[CANCER RESEARCH 61, 7603–7607, October 15, 2001]

Hypoxia-induced Enrichment and Mutagenesis of Cells That Have Lost DNA Mismatch Repair[1]

Akira Kondo, Roohangiz Safaei, Misako Mishima, Hannes Niedner, Xinjian Lin, and Stephen B. Howell[2]

Department of Medicine and the Cancer Center, University of California San Diego, La Jolla, California 92093-0058

ABSTRACT

Loss of DNA mismatch repair (MMR) increases the risk of spontaneous mutations. We sought to determine whether there was an interaction between hypoxia and MMR deficiency that might contribute to the phenomenon of tumor progression. Human colon carcinoma HCT116+ch2 (MMR-deficient) and HCT116+ch3 (MMR-proficient) sublines were exposed for varying periods of time to an environment of <0.1% O_2 and pH as low as 6.1. When a population containing 5% MMR-deficient cells and 95% MMR-proficient cells was subjected to hypoxia for 72 h, the MMR-deficient cells were enriched by a factor of 2-fold in the surviving population, whereas no enrichment was detected in cells maintained under aerobic conditions. The potential of hypoxia to destabilize the genome was determined by measuring the frequency of clones in the surviving population resistant to very high concentrations of 6-thioguanine or cisplatin. A 72-h exposure to hypoxia did not increase the frequency of resistant clones in the MMR-proficient cells but produced a 7.8-fold increase in 6-thioguanine-resistant clones and a 2.5-fold increase in cisplatin-resistant clones in the MMR-deficient cells. Loss of MMR increased the frequency of mutations in a reporter vector sensitive to frameshift mutations in a microsatellite sequence. Exposure to hypoxia for a time period as short as 48 h further increased the number of mutations in both cell types, but the absolute number of mutants was higher in the MMR-deficient cells. These results indicate that hypoxia and its accompanying low pH enrich for MMR-deficient cells and that loss of MMR renders human colon carcinoma cells hypersensitive to the ability of hypoxia to induce microsatellite instability and generate highly drug-resistant clones in the surviving population.

INTRODUCTION

As cancers grow, they often exhibit increasingly malignant behavior, presumably as the result of the acquisition of additional genetic alterations. The tumor microenvironment is characterized by regions of both fluctuating and chronic hypoxia, low pH, and nutrient deprivation (1, 2). The extent of hypoxia has been reported to affect tumor cell invasiveness, metastasis, and the risk of recurrence (3). Hypoxia and its associated low pH have also been observed to augment the emergence of drug resistance (4). One hypothesis advanced to explain these observations is that the microenvironment of a solid tumor is itself mutagenic and may be an important source of genetic instability. Reynolds *et al.* (5) reported that the frequency of *supF* reporter gene mutations arising in cells within *in vivo* tumors was 5-fold higher than that in otherwise identical cells grown in culture and that exposure of cultured cells to hypoxia produced an elevated mutation frequency and a pattern of mutation similar to that observed in the *in vivo* tumors. The mutation frequency in the *HGPRT* (hypoxanthine guanine phosphoribosyl transferase) gene in tumors was also reported to be higher than that found in cultured cells (6). Several groups have observed an increased frequency of specific types of drug resistance among cells exposed to hypoxia or low pH, effects attributed to amplification of genes encoding the targets of these drugs (7–9). However, the mechanisms by which the tumor microenvironment induces genetic instability and increased mutagenesis are not well understood.

It is known that hypoxic cells have numerous alterations in metabolic activity (10). These metabolic changes are assumed to lead to conditions that either cause increased damage to DNA (10) or compromise DNA repair processes (11). Cells subjected to repeated cycles of hypoxia and reoxygenation undergo transiently increased levels of intracellular reactive oxygen species that can damage DNA bases and may be a cause of mutation (12). In addition to generating mutations, the hypoxic environment may favor the outgrowth of clones with specific types of genetic defects. Graeber *et al.* (13) reported that hypoxia favors the expansion of p53-deficient variants that demonstrate reduced apoptosis in response to injury that is normally cytotoxic.

The MMR[3] system is particularly important to the maintenance of genomic integrity. Loss of MMR function results in microsatellite instability and underlies hereditary nonpolyposis colon cancer (14–18). We and others have demonstrated that loss of MMR causes resistance to the cytotoxic effects of many platinum-containing drugs (19–21) and also renders cells hypersensitive to the mutagenic effects of cisplatin (22). We recently showed that loss of MMR renders cells both resistant to the cytotoxicity of H_2O_2 and hypersensitive to the mutagenic effect of this oxidative stress (23). We report here that hypoxia, along with its associated low pH, enriches for MMR-deficient cells in the surviving population and makes cells sensitive to some forms of hypoxia-induced genomic instability.

MATERIALS AND METHODS

Cell Lines and Materials. Clones of the human colorectal adenocarcinoma hMLH1-deficient cell line HCT116 that had undergone chromosome 3 transfer (HCT116/3-6, identified here as HCT116+ch3) and chromosome 2 transfer (HCT116/2-1, identified here as HCT116+ch2) were obtained from Drs. C. R. Boland, M. Koi, and T. A. Kunkel (24). These cell lines were maintained in a 5% CO_2 atmosphere at 37°C in Iscove's modified Dulbecco's medium (Irvine Scientific, Irvine, CA) supplemented with L-glutamine, 10% heat-inactivated fetal bovine serum, and 400 μg/ml Geneticin (Life Technologies, Inc., Gaithersburg, MD). All cell counts were performed in the presence of 0.1% trypan blue. The absence and presence of hMLH1 expression in these cell lines was verified by immunoblot analysis (data not shown). All cell lines tested negative for contamination with *Mycoplasma* species. Cisplatin was kindly provided by Bristol-Myers Squibb Co. (Princeton, NJ). A stock solution of 1 mM cisplatin in 0.9% NaCl was stored in the dark at room temperature.

Hypoxia. Hypoxic conditions were created using plastic chambers (AnaeroPac jar; Mitsubishi Gas Chemical Co., Inc.) modified to permit gas flushing (25). Deoxygenation was accomplished by flushing the chamber for 15 min with a mixture of 95% N_2 and 5% CO_2. To further deplete oxygen, an ascorbic acid sachet (Mitsubishi Gas Chemical Co., Inc.) was added to each chamber just before it was flushed with nitrogen/CO_2. Anaerobic conditions were confirmed using Anaero-Indicator strips that documented the oxygen content in the chamber to be between 0 and 0.1% in all experiments. To prevent evaporation of medium from the dishes, dishes of distilled water were positioned in the chamber.

Received 3/22/01; accepted 8/15/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part byNIH Grant CA78648. This work was conducted in part by the Clayton Foundation for Research-California Division. X. L. and S. B. H. are Clayton Foundation investigators.

[2] To whom requests for reprints should be addressed, at Department of Medicine 0058, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0058. Phone: (858) 822-1110; Fax: (858) 822-1111; E-mail: showell@ucsd.edu.

[3] The abbreviations used are: MMR, DNA mismatch repair; GFP, green fluorescence protein; 6TG, 6-thioguanine.

Enrichment Assay. A cell population containing 5% HCT116 MMR-deficient cells constitutively expressing GFP (26) and 95% HCT116+ch3 cells was prepared by mixing appropriate numbers of the two cell types. Enrichment assays were performed by plating 20,000 cells in 35-mm tissue culture dishes for 24 h and then exposing one set of cultures to hypoxia for 72 h at 37°C while maintaining the other under euoxic conditions. Flow cytometric analysis for determination of the fraction of cells in the population expressing GFP was performed at various times up to 8 days later. Each experiment was performed using triplicate cultures a minimum of three separate times. There was no difference in the growth rate of HCT116 and HCT116-GFP cells under euoxic or hypoxic conditions (data not shown).

Assay for Frequency of Resistant Variants. Cells were grown in hypoxanthine-aminopterin-thymidine medium containing 0.4 μM aminopterin, 16 μM thymidine, and 100 μM hypoxanthine for a minimum of 14 days before the start of the experiment to rid the population of any preexisting *HGPRT* mutants. Two million cells were seeded in 10 ml of medium in 100-mm tissue culture dishes and allowed to attach for 24 h. Cells were incubated under euoxic or hypoxic conditions for up to 72 h at 37°C. Thereafter, fresh medium was added, and the cultures were allowed to recover under euoxic conditions for 10 days to permit full expression of any mutant phenotypes. During this 10-day period, the cultures were split 2:1 as needed to keep them from becoming confluent. All of the cells were then trypsinized and seeded into each of five 100-mm tissue culture dishes at 100,000 cells/dish. A concentration of 6TG or cisplatin that resulted in a cloning efficiency of approximately 0.0002% was added to one of the sets of five dishes. For the HCT116+ch2 and HCT116+ch3 cells, respectively, these concentrations were 20 μM for 6TG and 20 μM for cisplatin. At the same time, 250 cells were seeded into each of three 60-mm dishes in drug-free medium for determination of cloning efficiency. After 14 days, colonies were counted after staining with 0.1% crystal violet. The frequency of drug-resistant variants/clonogenic cell was calculated from the following equation: resistant frequency $= a/(b \times 10^6)$, where a is the number of colonies present in the five drug-treated dishes, and b is the cloning efficiency. Each experiment was performed a minimum of three times, and the data are presented as the mean $\pm$ SD.

Host Cell Microsatellite Instability Assay. The pZCA29 vector (27) was obtained from Dr. T. M. Runger (Department of Dermatology, University of Gottingen, Gottingen, Germany). Two million HCT116+ch2 and HCT116+ch3 cells were transfected with 50 μg of pZCA29 by lipofection using the LipofectAMINE 2000 Reagent (Life Technologies, Inc., Grand Island, NY) on day 1. Starting 24 h after transfection, the cells were incubated under euoxic or hypoxic conditions for 48 h, and replicated pZCA29 was recovered from the transfected cells on day 7. pZCA29 plasmid DNA was purified by a modified alkaline lysis procedure using the EndoFree Plasmid Maxi Kit (Qiagen, Malenicia, CA). Epicurian^R Coli XL1-Blue Supercompetent Cells (Stratagene, Cedar Creek, TX) were transformed with recovered pZCA29 and then selected on Luria-Bertani agar plates containing 5-bromo-4-chloro-3-indolyl-β-galactosidase and isopropyl-β-D-thiogalactoside (Life Technologies, Inc.), and ampicillin. The total number of blue and white colonies was counted, and the mutant frequency was calculated as the mean of the total number of blue colonies divided by the mean of the total number of colonies.

Statistical Analysis. Frequency and rate data were analyzed by use of a two-sided paired Student's t test with the assumption of unequal variances. Differences were considered statistically significant if P was <0.05.

RESULTS

Hypoxia Enriches for MMR-deficient Cells. Fig. 1*A* shows that when HCT116+ch2 and HCT116+ch3 cells were incubated under hypoxic conditions for up to 72 h, the pH of the medium dropped to 6.1, whereas it remained above 7.0 in cells cultured under euoxic conditions. Fig. 1*B* shows that the number of attached cells capable of excluding trypan blue in the cultures of both MMR-proficient and MMR-deficient cells was substantially decreased after 48 and 72 h of hypoxia, reaching a low of 8% of that in the simultaneously plated euoxic cultures. A population of 5% MMR-deficient GFP-expressing cells and 95% MMR-proficient cells was prepared by mixing the appropriate numbers of each cell type. Aliquots of the mixed population were then either subjected to 72 h of hypoxia or maintained under euoxic conditions. The fraction of MMR-deficient GFP-expressing cells in both treatment groups was measured at various time points up to 8 days later. Fig. 2 shows that there was little change in the fraction of MMR-deficient cells when they were grown under euoxic conditions. In contrast, whereas no enrichment for MMR-deficient cells occurred during the period of hypoxia *per se*, 6 and 8 days later, GFP-expressing MMR-deficient cells were 1.5- and 2.0-fold more abundant, respectively, ($P = 0.007$). Thus, although there was no difference in the reduction in cell number in the cultures during the period of hypoxia itself, there was substantial enrichment for the MMR-deficient cells during the recovery phase.

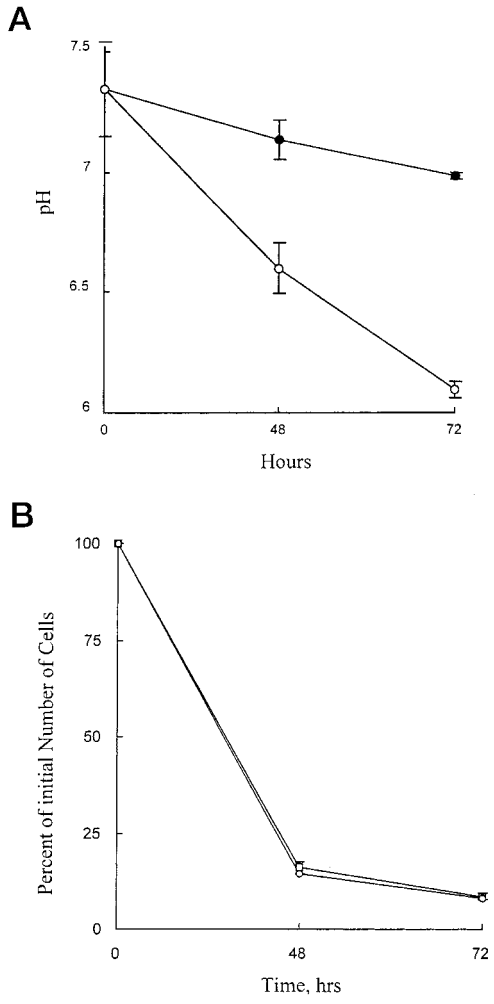

Fig. 1. Effect of hypoxia on pH and cell number. *A*, change in medium pH during culture of a population of 5% HCT116-GFP and 95% HCT116+ch3 cells under aerobic (●) or hypoxic (○) conditions. *B*, change in cell number during culture of HCT116+ch2 and HCT116+ch3 cells under hypoxic conditions. Each data point is the mean $\pm$ SD of three experiments performed with triplicate cultures. Where the SD bar is not visible, it is smaller than the symbol.

Hypoxia Generates Drug-resistant Variants in MMR-deficient Cells. As one measure of the potential of hypoxia to destabilize the genome, measurements were made of the fraction of clonogenic cells in the surviving population that were resistant to very high concentrations of 6TG or cisplatin 14 days after a hypoxic exposure of 48 or 72 h. At this late time point, the acute effects of the hypoxic exposure on proliferation rate, as measured by cell cycle phase distribution, had resolved (data not shown). Fig. 3*A* presents the ratio of the fraction of resistant colonies/10^6 clonogenic cells in the hypoxia-exposed relative to the euoxia-exposed populations. Neither a 48- nor 72-h exposure to hypoxia increased the frequency of 6TG-resistant clones in the MMR-

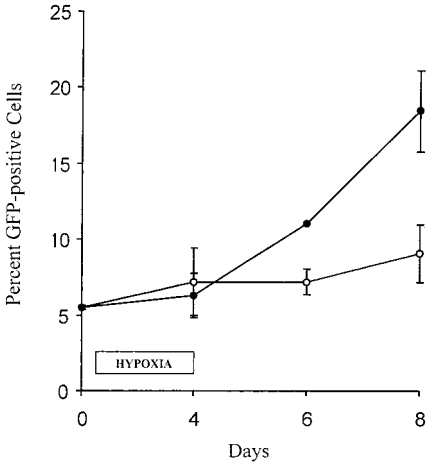

Fig. 2. Enrichment for MMR-deficient cells by hypoxia. A population containing 5% MMR-deficient GFP-expressing cells and 95% MMR-proficient cells was cultured for 48 h under hypoxic (●) or euoxic conditions (○), and the fraction of GFP-expressing cells in the population was monitored during subsequent growth under euoxic conditions. Each point is the mean ± SD of three experiments performed with triplicate cultures.

proficient HCT116+ch3 cells by a statistically significant amount. In contrast, in the MMR-deficient HCT116+ch2 cells, a 48-h exposure to hypoxia increased the frequency of 6TG-resistant clones by 2.3 ± 0.1-fold ($P < 0.02$), and a 72-h exposure to hypoxia augmented this effect further producing a 7.8 ± 2.0-fold increase ($P < 0.001$). As shown in Fig. 3*B*, a similar albeit smaller effect was observed for cisplatin-resistant clones. In the MMR-proficient cells, neither a 48- nor 72-h exposure increased the number of cisplatin-resistant clones in the surviving population. However, in the MMR-deficient cells, although a 48-h exposure to hypoxia did not increase the number of resistant clones, a 72-h exposure produced a 2.4 ± 0.3-fold increase relative to the number found in concurrently plated euoxic cells. Thus, loss of MMR function sensitized human colon carcinoma cells to the ability of hypoxia to generate clones in the surviving population that were highly resistant to two different classes of chemotherapeutic agents.

Hypoxia Augments Microsatellite Instability in MMR-deficient Cells. Another way to assess the effect of loss of MMR function on sensitivity to the mutagenic potential of hypoxia is to measure its ability to produce insertion/deletion mutations in a microsatellite sequence. The effect of hypoxia on the stability of a microsatellite sequence introduced in the form of an episomally replicating shuttle vector was compared in the MMR-proficient and -deficient cells. The pZCA29 vector contains a 94-bp insertion that includes a 28-bp CA repeat tract and a 30-bp GT repeat tract arranged palindromically. This insertion renders the coding sequences of a β-galactosidase reporter gene out of frame. The vector also contains the SV40 T antigen, origin, and enhancer to allow episomal replication in the human cells. In the absence of a frameshift mutation generated during replication of the vector in the tumor cell, β-galactosidase is not expressed when the plasmid is rescued from the mammalian cell and introduced into an appropriate bacterial strain. However, insertions or deletions that correct the reading frame permit expression of β-galactosidase when the vector is transferred to the bacteria. The fraction of the plasmids containing such a mutation after passage through the tumor cells can be quantified as the fraction of blue *versus* white bacterial colonies.

The pZCA29 vector was transfected into the tumor cells, and 24 h later, one aliquot was exposed to hypoxia for 48 h, whereas the other was maintained under euoxic conditions. The vector was recovered from the tumor cells 7 days after transfection. Fig. 4 shows that, as expected, the frequency of pZCA29 mutations under euoxic conditions was higher in the MMR-deficient cells than in the euoxic MMR-proficient cells. Interestingly, exposure to hypoxia increased the mutant frequency in both MMR-proficient and MMR-deficient cells. In the case of the MMR-proficient cells, the increase could not

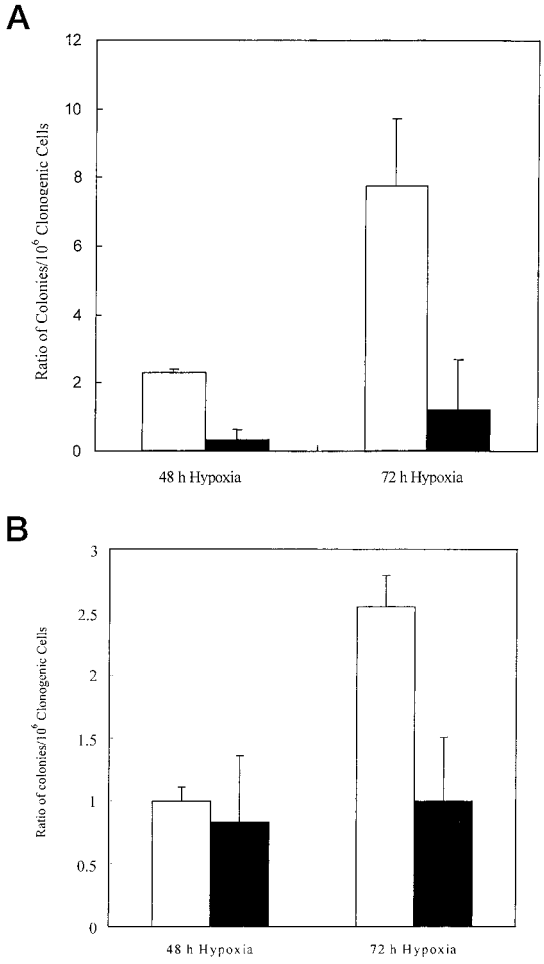

Fig. 3. Effect of the loss of MMR function on sensitivity to the mutagenic effect of hypoxia and low pH. The histogram shows the ratio of the number of 6TG-resistant (*A*) and cisplatin-resistant (*B*) colonies:10^6 clonogenic cells for the HCT116 sublines (□, MMR deficient; ■, MMR proficient). Each symbol represents the mean of three experiments performed with quintuplicate cultures. *Vertical bars*, SD.

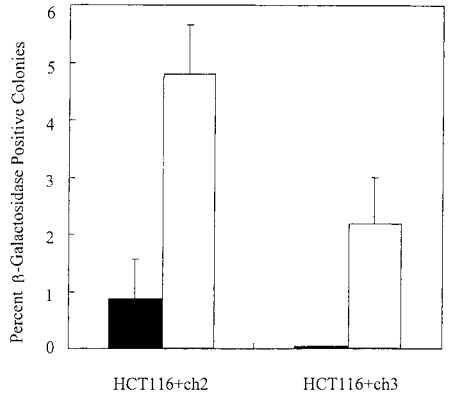

Fig. 4. Microsatellite instability in the HCT116 sublines. The histogram shows the frequency of blue bacterial colonies obtained after passage of pZCA29 in the tumor cells and subsequent transduction into permissive bacteria. Plasmid DNA was isolated from the cells at the 7 days after transfection. ■, euoxic conditions; □, 48 h hypoxia. Each symbol represents the mean of at least three experiments. *Vertical bars*, SD.

be quantified as a ratio because no blue colonies were obtained under euoxic conditions; however, in the case of the MMR-deficient cells, the increase was by a factor of 5.5-fold ($P < 0.0001$). Thus, whereas hypoxia induced microsatellite instability in both cell types, the absolute number of mutants obtained was substantially larger when MMR function had been disabled.

DISCUSSION

Marked fluctuations in oxygen availability and pH are common in tumor microenvironments, as are cells that have lost MMR function. In this study, we sought to determine whether there was an interaction between hypoxia and MMR deficiency that might contribute to the phenomenon of tumor progression. Using an assay sensitive to small changes in the fraction of MMR-deficient cells in a population, we found that even a single episode of hypoxia followed by reoxygenation was sufficient to produce a 2-fold enrichment for MMR-deficient cells in the surviving population. The significance of this for the emergence of drug resistance is underscored by the fact that MMR-deficient cells are directly resistant to several classes of chemotherapeutic agents (21) and hypersensitive to the ability of both exogenous (22) and endogenous mutagens (23) to generate clones with very high level resistance to yet other classes of cytotoxic drugs.

The mechanism by which hypoxia-induced enrichment occurs was not defined by the experiments reported here, but the available data are consistent with the concept that the MMR-deficient cells recover proliferation more rapidly after the hypoxic insult. Both hypoxia itself and the generation of reactive oxygen species during subsequent reoxygenation are capable of triggering apoptosis. It has already been established that cells in which the apoptotic mechanism is partially disabled due to loss of p53 function are resistant to hypoxia-induced cell death (13). Through its ability to function as a detector of DNA damage, the MMR system also plays an important role in activating apoptosis through both p53-dependent and -independent pathways (28, 29). Reactive oxygen species are formed during reperfusion of ischemic tissues (30, 31). Recently, it has been directly demonstrated that loss of MMR function causes resistance to the cytotoxic effect of H_2O_2 (23). The fact that loss of MMR substantially impaired the ability of H_2O_2 to signal an increase in p53 level indicates that the MMR proteins are not only capable of recognizing an 8-oxoguanine mismatch but are also capable of generating a proapoptotic injury signal (23).

The results reported here demonstrate that hypoxia, together with its accompanying acidosis, induces some degree of genomic instability and that the MMR system normally serves to limit hypoxia-induced mutations. In this study, the effect of a cycle of hypoxia followed by reoxygenation on genomic stability was measured by both its ability to increase the fraction of clonogenic cells that are highly drug resistant in the surviving population and its effect on microsatellite stability. Whereas hypoxia did not increase the frequency of 6TG-resistant clones in the MMR-proficient cells, it produced a marked increase in the frequency of such clones in the MMR-deficient cells, and this effect increased with the duration of hypoxia. The 6TG resistance of these clones was not formally demonstrated to be due to a mutation; however, mutation in the *HGPRT* gene is by far the most common etiology of very high level resistance to 6TG, and mutations in this gene have been reported previously in response to a hostile microenvironment (6). Hypoxia was also noted to increase the frequency of cisplatin-resistant clones in MMR-deficient cells. The genes that mediate such high level cisplatin resistance are not known; thus, this observation provides only indirect evidence of a genome-destabilizing effect of hypoxia. However, it is of substantial concern from the vantage point of the chemotherapist that such levels of cisplatin resistance can be generated by a single exposure to hypoxia.

The ability of a cycle of hypoxia and reoxygenation to induce microsatellite instability was clearly demonstrated by use of the pZCA29 vector. This reporter system was sensitive enough to detect a significant degree of hypoxia-induced microsatellite instability even in the MMR-proficient cells, although the degree of destabilization was substantially greater in the MMR-deficient cells. From the effects on the generation of 6TG- and cisplatin-resistant variants and the frameshift mutations reported by the pZCA29 vector, we infer that cells that survive an episode of hypoxia and low pH exposure are likely to carry an increased burden of mutations throughout their genome. Insertion/deletion mutations, such as those detected by pZCA29, are particularly devastating to the cell because when they occur in a coding sequence, they shift the reading frame, often resulting in complete loss of function of the protein encoded by that gene.

What is the mechanism by which loss of MMR permits hypoxia and low pH to be more mutagenic? Deletions and point mutations such as C:G→A:T and T:A→G:C transversions have been found to arise more frequently in cells exposed to hypoxia (5, 11). Generation of oxygen free radicals during reoxygenation can produce excess levels of 8-oxyoguanine in DNA, and this adduct has been shown to cause miscoding for A leading to C:G→A:T and T:A→G:C transversions (32). 8-Oxoguanine plays an important role in the mutagenesis generated by H_2O_2 (33, 34); recently, it has been directly demonstrated that loss of MMR function increases the mutagenic effect of H_2O_2 (23). MMR function prevents accumulation of DNA damage generated by oxidative stress (35, 36), and MMR can correct 8-oxoguanine:A mismatches in *Escherichia coli* (37). These observations indicate that the MMR system may play a role in the repair of 8-oxoguanine mismatches in DNA in addition to repairing single-base mismatches and small mismatched loops. Thus, with loss of MMR function, one would anticipate the persistence of these mutagenic lesions in the DNA and fixation of mutations at the next S phase. Recent reports have also disclosed a possible second mechanism by which MMR may prevent hypoxia-induced mutagenesis. A series of mammalian and lower organism DNA polymerases have now been identified that can bypass adducts in DNA, producing mutations as they do so (38, 39). For example, human polymerase η can efficiently bypass 8-oxoguanine adducts, incorporating an A or a C opposite the lesion with similar proficiency (40), and polymerase κ can incorporate A and, less frequently, C opposite such an adduct (41). There is some evidence that the MMR system may play a role in preventing such mutagenic bypass replication (42, 43).

Although the above discussion has focused on damage produced by reactive oxygen species as a putative mediator of the mutagenesis produced by a cycle of hypoxia and reoxygenation, there is evidence that the low pH that accompanies hypoxia is also important. Low pH can alter the structure and function of a variety of cellular proteins, including DNA polymerases (44, 45). Several studies have reported synergistic effects of low oxygen and low pH on cellular energy metabolism and cell viability (46, 47). In some models, decreased repair of damaged DNA and induction of cell death was very much greater when hypoxia was accompanied by low pH (10, 48). It is thus reasonable to hypothesize that in solid tumors, the influence of hypoxia and low pH on tumor progression may be even more important than either factor alone. Additional studies directed at dissecting the contributions of hypoxia, acidosis, and the formation of oxygen free radicals during reoxygenation would be of particular interest.

In this study, we used the MLH1-deficient HCT116 cells and a chromosome 3 complemented subline as models of MMR-deficient and MMR-proficient phenotypes. Because transfer of chromosome 3 introduced other genes in addition to a wild-type copy of MLH1, some

caution must be exercised in interpreting the results of this study. Nevertheless, this cell system has now been used very extensively in a wide variety of studies of MMR (24, 49–51), and with respect to the types of measurements made in this study, the differences between the phenotypes do in fact appear to be attributable to the restoration of a functional MMR system in the HCT116+ch3 cells.

Very hypoxic tumors often follow an aggressive clinical course (3). The ability of hypoxia and its accompanying low pH to simultaneously select for the overgrowth of MMR-deficient cells and more readily generate variants resistant to drugs commonly used to treat these tumors suggests that the presence of such cells in a tumor may predispose patients to the rapid emergence of drug resistance and treatment failure.

REFERENCES

1. Moulder, J. E., and Rockwell, S. Tumor hypoxia: its impact on cancer therapy. Cancer Metastasis Rev., *5:* 313–341, 1987.
2. Rockwell, S. Use of hypoxia-directed drugs in the therapy of solid tumors. Semin. Oncol., *19:* 29–40, 1992.
3. Rofstad, E. K. Microenvironment-induced cancer metastasis. Int. J. Radiat. Biol., *76:* 589–605, 2000.
4. Teicher, B. A. Hypoxia and drug resistance. Cancer Metastasis Rev., *13:* 139–168, 1994.
5. Reynolds, T. Y., Rockwell, S., and Glazer, P. M. Genetic instability induced by the tumor microenvironment. Cancer Res., *56:* 5754–5757, 1996.
6. Wilkinson, D., Sandhu, J. K., Breneman, J. W., Tucker, J. D., and Birnboim, H. C. Hprt mutants in a transplantable murine tumour arise more frequently *in vivo* than *in vitro*. Br. J. Cancer, *72:* 1234–1240, 1995.
7. Rice, G. C., Ling, V., and Schimke, R. T. Frequencies of independent and simultaneous selection of Chinese hamster cells for methotrexate and doxorubicin (Adriamycin) resistance. Proc. Natl. Acad. Sci. USA, *84:* 9261–9264, 1987.
8. Rice, G. C., Hoy, C., and Schimke, R. T. Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA, *83:* 5978–5982, 1986.
9. Schlappack, O. K., Zimmermann, A., and Hill, R. P. Glucose starvation and acidosis: effect on experimental metastatic potential. DNA content and MTX resistance of murine tumour cells. Br. J. Cancer, *64:* 663–670, 1991.
10. Yuan, J., and Glazer, P. M. Mutagenesis induced by the tumor microenvironment. Mutat. Res., *400:* 439–446, 1998.
11. Yuan, J., Narayanan, L., Rockwell, S., and Glazer, P. M. Diminished DNA repair and elevated mutagenesis in mammalian cells exposed to hypoxia and low pH. Cancer Res., *60:* 4372–4376, 2000.
12. Welbourn, C. R., Goldman, G., Paterson, I. S., Valeri, C. R., Shepro, D., and Hechtman, H. B. Pathophysiology of ischaemia reperfusion injury: central role of the neutrophil. Br. J. Surg., *78:* 651–655, 1991.
13. Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W., and Giaccia, A. J. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature (Lond.), *379:* 88–91, 1999.
14. Fishel, R., Lescoe, M. K., Rao, M. R. S., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M., and Kolodner, R. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell, *75:* 1027–1038, 1993.
15. Leach, F. S., Nicolaides, N. C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R. E., Peltomaki, P., Sistonen, P., Aaltonen, L. A., Nystrom-Lahti, M., Guan, X-Y., Zhang, J., Meltzer, P. S., Yu, J-W., Kao, F-T., Chen, D. J., Cerosaletti, K. M., Fournier, R. E. K., Todd, S., Lewis, T., Leach, R. J., Naylor, S. L., Weissenbach, J., Mecklin, J-P., Jarvinen, H., Petersen, G. M., Hamtilton, S. R., Green, J., Jass, J., Watson, P., Lynch, H. T., Trent, J. M., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell, *75:* 1215–1225, 1993.
16. Nicolaides, N. C., Papadopoulos, N., Liu, B., Wei, Y-F., Carter, K. C., Ruben, S. M., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Dunlop, M. G., Hamilton, S. R., Petersen, G. M., de la Chapelle, A., Vogelstein, B., and Kinzler, K. W. Mutations of two *PMS* homologues in hereditary nonpolyposis colon cancer. Nature (Lond.), *371:* 75–80, 1994.
17. Papadopoulos, N., Nicolaides, N. C., Wei, Y-F., Ruben, S. M., Carter, K. C., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Hamilton, S. R., Petersen, G. M., Watson, P., Lynch, H. T., Peltomaki, P., Mecklin, J-P., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. Mutation of a mutL homolog in hereditary colon cancer. Science (Wash. DC), *263:* 1625–1629, 1994.
18. Fishel, R., and Kolodner, R. D. Identification of mismatch repair genes and their role in the development of cancer. Curr. Opin. Genet. Dev., *5:* 382–395, 1995.
19. Anthoney, D. A., McIlwrath, A. J., Gallagher, W. M., Edlin, A. R. M., and Brown, R. Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumor cells. Cancer Res., *56:* 1374–1381, 1996.
20. Drummond, J. T., Anthoney, A., Brown, R., and Modrich, P. Cisplatin and Adriamycin resistance are associated with MutLα and mismatch repair deficiency in an ovarian tumor cell line. J. Biol. Chem., *271:* 19645–19648, 1996.
21. Fink, D., Nebel, S., Aebi, S., Zheng, H., Cenni, B., Nehme, A., Christen, R., and Howell, S. The role of DNA mismatch repair in platinum drug resistance. Cancer Res., *56:* 4881–4886, 1996.
22. Lin, X., and Howell, S. B. Effect of loss of DNA mismatch repair on development of topotecan-, gemcitabine-, and paclitaxel-resistant variants after exposure to cisplatin. Mol. Pharmacol., *56:* 390–395, 1999.
23. Lin, X., Ramamurthi, K., Mishima, M., Kondo, A., and Howell, S. B. p53 interacts with the DNA mismatch repair system to modulate the cytotoxicity and mutagenicity of hydrogen peroxide. Mol. Pharmacol., *58:* 1222–1229, 2000.
24. Koi, M., Umar, A., Chaudan, D. P., Cherian, S. P., Carethers, J. M., Kunkel, T. A., and Boland, C. R. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces *N*-methyl-*N*′-nitro-*N*-nitroguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res., *54:* 4308–4312, 1994.
25. Kamiya, T., Kwon, A. H., Kanemaki, T., Matsui, Y., Uetsuji, S., Okumura, T., and Kamiyama, Y. A simplified model of hypoxic injury in primary cultured rat hepatocytes. In Vitro Cell Dev. Biol. Anim., *34:* 131–137, 1998.
26. Fink, D., Zheng, H., Nebel, S., Norris, P. S., Aebi, S., Lin, T-P., Nehme, A., Christen, R. D., Haas, M., MacLeod, C. L., and Howell, S. B. *In vitro* and *in vivo* resistance to cisplatin in cells that have lost DNA mismatch repair. Cancer Res., *57:* 1841–1845, 1997.
27. Diem, C., and Runger, T. M. A novel plasmid shuttle vector for the detection and analysis of microsatellite instability in cell lines. Mutat. Res., *407:* 117–124, 1998.
28. Li, G. M. The role of mismatch repair in DNA damage-induced apoptosis. Oncol. Res., *11:* 393–400, 1999.
29. Hickman, M. J., and Samson, L. D. Role of DNA mismatch repair and p53 in signaling induction of apoptosis by alkylating agents. Proc. Natl. Acad. Sci. USA, *96:* 10764–10769, 1999.
30. Sussman, M. S., and Bulkley, G. B. Oxygen-derived free radicals in reperfusion injury. Methods Enzymol., *186:* 711–723, 1990.
31. Zimmerman, B. J., and Granger, D. N. Reperfusion injury. Surg. Clin. N. Am., *72:* 65–83, 1992.
32. Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., and Loeb, L. A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G—-T and A—-C substitutions. J. Biol. Chem., *267:* 166–172, 1992.
33. Bessho, T., Tano, K., Nishmura, S., and Kasai, H. Induction of mutations in mouse FM3A cells by treatment with riboflavin plus visible light and its possible relation with formation of 8-hydroxyguanine (7,8-dihydro-8-oxoguanine) in DNA. Carcinogenesis (Lond.), *14:* 1069–1071, 1993.
34. Demple, B., and Harrison, L. Repair of oxidative damage to DNA: enzymology and biology. Annu. Rev. Biochem., *63:* 915–948, 1994.
35. deWeese, T. L., Shipman, J. M., Larrier, N. A., Buckley, N. M., Kidd, L. R., Groopman, J. D., Cutler, R. G., Riele, H. T., and Nelson, W. G. Mouse embryonic stem cells carrying one or two defective Msh2 alleles respond abnormally to oxidative stress inflicted by low-level radiation. Proc. Natl. Acad. Sci. USA, *95:* 11915–11920, 1999.
36. Jackson, A. L., Chen, R., and Loeb, L. A. Induction of microsatellite instability by oxidative DNA damage. Proc. Natl. Acad. Sci. USA, *95:* 12468–12473, 1999.
37. Zhao, J., and Winkler, M. E. Reduction of GC→TA transversion mutation by overexpression of MutS in *Escherichia coli* K-12. J. Bacteriol., *182:* 5025–5028, 2000.
38. Paz-Elizur, T., Takeshita, M., Goodman, M., O'Donnell, M., and Livneh, Z. Mechanism of translesion DNA synthesis by DNA polymerase II. Comparison to DNA polymerases I and III core. J. Biol. Chem., *271:* 24662–24669, 96.
39. Eckert, K. A., and Opresko, P. L. DNA polymerase mutagenic bypass and proofreading of endogenous DNA lesions. Mutat. Res., *424:* 221–236, 1999.
40. Zhang, Y., Yuan, F., Wu, X., Rechkoblit, O., Taylor, J. S., Geacintov, N. E., and Wang, Z. Error-prone lesion bypass by human DNA polymerase ϵ. Nucleic Acids Res., *28:* 4717–4724, 2000.
41. Zhang, Y., Yuan, F., Wu, X., Wang, M., Rechkoblit, O., Taylor, J. S., Geacintov, N. E., and Wang, Z. Error-free and error-prone lesion bypass by human DNA polymerase κ *in vitro*. Nucleic Acids Res., *28:* 4138–4146, 2000.
42. Vaisman, A., Varchenko, M., Umar, A., Kunkel, T. A., Risinger, J. I., Barrett, J. C., Hamilton, T. C., and Chaney, S. G. The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts. Cancer Res., *58:* 3579–3585, 1998.
43. McGregor, W. G. DNA repair, DNA replication, and UV mutagenesis. J. Investig. Dermatol. *4:* 1–5, 1999.
44. Eckert, K. A., and Kunkel, T. A. Fidelity of DNA synthesis catalyzed by human DNA polymerase α and HIV-1 reverse transcriptase: effect of reaction pH. Nucleic Acids Res., *21:* 5212–5220, 1993.
45. Eckert, K. A., and Kunkel, T. A. Effect of reaction pH on the fidelity and processivity of exonuclease-deficient Klenow polymerase. J. Biol. Chem., *268:* 13462–13471, 1993.
46. Boyer, M. J., and Tannock, I. F. Regulation of intracellular pH in tumor cell lines: influence of microenvironmental conditions. Cancer Res., *52:* 4441–4447, 1992.
47. Rotin, D., Robinson, B., and Tannock, I. F. Influence of hypoxia and an acidic environment on the metabolism and viability of cultured cells: potential implications for cell death in tumors. Cancer Res., *46:* 2821–2826, 1986.
48. Schmaltz, C., Hardenbergh, P. H., Wells, A., and Fisher, D. E. Regulation of proliferation-survival decisions during tumor cell hypoxia. Mol. Cell. Biol., *18:* 2845–2854, 1998.
49. Davis, T. W., Wilson-Van Patten, C., Meyers, M., Kunugi, K. A., Cuthill, S., Reznikoff, C., Garces, C., Boland, C. R., Kinsella, T. J., Fishel, R., and Boothman, D. A. Defective expression of the DNA mismatch repair protein, MLH1, alters G_2-M cell cycle checkpoint arrest following ionizing radiation. Cancer Res., *58:* 767–778, 1998.
50. Chen, W. D., Eshleman, J. R., Aminoshariae, M. R., Ma, A. H., Veloso, N., Markowitz, S. D., Sedwick, W. D., and Veigl, M. L. Cytotoxicity and mutagenicity of frameshift-inducing agent ICR191 in mismatch repair-deficient colon cancer cells. J. Natl. Cancer Inst. (Bethesda), *92:* 480–485, 2000.
51. Lin, X., Ramamurthi, K., Mishima, M., Kondo, A., Christen, R. D., and Howell, S. B. p53 modulates the effect of loss of DNA mismatch repair on the sensitivity of human colon cancer cells to the cytotoxic and mutagenic effects of cisplatin. Cancer Res., *61:* 1508–1516, 2001.

Hypoxia-induced Enrichment and Mutagenesis of Cells That Have Lost DNA Mismatch Repair

Akira Kondo, Roohangiz Safaei, Misako Mishima, et al.

Cancer Res 2001;61:7603-7607.