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Journal of Nutritional Biochemistry 13 (2002) 355–363

THE JOURNAL OF Nutritional Biochemistry

Involvement of DNA mismatch repair in folate deficiency-induced apoptosis☆

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Received 30 November 2001; received in revised form 16 January 2002; accepted 30 January 2002

Abstract

Folate is a critical factor for DNA metabolism and its deficiency is associated with a number of human diseases and cancers. Although it has been shown that folate deficiency induces genomic instability and apoptotic cell death, the underlying mechanism is largely unknown. Given the role of mismatch repair in maintaining genomic integrity, mismatch repair was tested for its involvement in folate deficiency-induced genomic instability and cell death. Cells proficient in mismatch repair were highly sensitive to folate deficiency compared with cells defective in either hMutSα or hMutLα. Since wild-type cells but not mutant cells underwent apoptosis upon extensive folate depletion, the apoptotic response is dependent on a functional mismatch repair system. Our data also indicate that p53 is required for the folate depletion-induced apoptosis. *In vitro* biochemical studies demonstrated that hMutSα specifically recognized DNA damage induced by folate deficiency, suggesting a direct participation of mismatch repair proteins in mediating the apoptotic response. We conclude that while the mismatch repair-dependent apoptosis is necessary to protect damaged cells from tumorigenesis, it may damage a whole tissue or organ, as seen in patients with megaloblastic anemia, during extensive folate deficiency.

Keywords: Folate deficiency; DNA repair; Apoptosis

1. Introduction

Folate is an important cofactor in the transfer of one-carbon moieties and plays a key role in DNA synthesis, repair, and methylation. Classic folate deficiency causes a macrocytic anemia and conspicuous megaloblastic changes in the blood and bone marrow (for a review see [1]). Growing evidence indicates that folate deficiency is also associated with an increased risk of certain types of human cancer, including colon [2], cervix [3], breast [4], pancreas [5], and acute lymphocytic leukemia [6], as well as other diseases such as neural tube defects and heart disease (reviewed in [1,6]). Although the mechanism by which folate deficiency contributes to these diseases is largely unknown, it has been demonstrated that folate deficiency induces severe DNA damage including massive uracil misincorporation and chromosome breaks [7–10]. Since 5,10-methylenetetrahydrofolate (the folate cofactor for thymidylate synthase) is the primary methyl donor for the *de novo* biosynthesis of deoxythymine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP), folate deficiency results in an increased cellular dUMP/dTMP ratio, thereby leading to DNA polymerase-mediated dUTP incorporation into DNA. In addition to genetic damage, folate deficiency results in programmed cell death. However, the underlying mechanism for this apoptotic cell death remains elusive.

Given the fact that folate depletion induces severe DNA damage and increased mutagenesis, it has been postulated that folate deficiency may be associated with defective DNA repair [11]. It is intriguing that Choi et al. [12] have recently reported the impairment of DNA excision repair in rat colon cells by folate deficiency. Cravo et al. [13] demonstrated that the status of folic acid can dominate the microsatellite status in non-neoplastic mucosa in patients with ulcerative colitis. Since microsatellite instability is a hallmark of DNA mismatch repair (MMR) deficiency, these

☆This work is supported by grant CA72956 from the National Cancer Institute and funds from Lucille P. Markey Trust (to G-M.L.).

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0955-2863/02/$ – see front matter
PII: S0955-2863(02)00178-X

results suggest a link between folate deficiency and the MMR pathway.

MMR is a critical pathway for the maintenance of genomic integrity. In *Escherichia coli*, methyl-directed MutHLS- (MutH, MutL, and MutS) dependent MMR ensures chromosome fidelity by correcting mispairs generated from biosynthetic errors and homologous recombination [14]. In humans, a MutHLS homologous MMR pathway has been characterized and defects in the human pathway are the genetic basis for certain types of human cancer, including hereditary nonpolyposis colorectal cancer (HNPCC) (for reviews see [14–18]). Previous studies have identified two human MutS complexes (hMutSα and hMutSβ) and three human MutL complexes (hMutLα, hMutLβ, and hMutLγ), with each being a heterodimer. hMSH2 interacts with hMSH6 or hMSH3 to form hMutSα [19,20] or hMutSβ [21,22], respectively, while hMLH1 interacts with hPMS2, hPMS1, or hMLH3 to constitute the three hMutL heterodimers [23–27]. Although MMR components play an important role in stabilizing genomic integrity, it remains to be established whether MMR plays any role in folate deficiency-caused genetic instability.

In this study, we provide evidence that MMR system senses folate deficiency-induced DNA damage and mediates apoptosis, as judged by the fact that folate deficiency induces DNA fragmentation in MMR proficient cells, but not in hMutSα- or hMutLα-deficient cells. *In vitro* biochemical experiments show that hMutSα efficiently recognizes DNA containing A:U or G:U pairs, a condition caused by uracil incorporation during folate deficiency. Therefore, the interaction between these abnormal base pairs and MMR proteins (hMutSα and hMutLα) may be the signal for cells to activate apoptotic machinery.

2. Materials and methods

2.1. Cell lines and cell culture

Cell lines (TK6, MT1, WI-L2-NS, HCT116, and HCT116-Chr3) were cultured in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (HyClone) at 37°C in a humidified 5% CO_2 incubator. To simulate a folate deficient condition, cells were grown in folate-free medium (GIBCO) supplemented with 10% dialyzed fetal bovine serum, in which any folic acids in the serum were removed by dialysis.

2.2. Nuclear extracts, heteroduplexes, and MMR assay

Nuclear extracts were prepared from 2×10^9 folate-depleted or folate-supplemented cells and assayed for their *in vitro* MMR activity (see below). Since cells proficient in MMR are sensitive to folate depletion (e.g., TK6 cells), subclones were established from cells that had survived 12-days of folate depletion and were expanded to 2×10^9 cells in normal medium prior to harvest for nuclear extracts as described [28,29]. Heteroduplexes containing a single G-T mismatch or a CACA tetranucleotide insertion/deletion mispair (see Fig. 2A) were prepared as described [29] by hybridizing f1MR series DNA. MMR assays were performed using 50 μg of nuclear extracts and 100 ng (24 fmol) of heteroduplex as described [28,29].

2.3. Band shift analysis

hMutSα were purified from nuclear extracts of HeLa S_3 (purchased from Cell Culture Center, Minneapolis, MN) as described [19] with modifications [30]. SDS-polyacrylamide gel electrophoresis indicated that the purified protein is near homogeneity (>99% purity) and contained only two polypeptides, 160-kDa hMSH6 and 105-kDa hMSH2, based on Coomassie Brilliant Blue staining (data not shown). Oligoduplexes used for band-shift analysis were constructed from the following 4 oligonucleotides:

1. 5′-GCTAGCAAGCT**T**TCGATTCTAGAAAT-TCGGC-3′
2. 5′-GCTAGCAAGCT**U**TCGATTCTAGAAAT-TCGGC-3′
3. 5′-GCCGAATTTCTAGAATCGA**A**AGCTT-GCTAGC-3′
4. 5′-GCCGAATTTCTAGCCTCGA**G**AGCTT-GCTAGC-3′

Homoduplex (A:T) was obtained by annealing oligonucleotides 1 and 3, and heteroduplexes A:U, G:U, and G:T were constructed by combining oligonucleotides 2 and 3, 2 and 4, and 1 and 4, respectively. Band shift assays were performed as described [30] in 25 μl reactions containing 0.5 pmol of ^{32}P-labeled oligonucleotide duplex (oligonucleotides 3 or 4 labeled), 66 ng (about 0.25 pmol) of hMutSα, and 0.1 pmol (equivalent to 20× of labeled oligos) of double stranded f1MR3 DNA. Bands were detected by autoradiography.

2.4. Cell death and apoptosis analysis

Folate deficiency-induced cell death was determined by DNA fragmentation analysis using agarose gel electrophoresis and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling or TUNEL method as described [31]. Briefly, cells were cultured in medium with (control) or without folic acids at 37°C for 0 to 12 days and 50% of the cells were harvested for DNA fragmentation analysis. The remaining cells were fixed with 1% (w/v) of paraformaldehyde in phosphate-buffered saline, and labeled by propidium iodide (PI) and FITC-dUTP in the presence of TdT, all of which were provided in the APO-DIRECT™ kit (PharMingen, San Diego, California). The cells were then analyzed by flow cytometry.

Table 1
Intracellular folic acid levels in cells grown in folate-depleted medium

Cell line	Folic acid concentration (ng/ml)			
	Day 0	Day 4	Day 8	Day 12
TK6	22.1 ± 3.1	1.4 ± 0.29	1.25 ± 0.20	N.A.
MT1	20.5 ± 3.0	1.25 ± 0.20	1.21 ± 0.11	1.02 ± 0.10
WI-L2-NS	21.8 ± 2.5	1.17 ± 0.31	1.20 ± 0.19	1.16 ± 0.15

2.5. Determination of intracellular folate concentration

Intracellular folate levels were determined by a folic acid determination kit and device (IMMULITE 2000) from Diagnostic Products Corporation (Los Angeles, CA). This procedure is a solid-phase, competitive binding chemiluminescent immunoassay. Cells were lysed by a brief sonication in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, and 5 mM EDTA. Supernatant was collected by centrifugation and incubated first with ligand-labeled folic acid and 2 mM dithiothretol (DTT) at 37°C for 30 min, and then with NaCl/KCN. The treated sample was transferred to a fresh tube containing folate binding protein (FBP) and FBP antibody-coated polystyrene beads, and incubated at 37°C for another 30 min. The beads were washed to remove free folic acid and alkaline phosphatase labeled anti-ligand, which specifically binds to the ligand-labeled folate that was bound to the beads during the preceding incubation, was added to the reaction. After removal of the unbound alkaline phosphatase conjugate by centrifugation, the sample was incubated with the chemiluminescent substrate (a phosphate ester of adamantyl dioxetane) at 37°C for 5 min, during which time the substrate underwent hydrolysis to yield an unstable intermediate that emits light. The amount of light, which is inversely proportional to the concentration of folic acid in the sample, was measured using IMMULITE 2000, and the concentration of folic acid was calculated using a standard curve.

3. Results

3.1. Folate deficiency does not impair mismatch repair pathway

To determine if folate deficiency impairs the MMR pathway, two isogenic and MMR proficient cell lines, TK6 and WI-L2-NS, were cultured in media without folic acids for indicated period of time and the relative cellular folate levels were determined as described in Materials and Methods. As expected, cellular folate concentration in both cell lines decreased as the incubation time increased. The intracellular folate levels dropped almost 20-fold in the first 4-days of culture without folic acids (Table 1). These results are consistent with previous observations [32]. Nuclear extracts of both cell lines were obtained after 12-days of folate-deficient culture and compared with those derived from their control cells in normal culture for mismatch repair capability. In WI-L2-NS cells, there was no detectable difference in MMR activity between the control extracts and the folate-deficient extracts (Fig. 1B). These results suggest that folate depletion has no effect on MMR capability. However, MMR activity was not detected in TK6 cells after 8- or 12-days of folate-deficient culture (Fig. 1C, lanes 2 and 3). Trypan blue exclusion analysis indicated that more than 95% of TK6 cells died after 12-days of folate depletion, while only 15% of WI-L2-NS cells showed blue under the same condition (not shown).

To confirm that the loss of MMR function in TK6 cells is due to cell death but not the impairment by folate depletion, TK6 cells that had survived 12-days of folate depletion were subcloned in the normal growth condition and the subclones were subjected to the *in vitro* MMR analysis. As shown in Fig. 1C, nuclear extracts derived from all individual clones (lanes 5–9) repair heteroduplexes as efficiently as nuclear extract derived from untreated TK6 cells (lane 4). Therefore, folate deficiency does not impair MMR system.

3.2. Folate deficiency-induced cell death is dependent on functional mismatch repair and p53

Although the isogenic TK6 and WI-L2-NS cell lines are proficient in MMR, the former line possesses a wild-type *p53* gene and the latter carries a mutant *p53* gene [33,34]. Both p53 and MMR system have been implicated in DNA damage induced apoptosis [35,36]. Sensitivity of TK6 cells to folate deficiency prompted us to hypothesize that MMR and p53 may be also involved in folate deficiency-induced apoptosis. To explore this possibility, cell lines TK6, WI-L2-NS, and MT1 (derived from the TK6 cell line [37] and defective in hMutSα [38,39]), were cultured in folate deficient medium and subjected to TUNEL analysis. As shown in Fig. 2, TK6 cells were much more sensitive to folate deficiency compared with hMutSα-deficient MT1 and p53-deficient WI-L2-NS cells. While almost all (more than 99%) TK6 cells died after 12 days of folate depletion, only 55% of MT1 and 22% of WI-L2-NS cells were killed under the same conditions (Fig. 2A and B). These results suggest the involvement of both p53 and hMutSα in folate depletion-induced cell death.

To further explore the role of MMR in folate deficiency-induced cell death, similar analyses were applied to the *hMLH1*-deficient HCT116 cell line and its derivative, the MMR-proficient HCT116-Chr3 cell line, which received a copy of chromosome 3 carrying the wild-type *hMLH1* gene [40]. As observed in the TK6 and MT1 cells, repair deficient HCT116 cells are much more resistant to killing by folate deficiency than repair proficient HCT116-Chr3 cells. After 8-days of folate depletion, more than 98% of HCT116-Chr3 cells were killed, but the cell death in HCT116 cells was less than 10% (Fig. 2C and D). Taken together, these results

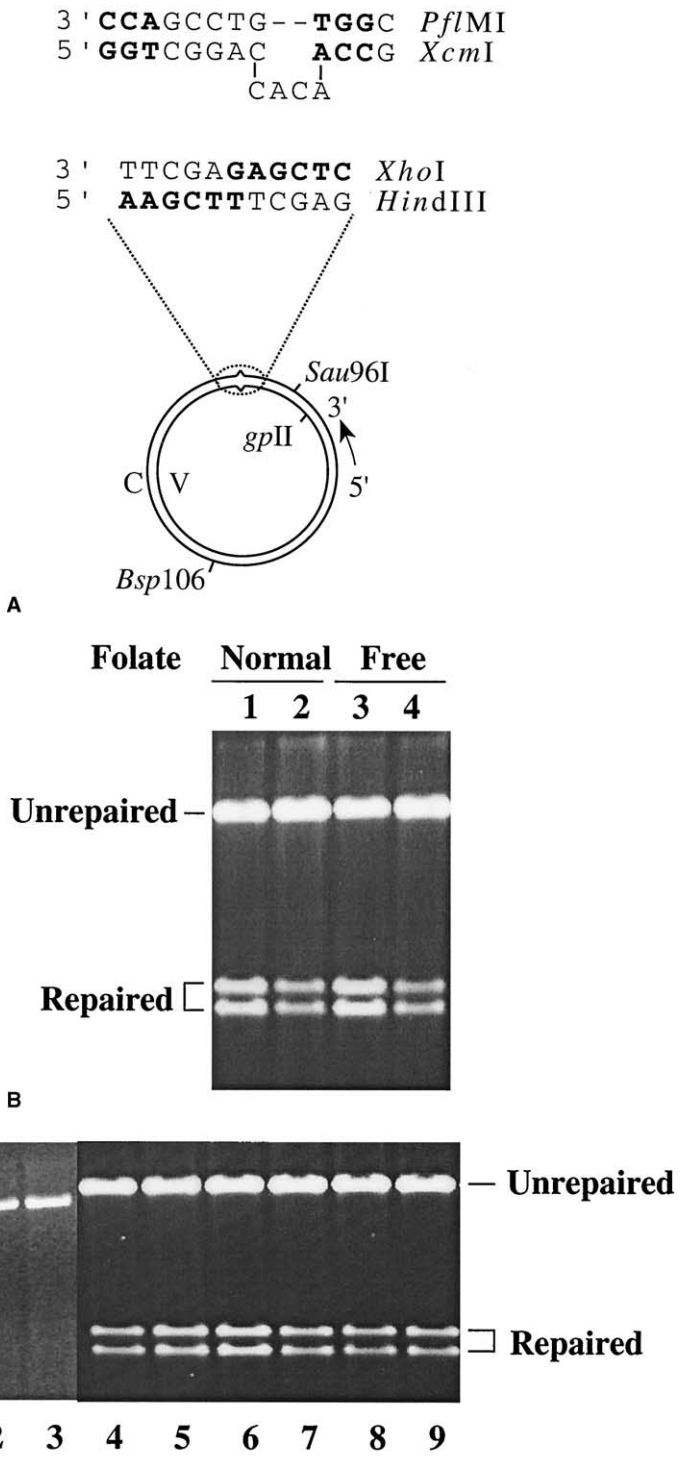

Fig. 1. Folate deficiency has no effect on MMR activity. A. Structure of heteroduplex substrates. The circular G:T substrate contains a strand break 125 base-pair (at the *Sau96*I site) 5′ to the mismatch in the complementary (C) strand, while the insertion/deletion (CACA) mispair possesses a viral (V) strand break 181 base-pair (at the *gp*II site) 3′ to the heterology. B. Repair of the G:T (lanes 1 and 3) and the insertion/deletion (lanes 2 and 4) substrates by nuclear extracts of WI-L2-NS cells that were grown in normal or folate-free medium. C. Repair of the G:T substrate by nuclear extracts of TK6 cells that were cultured in normal (lanes 1 and 4), folate-free medium (lanes 2 and 3), or TK6 subclones that had survived folate depletion and were subsequently cultured in normal medium (lanes 5–9). Mismatch repair assays were performed at 37°C for 15 min in reactions containing 50 μg of nuclear extracts and 100 ng (24 fmol) of heteroduplex as described [28]. Since MMR in human cells is nick-directed, the repair was scored by *Hin*dIII and *Bsp*106 for the G:T substrate and by *Bsp*106 and *Pfl*MI for the insertion/deletion substrate. *Bsp*106 cleaves both substrate and product DNA, whereas *Hin*dIII and *Pfl*MI only cleave repaired molecules. Thus, repaired DNA yields the two small fragments as indicated.

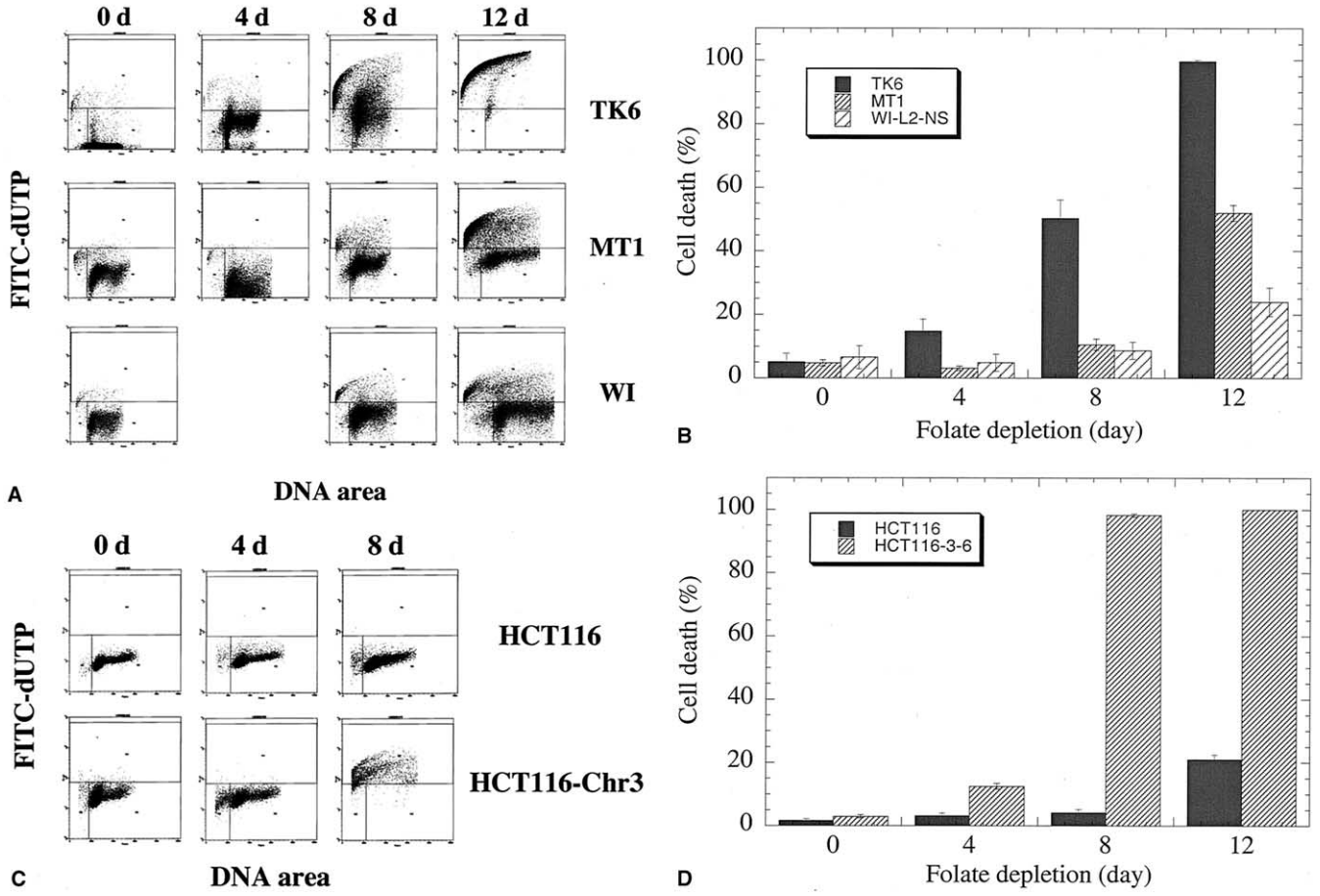

Fig. 2. Cells deficient in MMR are tolerant to folate deficiency. Cells were cultured in folate-free medium for indicated days and harvested for TUNEL analysis as described in Materials and Methods. The cells above the gate are apoptotic cells (TUNEL positive), while those below the gate are living cells (TUNEL negative). FL2-A (horizontal axis) indicates PI staining, and FL1H (vertical axis) indicates FITC-dUTP staining. A. Histogram of TUNEL analysis of TK6, MT1, and WI-L2-NS cells. B. Quantitation of the histogram in A. C. Histogram of TUNEL analysis of HCT116 and HCT116-Chr3 cells. D. Quantitation of the histogram in C.

indicate that folate deficiency-induced cell death is dependent on both human MutS and human MutL homologs.

To evaluate folate deficiency-induced cell death, cells treated with various degrees of folate depletion were analyzed by the classic DNA fragmentation analysis. As indicated in Fig. 3, DNA fragmentation was observed in TK6 cells after incubation for 12 days (lane 4) in medium without folic acids. However, DNA fragmentation was not clearly observed in MT1 cells under the same conditions. It is worth mentioning that although substantial (~50%) TUNEL-positive cells were observed in MT1 cell line after 12-day of folate depletion, no clear DNA fragmentation was observed. Instead, a fraction of DNA was smeared (Fig. 3, lane 8), suggesting that the cell death in MT1 cells is through a manner different from that in TK6 cells. Similar results were also observed in the isogenic pairs of HCT116/HCT116-Chr3, i.e., DNA fragmentation was detected in HCT116-Chr3 cells, but not in HCT116 cells (data not shown). The characteristics of the DNA fragmentation and the positive TUNEL analysis in repair proficient cells indicate that hMutSα- and hMutLα-dependent cell death occurs through active apoptosis.

3.3. hMutSα specifically recognizes DNA containing A:U or G:U base pairs

The requirement of hMutSα and hMutLα in folate deficiency-induced apoptosis suggests that MMR proteins play an important role in mediating folate deficiency-caused DNA damage. One possibility is that MMR proteins sense folate deficiency-induced DNA damage by directly binding to the damage site. Since folate deficiency induces massive misincorporation of uracil into DNA, leading to uracil-containing base pairs (such as A:U and G:U), we therefore tested hMutSα, the major form of mismatch recognition complex in human cells, for its ability to interact with DNA containing a single A:U or G:U base pair. Fig. 4 shows the interactions between these DNA substrates and hMutSα. While homoduplex DNA (lane 1) was poorly recognized by hMutSα, both A:U- and G:U-containing DNA substrates

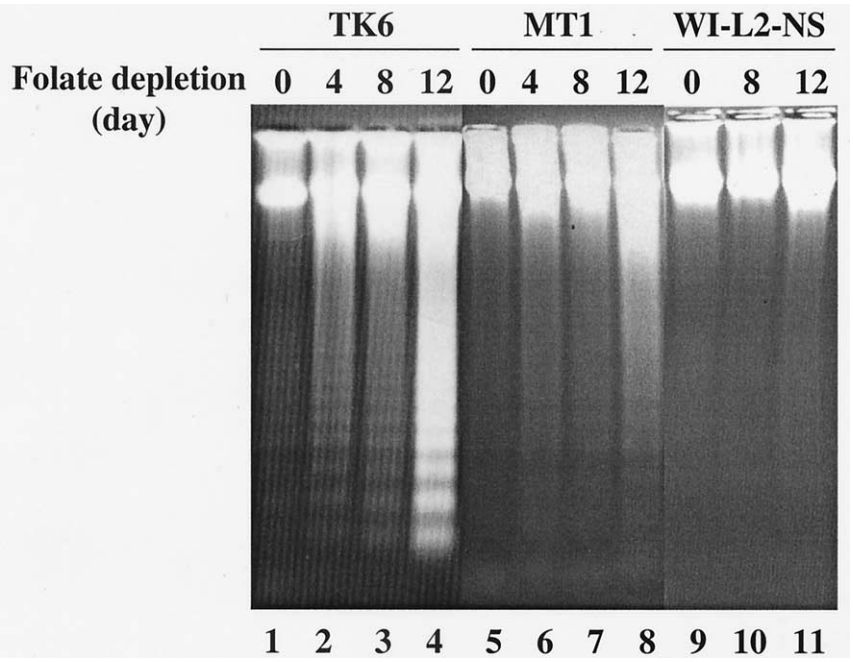

Fig. 3. Folate depletion-induced cell death is an MMR-dependent apoptosis. Cells were cultured in normal (day 0) or folate-free medium for indicated days and harvested for DNA isolation by protease K digestion and phenol-chloroform extraction. DNA was fractionated through 1.6% of agarose gels and visualized under UV light in the presence of ethidium bromide.

were efficiently bound by the protein (lanes 3 and 4). However, the interaction between hMutSα and the uracil-containing DNA was completely inhibited in the presence of 1 mM ATP (data not shown), a condition that also blocked the binding of hMutSα to a G:T mismatch [19]. If the nonspecific binding in homoduplex (A:T) is used as a binding affinity factor, then relative binding ratios of hMutSα with substrates A:T, A:U, G:U, and G:T were 1, 5.2, 13.4, and 18, respectively. The specific interaction between hMutSα and uracil-containing DNA suggests that MMR-proteins directly participate in mediating folate deficiency-induced apoptosis.

4. Discussion

It has been known for some time that folate deficiency induces apoptosis [32,41–43], but the mechanism underlying this process is poorly understood. In an effort to determine if DNA MMR plays any role in folate deficiency-caused genomic instability, we demonstrate here that folate deficiency-induced apoptosis is mediated by the MMR system. In addition, this work provides further evidence to support the notion that MMR maintains genomic stability through two distinct pathways: correcting mismatches generated from DNA metabolism and mediating apoptosis when DNA damage cannot be fixed [36].

Folate deficiency in humans has been linked with the development of certain types of cancer and many other diseases including megaloblastic anemia and developmental disorders. It is also associated with genomic instability characterized by misincorporation of uracil, DNA hypomethylation, and double strand breaks. A growing body of literature implicates an association of folate deficiency with DNA repair defects. Choi et al. [12] have shown that folate depletion can cripple the nucleotide excision repair pathway. Cravo et al. [13] have reported a correlation between folate deficiency and microsatellite instability in patients with ulcerative colitis, a condition well known to be associated with both folate deficiency and an increased risk of colorectal neoplasia. These authors have found that a low level (30–50% lower than the normal level) of folate concentration in these patients is coupled with microsatellite instability in their non-neoplastic mucosa. It is known that microsatellite instability is a hallmark of MMR deficiency and is usually found in cancer tissue. Very interestingly, microsatellite instability in colonic mucosa of the patients with ulcerative colitis disappeared after folate supplementation [13]. Given the relationship between microsatellite instability and MMR deficiency, it has been proposed that folic acid can regulate MMR capacity, i.e., MMR activity impaired by folate deficiency can be reactivated by folate supplementation [13,44].

This study investigates this issue by directly measuring MMR activity of cultured cells. The repair data in TK6 cells seem to be in agreement with the conclusion drawn by Cravo et al., since folate-depleted TK6 cells are defective and surviving TK6 cells that are cultured in normal medium are proficient in strand-specific MMR. However, we found that undetectable MMR activity in TK6 cells with folate depletion is due to cell

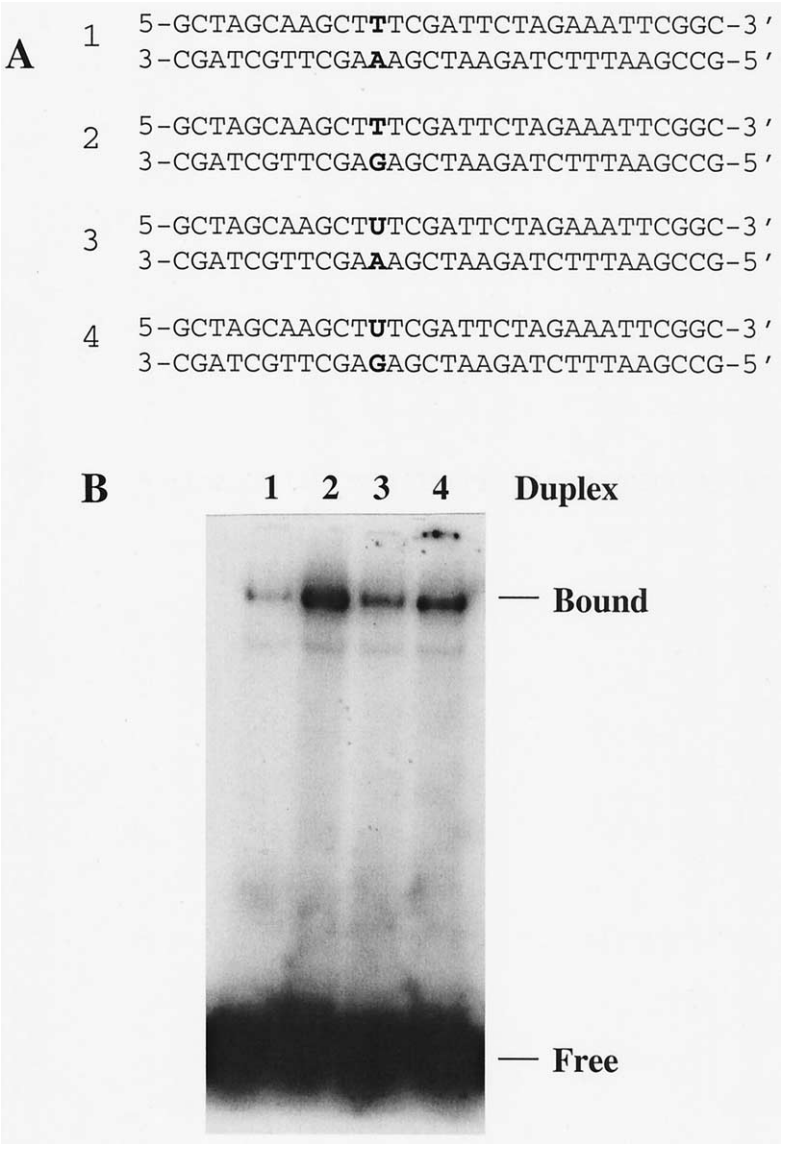

Fig. 4. Recognition of uracil-containing DNA by hMutSα. A) Sequences of oligonucleotide duplexes used in the gel-shift analysis. B) Gel-shift analysis. Gel-shift assays were performed in 25 μl reactions containing 0.5 pmol of ^{32}P-labeled oligonucleotide duplexes, 0.25 pmol of purified hMutSα, 0.1 pmol (0.4 μg) of double-stranded-f1MR3 DNA (competitor DNA), 10 mM Hepes-KOH (pH 7.5), 110 mM KCl, 1 mM EDTA, 1 mM DTT, and 4% glycerol. After 20-min incubation on ice, 5 μl of 50% sucrose was added. The samples were fractionated at room temperature through a 6%-non-denaturing polyacrylamide gel in 6.7 mM Tris-acetate (pH 7.5) and 1 mM EDTA with buffer recirculation.

death during the treatment, rather than to impairment. This is further confirmed by analyzing MMR activity in WI-L2-NS cells, which are derived from the same origin as the TK6 cells, but resistant to folate deficiency due to their defective p53. MMR activities in folate-depleted and folate-supplemented WI-L2-NS cells are comparable (see Fig. 1B), indicating that MMR activity is independent of the folate status in cultured cells. Therefore, we conclude that folate deficiency does not impair the MMR system and/or MMR activity is not regulated by folic acids. Instead, we demonstrate that MMR plays a crucial role in mediating folate deficiency-induced cell death. We show that MMR-proficient TK6 and HCT116-Chr3 cells undergo programmed cell death under the condition of folate depletion, confirming previous observations in other types of cells [32,41–43]. However, their corresponding mutant cells fail to elicit this apoptotic response regardless of whether the defect is in hMutSα (MT1) or hMutLα (HCT116). These findings strongly suggest that MMR proteins (at least hMutSα and hMutLα) are involved in folate depletion-induced apoptosis.

The role of MMR in folate deficiency is best demonstrated by our *in vitro* experiments, in which hMutSα, the major human mismatch recognition protein, specifically binds to DNA containing A:U or G:U base pairs, a condition derived from uracil incorporation during folate deficiency [7]. These results suggest that the apoptotic response induced by folate deficiency may be initiated by the binding of the human MutS protein to the damaged DNA.

Although the mechanism by which mismatch repair proteins mediate folate deficiency-induced apoptosis is unknown, we hypothesize, based on the data presented here, that the interaction between hMutSα and uracil-containing DNA can provoke strand-specific mismatch repair to excise uracils from DNA. However, given an unbalanced nucleotide pool in cells depleted of folate, uracils are immediately put back into the DNA during the resynthesis step of the MMR reaction, which will lead to another round of attempted repair by the MMR system. This futile repair cycle may signal cells to switch on apoptotic machinery. Alternatively, MMR proteins can function as signaling molecules to activate an apoptotic process, possibly through p53- and/or p73-dependent pathways [36,45]. However, this signaling pathway must start with the binding of MMR proteins (both hMutSα and hMutLα) to uracil-containing DNA. Although we did not test the involvement of p73, our data suggest that p53 may participate in the MMR-dependent apoptotic response induced by folate deficiency, as judged by the fact that wild type TK6 cells, not the *p53*-deficient WI-L2-NS cells, undergo apoptosis in response to folate depletion (Fig. 3). The requirement for p53 in MMR-dependent apoptosis has also been shown previously for other types of DNA damage [36,46,47]. Gong et al. [45] have recently demonstrated that the MMR-mediated apoptosis is dependent on p73, but not p53, in response to cisplatin-induced DNA damage, where the function of p73 is regulated by the tyrosine protein kinase c-Abl. Nevertheless, these findings suggest that MMR proteins may function as a sensor for server DNA damage and trigger signal transduction to switch on programmed cell death.

Given the role of MMR in folate deficiency demonstrated here, it is anticipated that MMR will have a great impact on tumorigenesis associated with folate deficiency. The incorporation of uracil into DNA during folate deficiency will induce mutations or chromosome breaks, thereby leading to genomic instability. However, cells with functional MMR system will be directed to programmed cell death so that there is little chance for these cells to develop progressive mutations. When MMR function is lost, cells cannot commit suicide, but accumulate mutations, which will eventually lead to tumorigenesis. On the other hand, the MMR-directed apoptosis can be problematic for a normal tissue or organ during a prolonged period of folate depletion, since extensive tissue or organ destruction by apoptosis may occur. Intramedullary apoptosis observed in megaloblastic anemia and other folate deficiency-related diseases is likely caused by the MMR-dependent apoptosis in response to severe folate deficiency.

Acknowledgments

We thank William Porter and Pam Jacobs for help in determining intracellular folate concentration, Jennifer Strange and Greg Bauman for help in TUNEL analysis, and Cecilia Ramilo for comments on the manuscript.

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