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Mutation Research 447 (2000) 187–198

Fundamental and Molecular Mechanisms of Mutagenesis

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Microsatellite instability induced by hydrogen peroxide in *Escherichia coli*

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Received 4 June 1999; received in revised form 11 October 1999; accepted 13 October 1999

Abstract

Damage to DNA by reactive oxygen species may be a significant source of endogenous mutagenesis in aerobic organisms. Using a selective assay for microsatellite instability in *E. coli*, we have asked whether endogenous oxidative mutagenesis can contribute to genetic instability. Instability of repetitive sequences, both in intronic sequences and within coding regions, is a hallmark of genetic instability in human cancers. We demonstrate that exposure of *E. coli* to low levels of hydrogen peroxide increases the frequency of expansions and deletions within dinucleotide repetitive sequences. Sequencing of the repetitive sequences and flanking non-repetitive regions in mutant clones demonstrated the high specificity for alterations with the repeats. All of the 183 mutants sequenced displayed frameshift alterations within the microsatellite repeats, and no base substitutions or frameshift mutations occurred within the flanking non-repetitive sequences. We hypothesize that endogenous oxidative damage to DNA can increase the frequency of strand slippage intermediates occurring during DNA replication or repair synthesis, and contribute to genomic instability.

Keywords: Microsatellite instability; Genomic instability; Oxidative damage; Repetitive sequence slippage; Hydrogen peroxide

1. Introduction

Genetic instability is a hallmark of human cancer and is manifested by multiple mutations that are believed to occur early during the course of tumorigenesis [1]. Reactive oxygen species represent an abundant source of endogenous mutagens. We have asked whether endogenous oxidative DNA damage can impact on genetic instability in the form of microsatellite instability.

Prominent among the mutations identified in tumors are expansions and contractions of short repetitive sequences termed microsatellites. Alterations in microsatellite sequences are found at high frequency in tumors of the hereditary non-polyposis colon carcinoma (HNPCC) syndrome [2–4], and at a lower frequency in a subset of sporadic cancers [4–16]. Alterations in microsatellite length are believed to result from strand slippage intermediates that arise during replication (Ref. [17] and references therein). In non-tumor cells, as well as in yeast and *Es-*

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0027-5107/00/$ - see front matter
PII: S0027-5107(99)00206-7

cherichia coli, these slippage intermediates are repaired by the mismatch repair system. Expansions and contractions of repetitive sequences within genes have been documented to occur in tumors, and result in somatic frameshift inactivation of tumor-associated genes including *TGF-βIIR* [18–20], *IGFIIR* [21], *BAX* [22], *E2F4* [23], *hMSH3* and *hMSH6* [24,25], *BRCA1* and *BRCA2* [24].

Defective mismatch repair, consisting primarily of alterations in *hMSH2* and *hMLH1*, is believed to be responsible for microsatellite instability in the majority of patients with HNPCC. However, several studies have failed to detect germline or somatic mutations in *hMSH2* or *hMLH1* in many sporadic colon cancers characterized by high frequency microsatellite instability [26–29]. Some of these colorectal tumor cells have recently been documented to lack hMlh1 protein expression due to promoter hypermethylation [30]. Thus, mutations in mismatch repair genes or reduced expression of mismatch repair proteins appears to correlate with the majority of HNPCC tumors exhibiting high frequency microsatellite instability. However, microsatellite instability is not unique to tumors of the HNPCC syndrome. The instability of microsatellite sequences has been reported in a variety of other malignancies, including cancers of the pancreas, ovary, prostate, breast, lung, and bladder. Alterations in the mismatch repair system have yet to be identified for these cases, thus, the underlying molecular basis for the observed microsatellite instability in many of these tumor systems remains to be established.

We have considered the possibility that factors other than mismatch repair deficiency may contribute to the instability of repetitive sequences. We reasoned that environmental or intracellular damage could impact on microsatellite instability through the induction of DNA slippage intermediates sufficient to overwhelm an intact mismatch repair system. Of significant interest in this regard is the documented instability of repetitive sequences in the non-neoplastic and inflammatory settings of ulcerative colitis [31] and pancreatitis [16]. These diseases are characterized by chronic inflammation with the generation of oxygen free radicals. The high degree of microsatellite instability in these settings suggests that the mismatch repair system may be insufficient to compensate for the stress of chronic inflammation. Indeed, we have previously demonstrated that oxidative damage to DNA alone is sufficient to induce microsatellite instability upon replication of the damaged DNA in wild type, repair-proficient bacterial cells [32]. In the present study, we extend these findings to examine the effect on microsatellite instability of oxidative damage in the context of the cell. Using a selective assay for microsatellite instability in *E. coli* [32], we demonstrate that oxidative damage to wild type bacterial cells significantly increases the frequency of microsatellite instability.

2. Materials and methods

2.1. Strains, plasmids, and media

Wild type strain BL21 (F *ompT* (lon) $hsdS_B$ ($r_B^- m_B^-$)) was from Novagen (Madison, WI). Wild type strain AB1157 (*his4 argE3 leuB6 proA2 thr-1 thi-1* rpsL31 *galK2 lacY1 ara-14 xyl-5 mtl-1 kdgK51 supE44 tsx-33*) was kindly provided by Leona Samson (Harvard School of Public Health). Wild type strain BW35 (*thi-1 relA-1 spoT1*) was a generous gift from Bernard Weiss (Emory University). All strains were routinely cultured in 2 × YT (2 × Yeast–Tryptone, Difco) media or agar (Difco Bacto agar) supplemented with the appropriate antibiotics.

The construction of plasmids containing microsatellite repeats has been described previously [32]. Briefly, dinucleotide repeats have been placed out-of-frame within the coding region of the β-lactamase gene of the low copy number plasmid pBR322, such that cells harboring the plasmid are sensitive to carbenicillin. Frameshift mutations that restore the reading frame of the β-lactamase gene confer antibiotic resistance. The plasmid additionally provides resistance to tetracycline, which serves as the marker for plasmid maintenance. Transformation of strains with microsatellite repeat plasmids was achieved by electroporation and selection for growth in the presence of 12.5 μg/ml tetracycline (Sigma). The antibiotic sensitivity of plasmids containing out-of-frame microsatellite sequence was confirmed by replica-plating transformed cells to solid 2 × YT agar containing 12.5 μg/ml tetracycline plus 100 μg/ml carbenicillin (Island Scientific). In addition, tetracycline-resistant, carbenicillin-sensitive clones were

sequenced by means of PCR sequencing with the Thermo Sequenase cycle sequencing kit (Amersham Life Sciences) using the sequencing primer described below. Transformed cells were routinely cultured in the presence of 12.5 μg/ml tetracycline to ensure maintenance of the plasmid.

2.2. In vivo hydrogen peroxide damage

E. coli strains containing microsatellite repeat plasmids were grown to saturation overnight in 2× YT supplemented with 12.5 μg/ml tetracycline. Fresh overnight cultures were diluted into fresh medium and grown to an $o.d._{600}$ of 0.5–0.6, indicative of mid-log phase. Cells were collected by centrifugation at 13,000 × *g*, washed with an equal volume of 1× M9 salts, and resuspended in an equal volume of 1× M9 salts. A total of 150 μl of cells was aliquoted to 1.7 ml eppendorf tubes and exposed to an equal volume of a 2× solution of hydrogen peroxide freshly diluted in 1× M9 salts. The final concentration of hydrogen peroxide in a 300 μl reaction was 1.25 or 2.5 mM. Stock hydrogen peroxide (30% solution (v/v), Fisher) is a 10 M solution. Hydrogen peroxide exposure was performed in open tubes at 37°C with shaking at 200 rpm for 30 min. The exposure was terminated by centrifugation of the cells followed by resuspension in 300 μl of 2× YT. Dilutions of the cells were immediately plated on 2× YT plates containing 12.5 μg/ml tetracycline and 2× YT plates containing 12.5 μg/ml tetracycline plus 100 μg/ml carbenicillin. The number of colonies recovered on each plate was normalized for the volume plated, and the mutation frequency for each strain was determined by dividing the number of $tet^R carb^R$ colonies per milliliter by the number of tet^R colonies per milliliter.

2.3. Modulation of hydrogen peroxide damage

2.3.1. Catalase

Inactivation of hydrogen peroxide was achieved by incubation in the presence of catalase. Catalase powder (Sigma, from Bovine liver, activity 10,000 units/mg protein) was freshly diluted in distilled water to a concentration of 10 mg/ml (100,000 units/ml). This solution was added to the resuspended cells at a final concentration of 100 units per reaction immediately prior to the addition of hydrogen peroxide.

2.3.2. Iron sulfate

Iron sulfate was included in the growth medium to increase the intracellular concentration of free iron, and therefore increase the production of the reactive hydroxyl radical. $FeSO_4$ (Sigma) was dissolved in distilled water and filter sterilized immediately prior to use. Cultures of cells were grown from single colonies to saturation overnight in 2× YT containing 12.5 μg/ml tetracycline and $FeSO_4$ at concentrations of 2.8, 8.3 or 25 μg/ml. The following day the cultures were diluted into fresh medium containing the same concentration of $FeSO_4$, grown to an $o.d._{600}$ of 0.5–0.6, and exposed to hydrogen peroxide in the absence of $FeSO_4$, as described above.

2.3.3. Sequencing

Following hydrogen peroxide damage, plasmid DNA was recovered from individual tet^R or $tet^R carb^R$ clones using the Wizard miniprep kit (Promega). Sequencing was performed manually by use of the Thermo Sequenase cycle sequencing kit (Amersham) using the primer ALJ20 (5′-ACAATAACCCT-GATAAATGC) end-labeled with ^{32}P by use of T4 polynucleotide kinase (New England Biolabs) and γ-^{32}P-ATP (3000 Ci/mmol, New England Nuclear). This primer anneals 26 nt upstream of the ATG of β-lactamase. Using this primer, we sequenced 150 nt downstream from the ATG, including the microsatellite sequence that begins 30 nt downstream of the ATG.

3. Results

3.1. Induction of microsatellite instability by oxidative damage in vivo

The induction of microsatellite instability by hydrogen peroxide damage to cells was investigated in three different wild type strains of *E. coli* (Fig. 1). Each strain was transformed with a plasmid containing a $(CA)_{11}$ repeat that renders the β-lactamase gene out-of-frame and non-functional [32]. This plasmid thus allows selection of microsatellite frameshift

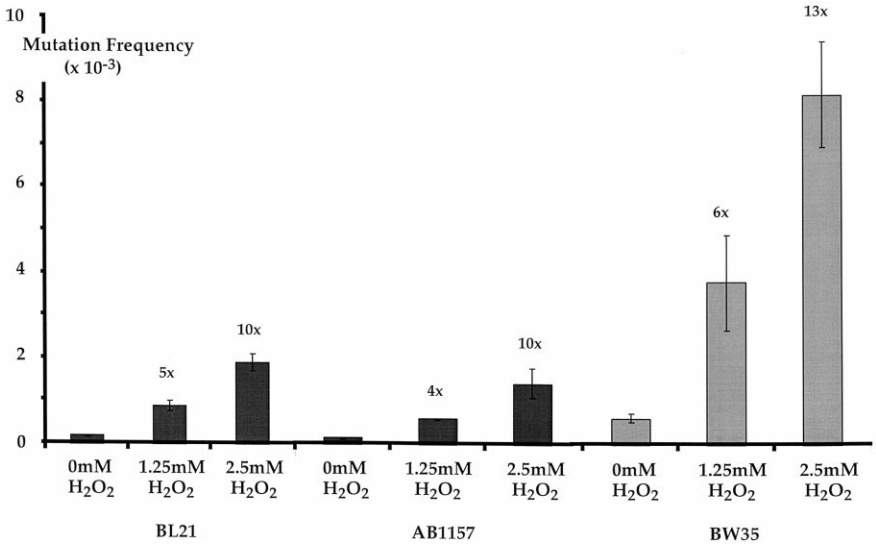

Fig. 1. Microsatellite instability is induced by hydrogen peroxide in *E. coli*. Log phase cultures of wild type *E. coli* harboring a plasmid containing a $(CA)_{11}$ microsatellite sequence in a selectable gene were exposed to hydrogen peroxide to induce oxidative stress. Mutation frequency was determined as the ratio of carbenicillin-resistant mutants recovered per tetracycline-resistant survivor to the damage. Results represent the mean of four to six independent experiments, and error bars represent standard error of the mean.

mutations that restore the reading frame of β-lactamase and confer resistance to carbenicillin. Hydrogen peroxide readily passes through the bacterial cell membrane, but the effective concentration of H_2O_2 within the cell is dependent on the concentration of catalase, as well as on the growth phase [33]. While H_2O_2 is itself unreactive, it is able to react with intracellular metal ions to produced mutagenic reactive oxygen species, such as hydroxyl radical [34]. The oxygen species derived from H_2O_2 are highly reactive and capable of damaging all macromolecules in the cell, including phospholipids, proteins, and DNA.

As reported by Imlay and Linn [35], two modes of killing of *E. coli* by hydrogen peroxide are apparent. One mode of killing occurs with lower, more physiological doses of H_2O_2 of less than 2.5 mM and is increased in repair-deficient cells, while the second occurs at doses higher than 10 mM. We investigated the induction of microsatellite instability by hydrogen peroxide at doses of 1.25 and 2.5 mM, which are characteristic of physiological mode one killing. Mode one lethality requires active DNA metabolism, is independent of oxygen tension during drug challenge, is abolished when drug exposure is performed at 4°C, and is enhanced in strains defective in base excision repair and recombination. Mode one killing was increased in *xth*, *pol*A, *rec*A, and *rec*B strains, and appeared to result from DNA damage that normally occurs at a low, non-lethal level during aerobic growth.

All three wild type strains displayed an increase in microsatellite mutation frequency of 4- to 13-fold following exposure to hydrogen peroxide, with the higher mutation frequency occurring at the higher dose (Fig. 1). The induction of microsatellite instability reflects the dose of hydrogen peroxide, and the magnitude of induction is similar in all three strains. Therefore, oxygen-induced instability is not specific to any particular strain background. Strains AB1157 and BW35, which are both K strains, displayed the increased mutation frequency with 50% survival following hydrogen peroxide exposure, while strain BL21, a B strain, displays the same mutation frequency with only 2.5–9% survival. The microsatellite mutation frequency is increased despite the fact that all three strains contain an intact mismatch repair system, suggesting that oxidative damage to cells at these doses of hydrogen peroxide is not efficiently repaired by mismatch repair. In contrast,

exposure of wild type strain BL21 to the alkylating agents methylmethane sulfonate or *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine resulted in only a threefold increase in microsatellite frameshift mutagenesis (data not shown).

3.1.1. Modulation of hydrogen peroxide damage

The integral role of reactive oxygen species in the generation of microsatellite instability was verified by modulating the concentration of reactive oxygen species in the reaction. Catalase is an enzyme scavenger that catalyzes the decomposition of hydrogen peroxide to water and oxygen. The inclusion of catalase during the hydrogen peroxide exposure was sufficient to abolish both the induction of mutations and the cytotoxicity incurred by hydrogen peroxide damage (Fig. 2). Thus, hydrogen peroxide itself or a reactive oxygen species generated from hydrogen peroxide is rate limiting for the generation of the resulting microsatellite instability.

In a separate experiment, cells were grown in the presence of $FeSO_4$ to increase the intracellular concentration of iron. Iron is a Fenton catalyst capable of catalyzing the reduction of hydrogen peroxide to the highly reactive hydroxyl radical via the reaction $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$ [36]. As a result, increasing the intracellular concentration of this iron species may be expected to increase the frequency of hydrogen peroxide-induced mutations. However, growing cells in the presence of iron sulfate failed to increase the spontaneous or hydrogen peroxide-induced mutation frequency in either of the *E. coli* strains used in this study (Table 1). One interpretation of these results is that the intracellular concentration of iron was not increased due to insufficient transport of $FeSO_4$ into the cell. This possibility is unlikely on the basis of previous reports in which preincubation in $FeSO_4$ dramatically increased the intracellular concentration of iron [37,38]. An alternative interpretation, consistent with previous

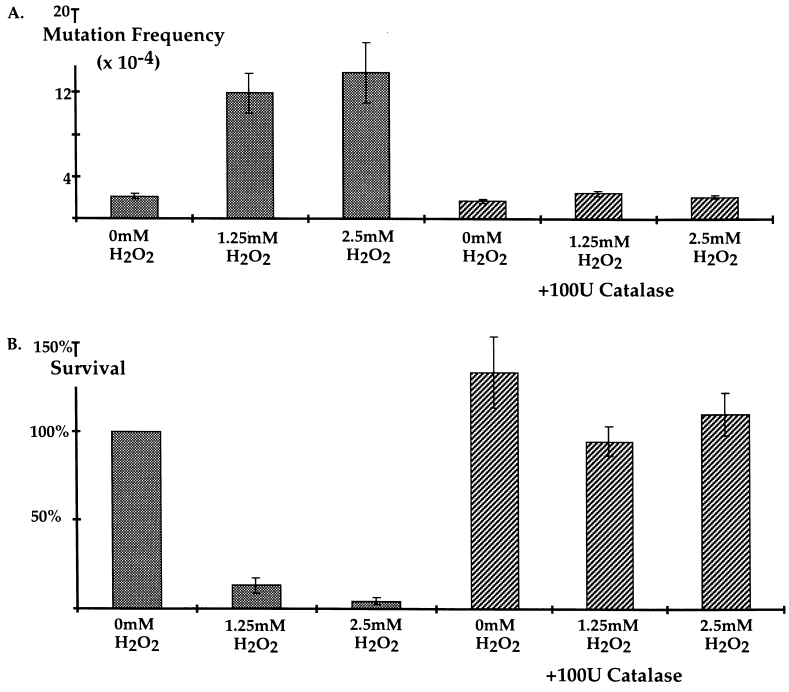

Fig. 2. Hydrogen peroxide induced microsatellite instability and cytotoxicity are abolished by catalase. Log phase cultures of *E. coli* strain BL21 were exposed to hydrogen peroxide in the presence or absence of 100 units of catalase, a hydrogen peroxide scavenger. Mutation frequency was calculated as the ratio of carbenicillin-resistant mutants recovered per tetracycline-resistant survivor to the damage. Survival was determined by plating dilutions of treated or untreated cultures on 2XYT or tetracycline plates. Results represent the mean of three independent experiments and error bars represent the error of the mean.

Table 1
Effect of iron on H_2O_2-induced cytotoxicity and microsatellite mutation frequency in vivo

	0 $FeSO_4$	Survival (%)	2.8 μg/ml $FeSO_4$	Survival (%)	8.3 μg/ml $FeSO_4$	Survival (%)	25 μg/ml $FeSO_4$	Survival (%)
BL21								
0 H_2O_2	1.7×10^{-4}	100[a]	1.7×10^{-4} (1×)	112	2.1×10^{-4} (1.2×)	95	1.7×10^{-4} (1×)	130
1.25 mM	15.7×10^{-4} (9×[b])	14	34.8×10^{-4} (20×)	7.2	12.1×10^{-4} (7×)	18	9.1×10^{-4} (5.4×)	18
2.5 mM	22.2×10^{-4} (13×)	3.4	25.7×10^{-4} (15×)	5.2	12×10^{-4} (7×)	17	13.5×10^{-4} (8×)	14
AB1157								
0 H_2O_2	5.5×10^{-4}	100	6.9×10^{-4} (1.2×)	94	6.5×10^{-4} (1.2×)	147	9×10^{-4} (1.6×)	117
1.25 mM	51.4×10^{-4} (9×)	29	6.9×10^{-4} (1.2×)	36	53.4×10^{-4} (10×)	52	47.4×10^{-4} (9×)	47
2.5 mM	74.6×10^{-4} (13×)	33	61.5×10^{-4} (11×)	27	47.8×10^{-4} (9×)	41	68.3×10^{-4} (12×)	30

[a] Percent survival is reported as relative to survival in the absence of hydrogen peroxide and iron sulfate.

[b] Mutation frequencies were calculated as carbenicillin-resistant mutants divided by the number of tetracycline-resistant survivors. Numbers in parentheses indicate the increase in mutation frequency relative to that observed in the absence of hydrogen peroxide and iron sulfate.

studies of H_2O_2 toxicity in *E. coli*, is that the responsible reactive oxygen species is not hydroxyl radical free in solution, but rather is a ferryl radical complexed to the DNA for localized damage [39]. If the same ferryl radical is responsible for the reduction in survival and the frameshift mutagenesis induced by H_2O_2, this explanation is consistent with our findings that increasing the intracellular concentration of iron free in solution does not result in increased microsatellite frameshift mutagenesis.

3.2. Spectrum of mutations induced by hydrogen peroxide exposure

3.2.1. Relative frequency of insertions vs. deletions

The induction of frameshift mutations generated with a β-lactamase construct containing the microsatellite sequence in the +2 frame was compared with those generated with a microsatellite sequence in the −2 frame. Frameshift mutations were significantly more frequent with the +2 frame $(CA)_{11}$ construct than with the −2 frame $(CA)_{12}$ construct (Fig. 3). Frameshift mutations in repetitive sequences are predominantly characterized by insertions or deletions of the smallest integral repeat unit. In the case of the dinucleotide repeat utilized in this study, the expected frameshift mutations would be insertions or deletions of 2 or 4 bp. In the case of the +2 frame $(CA)_{11}$, the smallest frameshift mutation required to restore functional β-lactamase would be a 2-bp deletion, while a 2-bp insertion or a 4-bp deletion would be required to restore the reading frame of the −2 frame $(CA)_{12}$ construct. Our results therefore suggest that the 2-bp deletion is a much more frequent event than the 2-bp insertion or 4-bp deletion following exposure of *E. coli* to oxidative stress.

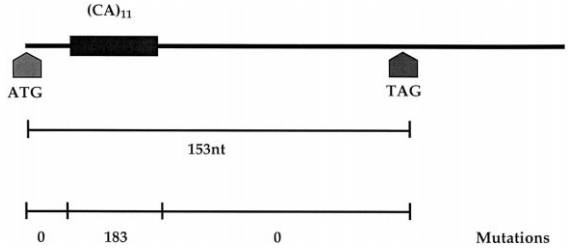

Fig. 4. Target for hydrogen peroxide-induced frameshift mutagenesis. The $(CA)_{11}$ microsatellite sequence produces a TAG stop codon 153 nt downstream from the ATG of β-lactamase, and a frameshift mutation at any location within this target could restore the reading frame and confer antibiotic resistance. Sequence analysis of 183 carbenicillin-resistant clones demonstrated that 100% of the frameshift mutations were targeted to the microsatellite sequence.

3.2.2. Sequence analysis of oxygen-induced mutations

Plasmids were recovered from carbenicillin-resistant clones and sequenced to determine the spectrum and location of the induced mutations, and to verify that the mutations induced by hydrogen perox-

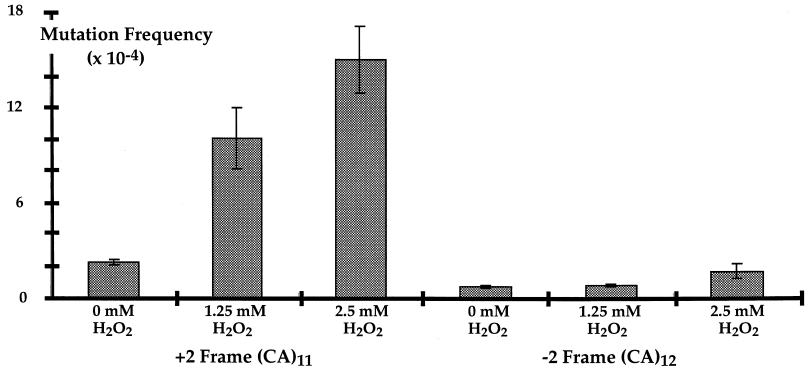

Fig. 3. Relative frequency of insertion and deletion mutations following exposure to hydrogen peroxide. The +2 frame $(CA)_{11}$ requires a 2-bp deletion or 4-bp insertion to restore resistance to carbenicillin. The −2 frame $(CA)_{12}$ requires a 2-bp insertion or a 4-bp deletion to restore carbenicillin resistance. The relative frequency of H_2O_2-induced insertions versus deletions was determined by comparing the mutation frequency of these two constructs. Results represent the mean of three independent experiments using wild type strain BL21. Results with strain AB1157 are not shown but were similar.

Table 2
Spectrum of microsatellite mutations induced by H_2O_2 in vivo

Strain	Plasmid	H_2O_2 dose (mM)	Mutation frequency ($Carb^r/Tet^r$)	Fold	Frameshift	Number
BL21	$(CA)_{11}$: +2 frame	1.25	$10 \times 10^{-4} \pm 1.9 \times 10^{-4}$	4.6×	−2 bp	24/24
		2.5	$15.3 \times 10^{-4} \pm 2 \times 10^{-4}$	7×	−2 bp	40/43
					+4 bp	1/43
					−8 bp	1/43
					+10 bp	1/43
	$(CA)_{12}$: −2 frame	1.25	$0.742 \times 10^{-4} \pm 9.4 \times 10^{-6}$	1.1×	−4 bp	18/18
		2.5	$1.56 \times 10^{-4} \pm 4.27 \times 10^{-5}$	2.3×	+2 bp	11/20
					−4 bp	9/20
AB1157	$(CA)_{11}$: +2 frame	1.25	$5.85 \times 10^{-4} \pm 1.9 \times 10^{-5}$	4.2×	−2 bp	20/20
		2.5	$8.95 \times 10^{-4} \pm 3.3 \times 10^{-4}$	6.4×	−2 bp	19/20
					+4 bp	1/20
	$(CA)_{12}$: −2 frame	1.25	$3.4 \times 10^{-4} \pm 6.7 \times 10^{-5}$	1.6×	+2 bp	10/18
					−4 bp	8/18
		2.5	$7.7 \times 10^{-4} \pm 2.8 \times 10^{-4}$	3.7×	+2 bp	9/20
					−4 bp	9/20
					+8 bp	1/20
					−10 bp	1/20

ide were consistent with the expectation for microsatellite instability. The target for frameshift mutagenesis to restore the reading frame to β-lactamase comprises 153 nucleotides from the ATG to the first stop codon incurred by the +2 frame $(CA)_{11}$ (Fig. 4). Despite this large target size, 100% of the hydrogen peroxide-induced mutations were targeted to the microsatellite sequence (Fig. 4). No mutations were observed in the flanking 131 nucleotides of non-repetitive DNA. Sequencing of non-selected, tetracycline-resistant clones confirmed the presence of unmodified $(CA)_{11}$ repeats (data not shown).

The vast majority of frameshift alterations consisted of deletions of repetitive units (Table 2). Following exposure to 1.25 mM H_2O_2, 100% of the frameshift alterations in the +2 frame construct and 78% of the frameshifts in the −2 frame construct consisted of deletions of the minimum number of integral repeats (Table 2). At the higher dose of H_2O_2, the proportion of deletion and insertion mutations was altered from that seen at the lower dose. In the case of the $(CA)_{11}$ microsatellite in the +2 frame, 93% of the frameshift alterations were 2-bp deletions, and only 2.3% were 4-bp insertions. One 8-bp deletion and one 10-bp insertion were observed at this higher dose of damage. In the case of the $(CA)_{12}$ microsatellite in the −2 frame, approximately 50% of the frameshift alterations were 2-bp insertions and 50% were 4-bp deletions. Again, this higher dose of damage produced a small percentage of larger frameshift mutations, including one 8-bp insertion and one 10-bp deletion. No significant differences were observed in the frequency or composition of frameshift mutations in the two strains analyzed, BL21 and AB1157, verifying that the microsatellite instability resulting from H_2O_2 damage is not strain-specific.

4. Discussion

The endogenous generation of H_2O_2 as a by-product of oxidative metabolism might be a significant source of mutagenesis in aerobic organisms. The intracellular level of H_2O_2 is normally maintained at acceptably low concentrations by antioxidant enzymes such as catalase. However, the production of these endogenous oxidants would be expected to be increased under conditions of oxidative stress, such as chronic inflammation, or in tumor cells that produce excess amounts of hydrogen peroxide [40].

Ulcerative colitis is a chronic inflammatory bowel disease in which cell and tissue damage is believed to be accompanied by increased production of oxygen radicals [41]. Decreased cellular antioxidant defenses have been reported in ulcerative colitis [42–

44], as have increased production of reactive oxygen intermediates that can be reduced in rat models by cyclo-oxygenase inhibitor or catalase, and in patients by catalase or 5-aminosalicylic acid [45]. Therefore, free radical mechanisms occurring in or near the colonic mucosa may contribute to the chronic inflammation and tissue damage, and may act as carcinogens or tumor promoters and contribute to the high incidence of colon carcinoma in patients suffering from chronic ulcerative colitis. Oxygen radicals mediate an important step in the initiation of acute pancreatitis. Heightened oxidative stress appears early in the course of acute pancreatitis and outlasts the clinical manifestations of the disease. The dependence of disease severity on the imbalance between reactive oxygen species and natural antioxidant defenses suggests that oxidative stress may play a pivotal role in the association of chronic pancreatitis with pancreatic cancer. Interestingly, microsatellite instability has been detected in 50% of patients with ulcerative colitis [31], and 100% of patients with pancreatitis [16]. We have therefore explored the possibility that oxidative stress is implicated in the genomic instability characterized by tumor cells and these pre-neoplastic inflammatory settings.

We have previously demonstrated that oxidative damage to DNA in vitro resulting from exposure of plasmid DNA to hydrogen peroxide is sufficient to promote microsatellite instability upon replication of the damaged DNA in *E. coli* [32]. Therefore, reactive oxygen species are capable of generating a modified DNA structure that gives rise to slipped intermediates either during DNA replication or during repair processing of the altered structure. In light of the fact that a high degree of microsatellite instability is significantly correlated with chronic inflammation [16,31], we investigated the possibility that microsatellite instability could result from oxidative damage to intact cells.

We report the induction of microsatellite instability in three independent wild type strains of *E. coli* exposed to hydrogen peroxide. The induction of microsatellite instability in these strains is notable because all of the strains utilized in this study are mismatch repair-proficient. Therefore, oxidative mutagenesis is capable of inducing DNA damage to an extent that is repaired inefficiently by the mismatch repair system, and the unrepaired lesions increase the frequency of mutations. The magnitude of mutagenesis in the in vivo system is approximately half of that observed with in vitro damage [32], most likely due to the induction of catalase in vivo. Two catalase enzymes exist in *E. coli*, one of which is induced in response to low levels of H_2O_2. Following exposure to H_2O_2, the transcription factor OxyR induces the expression of nine enzymes, including catalase and alkyl hydroperoxide reductase, which detoxify oxygen radicals [46]. Induction of this operon contributes resistance to H_2O_2 toxicity, and likely protects the cell from hydrogen peroxide-induced mutagenesis [47,48]. Despite this protective response to H_2O_2, mutagenesis was induced by H_2O_2 exposure in vivo.

The frameshift mutagenesis was abolished in the presence of catalase, verifying the integral role of reactive oxygen species in the induction of mutations, but was not increased when the intracellular concentration of free iron was increased. There are a number of possible explanations for the lack of increased H_2O_2-induced cytotoxicity and mutagenicity following exposure to $FeSO_4$. One possibility is that the intracellular iron concentration was not increased by the preincubation. This explanation seems unlikely, since two previous reports indicate that preincubation in media containing $FeSO_4$ progressively increases the iron content of bacterial cells [37,38], as much as 10-fold in the presence of 25 $\mu g/ml$ $FeSO_4$ [38]. Despite this considerable increase in intracellular iron concentration, the same study reported no increase in H_2O_2 toxicity [38], suggesting that the toxicity is independent of iron-catalyzed hydroxyl radical free in solution. This same conclusion was previously reached by Imlay et al. [39], who suggested that free hydroxyl radical was not the toxic oxidant, and rather that a ferryl radical complexed to the DNA was responsible for generating hydroxyl radical in the immediate vicinity of the DNA. If the ferryl radical is responsible for the mutagenesis in addition to the cytotoxicity of H_2O_2 damage, then increasing the concentration of iron free in solution would not be expected to increase the frequency of these mutagenic events. It is difficult to measure the amount of iron complexed to the DNA to determine whether increasing the amount of iron free in solution should also increase the amount of ferryl radical complexed to the DNA. A

third possibility is that following removal of the $FeSO_4$ medium prior to H_2O_2 damage, the intracellular iron concentration was returned to normal levels.

The oxygen-induced frameshift mutagenesis was highly specific for the microsatellite sequence, with 183 out of 183 mutants displaying frameshift mutations within the repetitive sequence. No frameshift mutations or base substitutions were identified in the 131 nucleotides of flanking non-repetitive sequence. Sequence analysis confirmed that all of the recovered mutants displayed expansions or deletions of integral repeat units consistent with the mechanism of microsatellite instability. Our previous results with in vitro H_2O_2 damage indicated that all microsatellite sequences investigated, including $(CA)_n$ dinucleotides, $(A/T)_n$ mononucleotides and $(G/C)_n$ mononucleotides were subject to oxygen-induced microsatellite instability, illustrating a lack of sequence-specificity. We therefore propose a mechanism involving an oxidative lesion that is common to all repetitive sequences, such as a single strand break. In the context of a repetitive sequence, a single strand break with a liberated terminus could give rise to slippage intermediates either due to simple denaturation and misalignment, or during repair processing of the break.

The mismatch repair system plays a critical role in genomic stability by correcting base pair misincorporations during replication, and by preventing frameshift mutations resulting from slippage intermediates in repetitive sequences. Although the traditional view holds that mismatch repair corrects mismatches between normal DNA bases, a recent report suggests that the mismatch repair system may recognize mispairs involving oxidatively damaged bases [49]. Using a set of isogenic yeast strains lacking mismatch repair and containing different point mutations in the *cyc1* gene, mutation rates were found to be highest with reversions likely to result from oxidative damage to DNA. These reversion rates were reduced dramatically when the strains were grown anaerobically to reduce spontaneous oxidative damage in the cells. Thus, mismatch repair appears to be involved in preventing mutations that result from oxidative damage to DNA. Our results complement those of Earley and Crouse by demonstrating that even in the presence of functional mismatch repair, oxidative damage is capable of producing mutations characteristic of mismatch repair deficiency. We have demonstrated that oxidative damage contributes to microsatellite instability both in vitro and in vivo. The most direct explanation is that the unrepaired oxidative lesions increase the frequency of slippage when the lesion is present in the template strand during DNA replication or repair. Therefore, oxidative damage appears to be capable of saturating the mismatch repair system, and may be a significant factor in the genomic instability associated with chronic inflammation and tumor progression.

Acknowledgements

We thank Ann Blank for critical analysis during the course of this investigation, Bernard Weiss, Susan Wallace, and Leona Samson for providing bacterial strains, and Greg Olsen for technical assistance. These studies were supported by National Institutes of Health/National Institute on Aging Grant CA80993 to LAL and by National Institutes of Health Grant CA69732-02 to ALJ.

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