The Mre11/Rad50/Nbs1 complex interacts with the mismatch repair system and contributes to temozolomide-induced G_2 arrest and cytotoxicity

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Abstract

The chemotherapeutic agent temozolomide produces O^6-methylguanine (O6MG) in DNA, which triggers futile DNA mismatch repair, DNA double-strand breaks (DSB), G_2 arrest, and ultimately cell death. Because the protein complex consisting of Mre11/Rad50/Nbs1 (MRN complex) plays a key role in DNA damage detection and signaling, we asked if this complex also played a role in the cellular response to temozolomide. Temozolomide exposure triggered the assembly of MRN complex into chromatin-associated nuclear foci. MRN foci formed significantly earlier than γ-H2AX and 53BP1 foci that assembled in response to temozolomide-induced DNA DSBs. MRN foci formation was suppressed in cells that incurred lower levels of temozolomide-induced O6MG lesions and/or had decreased mismatch repair capabilities, suggesting that the MRN foci formed not in response to temozolomide-induced DSB but rather in response to mismatch repair processing of mispaired temozolomide-induced O6MG lesions. Consistent with this idea, the MRN foci colocalized with those of proliferating cell nuclear antigen (a component of the mismatch repair complex), and the MRN complex component Nbs1 coimmunoprecipitated with the mismatch repair protein Mlh1 specifically in response to temozolomide treatment. Furthermore, small inhibitory RNA–mediated suppression of Mre11 levels decreased temozolomide-induced G_2 arrest and cytotoxicity in a manner comparable to that achieved by suppression of mismatch repair. These data show that temozolomide-induced O6MG lesions, acted upon by the mismatch repair system, drive formation of the MRN complex foci and the interaction of this complex with the mismatch repair machinery. The MRN complex in turn contributes to the control of temozolomide-induced G_2 arrest and cytotoxicity, and as such is an additional determining factor in glioma sensitivity to DNA methylating chemotherapeutic drugs such as temozolomide. [Mol Cancer Ther 2006;5(11):2757–66]

Introduction

Temozolomide is a commonly used agent for the treatment of a variety of malignancies including brain tumors, melanoma, and central nervous system lymphoma (1–3). The compound is a monofunctional methylating agent that methylates both protein and DNA (4). The DNA adducts, which include 3-methyladenine, N^7-methylguanine, and O^6-methylguanine (O6MG), are considered to be important in the cellular actions of the drug because all can result in the formation of DNA single-strand breaks (4). O6MG is believed to be the most therapeutically relevant lesion, however, because only O6MG mispairs with thymine during DNA replication, generating mismatches in the DNA. These DNA mismatches in turn are recognized by the DNA mismatch repair system that removes the misincorporated base opposite the O6MG lesion. Repair resynthesis of DNA then leads to reinsertion of thymine opposite O6MG and reinitiation of multiple rounds of futile mismatch repair. The DNA double-strand breaks (DSB) resulting from this process are thought to initiate a series of events including prolonged G_2 arrest, mitotic catastrophe, senescence, and ultimately cell death (5–7).

There are a variety of factors that influence the cellular response to temozolomide. One of the most critical of these is O^6-methylguanine DNA methyltransferase (MGMT), the DNA repair protein that stoichiometrically and selectively removes methyl lesions from the O^6 position of guanine and returns the DNA to its prelesioned state (8). Cells deficient in MGMT or depleted of MGMT activity by exposure to selective agents such as O^6-benzylguanine exhibit increased sensitivity to temozolomide (9–11). A second key regulator of temozolomide sensitivity is the mismatch repair system itself. Cells lacking mismatch repair do not recognize the temozolomide-induced DNA mismatches, and whereas these cells suffer from an accumulation of mutations, they do not undergo temozolomide-induced G_2 arrest and are resistant to temozolomide-induced cell death (12, 13). Whereas events controlling the ability of temozolomide to generate DNA damage can greatly influence temozolomide action and

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do contribute to temozolomide resistance *in vivo* (14, 15), we have also shown that alterations in pathways downstream of the initial DNA damage can alter temozolomide sensitivity (16). As an example, MGMT-deficient, mismatch repair–proficient glioblastoma multiforme cells can be made resistant to temozolomide by activation of Akt and suppression of temozolomide-induced G_2 arrest and mitotic catastrophe. These results suggest that although MGMT levels and the mismatch repair system play key roles in temozolomide resistance, other factors that sense temozolomide-induced DNA damage and link it to downstream cell death pathways can also play contributory roles.

A variety of systems exist to sense the types of DNA damage induced by temozolomide in mismatch repair–proficient cells. Temozolomide-induced DNA damage activates the DNA damage sensor ataxia telangiectasia mutated (ATM), which in turn activates both Chk1 and Chk2 (16). Because both Chk1 and Chk2 also interact with the mismatch repair protein Msh2, this pathway has been suggested as a means by which methylation-induced DNA damage is linked to mismatch repair and activation of the G_2 checkpoint (17, 18). In other systems, however, ATM and Rad3-related, c-jun NH_2-terminal kinase/stress-activated protein kinase, and p38α have also been linked to the activation of Chk1, Chk2, p53, and the G_2 checkpoint (19–23). Whereas multiple pathways may link DNA damage to downstream effectors, the earliest sensors of temozolomide-induced damage and their linkage to mismatch repair and the G_2 checkpoint have not been identified.

Accumulating data suggest that the protein complex containing Mre11, Rad50, and Nbs1 [also known as the Mre11/Rad50/Nbs1 (MRN) complex] plays an important and early role in the activation of the DNA damage response. The MRN complex is thought to serve as a flexible link between ends of broken DNA, and on binding to damaged DNA, the MRN complex undergoes a series of conformational changes that are transmitted through the FXF/Y-HEAT interface to activate ATM, increase ATM affinity for its substrates (24, 25), and retain active ATM at sites of DNA damage (26). Binding of MRN complex to DSB sites is ATM independent (27) and ATM association with these sites is dependent on functional MRN complex (28), suggesting that the MRN complex is upstream of ATM with regard to break detection. Because MRN-mediated activation of ATM is propagated to the ATM targets Chk1 and Chk2, the MRN complex may not only serve as an early DSB sensor but also as an activator of kinases critical for initiation of cell cycle arrest in response to DNA strand breaks. The central role of the MRN complex as a coordinator of DNA damage sensing and cell cycle arrest suggests that it may play a role in coordinating the cellular response to methylating agents such as temozolomide. To address these possibilities, we examined the kinetics of MRN complex foci formation in control and temozolomide-treated glioma cells, assessed the relationship of MRN foci formation to temozolomide-induced lesions, and assessed the consequences of MRN complex deficiency with respect to temozolomide action. The results of our studies show that the MRN protein complex is activated following temozolomide exposure, not by temozolomide-induced DNA DSB but rather at an earlier stage by mismatch repair processing of O6MG lesions. Suppression of MRN complex function decreases temozolomide-induced G_2 arrest and cytotoxicity, suggesting that the MRN complex may represent an important determinant in the sensitivity of gliomas to DNA methylating chemotherapeutic drugs such as temozolomide.

Materials and Methods

Cell Culture and Drug Treatment

The human glioblastoma cell lines U87, U251, SF188, and T98G were cultured in DMEM H-21 medium supplemented with 10% FCS. U87 cells stably expressing a retroviral construct encoding small inhibitory RNA (siRNA) targeting human Mlh1 were previously described (29). Temozolomide was supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutic Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, and was dissolved in DMSO. O^6-Benzylguanine was from Sigma (St. Louis, MO) and was dissolved in ethanol. Cells were exposed to temozolomide (0 or 100 μmol/L) for 3 hours and then incubated in drug-free medium throughout the course of the experiment. To inhibit MGMT in SF188 and T98G cells, 20 μmol/L O^6-benzylguanine was added 2 hours before temozolomide, followed by incubation with 100 μmol/L temozolomide for 3 hours in the presence of 10 μmol/L O^6-benzylguanine. The cells were then washed and incubated in medium containing 5 μmol/L O^6-benzylguanine for 0 to 3 days with O^6-benzylguanine being replenished every day. For all studies, the final concentration of DMSO or ethanol in the medium did not exceed 0.1% (v/v).

Antibodies

Rabbit antisera against Mre11 and Nbs1 were purchased from Novus Biologicals (Littleton, CO). Rabbit polyclonal anti-γ-H2AX and monoclonal anti-hMlh1 (Ab-1) were purchased from Calbiochem (San Diego, CA). The anti-Mlh1 (554073) used for immunoprecipitations was from BD PharMingen (San Diego, CA) whereas anti–proliferating cell nuclear antigen (PCNA; PC10) was from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-β-actin (AC15) was from Sigma. Mouse anti-53BP1 monoclonal antibodies were provided by Junjie Chen (Mayo Clinic, Rochester, MN; ref. 30).

Single-Cell Gel Electrophoresis (Comet Assay)

DNA damage in cells was measured using a comet assay kit (Trevigen, Gaithersburg, MD) as previously described (31). Briefly, cells were treated with temozolomide for 3 hours (100 μmol/L) and then medium was changed to normal growing medium. After incubation, cells were trypsinized, washed with PBS, resuspended in a 0.5% agarose, and mounted onto Comet Slides. After a 30-minute incubation at 4°C, the slides were sequentially incubated

with lysis solution and alkali buffer and subsequently subjected to electrophoresis in alkali buffer (alkali comet assay) or Tris-borate EDTA buffer (neutral comet assay) at 1 V/cm. After electrophoresis, the slides were dried, stained with SYBR Green I dye, and viewed under a fluorescent microscope. The presence of a tail (comet) reflects DNA damage in the cell (comet cell). For each condition, 100 cells were examined in triplicate random microscope fields and the percentage of comet cells was calculated.

Immunofluorescence

For detection of temozolomide-induced nuclear foci formation, cells were plated onto 18-mm coverslips for 24 hours. Then temozolomide was added (100 μmol/L, 3 hours) and, at time points following temozolomide removal, the cells were detergent extracted, fixed, and processed for immunofluorescence as previously described (27). Cells were incubated for 1 hour at room temperature with primary antibody followed by 30-minute incubation with secondary fluorescent antibody (antimouse Texas red and antirabbit Alexa-488; Molecular Probes, Eugene, OR). 4′,6-Diamidino-2-phenylindole counterstain was used at a final concentration of 0.05 μg/mL in the last wash. ProLong antifade reagent (Molecular Probes) was used for mounting. Controls with preimmune serum or secondary antibody alone gave no signal. Cells were viewed under a Zeiss Axioplan fluorescent microscope using AxioVision software. At least 200 nuclei were scored for each treatment and time point to calculate the percentage of cells displaying foci. The results were derived from at least three independent experiments.

Cell Cycle Analysis

At various time points following temozolomide exposure, cells were trypsinized and fixed in ice-cold 70% ethanol for at least 1 hour at −20°C, washed, and stained with the following solution: propidium iodide 30 μg/mL + RNase 0.6 mg/mL in PBS + 0.5% (v/v) Tween 20 + 0.1% (w/v) bovine serum albumin. Stained cells were analyzed on a Becton Dickinson FACScan (San Jose, CA) with 20,000 events per determination. ModFit LT software (Verity Software House, Inc., Topsham, ME) was used to assess cell cycle distribution.

Protein Lysates, Cellular Biochemical Fractionation, and Immunoblot Analyses

Cellular biochemical fractionation was done as previously described (32). Cells were lysed in ice-cold CSK buffer [10 mmol/L PIPES (pH 6.8), 100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.5% Triton X-100 supplemented with 1 mmol/L ATP, Halt EDTA-free protease inhibitor cocktail (Pierce, Rockford, IL), and phosphatase inhibitor cocktail (Set II, Calbiochem)] for 15 minutes at 2.5×10^7 cells/mL. The supernatant from low-speed centrifugation (5 minutes at 1,500 × *g*, 4°C) constituted the soluble fraction that was subsequently clarified by high-speed centrifugation (10 minutes at 16,000 × *g*, 4°C). The low-speed pellet, consisting of intact nuclei (verified microscopically), was washed once and incubated in CSK buffer containing 50 units of RNase-free DNase I (Roche, Nutley, NJ) at 1×10^8 nuclei/mL for 30 minutes at 37°C, centrifuged for 10 minutes at 16,000 × *g*, 4°C, and the supernatant was retained. The pellet was incubated in ice-cold CSK buffer containing 500 mmol/L NaCl for 10 minutes at 4°C. This extract was clarified by centrifugation (10 minutes at 16,000 × *g*, 4°C) and pooled with the supernatant from DNase-treated fraction, constituting the chromatin-associated fraction. Protein extracts were quantitated by a Bio-Rad DC protein concentration assay, resolved (25 μg for soluble fractions, 5 μg for insoluble fractions) by 8% SDS-PAGE, and immunoblotted onto Immobilon-P membrane (Millipore, Bedford, MA). Equal loading and transfer was monitored by Ponceau red staining of the membrane. The membranes were subsequently immunoblotted with primary antibodies followed by horseradish peroxidase–conjugated secondary antimouse or antirabbit antibodies (Pierce) and visualized by enhanced chemiluminescence Western blotting detection reagent (SuperSignal, Pierce). For quantification, the intensities of bands corresponding to each protein were determined using the AlphaImager v.5.5 software and normalized to the intensity of actin in the same lane.

Immunoprecipitation

U87 or U251 cells were plated in 100-mm tissue culture dishes the day before temozolomide addition. One to 4 days post temozolomide removal, cells (2×10^7) were fractionated into the soluble and chromatin-associated fractions as described above. Each fraction was divided into two equal aliquots, diluted to 0.4-mL volume, and incubated with anti-Mlh1 monoclonal antibodies (BD PharMingen) or Nbs1 antiserum (Novus Biological) for 30 minutes on ice. The protein-antibody complexes were captured by protein A/G-agarose beads (1-hour incubation at 4°C with rotation). The beads were washed thrice in the lysis (CSK) buffer, after which the immunoprecipitated proteins were eluted in 2× Laemmli buffer by heating (95°C for 5 minutes). The presence of MRN complex or Mlh1 protein in the immunoprecipitates was examined by Western blotting. For specificity controls, the immunoprecipitations with nonimmune rabbit serum or with isotype-matching mouse immunoglobulin G were carried out. Three independent experiments were done.

siRNA Studies

U87 cells were plated at 7×10^4/mL in 12-well plates the day before siRNA transfection in DMEM cell growth media without antibiotics. siRNAs targeting human Mre11 (SMARTpool) or nontargeting siRNAs as a negative control were purchased from Dharmacon. Each siRNA was used at 100 nmol/L concentration and transfected into cells using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). siRNA (2.5 μL each; 20 μmol/L stock) was mixed with 50 μL of OptiMEM serum-free medium and 1.75 μL of Plus reagent and incubated for 15 minutes at room temperature. Lipofectamine (2.5 μL) was mixed with 50 μL of OptiMEM serum-free medium, incubated for 15 minutes at room temperature, and combined with siRNA. Cell growth medium was replaced by serum-free DMEM (0.4 mL/well) and siRNA-Lipofectamine mixture was overlaid onto the cells. Three hours later, the transfection medium was

removed and replaced by cell growth medium containing 10% FCS. Effects of siRNA on protein expression were assessed by Western blotting.

Colony Formation Efficiency

The cells treated with temozolomide (0–50 μmol/L, 3 hours) were trypsinized, counted, and plated at a concentration of 2,000 per 100-mm culture dish. These cultures were then incubated for 14 days to allow colonies to form. Cells were visualized by staining with Coomassie blue and colonies of >50 cells were scored.

Results

Relocalization of the DNA Damage–Sensing MRN Complex into Nuclear Foci Is an Early Response to Temozolomide Exposure in Glioblastoma Multiforme

O6MG lesions form within minutes of temozolomide exposure, whereas in glioma cells, the formation of DNA single- and double-strand breaks is near maximal 24 and 72 hours post temozolomide exposure, respectively (7, 31, 33, 34). Consistent with these previous results, DNA single-strand breaks (detected by alkali Comet assay) in temozolomide-treated U87 cells were maximal by 18 hours post temozolomide exposure, whereas the extent of DNA DSBs (detected by neutral comet assay) in temozolomide-treated U87 cells was no different from that in untreated cells at 24 hours post temozolomide exposure and was only significantly increased 48 to 72 hours post temozolomide exposure (Fig. 1). Both temozolomide-induced DNA single- and double-strand breaks were significantly reduced by expression of an siRNA targeting the mismatch repair protein Mlh1, consistent with the role of the mismatch repair system in the generation of temozolomide-induced DNA damage. To address if and when the MRN protein complex is activated in response to temozolomide, we examined the subcellular dynamics of the MRN complex component Mre11, as well as that of γ-H2AX and 53BP1, proteins known to associate with sites of DSB (27, 35–37). 53BP1 and γ-H2AX formed foci similar in appearance to those induced by ionizing radiation (data not shown), and although the percentage of cells with 53BP1 and γ-H2AX foci was not significantly different from controls at 24 hours after temozolomide exposure, it gradually increased over 2 to 3 days (Fig. 2A and B), consistent with the time frame for formation of temozolomide-induced DNA DSB (Fig. 1; ref. 30). Mre11 also formed foci in response to temozolomide, but the percentage of cells with Mre11 foci was significantly different from that in controls as early as 6 hours post temozolomide exposure, with maximal Mre11 foci formation (70% of cells displaying foci) noted by 24 hours post temozolomide (Fig. 2C). Because the kinetics of Mre11 foci formation differed from the kinetics of 53BP1/γ-H2AX foci formation and from the kinetics of temozolomide-induced DNA DSB, these results suggest that activation of the MRN complex does not seem to be associated with temozolomide-induced DSBs but rather with some earlier event mediated by temozolomide exposure.

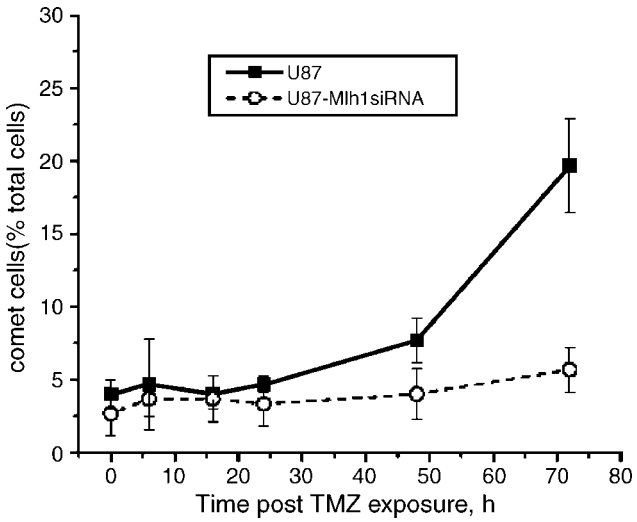

Figure 1. Time course of temozolomide-induced DNA damage in U87 cells and in mismatch repair–deficient U87 cells (stably expressing Mlh1 siRNA). Cells were treated with temozolomide (*TMZ*; 100 μmol/L, 3 h) and harvested at indicated time points after drug removal. DNA damage in the cells (comet cells) was detected by immunofluorescence as described in Materials and Methods. One hundred cells were scored for each data point to calculate the percentage of cells displaying comet tail. *Points,* mean of triplicates; *bars,* SD. Representative of three independent experiments.

MRN Complex Foci Formation Is O6MG Dependent

As a monofunctional methylating agent, temozolomide induces a wide variety of DNA adducts including 3-methyladenine, N^7-methylguanine, and O6MG. Whereas all the DNA adducts can indirectly lead to DNA single-strand breaks, only O6MG mispairs during replication and leads to DNA mismatches and DNA mismatch repair–dependent DNA damage. To determine if O6MG might be the source of MRN complex activation, T98G and SF188 glioblastoma multiforme cells proficient in MGMT (the protein that selectively removes O6MG but not other DNA adducts) were incubated with vehicle or the selective MGMT inhibitor O^6-benzylguanine, after which the cells were exposed to temozolomide and MRN foci formation was examined. As shown in Fig. 3A and B, MGMT-expressing T98 cells (*−BG* group) did not exhibit the type of temozolomide-induced Mre11 foci formation noted in the MGMT-deficient U87 cells (Fig. 2C), nor did these cells undergo the G_2 arrest typically noted in MGMT-deficient cells exposed to temozolomide (not shown). Depletion of MGMT in these cells by O^6-benzylguanine, however, not only allowed temozolomide-induced G_2 arrest (not shown) but also allowed rapid formation of temozolomide-induced Mre11 foci in a manner temporally and quantitatively similar to that noted in U87 cells. Similar results were also noted in a second MGMT-proficient glioblastoma multiforme cell line (SF188; data not shown). These results suggest that temozolomide-induced Mre11 foci formation is not a response to cell stress or generalized methylation damage but rather is a specific consequence of O6MG lesions in DNA.

Whereas O6MG lesions were clearly a stimulus for Mre11 foci formation, temozolomide-induced O6MG adducts

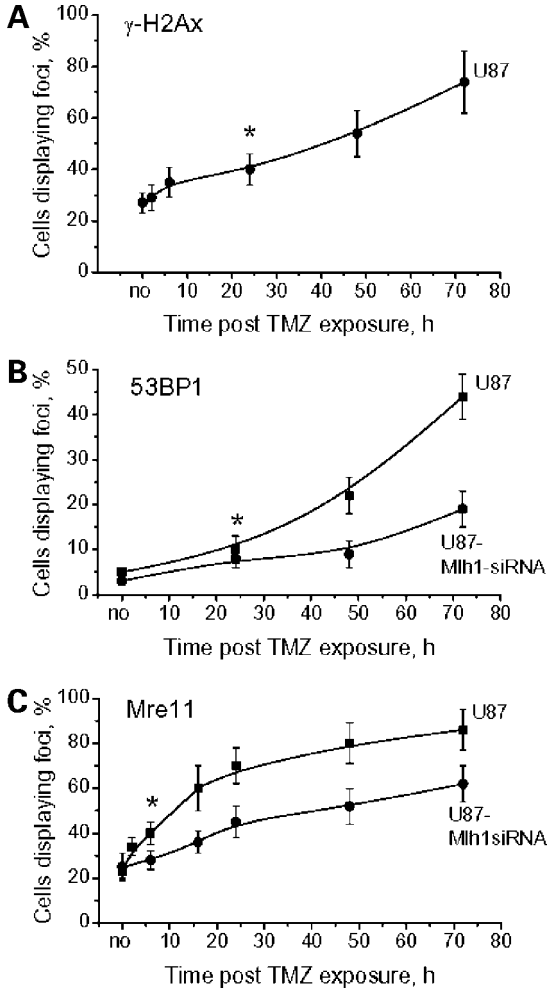

Figure 2. Time course of temozolomide-induced nuclear foci formation by Mre11 (**A**), 53BP1 (**B**), and γ-H2AX (**C**) in U87 cells and in mismatch repair–deficient U87 cells (stably expressing Mlh1 siRNA). Cells plated on coverslips were treated with temozolomide (0 or 100 μmol/L, 3 h) and fixed at indicated time points after drug removal. Nuclear foci were detected by immunofluorescence as described in Materials and Methods. Two hundred nuclei were scored for each data point to calculate the percentage of cells displaying foci. *Points,* mean of triplicates; *bars,* SD. Representative of three independent experiments. *, first time point when number of cells displaying foci was significantly different from control (untreated cells); $P < 0.05$, Student's *t* test.

form much more rapidly than temozolomide-induced MRN foci (minutes versus hours; ref. 31), suggesting that MRN foci form not in response to O6MG lesions themselves but rather in response to cellular processing of O6MG lesions, perhaps by the mismatch repair system. To address this possibility, we examined temozolomide-induced foci formation in paired MGMT-deficient U87 glioblastoma multiforme cells, which, by virtue of stable expression of an siRNA targeting the mismatch repair protein Mlh1, differed only in their ability to perform mismatch repair. U87-Mlh1 siRNA cells have ~20% residual expression of Mlh1 protein, as described in ref. 29. As shown in Figs. 1 and 2B, suppression of Mlh1 expression significantly decreased the percentage of cells exhibiting temozolomide-induced 53BP1 foci, consistent with the idea that DNA DSBs are generated by mismatch repair–dependent actions on mispaired O6MG. Suppression of Mlh1 expression, however, also significantly decreased the percentage of cells exhibiting temozolomide-induced Mre11 foci, and this effect was noted before the appearance of DNA DSBs and 53BP1 foci. Because the paired mismatch repair–proficient and mismatch repair–deficient U87 cells differ only in their ability to process O6MG mispairs, these results suggest that Mre11 foci formation is driven not by O6MG lesions themselves but by activation of the mismatch repair system in response to O6MG mismatches. Mre11 foci formation is therefore an early and mismatch repair–dependent response to temozolomide.

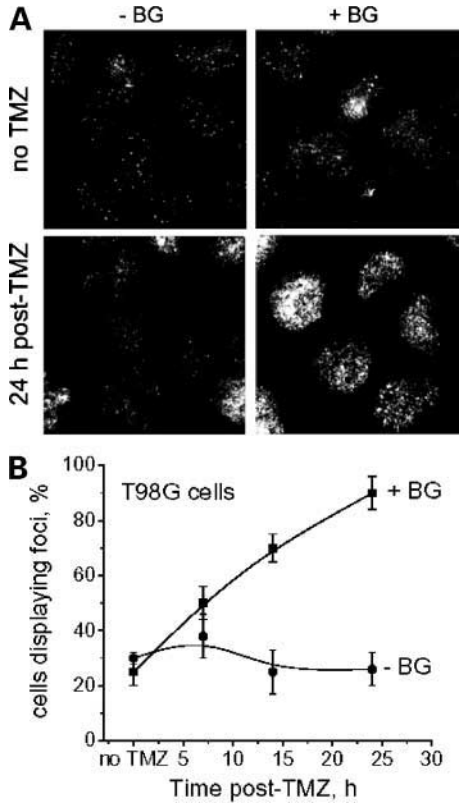

Figure 3. Temozolomide-induced Mre11 foci formation in MGMT-positive T98G cells in the presence or absence of MGMT inhibitor O^6-benzylguanine. Cells grown on coverslips were pretreated with O^6-benzylguanine (*BG*) 2 h before temozolomide addition and O^6-benzylguanine was present in the media throughout the course of the experiment. Cells were fixed at indicated time points after temozolomide removal and processed for Mre11 immunofluorescent detection as described in Materials and Methods and in legend to Fig. 1. **A,** photomicrographs of T98G cells showing temozolomide-induced DNA damage Mre11 foci formation in cells pretreated with MGMT inhibitor O^6-benzylguanine and the absence of such foci in MGMT-proficient cells (in the absence of O^6-benzylguanine). **B,** time course of Mre11 foci formation in T98G cells in the presence or absence of O^6-benzylguanine. Two hundred nuclei were scored for each data point to calculate the percentage of cells displaying foci. *Points,* mean of triplicate cultures; *bars,* SD.

In Response to Temozolomide, MRN Complex Is Deposited at Chromatin, Colocalizes with PCNA, and Interacts with Mlh1

Mre11 and PCNA have been shown to colocalize at replication forks in the insoluble chromatin fraction of cells during S-phase (38). PCNA, however, also interacts with the mismatch repair complex to provide a physical link between mismatch recognition and identification of the newly synthesized DNA strand at the replication fork (39). Because of the suggested connection between mismatch repair and Mre11 foci formation, we therefore addressed the association of the MRN complex with chromatin, PCNA, and the mismatch repair protein Mlh1 following temozolomide exposure. For chromatin studies, temozolomide-treated and untreated U87 cells were separated into the soluble and insoluble (chromatin-associated) fractions at various time points following temozolomide treatment. Cell extracts were then resolved by SDS-PAGE and immunoblotted sequentially with antibodies specific for Nbs1, Mlh1, α-tubulin, and β-actin (as a loading control). As shown in Fig. 4A, β-actin was found in both the soluble and chromatin fractions whereas α-tubulin was found only in the soluble fraction of control and temozolomide-treated cells, confirming that the conditions used for extraction distinguished soluble from chromatin-associated proteins. Whereas Nbs1 and Mlh1 were primarily found in the soluble fraction of untreated cells, temozolomide exposure significantly increased the amount of chromatin-bound Nbs1 and Mlh1 (Fig. 4A), and by 24 hours post temozolomide exposure, levels of chromatin-bound Nbs1 and Mlh1 were reproducibly two to three times those noted in untreated cells. These results show that components of both the mismatch repair and MRN complexes accumulate on chromatin in a similar and early time frame following temozolomide exposure.

To address the association of the MRN complex with PCNA, we did immunofluorescent detection of detergent-insoluble PCNA and Mre11 in temozolomide-treated U87 glioma cells at a time point (24 hours post temozolomide exposure) at which Mre11 foci formation and association of Nbs1 and Mlh1 with chromatin were near maximal. Approximately 20% of control cells exhibited colocalized PCNA/Mre11 foci, consistent with the known colocalization of these proteins in the chromatin of replicating S-phase cells (ref. 38; not shown). One day after temozolomide exposure, however, ~70% of the temozolomide-treated U87 cells exhibited both Mre11 foci (green) and PCNA foci (red), and in most cases PCNA foci colocalized with temozolomide-induced Mre11 foci (yellow foci, Fig. 4B) in a manner similar to that noted in S phase in undamaged human fibroblasts (38). These results suggest that components of the mismatch repair and MRN complexes colocalize and have the potential to spatially interact in an early time frame following temozolomide exposure.

Finally, to directly address the interaction between components of the mismatch repair and MRN complexes, control or temozolomide-treated U87 or U251 glioma cells were fractionated into soluble and chromatin-associated fractions, and the presence of Nbs1, Mlh1, or Nbs1/Mlh1 complexes was assessed. Consistent with the data in Fig. 4A, Nbs1 and Mlh1 were present in the soluble fraction of whole-cell lysates from both temozolomide-treated

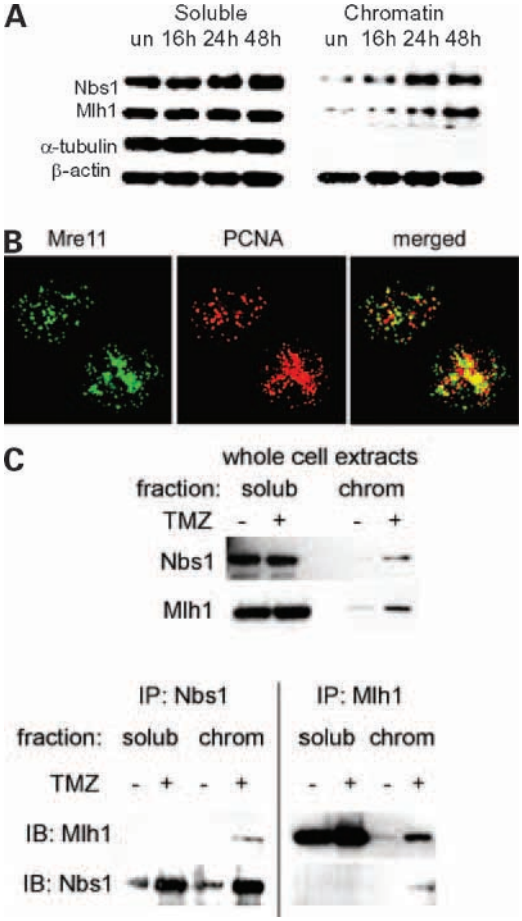

Figure 4. Temozolomide treatment results in enhanced accumulation of Nbs1 and Mlh1 on chromatin (**A**), colocalization of Mre11 with PCNA (**B**), and interaction of Nbs1 with Mlh1 (**C**). **A,** Nbs1 and Mlh1 accumulated on chromatin in temozolomide-treated cells. U87 cells treated with temozolomide were trypsinized, lysed, and fractionated in soluble and chromatin-associated fractions as described in Materials and Methods. Protein levels of Nbs1, Mlh1, α-tubulin (fractionation control), and β-actin (loading control) in soluble and chromatin fractions of U87 cells were assessed by Western blot and measured by densitometry relatively to actin expression. **B,** colocalization of Mre11 and PCNA in DNA damage foci 24 h post temozolomide exposure. Representative pictures of cells costained with Mre11 antiserum (*green*) and PCNA monoclonal antibody (*red*). Untreated cells displayed similar PCNA-Mre11 foci colocalization in S-phase cells (~25% of cells); in nonreplicating cells (75% of cells), PCNA staining was negative and Mre11 foci were absent (pictures not shown). **C,** Nbs1 associates with Mlh1 in chromatin fractions of temozolomide-treated cells. U87 cells were treated with temozolomide, harvested at time points after temozolomide exposure, lysed, and fractionated for soluble and chromatin fractions as described in Materials and Methods. *Top,* protein levels of Nbs1 and Mlh1 in the soluble and chromatin extracts prepared from the cells. *Bottom,* Nbs1 and Mlh1 proteins were immunoprecipitated from the same extracts, resolved by 8% SDS-PAGE, and immunoblotted sequentially with Nbs1 and Mlh1 antibodies. Representative immunoblots of day 4 post temozolomide exposure.

and untreated cells, but their association with chromatin was significantly increased in temozolomide-treated cells (Fig. 4C). Importantly, Mlh1 protein was significantly increased in the Nbs1-immunoprecipitates of the chromatin fraction of temozolomide-treated cells, compared with the same immunoprecipitates generated from untreated cells. Conversely, Nbs1 protein in Mlh1-immunoprecipitates of chromatin fractions was significantly higher in temozolomide-treated cells versus untreated cells (Fig. 4C). This increase in Mlh1-Nbs1 coimmunoprecipitation was detectable starting from 24 hours post temozolomide treatment in U87 cells and was most pronounced 2 to 4 days post temozolomide exposure in U87 and U251 cells. Taken together, these results suggest that in glioma cells, specific interaction of mismatch repair and MRN protein complexes takes place in response to processing of O6MG-mediated DNA mismatch lesions.

MRN Suppression Decreases Temozolomide-Induced G_2 Arrest and Enhances Cell Survival

Mismatch repair plays a key role in activation of the G_2 checkpoint and in temozolomide-induced cytotoxicity (29, 32). Because the mismatch repair complex also interacts with the MRN complex, we asked if the MRN complex might be linked to temozolomide-induced G_2 arrest and cell death. We therefore used an Mre11-targeted siRNA to suppress MRN complex function in U87 glioma cells, after which effects on temozolomide-induced G_2 arrest and cytotoxicity were measured. As shown in the Western blot in Fig. 5A, levels of Mre11 were significantly lower in cells that received the Mre11-targeted siRNA than in cells that received a nontargeted negative control siRNA, and this difference was apparent 3 days after transfection (0 days post temozolomide) as well as up to 6 days following temozolomide exposure (9 days after siRNA transfection). Temozolomide exposure of cells transfected with nontargeting siRNA resulted in accumulation of cells at the G_2-M boundary and an increase in the ratio of cells with 4N G_2-M DNA content versus 2N G_1-S DNA content (Fig. 5B and C) in a manner similar to that noted in temozolomide-treated cells that were not transfected or exposed to transfection agent alone (not shown). Temozolomide exposure of cells transfected with siRNA targeting Mre11, however, although activating the G_2 checkpoint at 2 days following temozolomide exposure, did not lead to sustained G_2 arrest, and these cells exhibited a significantly lower ratio of cells with 4N versus 2N DNA content than temozolomide-treated control cells at both 3 and 4 days post temozolomide exposure (Fig. 5B and C). To address whether the MRN complex suppression that resulted in inhibition of temozolomide-induced G_2 arrest would also affect glioma cell survival after temozolomide treatment, we measured the clonogenicity of the temozolomide-treated U87 cells transfected with a nontargeting siRNA and compared it to that of temozolomide-treated cells transfected with siRNA targeting Mre11. Cells with suppressed Mre11 levels, in addition to exhibiting less temozolomide-induced G_2 arrest, were ~2-fold more resistant to temozolomide compared with the control cells (Fig. 6).

Taken as a whole, these data show that temozolomide-induced O6MG lesions, acted upon by the mismatch repair system, drive formation of the MRN complex foci and the interaction of this complex with the mismatch repair machinery. The MRN complex in turn contributes to the control of temozolomide-induced G_2 arrest and cytotoxicity, and as such is an additional determining factor in glioma sensitivity to DNA methylating chemotherapeutic drugs such as temozolomide.

Discussion

The MRN complex is emerging as a central player in chromosome break metabolism (reviewed in ref. 40). It is involved in DSBs sensing, DNA recombinational repair, and cell cycle checkpoint signaling. Recent data show

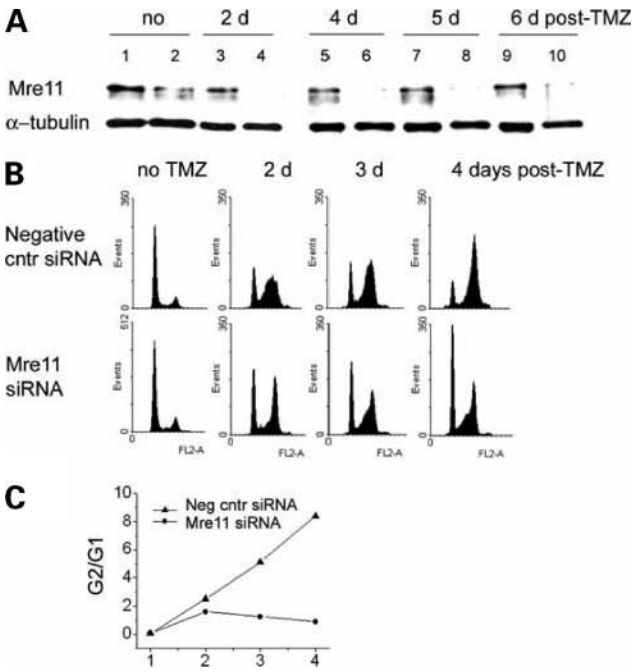

Figure 5. Suppression of Mre11 protein expression inhibits the ability of U87 cells to undergo temozolomide-induced G_2 arrest. siRNA targeting Mre11 (SMARTpool, Dharmacon) or negative control siRNA (siControl nontargeting siRNA pool, Dharmacon) were transfected into U87 cells using Lipofectamine reagent (Invitrogen). Three days after transfection, the cells were treated with temozolomide (100 μmol/L, 3 h) and fixed at indicated time points post temozolomide treatment. **A,** immunoblot showing suppression of Mre11 protein level after siRNA transfection. *Lanes 1, 3, 5, 7,* and *9,* cells transfected with negative control siRNA. *Lanes 2, 4, 6, 8,* and *10,* cells transfected with Mre11-targeting siRNA and harvested at indicated days post temozolomide exposure. Temozolomide was added 3 d after siRNA transfection. The level of Mre11 expression was suppressed in U87 cells by ~90% at 3 d post siRNA transfection as determined by densitometry of Western blot. **B,** cell cycle profiles of siRNA-treated U87 cells 2, 3, and 4 d post temozolomide treatment as determined by fluorescence-activated cell sorting analysis of 20,000 cells. The percentages of cells in G_1, G_2, and S phases were determined by DNA ModFit software. **C,** G_2/G_1 ratio versus time of post temozolomide exposure is plotted for U87 cells transfected with either Mre11 or negative control siRNA.

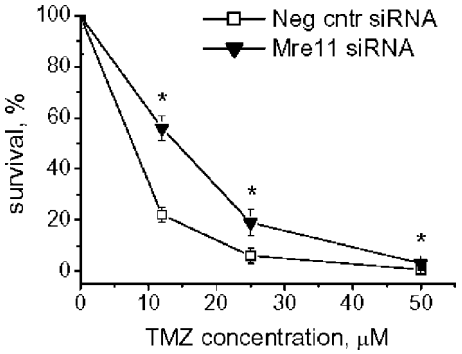

Figure 6. Mre11 deficiency results in increased survival of U87 cells after temozolomide exposure. U87 cells transfected with either Mre11 or negative control (nontargeting siRNA) were exposed to temozolomide (0, 12, 25, and 50 μmol/L, 3 h) 3 d after transfection. Cells were then plated at 2,000 per 10-cm culture dish in triplicate dishes and allowed to grow for 14 d. Colonies were stained with Coomassie blue and counted (containing >50 cells). Cell survival was calculated as a percentage of number of colonies grown in temozolomide-treated cultures versus number of colonies in untreated cultures. *Points,* mean of triplicates; *bars,* SD. *, $P < 0.05$, values differing statistically from controls (Student's *t* test). Each experiment was reproduced at least thrice.

that the MRN complex is involved in multiple interactions of proteins implicated in different aspects of DNA damage response as well as cell cycle regulation (27, 38, 41–43).

Our work reveals a novel function for the MRN complex involvement in the mismatch repair machinery activated by the DNA methylating agent temozolomide. The results of our studies show that temozolomide-induced O6MG lesions, acted upon by the mismatch repair system, stimulate the formation of the MRN complex foci. The MRN complex seems to form foci before the formation of DNA DSB, interacts with the Mlh1, and plays an important role in controlling both the temozolomide-induced G_2 checkpoint and temozolomide-induced cytotoxicity. The MRN complex therefore plays a key and early role in the response of glioma cells to temozolomide, and as such may represent an important determinant in the sensitivity of gliomas to DNA methylating chemotherapeutic drugs such as temozolomide.

The MRN complex has previously been shown to form foci at sites of DNA replication or DNA damage, the common link being DNA DSB and/or free DNA ends. The MRN complex binds and unwinds these ends and recruits and activates ATM and possibly other kinases involved in the intra-S-phase and G_2 checkpoints (25, 44, 45). Agents such as ionizing radiation generate immediate DNA DSB and, under these circumstances, the formation of MRN complex foci can occur within 10 to 20 minutes after the damage (27). Agents such temozolomide, however, induce DSB only after initial O6MG lesions mispair during replication and are acted upon by the mismatch repair system, a process that takes ~48 hours in most glioma cells (7). Whereas MRN foci would be expected to form in a gradual and delayed manner following temozolomide exposure, our studies show that Mre11 foci appear within 6 hours of temozolomide exposure and precede the formation both of DSB and of γ-H2AX and 53BP1 foci that are known to form at DSB sites (27, 35–37). Although it is possible that subdetectable amounts of early-forming DSB breaks could lead to early Mre11 foci formation, the results presented are most consistent with the idea that Mre11 foci are a response not to temozolomide-induced DNA DSB but rather to a very early temozolomide-induced lesion. The observation that Mre11 foci only formed in cells depleted of MGMT suggests that this lesion is O6MG and not any of the other possible temozolomide-induced DNA lesions. Because the formation of Mre11 foci is much slower than the formation of O6MG (which is very rapid in temozolomide-treated cells; ref. 46), the O6MG lesion per se must not be the stimulus for MRN foci formation. What lies between the initial O6MG lesion and frank DNA DSB is the DNA mismatch generated by replication past O6MG lesions and the futile processing of this lesion by the mismatch repair system. The present study suggests that these lesions, and not frank DNA DSB, are the driving force for formation of MRN complex foci following temozolomide exposure.

Consistent with the idea that the MRN foci form in response to mismatch repair–processed DNA lesions, Mre11 foci formation was mismatch repair dependent and the MRN complex colocalized with PCNA and interacted with Mlh1. Although physical association between Mre11 and Mlh1 was previously shown, the functional significance of their interaction was not addressed (41). Our work shows that the MRN-mismatch repair complex associations are significantly increased in the chromatin fractions following temozolomide, suggesting that specific interaction of mismatch repair-MRN complexes is induced after glioma exposure to temozolomide. We suggest that the MRN complex may serve as a scaffold to facilitate the interaction between other DNA damage response proteins, and as such MRN foci formation may be required for proper assembly of the mismatch repair complex at the sites of mismatch repair. Conversely, assembly of mismatch repair complexes may be required for the proper assembly of the MRN complex foci. Mre11 foci formation was shown to be suppressed in irradiated Msh2-deficient fibroblasts (17), and in our work, suppression of Mlh1 levels inhibited Mre11 foci formation. Although the exact linkage is yet to be defined, the present studies show that the MRN complex interacts with the mismatch repair system and is dependent on mismatch repair activity for its formation.

In addition to physically linking the mismatch repair and MRN complexes, our studies also provide a functional significance for the MRN complex in temozolomide action. In the present study, suppression of Mre11 had direct effects on temozolomide-induced G_2 arrest. The role of the MRN complex in the G_2-M checkpoint has been controversial, and whereas it has been shown that NBS cells have a defective G_2-M transition immediately after a low dose of ionizing radiation (47, 48), other reports have suggested that the early G_2-M checkpoint is independent of NBS1 (49, 50). Whereas Mre11 suppression did not seem to alter

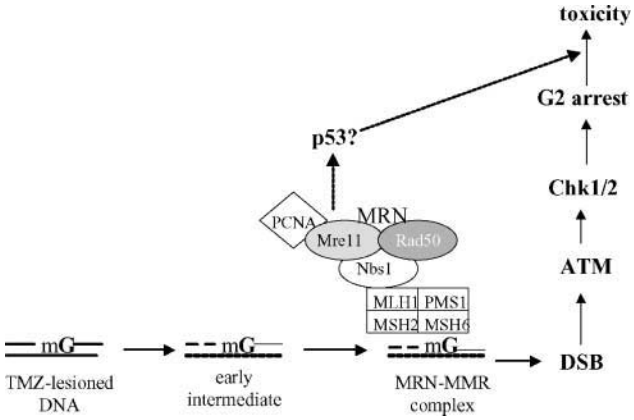

Figure 7. Schematic representation of the role the MRN complex plays in temozolomide action. An early lesion induced by temozolomide and involving O6MG (perhaps an early O6MG DNA mismatch in the DNA) facilitates the interaction and recruitment of both the DNA mismatch repair complex and the MRN complex (along with PCNA) to chromatin. The mismatch repair/MRN complex may serve as a signaling intermediate to p53 among other proteins, which in turn may modify the G_2 checkpoint that is activated by the ATM/Chk pathway in response to DSB created at later time points by the mismatch repair complex.

the ability of cells to undergo G_2 arrest in the present study, it significantly reduced the ability of the cells to maintain this arrest. Similar effects on the maintenance of the G_2 checkpoint have been noted in irradiated Msh2-deficient cells (17), suggesting that the effects of Mre11 suppression on the G_2 checkpoint may be a secondary consequence of effects on mismatch repair. Alternatively, because p53 controls the duration of G_2 arrest in response to temozolomide (7) and also may be indirectly activated by MRN (25), suppression of Mre11 may affect p53-mediated control of the duration of G_2 arrest. Suppression of Mre11 levels also made glioma cells ~2-fold more resistant to temozolomide-induced cytotoxicity. This finding is consistent with the idea that the MRN complex is a component of mismatch repair machinery because mismatch repair–deficient cells are insensitive to temozolomide (29). The results also agree with a recent study showing that Mre11 deficiency led to microsatellite instability and defective DNA mismatch repair (51). The present results, summarized in Fig. 7, suggest that the MRN complex, by influencing temozolomide-induced G_2 arrest and cytotoxicity, may ultimately influence the success of temozolomide-based therapies. The MRN complex may therefore be an additional determining factor in glioma sensitivity to DNA methylating chemotherapeutic drugs such as temozolomide.

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