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Mismatch repair-mediated G2/M arrest by 6-thioguanine involves the ATR–Chk1 pathway

Kazuhiko Yamane, Kerri Taylor, and Timothy J. Kinsella*

Department of Radiation Oncology, Case Western Reserve University, Case Comprehensive Cancer Center/University Hospitals of Cleveland, Cleveland, OH 44106-4942, USA

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Abstract

DNA mismatch repair (MMR) deficiency in human cancers is associated with resistance to a spectrum of clinically active chemotherapy drugs, including 6-thioguanine (6-TG). We and others have shown that 6-TG-induced DNA mismatches result in a prolonged G2/M cell cycle arrest followed by apoptosis in MMR^+ human cancer cells, although the signaling pathways are not clearly understood. In this study, we found that prolonged (up to 4 days) treatment with 6-TG (3 μM) resulted in a progressive phosphorylation of Chk1 and Chk2 in MMR^+ HeLa cells, correlating temporally with a drug-induced G2/M arrest. Transfection of HeLa cells with small interfering RNA (siRNA) against the ataxia telangiectasia-related (ATR) kinase or against the Chk1 kinase destroyed the G2/M checkpoint and enhanced the apoptosis following 6-TG treatment. On the other hand, the induction of a G2/M population by 6-TG was similar in $ATM^{-/-}$ and ATM^+ human fibroblasts, suggesting that the ATM–Chk2 pathway does not play a major role in this 6-TG response. Our results indicate that 6-TG DNA mismatches activate the ATR–Chk1 pathway in the MMR^+ cells, resulting in a G2/M checkpoint response

Keywords: Mismatch repair; 6-TG; G2/M checkpoint; ATR; Chk1

Although the principal function of DNA mismatch repair (MMR) is to correct DNA replication errors, it is also recognized to be involved in cellular responses to a wide variety of clinically active chemotherapeutic drugs. MMR-proficient (MMR^+) human cancer cell line cells are sensitive to several different classes of drugs including anti-metabolites (6-TG, 5-fluorouracil [5-FU], and 5-fluorodeoxyuridine [FUdR]), the platinum analogs (cisplatin and carboplatinum), and methylating agents [1–4]. The increased cytotoxicity in MMR^+ cells is usually preceded by an increased and prolonged G2/M arrest. In contrast, MMR-deficient (MMR^-) human cancer cell lines show variable levels of in vitro and in vivo drug resistance and show a reduced G2/M arrest. From a clinical perspective, a recent study demonstrates that MMR deficiency as determined by microsatellite instability in stage II and stage III colon cancers is associated with a reduced disease-free and overall survival following 5-FU based adjuvant chemotherapy [5]. Additionally, microsatellite instability is also recognized as a marker of poor prognosis in adult T-cell leukemias/lymphomas where 6-TG is often used [6].

Cell cycle checkpoints induced by DNA damage are essential for maintaining genomic integrity (for reviews, [7,8]). Some signals of DNA damage lead to a cell cycle arrest at the G1 and/or G2 phases, reduce the rate of DNA synthesis, or result in apoptosis, in order to prevent transfer of damaged genetic information to the daughter cells. These checkpoint responses and the induction of apoptosis are considered to be major mechanisms to reduce both initiation and progression of cancer as well as to repair some types of drug-induced damage.

The human mismatch repair protein, MLH1, has been shown to bind several proteins involved in DNA repair and cell cycle checkpoints, including methyl-CpG binding endonuclease 1 (MED1) [9], the proliferating cell nuclear antigen (PCNA) [10], BRCA1 [11], and ataxia telangiectasia mutated (ATM) [12]. We have already

* Corresponding author. Fax: 1-216-844-4799.
E-mail address: timothy.kinsella@uhhs.com (T.J. Kinsella).

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shown that MMR deficiency (both MLH1$^-$ and MSH2$^-$) in human tumor cell lines results in a reduced G2/M arrest following treatment with ionizing radiation and several different chemotherapeutic drugs, including the fluoropyrimidines (5-FU and FUdR), and 6-thioguanine (6-TG) [4,13–15]. However, the major functions of MMR in cell cycle regulation following chemically induced DNA mismatches are still unclear. In this study, we report that MMR-mediated processing of 6-TG mismatches can activate the ATR–Chk1 pathway, providing evidence of participation of a major checkpoint pathway in a chemically induced DNA mismatch response.

Materials and methods

Cells and cell culture. HeLa cells, a human cervical carcinoma cell line which is known to be MMR-proficient [16], were grown in RPMI 1640 medium supplemented with 10% fetal calf serum in a humidified atmosphere of 10% CO_2 and 90% air at 37 °C. Human ATM$^{-/-}$ fibroblast cells (FT169A) and its wild-type ATM transfected cells (ATM$^+$, YZ5) (generous gifts of Dr. Y. Shiloh) [17] were also grown under the conditions described above.

Transfection of siRNA. Transfection was performed with oligofectamine as recommended by the manufacturer (Invitrogen Life Technologies). The anti-ATR siRNAs (Dharmacon, Lafayette, CO) were 5′-GCCAAGACAAAUUCUGUGUdTdT and 5′-ACACAGAAUUUGUCUUGGCdTdT (the opposite strand). The scrambled control was 5′-GCUCAGAUCAAUACGGAGAdTdT and 5′-UCUCCGUAUUGAUCUGAGCdTdT. The target sequence of a premade anti-Chk1 siRNA was 5′-AAGCGUGCCGUAGACUGUCCA [18] and also purchased from Dharmacon. Transfection of the specific siRNAs was performed for 24–30 h and the HeLa cells were then trypsinized. Next, the cells from one well were divided into three wells (24-well plates) and RPMI medium was added. Two hours later after plating, 6-TG (0 or 3 μM) was added. To effectively reduce Chk1 protein levels in the HeLa cells during the 6-TG treatment, a second transfection (termed a tandem transfection) of anti-Chk1 siRNA was necessary. The second transfection in the tandem transfection was performed for 10 h in the absence of 6-TG after 2 days of treatment with 0 or 3 μM 6-TG. The transfection reagents were removed and the medium with or without 6-TG was added again. The 10 h period in the absence of 6-TG is included in the overall 6-TG treatment time for these Chk1-reduced cells.

Western blotting. Total cell extracts from the HeLa cells and the transfectants were prepared by trichloroacetic acid precipitation to detect ATR (PA1-450, Alexon Biopharma Research, Golden, CO) and MSH2 (Ab-2, Oncogene Research Products, San Diego, CA). After extraction of trichloroacetic acid with ether, DNA was sheared by sonication before loading onto gels. The lysates containing the checkpoint kinases were extracted as described [19]. Antibodies against the phosphorylated Chk1 and against the phosphorylated Chk2 were obtained from Cell Signaling Technology (Beverly, MA). Anti-total Chk1 and Chk2 antibodies were obtained from Santa Cruz (Santa Cruz, CA).

Flow cytometry. HeLa cells were fixed with 90% ethanol at −20 °C for 60 min to a few days, incubated with RNase, stained with propidium iodide, and then subjected to flow cytometry (Coulter, Epics XLMCL, Miami, FL). To measure the G2/M checkpoint response in HeLa cells transfected with either anti-ATR siRNA or anti-Chk1 siRNA, double staining with propidium iodide and mitotic protein monoclonal-2 (MPM-2) antibody (Upstate, Waltham, MA) was used [20]. For TUNEL staining, the Apo-Direct kit was used (eBioscience, San Diego, CA). For the G2/M checkpoint assay, nocodazole (0.3 mg/ml) was added to the medium and cells were cultured for 20 h, as a general method [21,22]. Nocodazole, a microtubule polymerization inhibitor, arrests cells in M phase without any cytotoxicity at the used concentration. If nocodazole is used, the M phase population is enhanced without 6-TG damage (typically, 1–3% to more than 40%). Following 6-TG treatment, nocodazole is used to quantitatively measure cells escaping a G2/M checkpoint in ATR- and Chk1-reduced HeLa cells.

Results

Chk1 and Chk2 are phosphorylated during 6-TG treatment

We used an antibody against phosphorylated serine 317 of Chk1 to examine the involvement of the checkpoint kinase Chk1 in HeLa cells following 6-TG

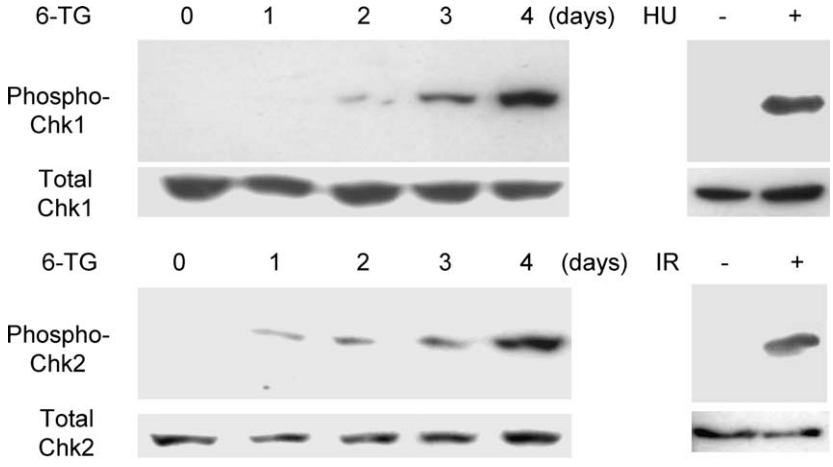

Fig. 1. Chk1 and Chk2 phosphorylation increase during 6-TG treatment. HeLa cells were treated with 3 μM 6-TG for 0–4 days. The cell lysates were subjected to Western blotting using antibodies against the phosphorylated serine 317 of Chk1 and against an anti-total Chk1 antibody, or using antibodies against the phosphorylated threonine 68 of Chk2 and against an anti-total Chk2 antibody. Control HeLa cells were treated with hydroxyurea (HU, 1 mM for 1 day) or ionizing radiation (IR, 10 Gy and then incubated for 3 h).

treatment. Phosphorylation of serine 317 is essential for the full activation of Chk1 [23]. Phosphorylation of Chk1 was gradually increased in HeLa cells during 4 days of treatment with 3 μM 6-TG (Fig. 1). The extent of Chk1 phosphorylation is temporally correlated with the 6-TG-induced G2/M peak induction (Fig. 2B), similar to our recently published data using another human cancer cell line (RKO) [15]. A positive control using hydroxyurea (HU) indicated that this phospho-specific antibody works well.

We also used an antibody against phosphorylated threonine 68 of Chk2. Chk2 is also phosphorylated in HeLa cells with a similar pattern to Chk1 following 6-TG treatment (Fig. 1). Ionizing radiation (IR) was used as a control treatment to induce the phosphorylation of Chk2. These data suggest that both ATR and ATM may play roles in the 6-TG damage response in HeLa cells.

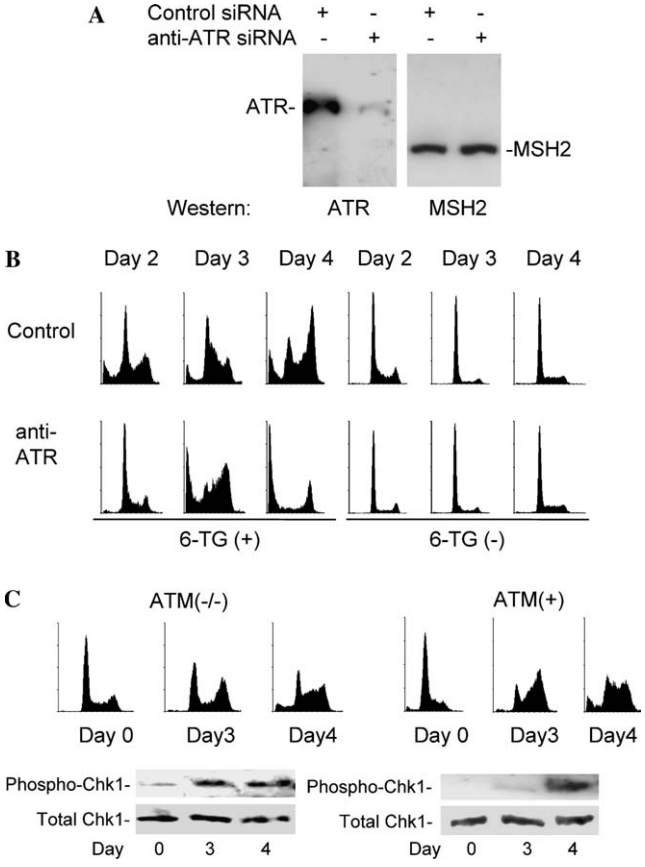

Fig. 2. Reduction of ATR protein level results in a reduced G2/M population and increased apoptosis after treatment with 6-TG. (A) The siRNA against ATR and its scrambled control were transfected into HeLa cells. The cells were cultured for 2 days, and proteins were detected by Western blotting using anti-ATR and MSH2 antibodies. (B) The transfectants were treated with 6-TG (0 or 3 μM) continuously for the indicated times. Cells were then fixed with ethanol and analyzed by flow cytometry. (C) ATM (−/−) cells (FT169A) and its wild-type ATM transfected cells (ATM$^+$, YZ5) were treated 3 μM 6-TG for 2 days and further cultured without drug for 1 day (Day 3) or 2 days (Day 4). The Western blotting against Chk1 was performed as described in the legend to Fig. 1.

Both kinases are known to regulate a G2/M checkpoint by phosphorylating and activating their downstream kinase Chk1 (by ATR) and Chk2 (by ATM) [24,25].

Reduction of ATR protein level in HeLa cells results in apoptosis and abrogation of G2/M checkpoint during treatment with 6-TG

To specifically examine whether ATR is involved in the G2/M checkpoint response to 6-TG, siRNA against ATR was transfected into MMR$^+$ HeLa cells. This single transfection resulted in a significant reduction of ATR protein levels (Fig. 2A). This strong but incomplete inhibition of the ATR expression did not affect the cell cycle distribution in the absence of 6-TG treatment (Fig. 2B). However, the transfectants of ATR siRNA showed a marked apoptotic response, as demonstrated

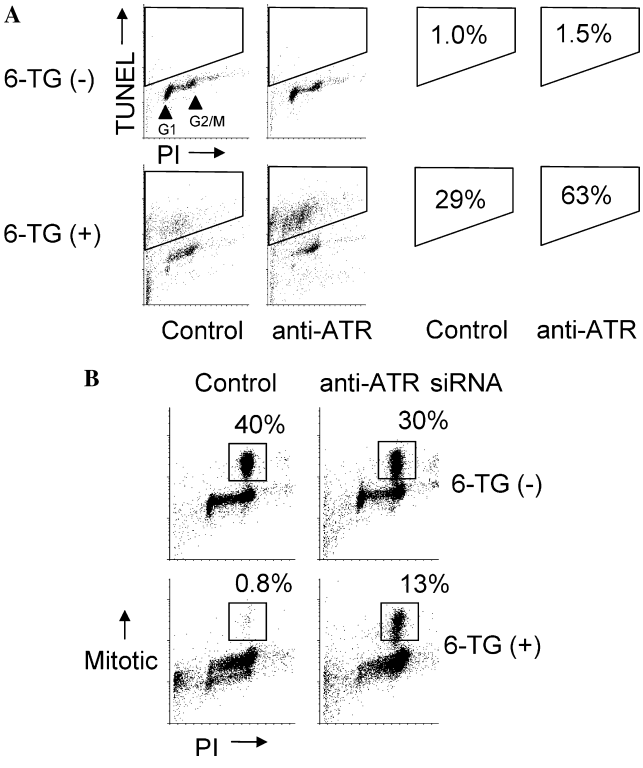

Fig. 3. ATR regulates the G2/M checkpoint activation induced by 6-TG DNA mismatches. (A) HeLa cells were transfected with siRNA against ATR or its scrambled control (Control), then treated with 6-TG (0 or 3 μM) continuously for 3 days, and fixed with formaldehyde and with ethanol. Cells were then labeled according to the TUNEL method using FITC-conjugated probes. The gated regions are TUNEL positive populations, which are also quantified as the percent TUNEL positive cell per total cell population in the accompanying panels. (B) Both transfectants were treated for 3.3 days with 6-TG (0 or 3 μM). After removal of 6-TG, nocodazole (0.3 mg/ml) was added to the medium and cells were cultured for 20 h. The cells were next fixed with formaldehyde (to suppress a subG1 population) and then stained with MPM-2 antibody conjugated with FITC and with propidium iodide, prior to analysis by flow cytometry. An antibody against the phosphorylated serine 10 of histone H3 gave similar results as the control (data not shown).

by an increased subG1 population and an increase in TUNEL positive cells following treatment with 6-TG (Figs. 2B and 3A). In contrast, transfectants with a control siRNA showed a major increase in the G2/M population by Day 4 of 6-TG treatment and a reduced subG1 population. It is highly possible that control transfectants also stop at G1 and at S, due to nucleotide pool reduction by 6-TG.

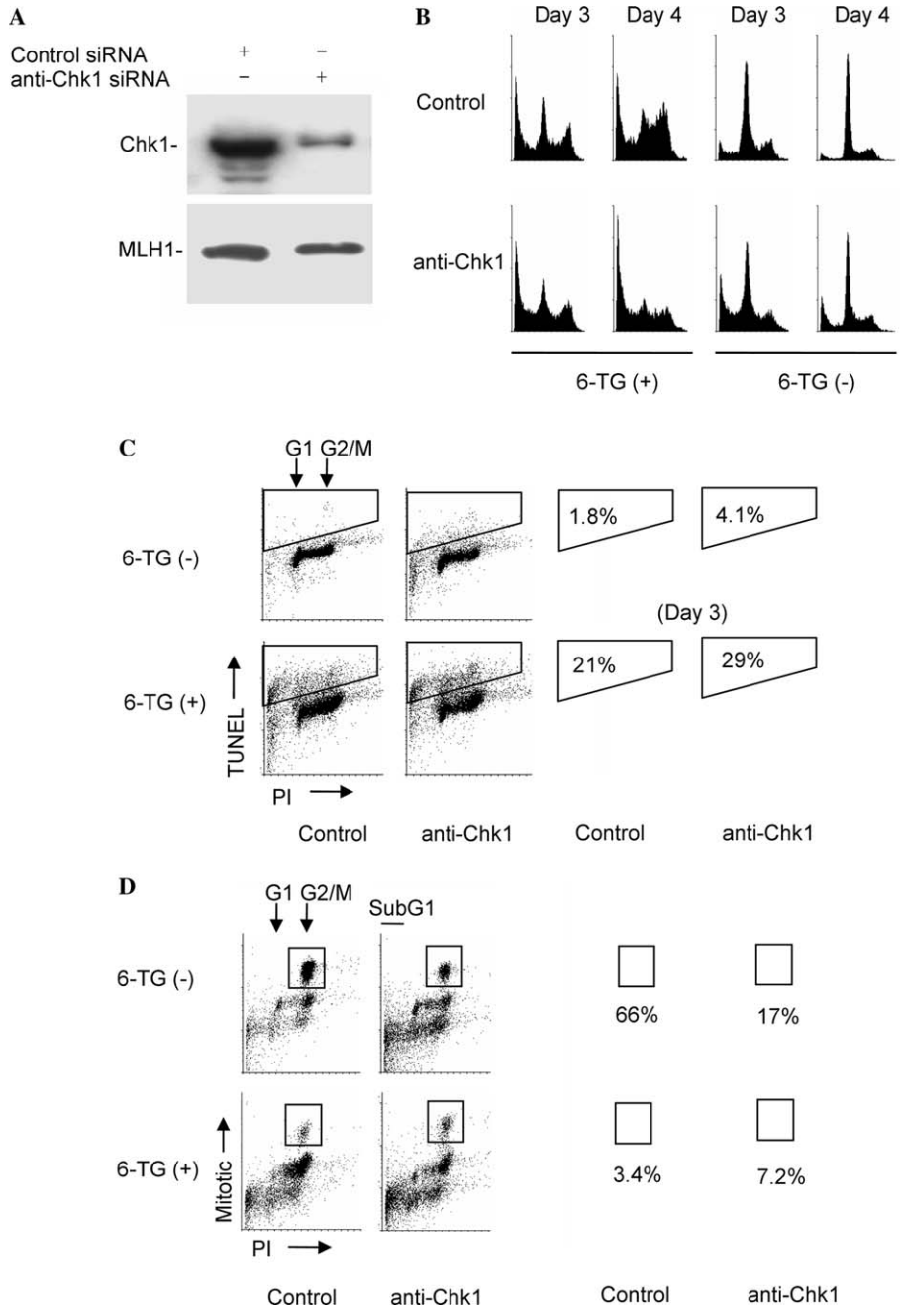

Fig. 4. Chk1 is required for G2/M peak induction and for inhibition of apoptosis after 6-TG DNA mismatch damage. (A) The siRNA against Chk1 and the control siRNA were transfected twice (tandem transfection) as described into HeLa cells. After culturing for 3 days, proteins were detected by Western blotting using anti-Chk1 and MLH1 antibodies. (B) The transfectants were treated with 6-TG (0 or 3 μM) for the indicated days, fixed with ethanol, stained by propidium iodide, and then analyzed by flow cytometry. The observed reduction of the subG1 population on Day 4 in the absence of 6-TG may be due to complete degradation of DNA. (C) The transfectants were treated with 6-TG (0 or 3 μM) for 3 days and fixed with formaldehyde and with ethanol. Cells were then labeled by the TUNEL method using FITC-conjugated probes. The gated regions are TUNEL positive populations, which are also quantified as the percent TUNEL positive cells per total cell population in the accompanying panels. (D) The transfectants were treated with 6-TG (0 or 3 μM) for 3 days. After removal of 6-TG, nocodazole (0.3 mg/ml) was added to the medium and cells were cultured for 24 h. The cells were then fixed with formaldehyde, stained with MPM-2 antibody conjugated with FITC and with propidium iodide, and analyzed by flow cytometry. The gated regions are mitotic populations, which are also quantified as the percent mitotic cells per total cell population in the accompanying panels.

To better examine the role of ATM in a 6-TG-induced G2/M checkpoint response, we compared the 6-TG responses in $ATM^{-/-}$ (FT169A) and ATM^{+} (YZ5) human fibroblast cells. We found that both the $ATM^{-/-}$ and ATM^{+} cells showed enhanced S phase and G2/M populations without a major subG1 fraction after 6-TG treatment (Fig. 2C), demonstrating clear differences from the ATR-reduced cells (Fig. 2B). Consistently, Chk1 was phosphorylated in both $ATM^{-/-}$ and ATM^{+} cells in Day 4. These results suggest that ATR but not ATM plays a major role in cell cycle profile in response to 6-TG. We do not exclude a minor role for ATM in a 6-TG response.

The G2/M checkpoint response in the ATR knock-down HeLa cells compared to control transfected HeLa cells was measured by double staining with propidium iodide and mitotic protein monoclonal-2 (MPM-2) antibody (a mitotic marker) after nocodazole treatment (Fig. 3B). Nocodazole, which induces a M phase arrest, is frequently used for a G2/M checkpoint assay [21,22]. While 6-TG treatment strongly inhibited the entry into mitosis in control cells (40–0.8%, a 50-fold reduction), a smaller but significant population of anti-ATR siRNA treated cells entered into mitosis following 6-TG treatment (Fig. 3B, 30–13%, a 2.3-fold reduction), suggesting that the ATR pathway is involved in a MMR-dependent G2/M checkpoint activation by 6-TG DNA mismatches. The smaller reduction in the ATR-reduced cells may be explained by the incomplete inhibition (80–90%) of ATR. The results in Figs. 3A and B suggest that even when control cells can stop at G2 with normal checkpoint function, cells are damaged by 6-TG, leading to generation of TUNEL positive cells.

Reduction of Chk1 in HeLa cells results in G2/M checkpoint abrogation and apoptosis following 6-TG treatment

Since Chk1 kinase is reported to be a critical downstream target of ATR, we were also interested in the response of Chk1 knock-down cells to 6-TG treatment. However, two sequential transfections (termed a tandem transfection) of anti-Chk1 siRNA were required to efficiently reduce Chk1 protein levels in HeLa cells for a relatively long time (4 days) for the 6-TG treatment (Fig. 4A and data not shown). Following 6-TG treatment for 4 days, the Chk1-reduced cells showed a significantly lower G2/M population compared to controls (Fig. 4B). 6-TG treatment also resulted in a marked apoptotic (subG1) population in the Chk1-reduced cells (Figs. 4B and C). However, the control transfected cells also had an increased subG1 population following 6-TG treatment, presumably due to cytotoxicity of the tandem transfection. This is the major difference between Figs. 2B and 4B. To specifically examine the G2/M checkpoint response, these cells were treated for 3 days with 6-TG and 1 day with nocodazole, and then stained with MPM-2 antibody and propidium iodide. As shown in Fig. 4D, only a 2.4-fold reduction (17–7.2%) in mitotic cells was found in Chk1-reduced cells following 6-TG treatment compared to a 19-fold reduction (66–3.4%) in the controls cells. Collectively, these data show a marked effect on the G2/M checkpoint in Chk1-reduced cells following 6-TG-induced DNA mismatch damage.

Discussion

In this study, we demonstrate that the ATR–Chk1 pathway participates in a G2/M cell cycle checkpoint activation by 6-TG-induced DNA mismatches. We show that a reduction of ATR kinase by siRNA strongly inhibits the G2/M checkpoint response and results in an induction of apoptosis after 6-TG treatment (Fig. 2). Our data also show that 6-TG treatment results in a critical phosphorylation of Chk1 (Fig. 1). Consistently, a reduction of Chk1 kinase results in failure of the formation of a major G2/M population after 6-TG treatment (Fig. 4). While we also found that Chk2 was also phosphorylated during 6-TG treatment (Fig. 1), the cell cycle responses to 6-TG were similar in $ATM^{-/-}$ and ATM^{+} human fibroblast cells (Fig. 2C).

Based on the results of this study and our prior study [15], we speculate that the interaction of MMR processing of 6-TG-induced DNA mismatches and the activation of ATR–Chk1 pathway is via a futile repair cycle model of MMR [26]. The possible sequential events are as follows. 6-TG is incorporated into a daughter DNA strand in the first cell cycle and subsequently methylated (6-me-TG) [27,28]. During the next S-phase, 6-me-TG on the parental strand is paired with a natural base T or C to form 6-me-TG mismatches. MMR then recognizes the mismatch and initiates repair of the daughter strand causing a DNA single-strand break. Since the 6-me-TG on the parental strand is not removed, MMR repeatedly processes this damage at the same site, leading to futile DNA single-strand break formation and repair in the daughter strand. We propose that the ATR–Chk1 pathway is then activated by this repetitive formation of DNA single-strand breaks, leading to a G2/M arrest. Alternatively, there may be a direct interaction of MMR proteins and ATR to signal a cell cycle arrest. Recently, it was reported that MSH2 can interact with ATR [29].

The double staining of ATR-reduced cells with TUNEL and propidium iodide showed that DNA mismatch-induced apoptosis is not concentrated in the G2/M fraction but is distributed widely from subG1 to G2 fractions (Fig. 3). Similar results were obtained in Chk1-reduced cells (Fig. 4). Our results comparing the cell cycle responses to 6-TG in ATM^{-} versus ATM^{+} fibroblasts also suggest that the ATM–Chk2 pathway does

not clearly affect on the G2/M checkpoint response after 6-TG-induced DNA mismatch damage (Fig. 2).

In conclusion, we show that MMR processing of 6-TG damage activates the ATR–Chk1 pathway to signal a G2/M cell cycle checkpoint. We previously reported that the 6-TG-mediated G2/M arrest in mismatch repair-proficient cells was dependent on MLH1 [4,13–15]. Further studies are underway to identify the downstream pathways of Chk1. A better understanding of how MMR processes chemotherapy-induced DNA damage may also provide insight into newer treatment approaches in MMR-deficient human cancers, including combinations of radiation therapy and halogenated thymidine analogs [30].

Acknowledgments

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