The Human MLH1 cDNA Complements DNA Mismatch Repair Defects in Mlh1-deficient Mouse Embryonic Fibroblasts

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Abstract

The DNA mismatch repair gene hMLH1 is reported to function in mutation avoidance, cell cycle checkpoint control, the cytotoxicity of various DNA-damaging agents, and transcription-coupled nucleotide excision repair. Formal proof of the involvement of hMLH1 in these processes requires single gene complementation. We have stably expressed hMLH1 from a transfected cDNA in Mlh1-deficient mouse embryonic fibroblasts. Expression of hMLH1 restored normal levels of mPMS2 protein, reduced spontaneous base substitution and microsatellite mutations, increased sensitivity to the toxic effects of 6-thioguanine (6-TG), and restored 6-TG-induced cell cycle arrest. Our studies confirm that hMLH1 has an essential role in the maintenance of genomic stability and the potentiation of 6-TG cytotoxicity and provide a system for detailed structure/function analysis of the hMLH1 protein.

Introduction

Maintenance of genomic stability plays an important role in the avoidance of cancer. The DNA MMR pathway reduces mutation by correcting mismatches arising during replication, from chemical damage such as spontaneous deamination of 5-methylcytosine, and during recombination (1). Alterations in the human MMR genes hMSH2, hMSH6, hMLH1, hPMS2, and hPMS1 are implicated in the development of hereditary and sporadic cancers (2, 3). The study of mammalian cell lines with mutations in MMR genes has provided insight into the functions of the MMR proteins. For example, various phenotypes have been associated with hMLH1 deficiency, including decreased levels of hPMS2 protein (4, 5), increased spontaneous mutation rate (6, 7), decreased sensitivity and lack of G2 cell cycle arrest in response to various chemotherapeutic agents and to γ-irradiation (8, 9), and loss of transcription-coupled repair (13). The extent to which each of these phenotypes contributes to increased risk of cancer is not known. In some studies (8–11), the phenotypes seen in the hMLH1-deficient cells were complemented after transfer of a normal human chromosome 3, which carries hMLH1. However, with chromosome transfer, there remains the possibility that correction of at least some phenotypes is due to other genes carried on chromosome 3. Correction of a hMLH1 defect by introduction of a single cloned gene or cDNA would allow a more precise assignment of hMLH1 functions. Here, we present the first successful single gene complementation of mammalian Mlh1 deficiency by stable expression of a transfected human MLH1 cDNA in Mlh1-deficient MEF cells.

Materials and Methods

Expression Vector Construction. The expression vector pCMV-Bam-Neo, provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD), was digested with BamHI and blunt-ended with Klenow (14). A full-length hMLH1 cDNA (15) was “blunt-end” cloned into pCMV-Bam-Neo. Expression constructions with the insert in either the correct or antisense orientation were identified by restriction mapping.

Cell Culture. Spontaneously immortalized MEF cells were established from embryos and cultured as described (6). To create the transfected cell lines, 20 μg of the sense or antisense expression vectors containing the hMLH1 cDNA were linearized by digestion with XmnI. DNA was electroperorated into ~10¹⁰ Mlh1-deficient MEFs using 0.4 cm gap cuvettes and a GenePulser apparatus (Bio-Rad, Hercules, CA) at 0.32 kV and a capacitance of 500 μFD. Transfected cells were selected and grown as clonal cultures in medium containing 15% serum and 400 μg/ml G-418 Sulfate (Life Technologies, Inc., Gaithersburg, MD). Transfected, clonal cultures subsequently were maintained without G-418 selection.

Methods for determination of the rate of forward mutation to ouabain resistance and frequency of mutation at dinucleotide microsatellite sequences have been described (6). For the microsatellite analysis, 8–10 subclones of each cell line were typed for the occurrence of a length alteration in five to eight dinucleotide repeat loci (D15Mit35, D17Mit93, D13Mit139, D16Mit4, D17Mit123, D15Mit59, D4Mit27, and D13Mit67; Research Genetics, Huntsville, AL). To calculate the mutation frequency, we assumed that there are two alleles present at each locus. DNAs were visualized after separation in polyacrylamide gels either directly by silver staining (16) or indirectly by Southern blot analysis.

Western Blot Analysis. Whole cell lysates from cultured cells were prepared by lysis in 1 × SDS-PAGE loading buffer (17). Approximately 50 μg of total cellular protein were electrophoresed in 8% or 10% SDS gels and transferred to Immobilon-P membrane (Millipore, Danvers, PA); hPMS2 and hMLH1 proteins were detected with monoclonal antibodies A16–4 and 728.2, respectively (PharMingen, San Diego, CA). Proteins were visualized with the enhanced chemiluminescence Western blotting detection system (Amersham Life Sciences, Arlington Heights, IL).

Treatment with 6-TG. To determine sensitivity to a “pulse” treatment of 6-TG (2-amino-6-mercaptopurine; Sigma Chemical Co., St. Louis, MO), exponentially growing cells were exposed to 6-TG diluted into complete medium for 24 h at 37°C, rinsed once with 5 ml of PBS and refed with complete medium. Eight to 10 days later, cells were fixed in 30% ethanol and stained with 0.25% methylene blue. Colonies containing >50 cells were counted. For each dose, four 100-mm plates were analyzed, two each seeded with 250 or 500 cells. Each cell line was tested in two to four experiments, and the CFUs at each dose were averaged.

For analysis of cell cycle responses to exposure to 6-TG, 1.5 × 10⁵ to 1.0 × 10⁶ cells were seeded into 100-mm dishes, depending on the planned day of harvest and the expected toxicity of the dose. Cells were refed with 6-TG-containing medium 18–24 h after seeding and were maintained in the 6-TG-containing medium for the course of the experiment. In parallel, cell survival (as measured by CFUs) for each dose was determined by treating in triplicate plates seeded with either 500, 1,000, or 10,000 cells.

Flow Cytometry. Adherent cells were harvested using trypsin and combined with any floating cells present in the growth medium. Cells were rinsed once with ice-cold Tris-saline [10 mM Tris (pH 7.0) and 50 mM NaCl], resuspended in 100 μl of Tris-saline, and fixed (for a minimum of 24 h at 4°C) by the addition of 900 μl of 100% ethanol. Fixed cells were spun out of ethanol, treated with phosphate-citric acid...
buffer (200 mM Na2HPO4, 100 mM citric acid (pH 7.8), and 400 μM for 5 min), and stained by resuspension in 500 μM of propidium iodide solution (0.2% NP-40, 7000 units/ml RNase A, and 33 μg/ml propidium iodide) for 24 h at 4°C. Cells were analyzed for DNA propidium fluorescence by using a FACScalibur fluorescence-activated cell sorter (Becton Dickinson) at a laser setting of 15.2 mV and an excitation wavelength of 488 nm. Data were quantified by using ModFit CT software version 2.0 (Verity Software House, Topsham, ME). Putative apoptotic cells or cell fragments were assigned as the peak with a DNA content less than G1 cells.

Results and Discussion

Expression of Human MLH1 in Mlh1-deficient Mouse Cells. To complement the MMR deficiency of MEFs lacking Mlh1, we introduced the human MLH1 cDNA into the cells by transfection. As determined by Western blot analysis of whole cell lysates, expression of hMLH1 was detectable at various levels in 9 of 10 transfectants examined (ME-1 through ME-10; Fig. 1A). No hMLH1 signal was detectable in 10 transfectants receiving the control vector (e.g., CT-5; Fig. 1B). The transfection efficiency was approximately the same with either the hMLH1-expression vector or with the control vector. Also, the expression of hMLH1 was stable without G-418 selection and did not reduce the growth rate or the cloning efficiency of the cells (data not shown). Thus, expression of hMLH1 in the Mlh1-deficient MEFs was not toxic.

As reported for human tumor cell lines with MLH1 deficiency (4, 5), we observed that mPms2 was reduced in Mlh1-deficient MEFs by ~95% relative to the level seen in the extracts from wild-type MEFs (Fig. 1B). We also observed a similar reduction in Pms2 in extracts of testes, thymus, and spleen isolated from Mlh1-deficient mice (data not shown). Our finding of reduced Pms2 in Mlh1-deficient mouse cells and tissues demonstrates that the presumed instability of Pms2 in the absence of Mlh1 is a general phenomenon in mammalian cells.

Expression of hMLH1 in the Mlh1-deficient MEFs increased the level of mPms2 (Fig. 1, A and B), consistent with the idea that mPms2 stability requires interaction with mMLH1. We could not determine whether the transfectants expressed hMLH1 at a level equivalent to the normal level of mMLH1 due to differences in the efficiency of antibody recognition of mouse versus human protein.

Reduced Mutation Rate in hMLH1-expressing MEFs Lacking Mlh1. To examine the effect of hMLH1 expression on the level of spontaneous base substitution mutation in the Mlh1-deficient MEFs, we measured the rate of forward mutation to ouabain resistance (18) in six transfectants, two each with low (ME-2 and -6), intermediate (ME-9 and -10), or high (ME-4 and -5) relative levels of expression of hMLH1. All of the hMLH1-expressing lines showed a reduced mutation rate (Table 1) approximately equal to that previously reported for two wild-type MEF cell lines (6). The mutation rates in two independent control transfectants (CT-1 and CT-3) were at least 20-fold higher (Table 1) and equal to that previously reported for the Mlh1-deficient parent cell line (6). Thus, stable expression of the mMLH1 significantly reduced the base substitution mutation rate in the Mlh1-deficient MEFs.

Next, we measured the frequency of mutation at five to eight dinucleotide microsatellite loci in three hMLH1-expressing lines. Pooled data from three cell lines expressing a low, intermediate, or high level of hMLH1 was compared with data generated from two control transfectants (Table 1). With the hMLH1-expressing lines, we observed length alterations at 2 of 416 alleles tested for a mutation frequency of 0.5%. In contrast, the control-transfected cell lines demonstrated allele length alterations in 21 of 216 alleles tested for a mutation frequency of 10%. Thus, expression of hMLH1 in Mlh1-deficient MEFs reduces the mutation frequency at dinucleotide microsatellite loci ~20-fold. The analyses of microsatellite instability and mutation to ouabain resistance demonstrate that expression of hMLH1 alone is sufficient to reduce the spontaneous mutation rate in the Mlh1-deficient MEFs. Interestingly, there was no apparent correlation

Table 1 Reduced mutation rate in hMLH1-expressing mouse Mlh1-deficient fibroblasts

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Base substitution rate ouabain resistance (× 10−6)</th>
<th>% microsatellite mutation dinucleotide (CA)n</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>mLlh1+/−</td>
<td>control</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>mLlh1+/−</td>
<td>hMLH1a</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>low expression</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>intermediate expression</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>high expression</td>
<td></td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

a The mutation rates (mutations/cell/generation) to ouabain resistance were calculated as described (6).

The mutation rates (mutations/cell/generation) to ouabain resistance were calculated as described (6).

Dinucleotide changes were assayed with DNA from 8–10 clonal cultures. Frequencies are shown as a percentage of total changes/total number of alleles detectable at 5–8 loci.

d Data from two wild type lines (6).

e Data from two control-transfected cell lines were combined, CT-1 and CT-3 for ouabain resistance and CT-1 and CT-8 for microsatellite mutation.

For ouabain resistance, data from two cell lines each expressing hMLH1 at low (ME-2 and -6), intermediate (ME-9 and -10), or high (ME-4 and -5) relative level were combined. For microsatellite mutation, data from three cell lines (ME-6, -9, and -5) were combined.
between level of expression of hMLH1 and the extent of reduction in the mutation rate, suggesting that even a low level of constitutively expressed hMLH1 is sufficient to restore Mlh1 function.

**Potentiation of 6-TG cytotoxicity in hMLH1-expressing Mlh1-deficient MEFs.** Others (10) have shown that hMLH1-deficient cells are resistant to killing by 6-TG. Therefore, we tested expression of hMLH1 in the Mlh1-deficient MEFs for effects on cell survival after 6-TG exposure. We determined the colony forming ability of wild type, Mlh1-deficient (parent cell line and CT-5), and hMLH1-expressing (ME-6, -9, and -5) cells exposed to 6-TG for 1–4 days. Representative peaks of cells with 2N (G1) and 4N (G2-M) DNA content are labeled. Hatched area, S phase cells. A-C, average percentage of cells with 4N DNA content (top right corner). Arrows, presumptive apoptotic cells. Average clonegenic survival with continuous exposure to 6-TG is indicated in parentheses.

**Restoration of G2 Cell Cycle Arrest in hMLH1-expressing Mlh1-deficient MEFs.** The potentiation of the cytotoxicity of 6-TG by MMR is associated with induced chromosomal rearrangements and growth arrest (10, 20, 21), although the precise nature of the signal generated by

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*Fig. 3. Cell cycle profiles of MEFs during exposure to 6-TG. Shown are representative flow cytometrograms of wild-type (A), Mlh1-deficient (B; CT-5), and hMLH1-expressing (C; ME-5) cells exposed to 6-TG for 1–4 days. Representative peaks of cells with 2N (G1) and 4N (G2-M) DNA content are labeled. Hatched area, S phase cells. A-C, average percentage of cells with 4N DNA content (top right corner). Arrows, presumptive apoptotic cells. Average clonegenic survival with continuous exposure to 6-TG is indicated in parentheses.*
MMR and how the signal is transduced is unknown. To study response to 6-TG in the hMLH1-expressing cell lines more fully, we used flow cytometry to analyze the cell cycle profile of MEFs cultured in different doses of 6-TG. In a dose- and time-dependent manner, the wild-type MEFs showed a significant increase in the 4N population, consistent with 6-TG-induced G2 cell cycle arrest (Fig. 3A). G2 cell cycle arrest was followed 1 day later by significant cell death, possibly by apoptosis as evident by the appearance of cells with a DNA content less than that of G1 (Fig. 3A). In contrast to the response of the wild type cells, the mMlh1-deficient cell line CT-5 did not show a specific G2 arrest at any dose tested, including doses where the survival of the cells was reduced dramatically (Fig. 3B). Similar responses were seen in two additional mMlh1-deficient cell lines (CT-1 and the parent cell line; data not shown). Therefore, our data using wild-type and Mlh1-deficient MEFs suggests that mMlh1 is required for 6-TG-induced G2 cell cycle arrest.

We next asked whether expression of hMLH1 can restore G2 cell cycle arrest in the Mlh1-deficient MEFs (Fig. 3C). At equitoxic doses of 6-TG, the responses of the hMLH1-expressing line ME-5 and wild type cells were similar. The ME-5 cell line showed a dose- and time-dependent accumulation of cells with a 4N DNA content (G2 arrest), followed by an induction of cell death. Whereas the wild type cells required 3 days of exposure to 6-TG, arrest in the ME-5 cells was obvious by day 2, most likely due to the faster doubling time of ME-5 cells (data not shown). Five additional hMLH1-expressing cell lines (ME-2, -6, -9, -10, and -4) also were tested in the assay. The clonogenic survival of all of the hMLH1-expressing cell lines with different doses of 6-TG was similar (Fig. 2 and data not shown). Cell lines ME-2, -10, and -4 showed a pattern of G2 arrest and induction of cell death similar to ME-5 (data not shown). Another line (ME-9) showed an equivalent induction of G2 arrest, but subsequent induction of cell death was reduced (data not shown). Even at the highest doses tested many of the ME-9 cells remained attached on the plate, and appeared permanently arrested. The final cell line tested (ME-6) did not show an accumulation of cells with a 4N DNA content (data not shown). Thus, five of the six hMLH1-expressing cell lines showed an induction of G2 arrest during exposure to 6-TG, suggesting that expression of hMLH1 is sufficient to restore 6-TG-induced G2 arrest in the Mlh1-deficient MEFs. Significantly, all of the hMLH1-expressing cell lines, including ME-6 and ME-9 showed a similar reduction in spontaneous mutation rate (Table 1), and similar 6-TG-induced cytotoxicity, suggesting first that mMlh1-dependent MMR was restored to the same level in all of the cell lines and, second, that restoration of MMR was sufficient to create a cytotoxic signal in response to 6-TG exposure. The lack of G2 arrest in ME-9 might reflect deficiencies in the G2 arrest in ME-9 might reflect deficiencies in the G protein, supporting the idea that mammalian Pms2 is unstable when not induced cell death (22). Similarly, Shin et al. (23) have reported that expression of hMLH1 in two different hMLH1-deficient tumor cell lines results in growth suppression. We have not noted any growth suppression caused by expression of hMLH1 in the Mlh1-deficient MEFs. Thus, the phenomenon of growth suppression by unregulated hMLH1 expression may be a property of human tumor cells, or tumor cell lines in general. Our findings, combined with biochemical assays, should facilitate studies of mammalian DNA MMR.

References

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