, exposed to 2 mM of caffeine immediately after infection, and cultured for 48 h. GFP flow cytometry analysis of 60,000 cells was carried out as described in the legend to Fig. 6. Approximately 70% inhibition of HRR was observed in the presence of caffeine. (B) Two additional experiments were performed under identical conditions and the average inhibition seen in the three experiments was determined. The average inhibition of HRR by caffeine was 73% (p< 0.001).
REFERENCES

Table 1
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<th>Exp 1</th>
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### Table 2
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Table 3
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Fig. 1
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Fig. 2
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Fig. 3
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Fig. 4

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Fig. 5
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Fig. 6A
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Fig. 6B
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Fig. 7A
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Fig. 7B
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Fig. 8A
Golding et al.
Fig. 8B
Golding et al.
Additions and Corrections


DsbB elicits a red-shift of bound ubiquinone during the catalysis of DsbA oxidation.

Kenji Inaba, Yoh-hei Takahashi, Nobutaka Fujieda, Kenji Kano, Hideto Miyoshi, and Koreaki Ito

The names of Drs. Miyoshi, Kano, and Fujieda were inadvertently omitted from the author list. All three of these authors are from the Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan. The corrected list is shown above.


Structure-function analysis of the reactive site in the first Kunitz-type domain of human tissue factor pathway inhibitor-2.

Hitendra S. Chand, Amy E. Schmidt, S. Paul Bajaj, and Walter Kisiel

Page 17505, Fig. 4: The amino-terminal sequence of the first Kunitz-type domain (KD1) of human TFPI-2 is incorrect at positions 7 and 8 where a transposition occurred. The correct sequence of the TFPI-2 KD1 is DAAQEPTG...KV. This correction does not have any impact on the conclusions drawn in the study.


Double strand break repair by homologous recombination is regulated by cell cycle-independent signaling via ATM in human glioma cells.

Sarah E. Golding, Elizabeth Rosenberg, Ashraf Khalil, Alison McEwen, Matthew Holmes, Steven Neill, Lawrence F. Povirk, and Kristoffer Valerie

Page 15403: There are errors in the primer sequences. In the right column, third line under “DNA Analysis” the primer sequence should be: “top strand, 5’-CGT GCT GGT TAT TGT GCT GTC T-3’; bottom strand, 5’-GAC TTG AAG AAG TCG TGC TG-3’.”

Page 15404: Under “Chromatin Immunoprecipitation (ChIP) Assay,” left column, lines 2 and 3 should read: “Primers used were 5’-GAA GTT CAT CTG CAC CAC C-3’ (top strand) and 5’-TTG AAG TTC ACC TTG ATG CC-3’ (bottom strand). . .”

Page 15406, Fig. 6A: The panels of the Western blot showing p53 phosphorylation were flipped. The correct Fig. 6A is shown below:

A

% inhibition 50
[CAFFEINE], mM: 0 2
pp53 (ser15) ➔
β-actin ➔

FIG. 6A

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Double-strand break repair by homologous recombination is regulated by cell cycle-independent signaling via ATM in human glioma cells
Sarah E. Golding, Elizabeth Rosenberg, Ashraf Khalil, Alison McEwen, Matthew Holmes, Steven Neill, Lawrence F. Povirk and Kristoffer Valerie

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