Methylation-induced G₂/M arrest requires a full complement of the mismatch repair protein hMLH1

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The mismatch repair (MMR) gene *hMLH1* is mutated in ~50% of hereditary non-polyposis colon cancers and transcriptionally silenced in ~25% of sporadic tumours of the right colon. Cells lacking hMLH1 display microsatellite instability and resistance to killing by methylating agents. In an attempt to study the phenotypic effects of hMLH1 downregulation in greater detail, we designed an isogenic system, in which hMLH1 expression is regulated by doxycycline. We now report that human embryonic kidney 293T cells expressing high amounts of hMLH1 were MMR-proficient and arrested at the G₂/M cell cycle checkpoint following treatment with the DNA methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), while cells not expressing hMLH1 displayed a MMR defect and failed to arrest upon MNNG treatment. Interestingly, MMR proficiency was restored even at low hMLH1 concentrations, while checkpoint activation required a full complement of hMLH1. In the MMR-proficient cells, activation of the MNNG-induced G₂/M checkpoint was accompanied by phosphorylation of p53, but the cell death pathway was p53 independent, as the latter polypeptide is functionally inactivated in these cells by SV40 large T antigen. **Keywords: cell cycle checkpoint/hMLH1/methylating agent/mismatch repair/TetOff**

Introduction

Mutations in mismatch repair (*MMR*) genes, predominantly *hMSH2* and *hMLH1*, segregate with hereditary non-polyposis colon cancer (HNPCC). Inheritance of a single mutated allele of a *MMR* gene predisposes to precocious cancers of the colon, endometrium and ovary. Analysis of HNPCC tumour cells showed that repeated sequence elements (microsatellites) in their genomic DNA are frequently mutated (for a review see Peltomäki, 2001). As microsatellite instability (MSI) is a hallmark of defective MMR in all organisms tested to date, and has been shown to be present in all tumour cell lines that have lost both alleles of *hMSH2* or *hMLH1* (Boyer *et al.*, 1995), it is assumed that the wild type alleles of the respective *MMR*

genes in cells of HNPCC tumours have been lost or inactivated by mutation. But mutations in *MMR* genes are not an absolute prerequisite for MSI. In recent years, a number of sporadic colon tumours and tumour cell lines displaying MSI have been described that are MMR-deficient due to silencing of the *hMLH1* promoter by hypermethylation (reviewed in Esteller, 2002).

Once both *MMR* gene alleles have been inactivated, the cell's propensity towards acquiring mutations increases, especially in genes carrying microsatellite repeats. Should the mutated genes be involved in the control of cell proliferation, the mutator cell in, for example, the colonic epithelium would be able to divide in an uncontrolled manner and thus give rise to an adenomatous polyp. As the cells in this benign growth acquire further mutations with subsequent cell divisions, the adenoma would rapidly become transformed into a carcinoma. That such a path to transformation can be followed *in vivo* was demonstrated when numerous HNPCC colon cancers were shown to carry frameshift mutations in a run of 10 adenines within the coding sequence of the transforming growth factor β receptor type II (*TGF β RII*) gene, as well as in other genes involved in growth control or apoptosis (reviewed in Markowitz *et al.*, 2002). Further support for this hypothesis comes from the finding that adenomas of HNPCC kindred transform to carcinomas with a much higher frequency than those associated with sporadic disease (Kinzler and Vogelstein, 1998), presumably due to a more rapid acquisition of transforming mutations.

The above findings help explain how the loss of MMR might accelerate cellular transformation and tumour progression. What is unclear to date, however, is whether the transformation process begins only following the inactivation of both *MMR* gene alleles, or whether it commences already at the stage when only one allele is affected or when the expression of the given *MMR* gene is only attenuated, rather than shut off, such as might be the case in cells where the *hMLH1* promoter is only partially methylated. The notion that a reduction in MMR protein levels might promote tumorigenesis originates in studies with *Msh2*^{+/-} mice. Although the *Msh2*^{+/-} embryonic stem cells were apparently normal in terms of their MMR capacity as measured by MSI (de Wind *et al.*, 1995), the heterozygous animals were cancer prone, and presented with tumours that often still contained the wild-type *Msh2* allele (de Wind *et al.*, 1998). The propensity of the *MMR* heterozygous cells to transformation would thus appear to be linked to a process distinct from the correction of replication errors. What might the nature of these processes be?

In recent years, MMR defects have been linked to several other phenomena, such as transcription-coupled repair and recombination—both mitotic and meiotic (reviewed in Harfe and Jinks-Robertson, 2000). In

Fig. 1. Inducible *hMLH1* expression in 293T $L\alpha$ cells. (A) In the Tet-Off system, *hMLH1* is expressed in the absence of Dox, because the tTA factor binds to the promoter of the expression vector and thus activates transcription. Addition of Dox to the culture medium causes a conformational change in tTA, which leads to its dissociation from the promoter and thus to an inactivation of *hMLH1* transcription. (B) Western blot analysis of cytoplasmic (CE) and nuclear (NE) extracts of cells cultured in the absence (–) or presence (+) of 50 ng/ml Dox. *hMLH1* and hPMS2 were visualized using anti-*hMLH1* or anti-hPMS2 antibodies as described in Materials and methods. Total extract (TE) of MMR proficient HeLa cells was used as a positive control. (C) Stability of hMut $L\alpha$. The cells were cultured without Dox (–) to induce maximal *hMLH1* expression. Following the addition of 50 ng/ml Dox (+), total cell extracts were isolated after 1, 2, 3, 4, 6 and 8 days. Western blot analysis was performed using anti-*hMLH1* and anti-hPMS2 antibodies as in (B).

addition, the MMR system was implicated in activation of cell cycle checkpoints and apoptosis, as witnessed by the increased resistance of MMR-deficient cells to the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or cisplatin (reviewed in Bellacosa, 2001). Thus, while MMR^{+/–} cells, or cells expressing low amounts of MMR proteins, may not display a mutator phenotype, they might have at least a partial defect in one of the above processes, specifically in the DNA damage signalling pathway, which we judged to be of the greatest relevance to cancer. We wanted to study these processes in detail, but we lacked isogenic cells expressing varying amounts of MMR proteins. Cells in which the MMR defect was corrected either by transfer of a chromosome carrying a single wild-type copy of the mutated *MMR* gene (Koi *et al.*, 1994) or its cDNA (Risinger *et al.*, 1998; Buermeier *et al.*, 1999; Lettieri *et al.*, 1999; Claij and Te Riele, 2002) were unsuitable for our studies, because they express similar or even higher amounts of the complementing MMR proteins than MMR-proficient controls. Thus, in order to be able to study the phenotypic consequences associated with reduced levels of MMR proteins, we had to generate a new line, preferably of epithelial origin, in which the expression of a selected *MMR* gene could be regulated. We now describe the construction and characterization of a line in which the expression of *hMLH1* can be tightly regulated by doxycycline with the help of the TetOff system.

Results

Construction of cells with inducible *hMLH1* expression

The human embryonic kidney cell line 293T is MMR deficient, because the *hMLH1* gene in these cells is

epigenetically silenced by promoter hypermethylation (Trojan *et al.*, 2002). We set out to correct its MMR defect through the expression of exogenous *hMLH1* using the TetOff expression system, which can be tightly regulated. We first generated the 293T-TetOff cell line by stable transfection of the 293T cells with a DNA vector encoding the tetracycline-controlled transactivator (tTA). In the second step, we stably transfected the 293T-TetOff cells with a vector carrying the *hMLH1* cDNA under the control of the tetracycline response element (TRE), thus creating 293T $L\alpha$ cells. In the absence of tetracycline, or its more stable analogue doxycycline (Dox), the tTA protein binds to the TRE and activates transcription of *hMLH1*; conversely, addition of the drug induces a conformational change in tTA, which loses its ability to bind DNA and the transcription of *hMLH1* is thus turned off (Figure 1A). During the initial screening, we used Dox at a concentration of 2 μ g/ml, as recommended by the manufacturer, but later we found that a concentration of 50 ng/ml was sufficient to turn off the expression of *hMLH1* below the limit of detection by western blotting (see below).

In vivo, *hMLH1* interacts with hPMS2 to form the heterodimer hMut $L\alpha$, which is essential for MMR. Our previous studies have shown that hPMS2 is unstable in the absence of its cognate partner (Räschle *et al.*, 1999). Indeed, no *hMLH1* could be detected in the extracts of 293T cells, and hPMS2 was hardly detectable (Trojan *et al.*, 2002). A similar situation also existed in our 293T $L\alpha$ clone grown in the presence of Dox, i.e. under conditions where the *hMLH1* promoter is shut off (Figure 1B). However, expression of *hMLH1* brought about hPMS2 stabilization through the formation of hMut $L\alpha$, such that the levels of the latter protein were

comparable to those seen in extracts of MMR-proficient cell lines (Figure 1B).

The expression of hMLH1 in the 293T L α cells grown in the absence of Dox was substantially higher than in any MMR-proficient cell line tested by us to date (Figure 1B; data not shown). Interestingly, this overexpression did not appear to be toxic to the cells: we detected no increase in the rates of apoptosis, as described for cells microinjected with expression vectors encoding hMSH2 and hMLH1 (Zhang *et al.*, 1999). Moreover, cells grown in the absence or presence of Dox divided roughly once every 24 h (data not shown), unlike HCT116 and SNU-1 cells, in which the stable expression of hMLH1 was reported to result in substantially slower growth rates (Shin *et al.*, 1998). When the expression of the transgene was turned off by the addition of Dox, the hMLH1 and hPMS2 proteins were present in the cell extracts in a 1:1 ratio only 24 h later (Figure 1C) and decayed with similar kinetics. This experiment showed that hMutL α is extremely stable, as it was detectable in the extracts of 293T L α cells even 6 days after the expression of hMLH1 was shut off.

In the following text, cells grown in the presence of 50 ng/ml Dox that do not express hMLH1 and thus lack hMutL α will be referred to as 293T L α ⁻ cells. Those grown in the absence of Dox, which express hMLH1 and thus contain functional hMutL α , will be referred to as 293T L α ⁺ cells.

hMLH1 expression in 293T L α cells restores MMR *in vitro*

Extracts of the 293T L α cells were tested for MMR activity *in vitro* using two different MMR assays (see Materials and methods). No MMR activity was detected in extracts of 293T L α ⁻ cells, but as the defect could be complemented by the addition of the recombinant wild-type hMutL α , we concluded that this heterodimer was the only factor missing in these extracts (Figure 2). In contrast, extracts from 293T L α ⁺ cells were MMR proficient in both assays (Figure 2). Importantly, these results showed that the excess partnerless hMLH1 in the 293T L α line does not inhibit MMR, at least not in our *in vitro* system. This differs from the situation in *Saccharomyces cerevisiae*, where overexpression of MLH1 gave rise to a mutator phenotype associated most likely with the inhibition of MMR through the homodimerization of this polypeptide (Shcherbakova and Kunkel, 1999; Shcherbakova *et al.*, 2001). The MMR proficiency of the 293T L α ⁺ cells in our *in vitro* assay was similar irrespective of whether the extracts were prepared from cells grown in the absence of Dox, or 24 h after the addition of the drug (data not shown), at which time point the ratio of hMLH1 to hPMS2 was 1:1 (Figure 1C).

Inducible hMLH1 expression restores sensitivity to alkylating agents

In order to determine the effect of hMLH1 expression on the sensitivity of 293T L α cells to MNNG, we used clonogenic assays to quantify the surviving fraction of 293T L α ⁻ and 293T L α ⁺ cells following treatment with 5 μ M MNNG. [Note that 293T L α cells do not express MGMT, the enzyme responsible for the detoxification of methylation damage (G.Marra, unpublished data). For this reason, the experiments described below were carried out

Fig. 2. MMR proficiency of 293T L α cell extracts. **(A)** Repair efficiency of a G/T mismatch in the M13mp2 vector carrying a strand discrimination signal 3' from the mismatch, using cytoplasmic extracts of the 293T L α ⁺ and 293T L α ⁻ cells, supplemented or not with recombinant hMutL α (see text for details). Error bars show standard errors. **(B)** Correction of a G/T mismatch within a *Bgl*II restriction site of a pGEM vector, following incubation with nuclear extracts of 293T L α ⁺ or 293T L α ⁻ cells, supplemented or not with recombinant hMutL α . The strand discrimination signal in this heteroduplex substrate was 5' from the mismatch. Efficient repair resulted in the restoration of a *Bgl*II site and in the generation of two DNA fragments that co-migrate with those observed in the reference digest of the homoduplex molecule carrying a bona fide *Bgl*II site.

in the absence of the MGMT inhibitor O⁶-benzylguanine.] As shown in Figure 3A, 293T L α ⁺ cells were very sensitive to killing by MNNG, and the surviving fraction was indistinguishable from that obtained after MNNG treatment of the related MMR-proficient 293 cell line. In contrast, 293T L α ⁻ cells were resistant to killing by MNNG, just like the parental, MMR-deficient 293T cells. The presence of Dox in the culture medium had no effect on the survival of any of the control cell lines used in this study (Figure 3A).

The sensitivity of 293T L α cells to MNNG was further examined using the MTT assay, which is based on the cleavage of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by the action of mitochondrial dehydrogenases to form a violet formazan dye. As this reaction takes place only in living cells, these can be distinguished from non-viable cells in a simple colorimetric assay. As shown in Figure 3B, 293T L α ⁻ cells were 125-fold more resistant

Fig. 3. Sensitivity of 293T Lα cells to MNNG. (A) Survival of 293T Lα⁺ and 293T Lα⁻ cells following treatment with 5 μM MNNG. 293 and 293T cells were used as MMR-proficient and -deficient controls, respectively. The presence of Dox (+Dox) in the culture medium did not affect the control cells, but had a dramatic effect on the survival of the 293T Lα⁻ cell populations. (B) IC₅₀ values of 293T Lα⁺ and 293T Lα⁻ cells. Each value represents the mean ± SE. (C) Cell cycle profiles of 293T Lα⁺ and 293T Lα⁻ cells treated with 0.2 μM MNNG. Shown are representative cytometrygrams of cells expressing (293T Lα⁺) and not expressing (293T Lα⁻) hMLH1. G₁, cell population in the G₁ phase of the cell cycle with a 2n DNA content; G₂, cells in the G₂ and M stages of the cell cycle with a 4n DNA content; S, cells in various stages of DNA synthesis with a DNA content between 2n and 4n.

to killing by MNNG than the same cells in a MMR-proficient mode (i.e. 293T Lα⁺ cells).

Expression of hMLH1 in 293T Lα cells leads to activation of a methylation damage induced cell cycle arrest

To determine whether the increased sensitivity of 293T Lα⁺ cells to MNNG resulted from induction of cell cycle arrest and cell death, the treated 293T Lα⁺ and 293T Lα⁻ cell populations were analysed by flow cytometry. As shown in Figure 3C, 2 days after treatment with 0.2 μM MNNG, the 293T Lα⁺ cells were mostly arrested in the G₂/M phase of the cell cycle. One day later, cells containing sub-G₁ amounts of DNA became detectable, and this population increased with time. In contrast, no increase in the population of cells either arrested in G₂/M or with a lower than 2n DNA content was detected in cultures of treated 293T Lα⁻ cells.

In order to further characterize the response of cells to MNNG, we analysed the phosphorylation status of Cdc2. As shown in Figure 4A, Cdc2 phosphorylated on Tyr15 accumulated exclusively in 293T Lα⁺ cells treated with 0.2 μM MNNG. This provides molecular evidence for a G₂/M arrest, because so long as this kinase remains phosphorylated, entry into mitosis should be blocked. No difference in Cdc2 phosphorylation was observed in the extracts of MNNG-treated 293T Lα⁻ cells (Figure 4A).

The above results thus show that induction of hMLH1 expression in the 293T Lα cells was necessary and sufficient to endow them with a MMR-proficient status, which also enabled them to respond to DNA damage induced by MNNG. What is presently unclear is the role of the MMR system in this checkpoint activation. DNA damage signalling is known to be mediated via several protein phosphorylation cascades, which involve primarily the DNA-dependent protein kinase (DNA-PK), or the ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR) kinases. The downstream target of the latter enzymes is the p53 tumour suppressor protein, the phosphorylation of which on Ser15 is known to lead to its stabilization and subsequent activation as a transcription factor (Tibbetts *et al.*, 1999). Phosphorylation of p53 has indeed been shown to take place upon MNNG treatment, and was shown to be dependent on functional hMutSα and hMutLα (Duckett *et al.*, 1999; Hickman and Samson, 1999; Adamson *et al.*, 2002). However, as the latter experiments were carried out with drug concentrations 25- to 125-fold higher than those used in our study, we wanted to test whether Ser15 phosphorylation also took place in the 293T Lα cells treated with 0.2 μM MNNG. These cells overexpress the SV40 large T antigen and thus contain large amounts of stabilized p53 polypeptide. This system is ideally suited for the study of post-translational modification of p53, as the steady-state levels of the latter protein remain unaltered during the experiment (Tibbetts *et al.*, 1999). As anticipated, the p53 steady-state levels in the 293T Lα cell extracts were high, irrespective of whether hMLH1 was expressed or not, or whether extracts of treated or untreated cells were examined (Figure 4A). However, following MNNG treatment, phosphorylation of p53 with a Ser15-specific antibody could be detected exclusively in the MMR-proficient 293T Lα⁺ cells. Notably, and in contrast to the study by Adamson *et al.* (2002), where the phosphorylation of p53 became detectable already just minutes after MNNG treatment, the MMR-dependent post-translational modification of p53 observed in our cells peaked at 48 h, i.e. at a time point where most cells were arrested at G₂/M (Figure 3C). This difference is probably linked with the high concentration of MNNG (25 μM) used in the latter study, which would be expected to introduce numerous single- and double-strand breaks into DNA that arise through the spontaneous loss of methylated purines and the subsequent breakage of the sugar-phosphate DNA backbone by β-elimination at the resulting abasic sites (Loeb, 1985). DNA strand breaks rapidly activate the ATM/ATR kinases that subsequently phosphorylate a number of downstream targets, one of which is histone H2AX (Redon *et al.*, 2002). This histone modification is thought to aid the recruitment of DNA repair factors to the sites of damage (Paull *et al.*, 2000). H2AX is phosphorylated in the 293T Lα cells upon

Fig. 4. Post-translational protein modification and strand break processing in MNNG-treated 293T Lα cells. (A) Phosphorylation status of p53 and cdc2 in 293T Lα⁺ and 293T Lα⁻ cells 1–4 days after treatment with 0.2 μM MNNG. P-p53, P-cdc2, phosphorylated p53 and cdc2 proteins, respectively; C, untreated control cells; β-tubulin, internal standard used to ascertain equal gel loading. (B) γ-H2AX foci formation in MNNG-treated 293T Lα cells. In the control cell population, <10% of cells displayed H2AX foci. Following MNNG treatment, all cells contained foci until 24 h post-treatment. See text for details and Materials and methods for experimental procedures.

treatment with 0.2 μM MNNG, as witnessed by the formation of phospho-H2AX foci (Figure 4B). However, these foci arise in both 293T Lα⁺ and 293T Lα⁻ cells soon after treatment. Thus, damage caused by direct modifications of DNA at low concentrations of MNNG does not trigger the G₂/M checkpoint. The activation of the checkpoint machinery must take place after H2AX phosphorylation, in the second cell cycle post-treatment (Kaina *et al.*, 1997), and must involve the MMR system, perhaps in conjunction with another pathway of DNA metabolism that remains to be identified. Thus, the lesions that trigger the checkpoint machinery are distinct from those that bring about phosphorylation of H2AX.

MMR proficiency and response to MNNG treatment require different levels of hMLH1 expression

The principal goal of this study was to investigate the phenotypic effects of reduced expression of MMR proteins, such as might be encountered when expression of the gene is attenuated by cytosine methylation. In order

to achieve this goal, we attempted to modulate hMLH1 expression in the 293T Lα cells. This could be achieved by varying the Dox concentration in the culture media. Thus, cells grown in the presence of 0.1, 0.2, 0.4, 0.8 and 1.5 ng/ml Dox contained steadily decreasing amounts of hMLH1 and hPMS2, as compared with cells grown in the absence of the drug (Figure 5A).

When we tested how this variation in the amount of hMutLα affected MMR efficiency, we found that extracts of cells expressing as little as 10% of the amounts found in cells grown in the absence of Dox were still proficient in the *in vitro* MMR assays. Cells cultivated with 0.1 and 0.2 ng/ml Dox showed MMR activities comparable to those of the MMR-positive 293T Lα⁺ cells grown in the absence of Dox, and even extracts of cells cultivated with 0.4 ng/ml Dox were still able to repair mismatches *in vitro*, albeit with lower efficiency (Figure 5B). MMR proficiency was lost only in cell extracts in which the hMLH1 and hPMS2 proteins became difficult to detect by western blotting (Figure 5A). To test whether the results of the *in vitro* MMR assays were reflected also in the MSI

Fig. 5. Mismatch correction efficiency and MNNG-induced G₂/M arrest in cells expressing different amounts of hMLH1. (A) Dependence of hMLH1 expression on Dox concentration. hMLH1 and hPMS2 were visualized as described in Materials and methods. β-tubulin, internal standard used to ascertain equal loading. (B) MMR efficiency of a G/T mispair in an M13mp2 substrate carrying a strand-discrimination signal 3' from the mispair. Error bars show standard errors. (C) Variation in doubling times of 293T Lα cells grown in the indicated Dox concentrations following treatment with 5 μM MNNG. (D) FACS analysis of 293T Lα cell populations grown in the indicated Dox concentrations, either untreated (Control), or 72 h after treatment with 0.2 μM MNNG (see also Figure 3C). (E) Phosphorylation of p53 and cdc2 48 h after treatment of cells (grown in the indicated Dox concentrations) with 0.2 μM MNNG. β-tubulin, internal standard used to ascertain equal loading.

Table I. Instability of the BAT26 chromosomal locus in 293T Lα cells expressing varying amounts of hMLH1

Dox (ng/ml)	MSI ⁺ /total	% MSI
0	2 (0)/131	1.5
0.2	1 (0)/80	1.3
50	4 (2)/73	5.5 (2.7)

MSI⁺ clones were defined as those displaying more than three extra peaks in the sequence of the PCR product. Numbers in parentheses refer to clones with more than four extra peaks.

phenotype of the cells, we analysed the BAT26 microsatellite marker, which contains a repeat of 26 deoxyadenosines, and which is considered to be a reliable indicator of MSI. Because the 293T Lα cells are hypotriploid, and because this cell line was MMR deficient for many generations prior to our intervention, the BAT26 locus was found to be highly heterogeneous. The product of PCR amplification had on average eight peaks, and we therefore applied the HNPCC criteria of MSI (Loukola *et al.*, 2001), whereby only PCR products that differed by three or more peaks at this locus were considered to be a sign of MSI. By these criteria, the BAT26 instability in the cells propagated for 35 generations in 0 or 0.2 ng/ml Dox was ~1%, whereas cells grown with 50 ng/ml Dox displayed MSI that was ~5-fold higher (Table I). However, closer inspection of the data revealed that cells propagated in 0 or 0.2 ng/ml Dox displayed no alleles (0/211) that differed by more than 4 bp from the median. In contrast, two such alleles (two out of 73; 2.7%) were found in the cells grown with 50 ng/ml Dox (Table I, numbers in parentheses). This suggests that MSI at the BAT26 locus in the 293T Lα cells is substantially greater than in cells expressing hMLH1, and thus that expression of even low amounts of hMutLα are sufficient to correct the MMR defect in these cells, both *in vitro* (Figure 2) and *in vivo* (Table I).

We were interested to determine whether the low amounts of the hMLH1/hPMS2 heterodimer that were shown to restore MMR proficiency were also able to activate the DNA damage-induced cell cycle arrest in 293T Lα cells. To this end, we treated the cells with 5 μM MNNG and calculated the average doubling time over a period of 5 days. In accordance with our previous experiments, only cells expressing the highest amounts of hMLH1 (i.e. 293T Lα⁺ cells grown without Dox) ceased to grow, as suggested by their increased doubling time. Cells grown in 0.1 ng/ml Dox were only partially affected, and cells cultivated with 0.2 ng/ml Dox or more grew similarly to 293T Lα⁻ cells (Figure 5C). To test whether this growth retardation was due to checkpoint activation, we analysed the DNA content of the cells 3 days after treatment with 0.2 μM MNNG. As shown in Figure 5D, FACS analysis showed that only cells expressing the highest amounts of hMLH1 (i.e. cells cultured without Dox) displayed a strong G₂/M arrest (an average of 63% of the cells were in G₂/M). The response of cells cultivated with 0.1 ng/ml Dox was substantially weaker (~27% cells in G₂/M), and the cell cycle profiles of cells grown with 0.2 ng/ml Dox or more were indistinguishable from those of the untreated controls (~22% cells in G₂/M). Notably, whereas cells grown in the absence of Dox activated the MNNG-induced G₂/M checkpoint, while those grown in

0.2 ng/ml Dox failed to do so, phosphorylated forms of p53 and cdc2 could be detected in both cell populations (Figure 5E). The extent of cdc2 phosphorylation in particular would predict that a detectable proportion of the treated cells should be at the G₂/M boundary. This was clearly not the case, as judged by FACS analysis (Figure 5D; also see Figure 3C).

Taken together, these experiments show that although only low amounts of hMutL α are required for MMR proficiency, DNA damage-induced G₂/M arrest and cell death in response to MNNG treatment require a full complement of this heterodimer. The fact that the 293T L α ⁺ cells arrest and die with kinetics and efficiency similar to other MMR-proficient cells confirms that p53, which is inactive in these cells, is not required for either of these processes (Hickman and Samson, 1999). Thus, the molecular pathways controlling the MNNG-induced G₂/M checkpoint in these cells require further study.

Discussion

We show that expression of hMLH1 in 293T L α cells corrected their MMR defect *in vitro* and *in vivo*. The 293T L α ⁺ cells were also found to be >100-fold more sensitive to killing by MNNG than the isogenic cells lacking hMLH1. MNNG treatment arrested the MMR-proficient cells in the G₂/M phase of the cell cycle, and this arrest was entirely and solely dependent on the function of hMLH1. This latter statement is supported by the finding that expression of hMLH1 in 293T L α ⁺ cells did not affect the transcriptional activity of other genes, as demonstrated by Affymetrix GeneChip™ analysis (data not shown).

This study also showed that the steady-state levels of the hMLH1/hPMS2 heterodimer required for MMR proficiency and DNA damage response were significantly different. In earlier experiments (Lettieri *et al.*, 1999) we generated a cell line derived from hMSH6-deficient HCT15 cells, which expressed low levels (~20%) of wild-type hMSH6. This line was MMR proficient, but remained as resistant to killing by methylating agents as the parental cell line. Similarly, a recent study described a *Msh2*^{-/-} mouse embryonic stem cell line in which the MMR defect was largely corrected by the expression of low levels (10% of control) of exogenous Msh2, but the response of these cells to methylating agents was comparable to that observed with the parental *Msh2*^{-/-} cells (Claij and Te Riele, 2002). This damage signalling defect was suggested by the authors to be linked to poor recognition of ^{Me}G/T mispairs, which arise through the mispairing of O⁶-methylguanine (^{Me}G) with thymine during DNA replication (Karran and Bignami, 1996), and which are bound less efficiently than bona fide mispairs by the hMSH2/hMSH6 (hMutS α) heterodimer (Duckett *et al.*, 1996). Constant loading of hMutS α sliding clamps at ^{Me}G/T mispairs was proposed to be responsible for transmission of the DNA damage signal to the checkpoint machinery *in vivo* (Fishel, 1999), and it might be expected that this process is substantially less efficient in cells expressing only low amounts of the mismatch binding factor hMutS α . However, the amounts of hMutS α in 293T L α ⁺ and 293T L α ⁻ cells are equal, and similar to those found in other MMR-proficient cells. Our results thus extend the above hypothesis by showing that the signal

transduction process also requires the hMLH1/hPMS2 heterodimer, thought to act downstream of damage recognition. Moreover, our result show that the recognition of ^{Me}G/T mispairs *per se* is insufficient to activate the checkpoint machinery. The G₂/M checkpoint is thought to be controlled by the phosphoinositide-3 (PI3) kinases ATM/ATR, which are principally responsible for the phosphorylation of p53 on Ser15 (Osborn *et al.*, 2002). The ^{Me}G/T mispairs arise already during the first round of replication, yet no p53 phosphorylation is detectable until 24 h after treatment, at which point the cells are beginning to enter the second S phase (Figure 3C; data not shown). Notably, the peak of signalling activity coincides with that of chromosomal rearrangements (sister chromatid exchanges and recombinations) induced by MNNG (Kaina *et al.*, 1997). Thus, MMR-dependent processing of the ^{Me}G/T mispairs that arise during the first S phase apparently does not activate the checkpoint machinery, but leads instead to the generation of intermediates that result in aberrant recombination events during the subsequent round of DNA replication, which then signal. What the exact nature of these intermediates may be is currently the subject of intensive studies.

The evidence presented here shows that cells with lower than wild-type levels of MMR proteins are not phenotypically normal, despite being MMR proficient. The observed defect in DNA damage signalling may be relevant to cellular transformation and cancer, particularly in epithelial cells that are rapidly proliferating and that may be exposed to stress or carcinogens. In the colon, the epithelial stem cells that are near the bottom of the crypts give rise to daughter cells that begin to differentiate during their migration towards the surface of the colon. Upon reaching the apex of the crypt, these cells undergo apoptosis and are shed. When the colonic epithelial cells become damaged, they should undergo apoptosis and thus give rise to no mutant progeny. In contrast, cells with a defect in DNA damage signalling, such as those expressing suboptimal amounts of MMR proteins, would not activate cell cycle checkpoints and apoptosis in response to DNA damage. Instead, they might acquire mutations that allow them to continue to proliferate and give rise to an adenoma.

The relevance of this hypothesis to the situation *in vivo* hinges on two points. First, there are currently no experimental data documenting instances where colonocytes or other epithelial cells that are prone to transformation express low MMR protein levels. We obtained some evidence of lower than normal steady-state levels of hMSH2 and increased resistance to methylating agents in the immortalized lymphoblasts of HNPCC patients, which are heterozygous in the *hMSH2* locus, but the *hMLH1*^{-/-} cells were normal in all assays (Marra *et al.*, 2001). It is not known whether hMSH2 and hMLH1 levels in heterozygous colonocytes of HNPCC kindred are lower than in similar cells of normal individuals, even though some fluctuations might be expected. However, the recent characterization of the *hMLH1* promoter as a frequent target of DNA hypermethylation (Esteller, 2002) implies that there must be cells with only partially methylated promoters, because *de novo* methylation of CpG islands is a gradual process. These cells, such as the 293T L α cells grown in low concentrations of Dox (Figure 5A), would

contain decreased levels of hMutL α and would therefore be likely to also have a defective response to DNA damage.

The second point concerns the nature of the endogenous DNA damage that might trigger the transformation process. It is conceivable that normal colonocytes which become damaged by endogenous or exogenous DNA modifying agents would arrest and, in cases where the extent of the damage is beyond repair, activate cell death processes, while those expressing reduced levels of hMutL α would continue to proliferate and thus acquire mutations. Although human DNA is aberrantly modified by *S*-adenosyl methionine and other methyl group donors, the extent of such modifications might be too low to trigger cell death. However, the deleterious effects of the checkpoint defect could become evident also in response to other types of DNA damage; experimental evidence implicates the MMR system in the processing of DNA modifications ranging from oxidative damage to bulky moieties such as cisplatin and AAF (reviewed in Bellacosa, 2001).

We have described a cell line in which the MMR status can be controlled by the concentration of doxycycline in the culture medium. Our current results show that the activation of transcription of exogenous *hMLH1* complements not only the MMR defect of the 293T cells, but also reactivates their responsiveness to treatment with methylating agents, providing that the levels of the MMR proteins are sufficiently high to activate the DNA damage-induced checkpoint. This fully isogenic system is clearly open to further exploitation, and should allow us to study the involvement of the MMR system in other pathways of DNA metabolism, such as response to other types of DNA damaging agents ranging from ionizing radiation to crosslinking chemotherapeutics, where the involvement of MMR was found to be only marginal and where it could not be ruled out that the observed effects (or lack thereof) were linked to a selection of an atypical clone from the stably transfected population. The 293T L α line could also be used in the screening for substances that preferentially kill MMR-deficient cells. This should prove invaluable in the treatment of tumours, both hereditary and sporadic, with defective MMR.

Materials and methods

Cell lines

The 293T cells (a kind gift of K.Ballmer) were grown in Dulbecco's modified Eagle's medium with Eagle salts (Gibco-BRL, Gaithersburg, MD), supplemented with 10% Tet System Approved Fetal Bovine Serum (Clontech, Palo Alto, CA), 2 mM L-glutamine (Gibco-BRL), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Gibco-BRL). For 293T-TetOff or 293T L α cells, 100 μ g/ml Zeocin (Invitrogen, San Diego, CA) or 100 μ g/ml Zeocin and 300 μ g/ml Hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany) were added, respectively.

Plasmid construction

The pTetOff-Zeo plasmid was constructed by ligation of the following DNA molecules: the first, coding for tTA, was obtained by digestion of pTetOff (Clontech) with *Xho*I (New England Biolabs, Beverly, MA) followed by filling-in with dCTP and dTTP using the Klenow fragment of DNA polymerase I (New England Biolabs). The second, coding for Zeocin resistance protein, was obtained by digestion of pVgR XR (Invitrogen) with *Bam*HI (New England Biolabs) followed by filling-in with dGTP and dATP. The pTRE2-hMLH1 plasmid was generated by

subcloning hMLH1 cDNA (a kind gift of R.Michael Liskay) into the *Bam*HI and *Not*I sites of pTRE2 (Clontech).

Calcium phosphate transfections

One day before transfection, 250 000 cells were plated in 6-well plates in 3 ml of cell culture medium. The cells reached ~50% confluency on the day of transfection. Three hundred microlitres of solution A (250 mM CaCl₂) was carefully mixed with 15 μ g DNA and 300 μ l of solution B (140 mM NaCl, 50 mM HEPES, 1.4 mM Na₂HPO₄ pH 7) in an Eppendorf tube. Exactly 1 min after mixing, 300 μ l of the precipitation cocktail was added to the medium. The plates were incubated for 4 h at 37°C. The medium was then removed, the cells were washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄) and fresh cell culture medium was added.

Generation of the 293T L α cell line

293T cells were transfected with pTetOff-Zeo using the calcium phosphate method (see above). The selection of stable cell lines was initiated 2 days later using 400 μ g/ml Zeocin. After 3 weeks, ~50 colonies were isolated and screened by transient transfection with pTRE2-Luc (Clontech) for the expression of luciferase in induced and noninduced cells (with or without 2 μ g/ml Dox; Clontech). The clone with the lowest background and high induction of luciferase (293T-TetOff) was then transfected with pTRE2-hMLH1 and pTK-Hyg (ratio 15:1). Selection of stable cell lines was initiated 2 days post-transfection using 400 μ g/ml hygromycin-B. After 3 weeks, ~160 colonies were isolated and their extracts were screened by western blotting using antibodies against hMLH1, hPMS2 and β -tubulin. The clone 293T L α was selected for further study, as it displayed the highest induction of hMLH1 in the absence of Dox, and no background expression with 2 μ g/ml Dox.

Regulation of hMLH1 expression

293T L α cells were grown in the presence of 50 ng/ml Dox to keep hMLH1 expression turned off; fresh Dox was added every second day. To induce hMLH1 expression, the cells were transferred to a Dox-free medium, and the cells were cultivated for at least 6 more days. To obtain cells completely free of hMLH1, cells grown in the absence of Dox were kept for at least 7 days in a medium containing 50 ng/ml Dox. To obtain intermediate levels of hMLH1, the cells were cultivated with 1.5, 0.8, 0.4, 0.2 or 0.1 ng/ml Dox for at least 7 days.

Preparation of total protein extracts for western blots

Cells were harvested, transferred to a 1.5 ml Eppendorf tube and washed twice with PBS. Cell lysis was performed on ice in 50 mM Tris-HCl pH 8, 125 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 $\times$ complete protease inhibitory cocktail (Roche Molecular Biochemicals) for 25 min. Insoluble material was pelleted by centrifugation at 18 000 *g* for 3 min at 2°C. Protein concentration was determined using the Bradford assay (Bio-Rad, Munich, Germany).

Western blot analyses

The primary antibodies used in this study were: anti-hMLH1 [PharMingen, San Diego, CA], 1:2000 in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20 with 2.5% non-fat dry milk), hPMS2 (Calbiochem; 1:500), β -tubulin, p53 (Santa Cruz Biotechnology; 1:1500 and 1:2000, respectively), cdc2 (Upstate Biotechnology; 1:1000) and phospho-p53-Ser15, phospho-cdc2-Tyr15 (Cell Signalling Technology; 1:1000 and 1:5000, respectively). The proteins (20–50 μ g) were denatured, reduced, separated by SDS-PAGE (7.5–12.5%) and transferred to Hybond-P PVDF membrane (Amersham Pharmacia Biotech) according to standard protocols (Sambrook *et al.*, 1989). The membranes were blocked with 5% non-fat dry milk in TBST for 60 min, incubated with primary antibodies for 60 min, washed three times with TBST for 10 min, incubated with the peroxidase-conjugated secondary antibody (anti-mouse IgG, 1:5000 in TBST with 2.5% non-fat dry milk) for 60 min and washed three times with TBST for 10 min. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

Indirect immunofluorescence experiments

Cells grown on coverslips were treated or mock-treated with MNNG (0.2 μ M end concentration) and incubated for 6, 12 and 24 h (Figure 4B). Foci of phosphorylated histone H2AX were visualized using an anti-phospho-H2AX rabbit polyclonal antibody (Upstate Biotechnology) at +4°C, over night, at a dilution of 1:100. The procedure was as described previously (Kleczkowska *et al.*, 2001). To allow direct comparisons, all

the cells were treated and processed simultaneously, and all the images were obtained using the same magnification, brightness and contrast settings.

MMR assays

The cell extracts were prepared as described previously (Marra *et al.*, 2001; Nystrom-Lahti *et al.*, 2002). Two different *in vitro* assays were used. The first, adapted from Holmes *et al.* (1990), is based on a circular 3' 193 bp DNA molecule containing a G/T mismatch within a unique Bg/II recognition site, a single-strand nick 369 nucleotide residues 5' from the mismatch in the G-containing strand, and a unique BsaI site. This molecule is refractory to cleavage with Bg/II, unless the mismatch is corrected to an A/T. Thus, the unrepaired heteroduplex digested with both endonucleases gives rise to only a single fragment of 3' 193 bp, whereas the repaired homoduplex is cleaved into two fragments of 1' 833 and 1' 360 bp (Nystrom-Lahti *et al.*, 2002).

The second method, originally described by Thomas *et al.* (1991), makes use of an M13mp2 DNA heteroduplex containing G/T mismatch within lacZ α complementation gene, obtained by hybridizing single-stranded viral (+) DNA with the replicative form I (–) strand. The repair is corrected to the (–) strand by the presence of a nick. The method was described in detail elsewhere (Marra *et al.*, 2001). In the complementation studies, extracts were supplemented with purified recombinant hMutL α (0.1 μ g).

MTT assays

Two thousand cells/well were plated in 96-well plates, treated the next day with various concentrations of MNNG (Sigma; diluted in dimethyl sulfoxide and stored at –20°C in the dark) and incubated for 5 days. Then, 20 μ l of MTT solution (5 mg/ml MTT; Sigma; in PBS, sterile filtered) was added, and the plates were incubated for 4–5 h at 37°C. One volume of lysis solution was then added (20% SDS, 50% dimethylformamide pH <4.7), and the plates were incubated overnight at 37°C. The solubilized formazan was quantified at 570 nm, using the Versamax microplate reader (Molecular Devices, Sunnyvale, CA). The optical density values were plotted against logarithm of MNNG concentrations and IC₅₀ values were calculated from the regression curve.

Colony-forming assays

Cells in log phase (50–80% confluent) were treated with 5 μ M MNNG, harvested after 2 h, and 200 or 2000 cells per duplicate were plated in 10 cm plates. Colonies were counted after 15–20 days of incubation. Survival was calculated as the ratio of the number of colonies from treated versus untreated samples.

Doubling time assessment

Cells (35 000) were plated in 35 mm plates. The cell number was determined daily for 4 days. The doubling time was calculated from the numbers of cells between the first and the fourth day after plating.

Cell cycle analyses

Cells (both attached and floating) were harvested, counted, washed with PBS, fixed with 70% ethanol and stored up to 1 week at 4°C. The cells were then washed once with PBS, incubated in PBS containing RNase A (100 μ g/ml, Sigma) for 1 h at 37°C, stained with propidium iodide (20 μ g/ml, Sigma) and incubated on ice in the dark for 30 min. DNA content was analysed by Coulter Epics Altra Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA). DNA cell cycle analysis software (MultiCycle, Phoenix Flow Systems, Inc., San Diego, CA) was used to quantify cell cycle distribution.

MSI analysis

293 L α cells grown with 50, 0.2 and 0 ng/ml Dox were subcloned, and grown independently for 35 generations. The chromosomal DNA was extracted using the TRI Reagent (Molecular Research Center, Lucerne, Switzerland). MSI was assessed at the mononucleotide repeat locus BAT26. PCRs were carried out in a total volume of 25 μ l containing ~100 ng of genomic DNA, as described by Loukola *et al.* (2001). The PCR products were diluted 1:4 and 0.5 μ l was added to 10 μ l deionized formamide (including 0.5 μ l GS size standard 400 ROX), denatured at 95°C for 5 min, chilled on ice and loaded on a 96-capillary ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems). MSI was defined as the occurrence of novel alleles that differed by ± 3 nucleotides from the control (Loukola *et al.*, 2001).

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