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Oxidative Stress Increases Frameshift Mutations in Human Colorectal Cancer Cells[1]

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ABSTRACT

Chronic inflammation in the gastrointestinal tract increases the risk for development of cancer by an incompletely understood pathway, which may involve microsatellite instability (MSI). Low frequency of MSI referred to as "MSI-L" occurs frequently in chronically inflamed nonneoplastic tissue. In this work, we have tested the hypothesis that oxidative stress may induce the accumulation of frameshift mutations in human microsatellite DNA. Mismatch repair (MMR)-proficient HCT116+chr3 and MMR-deficient HCT116 cells were transfected with pCMV-$(CA)_{13}$-EGFP, a plasmid that contains a $(CA)_{13}$ dinucleotide repeat, which disrupts the reading frame of the downstream enhanced green fluorescent protein gene. A dose-dependent increase in frameshift mutations restoring the enhanced green fluorescent protein reading frame was detected in HCT116 by flow cytometry. At 1 mM H_2O_2, the mutant fraction was 9-fold higher than that in mock-treated control cells. Although demonstrating stability at lower H_2O_2 concentrations, MMR-proficient HCT116+chr3 cells accumulated mutations at the 1 mM H_2O_2 level (4.1-fold above mock-treated control). No significant mutations were detected when HCT116 cells were transfected with the pCMV-$(N)_{26}$-EGFP construct that contains 26 nucleotides in a random sequence. These data indicate that oxidative stress is a potential mutagen leading to accumulation of frameshift mutations and may contribute to MSI in the setting of chronic inflammation.

INTRODUCTION

ROS[5] are generated in cells as a byproduct of normal cellular metabolism. In the setting of inflammation, ROS are produced in increased abundance. ROS react with lipids and proteins. ROS also cause DNA base modifications, abasic sites, deoxyribose damage, and single-stranded as well as double-stranded breaks (reviewed in Ref. 1). Oxidative DNA damage that results from chronic inflammatory states has been implicated in mutagenesis and carcinogenesis. This is particularly evident in the gastrointestinal tract. Patients with Barrett's esophagus are at increased risk for development of esophageal cancer, patients with chronic gastritis are at increased risk for development of gastric cancer, patients with chronic pancreatitis are at increased risk for development of pancreatic cancer, patients with primary sclerosing cholangitis are at increased risk for development of cholangiocarcinoma, and in particular, patients with ulcerative colitis are at increased risk for development of colorectal cancer. The accumulation of cancers in the setting of chronic inflammation does not seem to be organ specific but is generally associated with the inflammatory process. The molecular mechanism of the underlying pathway is not fully understood.

The human genome is well protected against mutagenesis because of the existence of several DNA repair systems. Over the past several years, the mechanisms underlying many of these processes have been identified. The MMR system has been found to ensure DNA fidelity by correcting newly acquired mutations after DNA replication immediately before mitosis. The MMR system requires cooperation of several protein components, which can recognize and remove DNA mismatches that may occur in the newly synthesized DNA strand.

Insertion/deletion loops are most likely to occur in repetitive DNA sequences called microsatellites. Microsatellites are widely dispersed throughout the eukaryotic genome, primarily within noncoding regions but occasionally within coding regions. Many microsatellites are highly polymorphic throughout the population. They serve as valuable markers in genetic mapping, identity testing, and genetic linkage analysis. About half of the hereditary nonpolyposis colorectal cancer families have been linked to germ-line mutations in the DNA MMR genes *hMSH2* and *hMLH1* (2–5). Most tumors in patients with hereditary nonpolyposis colorectal cancer display MSI, which is considered a fingerprint of MMR deficiency (6) and the mutator pathway (7, 8).

MSI has also been detected in colorectal cancer samples from patients with ulcerative colitis (9). There are two forms of MSI, which have different implications. High frequency of MSI, referred to as "MSI-H," is found when the DNA MMR system is totally inactivated by mutation or hypermethylation of the *hMLH1* promoter. MSI-L (10) has been found not only in the tumors themselves but also in chronically inflamed nonneoplastic colonic tissue (11). Silencing of *hMLH1* expression by promoter hypermethylation was recently reported in about half of ulcerative colitis-associated tumors that displayed MSI-H, but in only one-sixteenth of the MSI-L tumors (12). *hMLH1* promoter hypermethylation may partially explain the pathway by which MSI-H arises in such tumors, but the mechanism that accounts for MSI-L is still undetermined and is due to neither mutational inactivation nor promoter silencing of any of the major DNA MMR genes. Here we hypothesized that oxidative stress may alter MMR function and allow mutations to accumulate in microsatellite sequences.

MATERIALS AND METHODS

Microsatellite-Green Fluorescence Protein Reporter Plasmid. The plasmid pcDNA3.1-EGFP allows the constitutive expression of an EGFP (13) under the control of a cytomegalovirus promoter. The pcDNA3.1-$(CA)_{13}$-EGFP and pcDNA3.1-$(CA/T)_{12}$-EGFP plasmids were kindly provided by Ilan R. Kirsch (National Cancer Institute-Medicine Branch, Bethesda, MD; Ref. 14) and are henceforth referred to as pCMV-$(CA)_{13}$-EGFP and pCMV-$(CA/T)_{12}$-EGFP. We constructed the pCMV-$(N)_{26}$-EGFP plasmid by inserting a nonrepetitive $(N)_{26}$ sequence (GCGCTTATACTAAGCGGAAATCGTTA) immediately adjacent to the start codon of EGFP before cloning into the pIREShyg vector (Clontech, Palo Alto, CA). Similar to pCMV-$(CA)_{13}$-EGFP, expression of the out-of-frame EGFP in pCMV-$(N)_{26}$-EGFP is under the control of a cytomegalovirus promoter. Sequences of all three plasmid con-

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[5] The abbreviations used are: ROS, reactive oxygen species; MMR, mismatch repair; MSI, microsatellite instability; EGFP, enhanced green fluorescence protein; MSI-H, MSI-high (≥40% of microsatellites are unstable); MSI-L, MSI-low (<40% of investigated microsatellite markers show instability).

structs were confirmed by DNA sequencing analyses using an ABI Prism 310 genetic analyzer (Perkin-Elmer Biosystems, Foster City, CA).

Transfection. HCT116 colorectal cancer cells (ATCC CCL-247) are MMR deficient by homozygous mutation of the *hMLH1* gene. HCT116+chr3 are MMR-proficient through stable transfer of chromosome 3 (15). The MMR-deficient HCT116+chr3-M2 clone was derived from HCT116+chr3 after exposure to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (16, 17). All these cells were grown in Iscove's modified Dulbecco's medium (Life Technologies, Inc., Rockville, MD) containing 2 nM glutamine and 10% fetal bovine serum (Life Technologies, Inc.) at 5% CO_2 and 37°. The medium for HCT116+chr3 and HCT116+chr3-M2 also contained 400 μg/ml G418 (Life Technologies, Inc.). Cells were plated at 4×10^5 cells/well in 6-well plates. The next day, cells were transiently transfected with pCMV-$(CA)_{13}$-EGFP, pCMV-$(N)_{26}$-EGFP, or pCMV-$(CA/T)_{12}$-EGFP construct using Effectene (Qiagen, Hilden, Germany).

H_2O_2 Treatment. H_2O_2 (30.9%; Sigma Chemical Co., St. Louis, MO) was diluted in PBS, and the concentration was determined by absorbance at 240 nm as described previously (18). Forty-eight h after transfection, triplicate cultures were washed with warm PBS and subsequently treated with various concentrations of H_2O_2 (0.001, 0.01, 0.1, 1, and 10 mM) in PBS for 60 min. Control cultures were mock-treated with PBS alone. At the end of treatment, cells were placed in the growth medium and allowed to recover from the oxidative stress.

Flow Cytometry. Twenty-four h after H_2O_2 treatment, cells were trypsinized, washed in PBS, and resuspended in PBS containing 2% fetal bovine serum and propidium iodide (0.5 μg/ml). Cells were analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). The propidium iodide-negative, EGFP-positive cell population (*i.e.*, the mutant fraction) was quantitated. The corresponding transfection efficiency was measured in parallel experiments with pCMV-$(CA/T)_{12}$-EGFP transfected cells. Data are shown as mean ± SD of the mutant fraction after normalization for corresponding transfection efficiency (*e.g.*, positive pCMV-$(CA)_{13}$-EGFP transfected cells × 100/mean of positive pCMV-$(CA/T)_{12}$-EGFP-transfected cells).

Southern Blot Analysis. Total cellular DNA was isolated from the transfectants using phenol and chloroform (19), 10 μg of total DNA were digested with *Hin*dIII or with *Hin*dIII and BsgI (New England Biolabs, Beverly, MA), resolved on a 1.2% agarose gel, and transferred onto a charged nylon membrane (Hybond-N+; Amersham Pharmacia Biotech, Piscataway, NJ). pCMV-$(CA)_{13}$-EGFP was digested with *Hin*dIII and *Eco*RI (New England Biolabs), and a 787-bp fragment containing the EGFP gene was gel-purified, labeled with [α-^{32}P]dCTP using random primer DNA labeling (Life Technologies, Inc.), and further purified using a G-50 microcolumn (ProbeQuant; Amersham Pharmacia Biotech). After prehybridization with aqueous prehybridization-hybridization solution (20), the probe was added and hybridized overnight at 68°C. Subsequently, the blot was washed twice with 2× SSC/0.1% SDS at room temperature, washed twice with 0.2× SSC/0.1% SDS at 45°C, washed once for 30 min with 0.2× SSC/0.1% SDS at 68°C, and subjected to autoradiography.

Western Blot Analysis. Of total proteins extracted from each cell sample after H_2O_2 treatment, 50 μg were resolved by 10% SDS-PAGE before being transferred onto a polyvinylidene difluoride membrane, as detailed previously (21). *Aequorea victoria* monoclonal antibody (clone JL-8; Clontech) was used for immunodetection of the EGFP protein using a chemiluminescent detection method (ECL-plus; Amersham Pharmacia Biotech).

Statistics. Paired and unpaired Student's *t* tests were used for comparison within and between groups. *P*s ≤ 0.05 were regarded as significant.

RESULTS

Oxidative Stress and Cell Viability. HCT116 and HCT116+chr3 were chosen as a model to study the effect of oxidative stress on MMR and MSI in the colon. We aimed to adjust our experimental H_2O_2 concentrations to achieve partial cell damage. Oxidative stress was produced by a 1-h incubation with various concentrations of H_2O_2. Five days later, cells were trypsinized, stained with propidium iodide, and analyzed by flow cytometry. Viability was unaffected when cells were treated with <1 mM H_2O_2. At 1 mM, cell viability was moderately reduced; at 10 mM, H_2O_2 was almost completely cytotoxic. The respective data were slightly different with HCT116+chr3, which was less susceptible to oxidative stress [1 mM viability, 54.3 ± 9.0% (HCT116) *versus* 64.7 ± 3.3% (HCT116+chr3), $P = 0.049$; 10 mM viability, 0.3 ± 0.1% (HCT116) *versus* 1.3 ± 0.8% (HCT116+chr3), $P = 0.038$]. We considered the H_2O_2 range above 0.1 mM and below 10 mM as potentially relevant to the clinical setting of chronic inflammation.

Spontaneous Frameshift Mutations in HCT116 and HCT116+chr3. Because MMR is inactive in HCT116 cells, these cells were considered to reflect the capacity of oxidative stress to induce frameshift mutations in colonic cells. By comparing data from HCT116 and from HCT116+chr3, the ability and limits of the MMR system to repair frameshift mutations were estimated. We used the EGFP as a reporter by transfecting cells with pCMV-$(CA)_{13}$-EGFP and pCMV-$(CA/T)_{12}$-EGFP. The pCMV-$(CA)_{13}$-EGFP plasmid contains 13 CA dinucleotide repeats inserted immediately after the start codon of the EGFP gene, which shifts the reading frame to a +2 position, resulting in a nonfluorescent truncated polypeptide. The pCMV-$(CA/T)_{12}$-EGFP plasmid was used to quantitate transfection efficiency, and it contains a nonperfect run of CA repeats $(CA/T)_{12}$ (5′-CACACTCACACACACACACACACA) inserted in the same reading frame with EGFP.

Three days after transfection of HCT116 with the pCMV-$(CA)_{13}$-EGFP plasmid, 1.9 ± 0.1% of cells were positive for fluorescence compared with 26.3 ± 0.7% of cells transfected with the pCMV-$(CA/T)_{12}$-EGFP plasmid. After normalization for parallel transfection efficiency, the fraction of mutant cells was 7.2 ± 0.3%. Six days after transfection, the mutant fraction decreased to 1.0 ± 0.2%. This was most likely due to loss of plasmid DNA because the number of controls expressing EGFP had also decreased to 10.1 ± 0.7% at the same time point. Southern blot analysis confirmed that this reduction in spontaneous reversion rate was accompanied by a significant loss of transiently integrated and nonintegrated plasmid DNA (Fig. 1). When HCT116+chr3 cells were transfected, the same pattern of spontaneous mutations was observed. At day 3 and day 6, 4.6 ± 0.2% and 1.2 ± 0.1% of cells showed restoration of the correct reading frame of EGFP, respectively. At day 3, the spontaneous mutant fraction in HCT116+chr3 was significantly lower than that in HCT116 ($P = 0.004$), indicating the contribution of the MMR system in repair of such mutations.

Induction of Frameshift Mutations by H_2O_2. In the following experiments, cells were treated with various concentrations of H_2O_2 and analyzed by flow cytometry 24 h later. In MMR-deficient HCT116 cells, H_2O_2 caused a dose-dependent increase in the EGFP-expressing mutant fraction (Fig. 2*A*, *dotted line*). This increase reached a plateau at 0.1 mM, possibly saturating the total capacity of H_2O_2-induced frameshift mutations in our model. HCT116+chr3 showed no change of H_2O_2-induced frameshift mutations at concentrations up to 0.01 mM (Fig. 2*A*, *solid line*). At concentrations above 0.01 mM, this MMR-proficient cell line showed a dose-dependent increase of the mutant fraction. For both HCT116 and HCT116+chr3, the maximum increase was 1.8-fold above baseline. To rule out the possibility that the increase in fluorescence-positive cells was due to an increase in H_2O_2-induced autofluorescence, total cellular protein was extracted from HCT116+chr3 and subjected to Western blotting using a monoclonal antibody against EGFP. A 29-kDa EGFP showed a dose-dependent increase in density after 0.1 and 1 mM H_2O_2 treatment (Fig. 2*B*). These findings indicate that oxidative stress can induce frameshift mutations in human cells.

To further support this conclusion, we transfected HCT116 with the pCMV-$(N)_{26}$-EGFP plasmid carrying a nonrepetitive $(N)_{26}$ oligonucleotide (GCGCTTATACTAAGCGGAAATCGTTA) that shifts EGFP out of the correct reading frame, resulting in a nonfluorescent truncated polypeptide. With this construct, we did not detect any

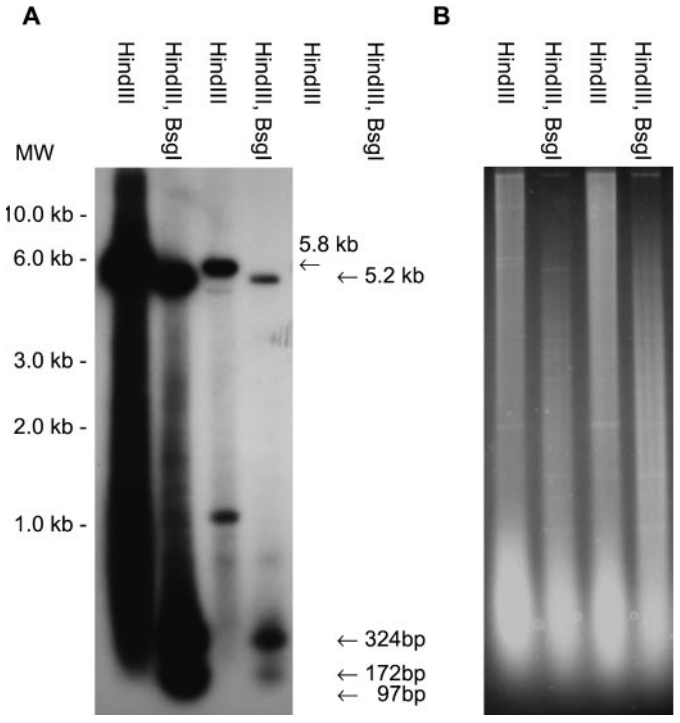

Fig. 1. Autoradiograph (*A*) showing Southern blot analysis of HCT116 cells, which was performed 3 (*two left lanes*) and 6 days (*two right lanes*) after transient transfection with pCMV-$(CA)_{13}$-EGFP. Ten μg of total DNA were digested with *Hin*dIII or with *Hin*dIII and *Bsg*I, separated on a 1.2% agarose gel, transferred and hybridized with a 787-bp EGFP probe, and washed under high stringency conditions. Digestion of nonintegrated plasmid DNA with *Hin*dIII results in a single 5.8-kb fragment. After double digestion with *Hin*dIII and *Bsg*I, four fragments are expected (*arrows*): the 5.2-kb fragment results from the nonintegrated episomes; and the 324-, 174-, and 97-bp fragments result from digestion of both integrated and nonintegrated plasmid DNA. The alterations in fragment density (*e.g.*, 5.8 kb, 5.2 kb, 324 bp, 174 bp, and 97 bp) are due in part to lower melting temperatures of shorter DNA:DNA hybrids (19). The intense smears in *Lanes 1* and *2* indicate random genomic integration of plasmid DNA at day 3. At day 6, the majority of plasmid DNA (transiently integrated and nonintegrated) is lost. Two *Hin*dIII fragments at ≈1.1 and ≈4.8 kb (*Lane 3*) disappear on double digestion (*Lane 4*) and possibly correspond to nonrandom residual integration sites. The ethidium bromide staining of the gel before transfer (*B*) shows equal loading of all lanes.

significant spontaneous or H_2O_2-induced EGFP expression because the mutant fractions for 0 and 1 mM H_2O_2 were 0.01 ± 0.02% and 0.03 ± 0.04%, respectively. These findings strongly support our hypothesis that oxidative stress may induce frameshift mutations in microsatellites. At a critical level of oxidative stress, the MMR system fails to repair such mutations, which permits the cells to accumulate frameshift mutations.

Repeated Oxidative Stress and MSI. We further intended to simulate chronic inflammation by repeated H_2O_2 treatment. The limiting factor of such an experiment, however, was that plasmid DNA in the transfectants was lost a few days after transient transfection. The MMR-deficient HCT116+chr3-M2 clone (16) was included in this experiment to rule out the possibility that the observed difference between HCT116 and HCT116+chr3 was due to other genes besides *hMLH1* that had been transferred on chromosome 3. HCT116+chr3-M2, a subclone of MMR-proficient HCT116+chr3 cells, has lost *hMLH-1* expression by mutational inactivation after two successive treatments with 5 μM *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (16, 17).

We kept the experimental conditions unchanged and added a second 60-min course of H_2O_2 two days after the primary treatment, followed by analysis of the cells 3 days later. Based on flow cytometry analysis, mock-treated cells displayed a small mutant fraction (*i.e.*, 0.4 ± 0.2%, 0.3 ± 0.06%, and 0.7 ± 0.03% for HCT116, HCT116+chr3, and HCT116+chr3-M2, respectively). After exposure to 0.001 and 0.01 mM H_2O_2, no increase in the mutant fraction was detected (Fig. 3). At 0.1 mM H_2O_2, only HCT116 showed an increase in the mutant fraction, whereas HCT116+chr3 and HCT116+chr3-M2 did not show an increase in the mutant fraction. At the 1 mM level, however, all cell lines accumulated mutations. HCT116+chr3-M2 cells were the most susceptible to frameshift mutations (16.4-fold above mock-treated control; $P = 0.005$), ruling out the possibility that other genes besides *hMLH1* that had been transferred on chromosome 3 are responsible for the observed difference between HCT116 (9-fold above control; $P = 0.013$) and HCT116+chr3 (4.1-fold above control; $P = 0.029$). These data indicated that repeated oxidative stress might produce frameshift mutations in microsatellites, which escape MMR and may accumulate over time.

DISCUSSION

The results reported in this study support our hypothesis that oxidative stress may interfere with MMR function and may allow frameshift mutations to accumulate in human colon cancer cells. Our

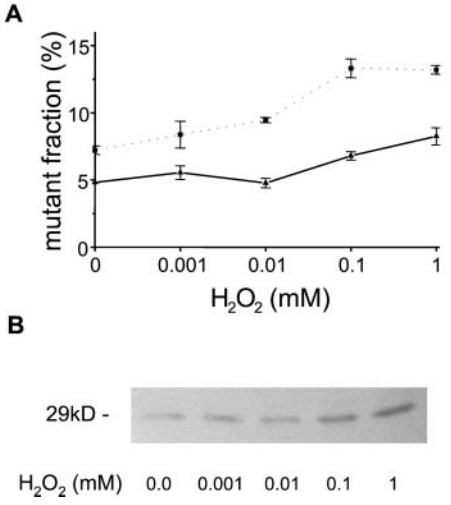

Fig. 2. Forty-eight h after transfection with the EGFP out-of-frame construct, cells were treated with various concentrations of H_2O_2. Flow cytometry was performed 24 h later, and data were normalized for transfection efficiency (measured in parallel experiments). *A*, in MMR-deficient HCT116 (*dotted line*), H_2O_2 caused a dose-dependent increase in the mutant fraction. MMR-proficient HCT116+chr3 (*solid line*) showed repair of H_2O_2-induced frameshift mutations at concentrations up to 0.01 mM. At concentrations above 0.01 mM, a dose-dependent increase of mutant cells was observed. *B*, total cellular protein was extracted from HCT116+chr3 and subjected to Western blotting with anti-EGFP. With 0.1 and 1 mM H_2O_2 treatment, more EGFP protein is expressed.

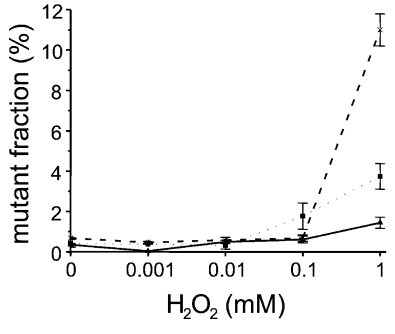

Fig. 3. Three days after repeated H_2O_2 treatment, no change in the mutant fraction was found at concentrations below 0.1 mM H_2O_2. At or above 0.1 mM, MMR-deficient HCT116 cells (*dotted line*) showed an increase in the mutant fraction. After repeated treatment with 1 mM H_2O_2, MMR-deficient HCT116+chr3-M2 (*dashed line*) displayed the highest mutant fraction, followed by HCT116 and the MMR-proficient HCT116+chr3 (*solid line*).

data indicate that oxidative stress is a potential mutagen leading to frameshift mutations even in MMR-proficient cells. We present a likely mechanism to explain how MSI-L could arise in chronically inflamed nonneoplastic tissue. The same mechanism could also contribute to the accumulation of mutations in neoplastic cells in the setting of chronic inflammation. Furthermore, MMR-proficient HCT116+chr3 cells were less susceptible to H_2O_2-induced frameshift mutations. This indicates that the mammalian MMR system plays a crucial role in the repair of frameshift mutations after oxidative stress.

Frameshift mutations in HCT116 and HCT116+chr3 involve two important steps: (*a*) first, H_2O_2 induces the mutations (as shown from our results in MMR-deficient HCT116 and HCT116+chr3-M2 cells); and (*b*) second, the mutation must escape the repair mechanisms of the MMR system (as shown in MMR-proficient HCT116+chr3 cells). Exposure of plasmid DNA to H_2O_2 *in vitro* is effective in producing frameshift mutations on replication of the damaged DNA in *Escherichia coli* (22). Interestingly, such mutations occur preferentially in microsatellites. Exposure of *E. coli* to low levels of H_2O_2 also increased the frequency of expansions and deletions within dinucleotide repetitive sequences (23). ROS are capable of generating and modifying DNA structures that may give rise to slip intermediates during either DNA replication or repair processing of the altered structure. Outside of microsatellites, H_2O_2-induced frameshift mutations have been found to be uncommon in mammalian cells (24). We assume that the frameshift mutations in our experiments also occurred within the dinucleotide repeat that had been cloned into the EGFP cDNA immediately after the start codon. Due to the nature of our experiments, however, we were unable to identify the position and nucleotide sequence of the frameshift mutation in the individual fluorescent cells. It was also not possible to calculate the exact mutation rate. These questions might have been addressed by working with stably transfected cell lines, which would enable us to further study repetitive exposure to H_2O_2. Unfortunately, we were unable to select HCT116 or HCT116+chr3 cells that are stably transfected with pCMV-$(CA)_{13}$-EGFP.

As shown in the Southern blot experiments, a high copy number of plasmid DNA was present in the cells in both its episomal and transiently integrated forms. We do not know from our experiments whether the frameshift mutations occurred in an episomal or an integrated form. However, the pCMV-$(CA)_{13}$-EGFP construct does not replicate episomally in HCT116 or HCT116+chr3. H_2O_2 may have caused modifications of DNA structure before integration, which could have given rise to frame slippage after integration during replication. It is also possible that H_2O_2 altered plasmid DNA after integration into the host genome. Because histones tend to protect the genomic DNA from oxidative damage, it seems more likely that mutations may have occurred during the nonintegrated condition (25). In any scenario, the high intracellular copy number found shortly after transfection provides a better target for mutation to occur and increases the likelihood of detecting such mutations with our assay. Theoretically, restoration of the EGFP reading frame in a single copy of plasmid could be sufficient for EGFP expression and detection of the cell by flow cytometry.

Here we report that oxidative stress can induce frameshift mutations in near-isogenic human colon cancer cell lines, HCT116, HCT116+chr3, and HCT116-chr3-M2. It is likely that these mutations occur within the dinucleotide repeat. The induction of frameshift mutations in HCT116+chr3 is notable because this cell line is MMR proficient. It might be asked how frameshift mutations escape recognition by the MMR system in HCT116+chr3. Our group has reported previously that oxidative stress can inactivate MMR function, which can be restored by adding recombinant MMR complexes.[6] An alternative explanation would be that the higher mutation rate during oxidative stress may alter the MMR capacity and may allow replication errors to pass the G_2 cell cycle checkpoint (16). No matter what the exact pathway might be, our data demonstrate that oxidative stress can produce frameshift mutations in a MMR-proficient colonic epithelial cell line, which raises a possible mechanism for MSI-L in the setting of chronic inflammation.

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