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Using methyl methanesulfonate (MMS) to stimulate targeted gene repair activity in mammalian cells

Research Article

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Abbreviations: camptothecin, (CPT); double strand breaks, (ds breaks); homologous recombination, (HR); Methyl methanesulfonate, (MMS); non-homologous end joining, (NHEJ); pulsed-field gel electrophoresis, (PFGE); single strand breaks, (ss breaks); targeted-nucleotide exchange, (TNE)

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Summary

We describe a simple, efficient and inexpensive means of stimulating targeted gene repair in mammalian cell lines. Methyl methanesulfonate (MMS) is an alkylation agent capable of inducing DNA lesions and subsequent single strand breaks (ss breaks) and double strand breaks (ds breaks). This damage activates the non-homologous end joining (NHEJ) and homologous recombination (HR) DNA repair pathways, a response that can enhance the frequency of synthetic oligonucleotide-directed gene repair. We show in mammalian cells that the conversion frequency of a mutant base pair to wild-type in an integrated enhanced green fluorescent protein gene is increased by pretreatment with MMS. This stimulation is dose-dependent and correlates to the level of ds breaks induced by MMS treatment. These ds breaks result in Rad51p nuclear-relocalization and foci formation, a specific indication that HR pathway has been activated. In presence of MMS the cell cycle is slowed with an accumulation in S phase where coincidently, the highest level of gene repair takes place. Our data suggest that MMS-induced DNA damage elicits a cellular response that stimulates gene repair in mammalian cells and provides a direct method for elevating levels of gene correction.

I. Introduction

Targeted gene repair is a technique that can be used to correct single base mutations directly in the chromosomal copy of certain genes. Nucleotide alteration is directed by a short single-stranded oligonucleotide which also serves as a template to provide the information for the base exchange reaction. The mechanism of action is still being elucidated but the general scheme involves a DNA pairing step in which the oligonucleotide aligns in homologous register with the target site and a correction step in which the genetic information in the oligonucleotide is transferred to the target gene (Andersen et al, 2002; Liu et al, 2003; Igoucheva et al, 2004). The overall reaction appears to be catalyzed by enzymes involved in homologous recombination but may be influenced by proteins that regulate the cellular response to DNA damage (Igoucheva et al, 2002; Thorpe et al, 2002; Ferrara and Kmiec, 2004; Ferrara et al, 2004). Such damage in mammalian genomes can occur naturally by mistakes in DNA synthesis or from exogenous sources such as chemicals or irradiation. Anticancer drugs such as VP16 or camptothecin (CPT) or reagents such as hydroxyurea or thymidine that cause lesions at replication forks (Essers et al, 2000; Rice et al, 2001; Saintigny and Lopez, 2002; Olsen et al, 2003) are often used to induce DNA damage in experimental systems. These lesions result in a cessation of fork movement and stall cells in S phase. The damage can be repaired by a variety of mechanisms, and once the repair is completed the block is released and progression through the cell cycle resumes.

In a resent study, Hu *et al.* (Hu et al, 2005) found that the synchronization of cells at the G1/S border and subsequent release enables a higher level of gene repair. The correction levels peak when the majority of cells are in S phase, results which align with earlier data of Majumdar *et al.* (Majumdar et al, 2003). Along the same lines, Brachman and Kmiec (Brachman and Kmiec, 2004; Brachman and Kmiec, 2005) demonstrated that DNA

replication can influence the rate and frequency of gene repair. By slowing fork movement, these workers were able to elevate the frequency by activating the homologous recombination (HR) pathway and enabling the correction process to occur with greater efficiency. These data are consistent with earlier reports that had already pointed to HR as a regulatory process of gene repair (Thorpe et al, 2002; Olsen et al, 2003). Based on these observations and the need to establish an induction methodology, we sought to develop a simple protocol for raising the HR response level and stabilizing the frequency of repair in various cell types. To meet this goal we utilized the well known mutagen, methyl methanesulfonate (MMS), that can be added directly to cells in culture to induce DNA damage and perhaps prime correction events; thus, developing a simple method of elevating gene repair activity.

MMS is a commonly used carcinogen that modifies DNA through nucleotide alkylation (Kim and LeBreton, 1994). This damage occurs through strand cleavage with single and double strand breaks created at random in the genome. Broken DNA halts the initiation of replication by blocking the firing of origins and stalled replication forks transduce a signal to cellular checkpoints that prevent transition to M phase and cell division. The delay is primarily imposed so that DNA repair/recombination activities can reverse the damage in the genome so that mutations are not passed on to daughter cells. It is likely that MMS activates this damage response pathway by inducing the production of an inhibitor of DNA replication which prevents PCNA from loading onto the template strand (Stokes and Michael, 2003). Thus, MMS is a well-characterized DNA damaging agent that induces the transitory stalling of DNA replication forks. Its ease of use and its rapid uptake by cells make it potentially useful as a reagent for elevating correction in mammalian cells.

In this paper, we describe the use of MMS as a stimulant of gene repair activity in two unrelated mammalian cell types. We find that pretreating cells for 24 hours prior to the introduction of the oligonucleotide leads to an elevation in correction. This response is due, in all likelihood, to the activation of homologous recombination by DNA damage that manifests in the form of double strand breaks. By harnessing the HR response, we were able to utilize recombination activities to upregulate the repair of point mutations.

II. Materials and methods

A. Cell line and culture conditions

The DLD-1 cell line was acquired from ATCC (American Type Cell Culture, Manassas, VA). The DLD-1 clone 1, DLD-1(1), was created by the integration of a pEGFP-N3 vector (Clontech, Palo Alto, CA) containing a mutated eGFP gene, as described by Hu *et al.* (Hu et al, 2005). A nonsense mutation at position +67 results in nonfunctional eGFP protein; the oligonucleotide directs the conversion of the stop codon to a tyrosine (wild type eGFP), allowing the expression of functional eGFP. This cell line was grown in RPMI 1640 medium with 2 mM glutamine (GIBCO, Invitrogen, Carlsbad, CA), 25 mM glucose, 1mM sodium pyruvate, 10 mM HEPES, and 10% fetal bovine serum. Another clonal cell line, DLD-1(3), had the mutant eGFP gene target integrated into these cells and a clonal line containing a single copy was isolated by neo-selection. Both cell lines were maintained under selection in media containing 200 μg/ml Geneticin (GIBCO, Invitrogen, Carlsbad, CA).

B. eGFP gene targeting

Cells grown in complete medium supplemented with 10% FBS were trypsinized and harvested by centrifugation. 2 x 10^6 cells were resuspended in 100 μl serum-free medium and transferred to a 4 mm gap cuvette (Fisher Scientific, Pittsburgh, PA). The oligonucleotide was added to a final concentration of 4 μM and the cells were electroporated (250V, 13ms, 2 pulses, 1s interval) using a BTX Electro Square Porator™ ECM 830 (BTX Instrument Division, Holliston, MA). The electroporated cells were then transferred to a 60mm dish, recovered in complete medium supplemented with 10% FBS, and incubated at 37°C for 48 h prior to FACS analysis.

C. MMS treatment

Cells were seeded at a density of 1.5 x 10^6 in a 100 mm dish in complete medium supplemented with 10% FBS and desired concentration of methyl methanesulfonate (MMS, Sigma-Aldrich, St. Louis, MO) 24 h prior to electroporation of the oligonucleotide. An MMS solution of 75 mM in RPMI medium was made fresh for every experiment from an 11.8 M stock. All cells were washed twice in 1X PBS (GIBCO, Invitrogen, Carlsbad, CA) immediately prior to harvesting for electroporation.

D. Flow cytometry analysis

Forty-eight hours after electroporation, the cells were harvested and resuspended in FACS buffer (0.5% BSA, 2 mM EDTA, 2 μg/ml propidium iodide in PBS) and immediately processed. eGFP fluorescence was measured by a Becton Dickinson FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). More specifically, the program was set for the appropriate cell size (forward scatter versus side scatter) and the population of single-cells was gated for analysis. Using the negative control (minus PI, minus GFP) the background fluorescence was set by positioning the cells in the 10^1 decade of the dot plot by adjusting the voltage for FL1 (GFP) and FL2 (PI). The machine composition was then set for multi-fluorochrome experiments using a GFP control sample containing no PI and increasing the compensation to bring the signal toward the FL1 parameter. Finally, the last control, PI and no GFP was used to increase the compensation to bring the signal toward the FL2 parameter. Samples of 50,000 cells each were analyzed and those cells being GFP positive and PI negative were scored as corrected cells.

To analyze cell cycle, cells were seeded at a density of 1.5 x 10^6 in a 100 mm dish in complete medium supplemented with 10% FBS and ± 150 μM MMS. After 24 h cells were trypsinized, resuspended in 300 μl cold PBS and fixed by addition of 700 μl cold 95% ethanol. Cells were incubated a 4°C for 16 hours, subsequently washed and resuspended in 500 μl of PBS containing 50 μg/ml propidium iodide and analyzed by FACScalibur for DNA content.

E. MTT cytotoxicity assay

Sensitivity to MMS was analyzed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) viability assay. Cells were plated in a 24-well plate at a density of 1 x 10^5 cells per well using complete medium supplemented with 10% FBS and MMS at concentrations of 75, 150, 225, 300, and 375 μM respectively. After 24 h of treatment, cells were washed in PBS and 500 μl (5 mg/ml) MTT in RPMI was added to each well and incubated for 2 h at 37°C, in the dark. This assay is based on a reaction in

which MTT is reduced and converted into purple formazan only by living cells. After the incubation period, media containing MTT was removed, cells were washed in PBS, and 300 µl DMSO (Fisher Scientific, Pittsburgh, PA) was added to each well followed by incubation for 1 h at room temperature, on an orbiting shaker protected from light. Samples were then transferred to 96-well plate and absorbance at 570 nm was determined using a Wallac 1420 Victor^{3}V micro-plate reader (PerkinElmer, Shelton, CT). Each data point represents three (±S.D.) independent results; all data points were normalized to untreated cells.

F. Pulsed-field gel electrophoresis

Cells were seeded at a density of 1.5 x 10^6 in a 100 mm dish in complete medium supplemented with 10% FBS and ± 150 µM MMS. After 24 h, the cells were trypsinized and 1 x 10^6 cells were melted in 1% low melt agarose (GIBCO, Invitrogen, Carlsbad, CA) in 50 mM EDTA. These agarose inserts were incubated in 50 mM EDTA, 1% N-laurosylsarcosine, 1 mg/ml proteinase K at 50°C, shaking, for 48 hours and subsequently washed four times in 1X TE buffer before loading onto a 1% pulsed field certified agarose gel (Bio-Rad, Hercules, CA). Samples were separated by electrophoresis for 24 h using a 120Æ field angle, 60 to 240s switch time, 4 V/cm (Bio-Rad, Hercules, CA), and visualized by ethidium bromide staining on an AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA).

G. RAD51 foci staining

1 x 10^5 cells were plated on microscope slides and treated with 150 µM MMS for 24 h. After treatment cells were washed twice in PBS and fixed in 3% (w/v) paraformaldehyde for 20 minutes. Cells were washed in PBS containing 0.1% (v/v) Triton X-100 (PBS-T) and normal goat serum (Zymed Laboratories, San Francisco, CA) was added 30 minutes prior to incubation with the anti-RAD51 antibody (H92, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000 in PBS-3% BSA for 16 h at 4°C. Cells were rinsed 4 times for 15 minutes each in PBS-T and incubated for one hour at room temperature with a Cy-3-conjugated goat anti-rabbit antibody (Zymed Laboratories, San Francisco, CA) at a dilution of 1:500 in PBS-3% BSA. The chambers were washed four times for 15 minutes each in PBS-T and mounted using a SlowFade Antifade kit containing DAPI (Molecular Probes, Eugene, OR). Images were obtained with a Zeiss 510 NLO inverted confocal microscope using 40X Plan-Neofluar (NA 1.3) oil immersion. A 543 nm helium neon laser with a 560 long pass emission filter and a 747 nm 5W Mira 900 fs mode-locked Ti:sapphire laser with a 390-465 IR band pass emission filter were used to detect the antibody and DAPI fluorescence, respectively.

III. Results

A. The assay system

A standard assay system based on the correction of an integrated mutant eGFP gene was employed to measure gene repair activity. A single clonal isolate (identified as clone 1) containing 2-3 copies of the mutant eGFP gene from DLD-1 cells was used for these experiments. Specific oligonucleotides were designed to correct the TAG point mutation at amino acid position 67 which lies in the chromophore region of the eGFP gene and conversion to TAC enables expression of wild-type eGFP, detectable by FACS analysis. The standard work horse vector, EGFP3S/72NT, is a 72-mer containing three phosphorothioates at each terminus to protect against nuclease activity. **Figure 1** illustrates the mutant and wild-type eGFP sequence surrounding the target sites as well as the oligonucleotides used for these correction experiments. This system is well established and has been used multiple times to evaluate gene repair activity (Brachman and Kmiec, 2004; Ferrara et al, 2004; Ferrara and Kmiec, 2004; Hu et al, 2005).

B. Measuring strand breaks in genomic DNA and relocalization of hRad51 to the nucleus of DLD-1 cells directed by MMS treatment

MMS is a cytotoxic agent that introduces strand breaks into DNA by altering the N^3 or N^7 position of adenosine and guanosine respectively (Kim and LeBreton, 1994) (**Figure 2A**). It causes random lesions that manifest as single-strand (Fortini et al, 2000) or double-strand breaks (Pascucci et al, 2005). The latter are likely to be replication-dependent and occur as a result of the replication machinery encountering MMS-generated N-

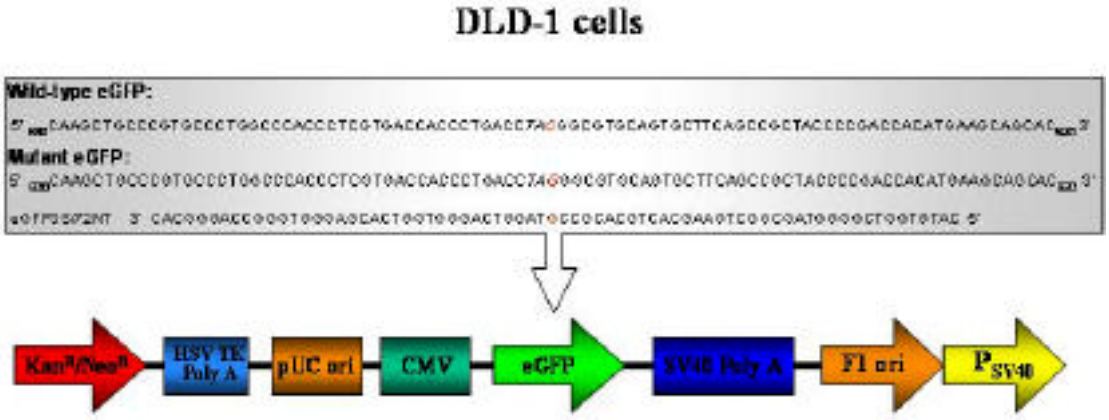

Figure 1. Integration of eGFP vector in the DLD-1 cells. Shown here is the wildtype and mutant sequence of the eGFP gene, where the point mutation is in the eGFP cassette, TAG. This is the target for the 72-mer oligonucleotide, EGFP3S/72NT, directed to the nontranscribed strand of the eGFP gene. EGFP3S/72NT is a single-stranded oligonucleotide with three phosphorothioate modifications on each terminus.

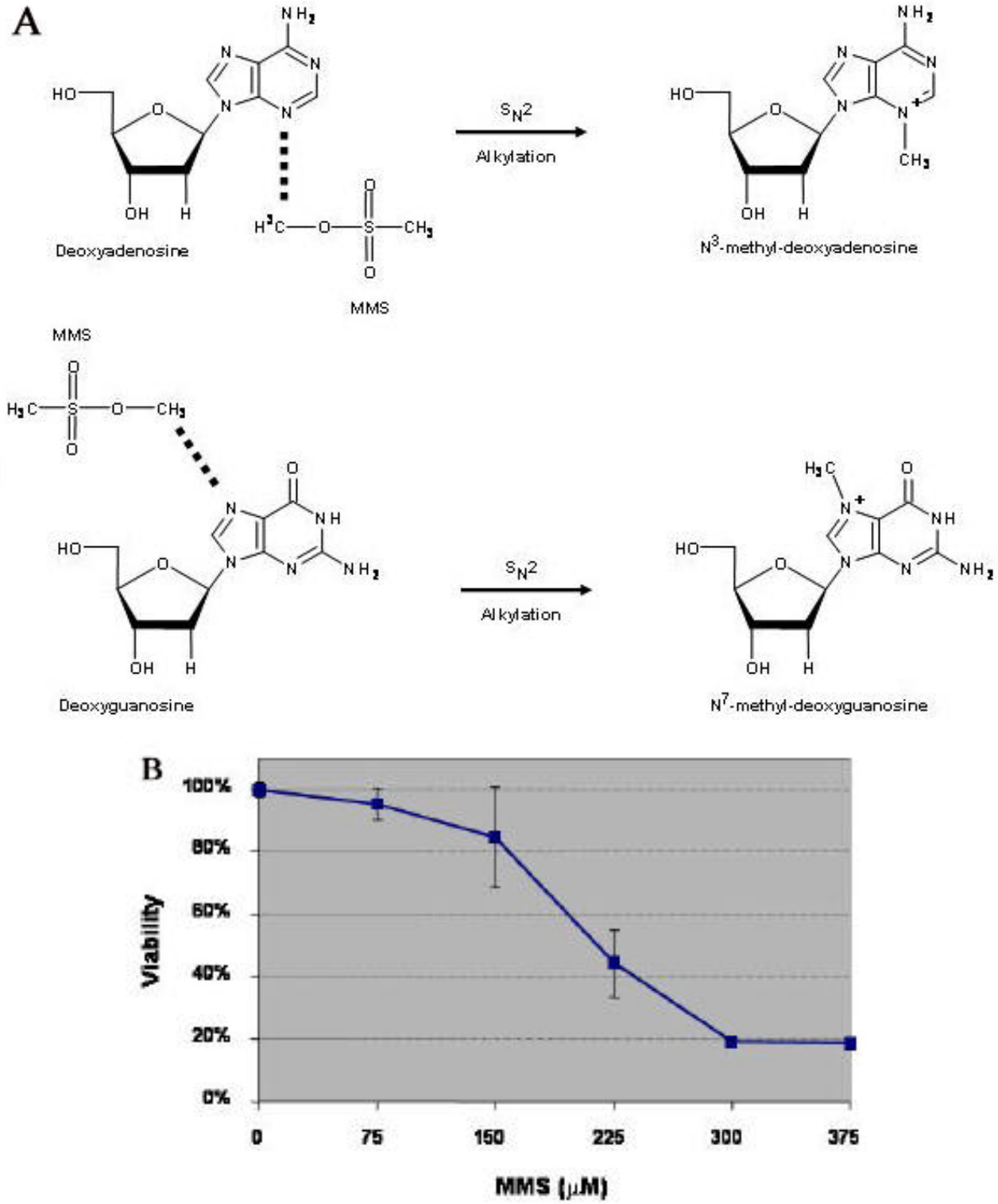

Figure 2. Effect of MMS on DLD-1 cells. **(A)** Mechanism of MMS-induced alkylation of purine bases in DNA. Methyl groups are transferred via SN2 reaction from methyl methanesulfonate (MMS) to N^3 in adenosine and N^7 in guanine, resulting in DNA lesions. **(B)** Viability, DNA damage, and DNA damage response in DLD-1(clone 1) cells. DLD-1 cells were treated with MMS for 24 h at concentrations of 75, 150, 225, 300, and 375 μM. Viability was determined by an MTT-reduction assay; each data point represents mean of three (±S.D.), non-treated cells are normalized to 100% viable.

methylpurines; such encounters lead to fork arrest. With the collapse of the replication fork, the cells stall in S phase until the damage is repaired or the cells die. **Figure 2B** illustrates that the cytotoxic effect of MMS measured by an MTT reduction assay is dose-dependent with a high percentage of the DLD-1 cells surviving at lower concentrations (150μM) after exposure for twenty-four hours. A sharp decline in viability is observed at concentrations between 150μM and 225μM respectively. This experiment sets the viability parameters and indicates that a 24 h pretreatment with 150 μM MMS does not lead to significant cell death. At this concentration, however, DNA breakage is induced evidenced by the multiple bands in the pulse field gel (PFGE) presented in **Figure 3A**. As compared to an untreated population of cells, genomic DNA isolated from MMS-treated cells exhibits fragmentation. The bright band near the top of the damaged DNA region represents genomic DNA

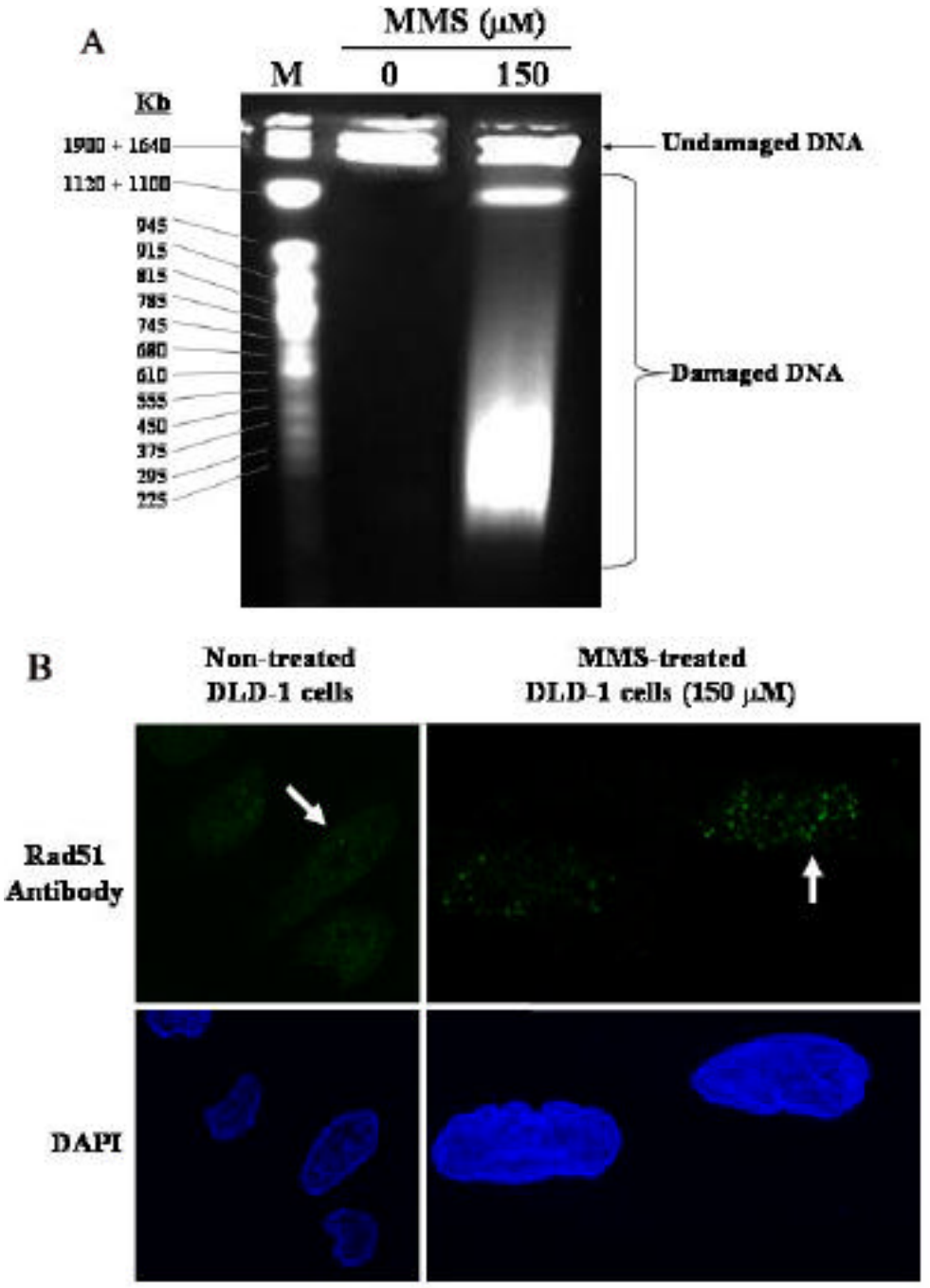

Figure 3. Exposure to MMS induces ds breaks in DLD-1 cells. **(A)** Cells were treated with MMS (150 μM) for 24 h prior to harvesting and prepared for pulsed-field gel electrophoresis (PFGE). The gel was run for 24 h and subsequently stained with ethidium bromide. Lane M, S. cerevisiae chromosomal DNA size marker presented in kilobases; Lane C, non-treated control DLD-1 genomic DNA; Lane MMS, MMS-treated DLD-1 genomic DNA. **(B)** Elevated levels of RAD51 nuclear localization and foci formation in MMS-treated cells. Cells were treated with MMS (150 μM) for 24 h prior to staining a RAD51-directed antibody. The RAD51 antibody is visible by green fluorescence; the nucleus is visualized by a DAPI counter stain (blue). White arrows denote RAD51 nuclear foci.

containing only a few strand breaks while the bright smear represents DNA containing an extensive number of breaks. Such DNA damage is most likely inducing a cellular response which works to repair the breaks using two pathways; nonhomologous endjoining (NHEJ) and homologous recombination (HR) with prolonged exposure favoring HR-assisted recombinational repair (Saintigny and Lopez, 2002). Similar DNA lesions resulting from exposure to etoposide (VP16) or camptothecin were found to be repaired primarily by homologous recombination (Arnaudeau et al, 2001). With regard to the gene repair reaction, the gel in **Figure 3A** illustrates the status of the genomic DNA at the time when the oligonucleotide is introduced into the cells by electroporation. It should be noted that Lundin *et al.* (Lundin et al, 2005) have reported that MMS-induced ds breaks measured by PFGE results from a heat-labile reaction occurring during the preparation of the genomic DNA samples. This data suggests that the ds break itself may not be the key inducer of gene repair in agreement with our own work (Ferrara and Kmiec, 2004). The ds break is simply a sign that the DNA damage pathway has been activated (see below).

Formation of nuclear foci containing human Rad51 has been, by and large, taken as evidence that the HR pathway has been induced by DNA damage (Haaf et al, 1995; Scully et al, 1997). Rad51, a prominent member of the HR response pathway, relocalizes and concentrates in the nucleus forming distinct foci. (Haaf et al, 1995; Scully et al, 1997) Thus, to confirm HR activation, we assessed Rad51 relocalization in DLD-1 cells, in response to MMS, using immunofluorescence (**Figure 3B**). Treated and untreated cells were stained with an anti-Rad51 antibody (H92, Santa Cruz Biotechnology) followed by a Cy-3 conjugated goat anti-rabbit antibody (Zymed Laboratories), nuclei were counter-stained with DAPI to visualize the relocalization event more accurately. Untreated cells were examined by confocal microscopy using a 390-465nm IR bandpass emission filter, and the results reveal that Rad51 is distributed in various cellular compartments including the nucleus as expected in untreated cells. In contrast, cells treated for 24 h with MMS exhibit distinct nuclear foci indicating Rad51 has indeed responded to the DNA damage, and relocalized to the nucleus (**Figure 3B**).

C. Quantifying gene repair activity stimulated by MMS

As described above, MMS alkylation creates DNA lesions which are repaired, at least in part, by the HR pathway. Such a scenario has been previously documented to elevate the level of gene repair activity (Ferrara and Kmiec, 2004; Ferrara et al, 2004). Thus, we tested for enhancement of gene repair by pretreating the cells with varying doses of MMS for 24 h, followed by a wash-out and the electroporation of the specific oligonucleotide, EGFP3S/72NT. Gene repair was evaluated after forty-eight hours of incubation by FACS as described previously (Parekh-Olmedo et al, 2003; Ferrara and Kmiec, 2004; Ferrara et al, 2004). The data reveal a peak of stimulation at 150 μM MMS (**Figure 4**), the concentration seen to induce DNA damage without a significant loss of viability. Interestingly, gene repair activity is also stimulated at 225 μM MMS which had been shown to induce a high level of toxicity in the MTT assay (**Figure 2B**).

The gene repair reaction requires the addition of the specific oligonucleotide, EGFP3S/72NT. In control reactions, oligonucleotides bearing perfect complementarity (EGFP3S-PM/72NT) to the target sequence or no complementarity to the target sequence (Kan3S/70NT) were found to be nonfunctional in promoting gene repair (**Table 1**).

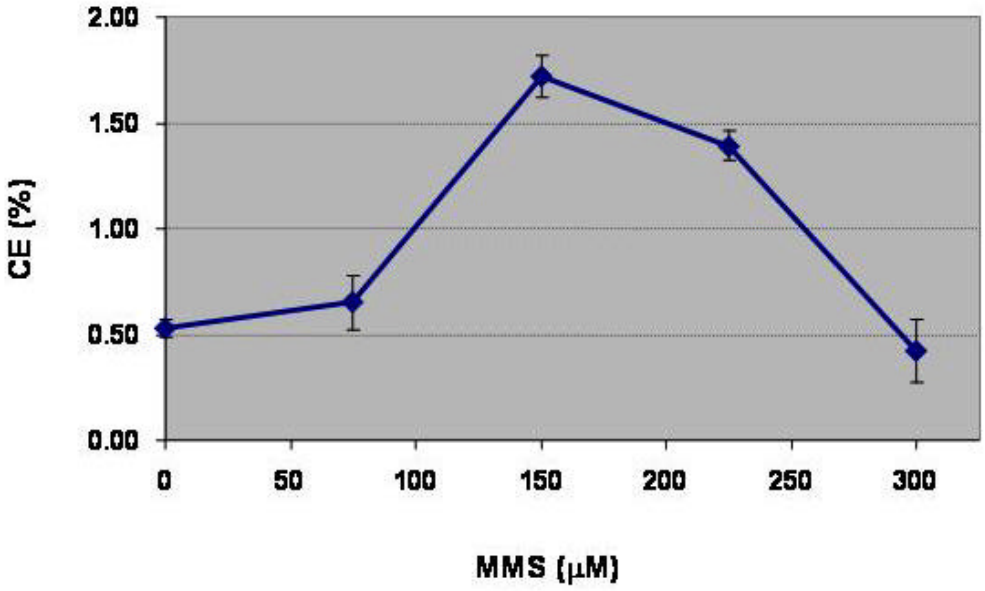

Figure 4. MMS stimulates targeted-nucleotide exchange (TNE). DLD-1 cells were treated with MMS at different doses (0-300 μM) for 24 h prior to electroporation of the oligonucleotide. The cells were analyzed by FACS 48 h after electroporation.

Table 1.

Oligonucleotide	Treatment	CE%
No Oligonucleotide	None	0.01 +/- 0.006
EGFP3S-PM/72 NT	None	0.01 +/- 0.007
Non-Specific Kan 70 NT	None	0.01 +/- 0.000
EGFP3S/72 T	None	0.01 +/- 0.007
EGFP3S/72 NT	None	0.53 +/- 0.042
No Oligonucleotide	150 μM MMS	0.01 +/- 0.006
EGFP3S-PM/72 NT	150 μM MMS	0.01 +/- 0.014
Non-Specific Kan 70 NT	150 μM MMS	0.02 +/- 0.015
EGFP3S/72 T	150 μM MMS	0.05 +/- 0.007
EGFP3S/72 NT	150 μM MMS	1.72 +/- 0.099

Comparison of oligonucleotides in TNE. DLD-1 cells were either untreated or were treated with MMS (150 μM) for 24 h prior to electroporation of the respective oligonucleotide. **T**, transcribed strand of target; **NT**, non-transcribed strand of target; **PM**, 100% homology to target strand (no targeted mismatch present); **Non-Specific**, not homologous to target strand; (CE – correction efficiency). Each data point arises from the results of three independent experiments performed in duplicate.

Likewise, the previously reported strand bias effect (Ferrara and Kmiec, 2004; Ferrara et al, 2004) favoring the nontranscribed strand of the mutant eGFP gene as a target over the transcribed strand was confirmed as the specific oligonucleotide, EGFP3S/72T, which hybridizes to the transcribed strand, was found to promote low levels of gene repair in both untreated and treated cells. We also added MMS at varying dosages to the cell culture *after* the oligo was electroporated into the cells. No stimulation in gene repair activity was seen and after 48 h of exposure, an increase in cell toxicity was observed (data not shown). Thus, we believe that a pretreatment of MMS at a sub-lethal concentration stimulates gene repair activity perhaps by prompting the cells to increase HR in response to DNA damage.

D. Analyzing the effect on cell cycle progression: MMS treatment stalls cells in S phase under conditions that stimulate gene repair activity

Since, cells bearing genomic damage must be repaired prior to mitosis; one might predict that treatment with MMS would result in an S phase accumulation under conditions that have been found to support high levels of gene repair. As shown in **Figure 5**, cells treated with MMS for 24 h progress through the cell cycle but, in contrast to untreated cells, their time in S phase appears to be extended. FACS analysis reveals that the profile of untreated cells includes 30% population in S phase while the percentage of MMS-treated cells in this phase is greater than 60% (**Figure 5**). These data are similar to FACS profiles obtained with cells treated with CPT, which also causes DNA damage, under conditions that enable high levels of correction (Ferrara and Kmiec, 2004). Taken together, the results suggest that agents that damage DNA also arrest cells in S phase, the part of the cell cycle that has previously been shown to be the most amenable to gene repair (Majumdar et al, 2003; Hu et al, 2005).

E. Measuring MMS-induced effects in other clonal isolates

We have shown that MMS can stimulate gene repair in DLD-1(1) cells presumably by inducing the HR response and stalling the cells in S phase. The cellular effects observed here are consistent with the results of earlier studies (Ferrara and Kmiec, 2004) and have helped to shape our thinking about the regulation of correction. Suzuki et al, (2003) had shown previously that agents similar to MMS induce higher levels of targeted gene repair in other cell types including Melan-c cells. These studies suggest that this method of stimulation may be universal for mammalian cells. We have also used MMS to stimulate gene repair frequencies in primary mouse cells, BNL-18 (T. Schwartz, unpublished observations). To broaden the application of this technology even further, we chose to test the stimulatory activity of MMS in a second DLD-1 clonal isolate that differs from the first in several ways. DLD-1(3) contains a single copy of the integrated mutant eGFP gene and has a slightly longer doubling time (Hu et al, 2005). The oligonucleotide, EGFP3S/72NT, was electroporated into DLD-1(3) cells and the gene repair activity was assessed by FACS after 48 h. As seen in Table 2, DLD-1(3) cells exhibit a low level of endogenous gene repair; the frequency is approximately 0.04% in contrast to DLD-1(1) cells which exhibit ten-fold higher correction efficiency. But preincubation with 150 μM MMS leads to a 4-fold increase in gene repair in DLD-1(3) cells. While the overall frequency is lower that that found in DLD-1(1) cells. The fold stimulation is approximately the same. We have observed that the 150 μM concentration of MMS is

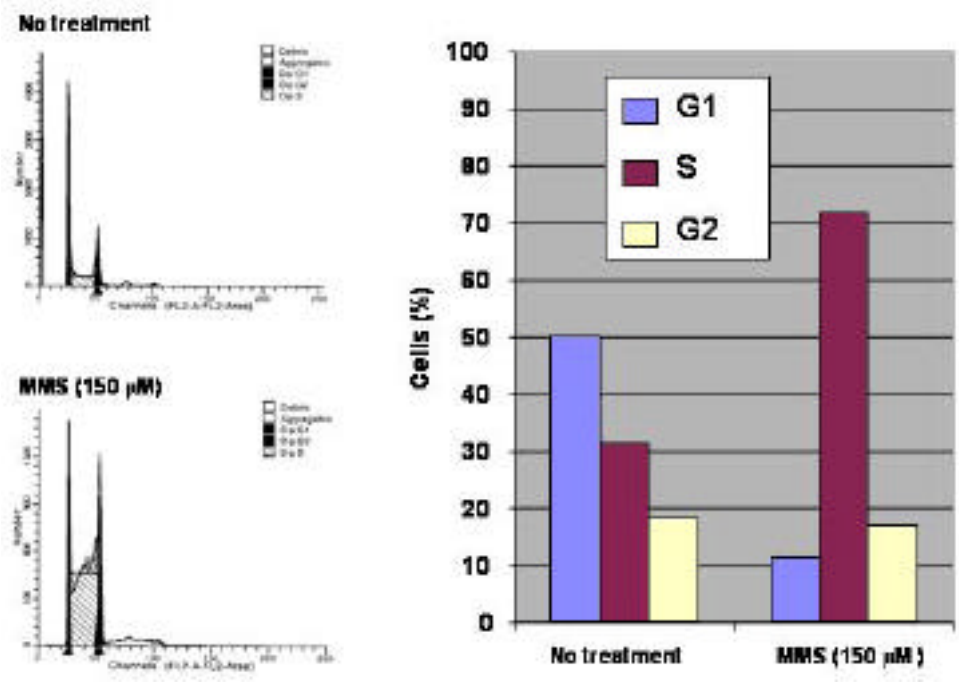

Figure 5. MMS delays cells in S-phase, enhancing targeted-nucleotide exchange (TNE). Cell cycle analysis of MMS-treated DLD-1 cells. DLD-1 cells were pre-treated with MMS (150 μM) for 24 h and processed for cell cycle analysis. The profiles were generated using ModFit LT software. Untreated cells: G1, 54.91%±6.48; S, 30.89%±0.78; G2, 14.21%±5.69; MMS-treated cells: G1, 14.40%±4.49; S, 60.93%±15.20; G2, 24.68%±10.70.

Table 2

Cell line	Oligonucleotide	MMS (μM)	C.E (%)
DLD-1(3)	-	0	0.00
DLD-1(3)	+	0	0.04 +/- 0.032
DLD-1(3)	+	150 μM	0.17 +/- 0.085

The indicated cell line was treated with MMS for 24 h prior to electroporation of the eGFP targeting oligonucleotide targeted to the nontranscribed strand (Figure 1). Cells were processed after 48 h and analyzed for correction by FACS. These data represent the results of three independent experiments performed in duplicate.

also optimal for stimulating gene repair in DLD-1(3) cells (data not shown) and the resulting viability is the same in DLD-1(1) cells. Based on these results, we conclude that MMS can be used effectively to stimulate gene repair frequencies in cell lines that exhibit an inherently low rate of correction.

IV. Discussion

Gene repair is an exciting method for correcting single base mutations in mammalian cells. Many cell types and animal models have been used to validate the overall technique (Bertoni et al, 2003; Lu et al, 2003; Kenner et al, 2004; Nakamura et al, 2004; Bertoni et al, 2005), but in some cases, the frequency of correction is low and difficult to measure. Therefore, we have developed a method for stimulating gene repair activity in mammalian cells. The goal was to find a reagent-based protocol that did not involve a sophisticated synthesis pathway or the purchase of an expensive anti-cancer drug.

Previous data from our own lab (Brachman and Kmiec, 2004, Brachman and Kmiec, 2005; Ferrara and Kmiec, 2004; Ferrara et al, 2004) and others (Suzuki et al, 2003) have shown that reagents which induce DNA damage wither at replication forks or throughout the genome stimulate the repair reaction and elevate repair frequencies. While it is not entirely clear how this happens, our current thinking centers on the effect of DNA damage or replication. In order to identify and reverse the damage, cells freeze DNA synthesis by stalling replication forks (Avemann et al, 1988; Tsao et al, 1993). Such stalling enables the assimilation of the correcting oligonucleotides into the daughter strand of the replicating helix and the evolution of a converted or corrected template (Brachman and Kmiec, 2005; Parekh-Olmedo et al, 2005). Thus, one can use an exogenously added reagent to induce a minimal amount of DNA damage including a temporary stalling of the fork. As we have shown in this paper, MMS induces an HR response to DNA damage, leads to a stalling of replication through an elongation of S phase and a concerted stimulation of gene repair frequencies. The MMS treatment is simple, requires little preparation and uses a reagent that is widely available. MMS and other reagents that induce low levels of ds breaks are already exhibiting universal activity in multiple

cell lines providing a measured level of confidence that such treatments can be applied to systems where the levels of repair are problematic. The mode of action of MMS has recently been re-evaluated and ds breaks, indicative of MMS activity may be a result of sample preparation (Lundin et al, 2005). What is clear about the gene repair stimulation is that MMS induces a relocalization of Rad51 to the nucleus, a hallmark feature of the activation of HR and DNA damage response pathway. These events alone are sufficient to enable correction even in the absence of strand breakage (Ferrara and Kmiec, 2004; Ferrara et al, 2004). We illustrate two clonal isolates from the same parent cell line that contain different copy numbers of the target gene and exhibit very different correction efficiencies. Yet, both are stimulated to approximately the same extent by preincubation with MMS. From a protocol standpoint, we suggest that workers studying gene repair reactions in other cell lines carry out an MMS dose response curve ranging from 100 μM-400 μM in order to pinpoint the optimal concentration of MMS for stimulation.

This method results in the amplification of gene repair events in cells that may not naturally support high levels. This method will also enable studies in cells with interesting genetic backgrounds which can provide unique insight into the mechanism of gene repair. In addition, supplementation of basic gene repair reactions with MMS will help to define the limits of the technology and its application to functional genomics.

Acknowledgements

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