DNA Mismatch Binding Defects, DNA Damage Tolerance, and Mutator Phenotypes in Human Colorectal Carcinoma Cell Lines

Pauline Branch, Richard Hampson, and Peter Karran

Imperial Cancer Research Fund, Clare Hall Laboratories, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3LD, United Kingdom

ABSTRACT

DNA mismatch binding in vitro, resistance to DNA methylation damage, and spontaneous mutation rates were examined in human colorectal adenocarcinoma cell lines. Of 11 cell lines, 3 (DLD1, HCT15, and LoVo) were defective in mismatch binding. All three lines had a mutator phenotype. These properties indicate that DLD1 and HCT15 may, like LoVo, carry mutations in the mismatch recognition protein hMSH2. Mismatch binding was normal in the remaining eight lines, including HCT116 in which a second mismatch repair protein, hMLH1, is defective. Two lines, SW620 and SW48, did not express detectable levels of the DNA repair enzyme O\(^6\)-methylguanine-DNA methyltransferase. SW620 exhibited the expected sensitivity to N-methyl-N-nitrosourea. In contrast, SW48 cells were highly resistant to N-methyl-N-nitrosourea and also slightly to methyl methanesulfonate, indicating that they are tolerant to DNA methylation damage. SW48 exhibited the spontaneous mutator phenotype and microsatellite instability that are hallmarks of a defect in mismatch repair. This cell line provides evidence for the association between methylation tolerance and defective mismatch correction in human colorectal carcinoma cells. The properties of methylation-tolerant, mismatch repair-defective cells identify possible selective pressures that might facilitate the natural selection of mismatch repair-defective tumors.

INTRODUCTION

A significant proportion of human cancer is associated with defects in a DNA mismatch repair pathway that corrects replication errors. HNPCC\(^2\) comprises 1–5% of all colorectal cancer and is associated with an increased incidence of endometrial and other malignancies (reviewed in Ref. 1). HNPCC tumors, and about 15% of sporadic colon tumors, exhibit a characteristic phenotype that includes high rates of mutation in DNA microsatellites as a direct consequence of uncorrected replication errors (2–4). HNPCC individuals have germ line mutations in one of four genes, each encoding a homologue of a prokaryotic DNA mismatch repair protein. The most commonly affected locus, hMSH2 on chromosome 2p (5), is homologous to the Escherichia coli mutS\(^+\) gene. The remaining known HNPCC genes map to chromosomes 2q, 3p, and 7q (6–8) and their products, hMSH2, hMLH1, and hPMS2, are all homologues of the E. coli MutL protein. The E. coli MutS and MutL proteins and their yeast counterparts, MSH2, PMS1, and MLH1, are involved in the initial stages of mismatch repair (9, 10). A major function of the mismatch repair pathway is to correct DNA replication errors that escape proofreading, and mismatch repair mutants have a spontaneous mutator phenotype (11).

The properties of many established colorectal adenocarcinoma cell lines are consistent with inactive mismatch repair genes. Microsatellite instability (12–14), defects in mismatch repair functions in cell extracts (14, 15), mutator phenotypes (13, 16), and mutations in mismatch repair genes (7, 14) have variously been observed. One line, LoVo, is homozygous for a partial deletion of the hMSH2 gene (14) and lacks a mismatch binding activity (17) that involves the hMSH2 protein (18). LoVo cells also exhibit microsatellite instability (12) and are unable to correct mismatches in an in vitro assay (14). Defects in mismatch repair, a mutator phenotype, and microsatellite instability are also associated with a mutated hMLH1 gene in HCT116 cells (7, 15).

Defective mismatch repair is also implicated in acquired resistance to some cytotoxic drugs. Methylating agents such as MNUN and MNG produce cytotoxic DNA damage. Human cells are normally protected against the most prevalent cytotoxic lesion, O\(^6\)-meGua, by the DNA repair enzyme MGMT. MGMT is susceptible to epigenetic silencing (19, 20), and Mex\(^-\) cells, in which it is not expressed, are very sensitive to killing by MNUN and MNG (21). After exposure to methylating agents, Mex\(^-\) cells often acquire specific resistance, or tolerance, to O\(^6\)-meGua in DNA. O\(^6\)-meGua residues persist in the DNA of tolerant cells but are no longer lethal. One current hypothesis is that methylation tolerance arises through loss of a mismatch repair pathway, and many of the properties of methylation tolerant cells are consistent with a mismatch repair defect (reviewed in Ref. 22). The first examples of defective mismatch binding in the in vitro assay were two methylating-agent tolerant cell lines that exhibited a mutator phenotype and microsatellite instability (17, 23). A third methylating-agent tolerant mutator cell line with microsatellite instability (14) was found to be defective in mismatch repair in an in vitro assay (24). The recent observation that transfer of human chromosome 3 encoding a normal hMLH1 gene into hMLH1-defective HCT116 cells corrects their mismatch repair defect, stabilizes microsatellite sequences, and apparently induces concomitant sensitization to MNG (25), also supports the hypothesis that methylation resistance is acquired by loss of mismatch repair functions.

The similarities between the properties of methylation-tolerant and mismatch repair-defective colorectal cells prompted us to examine whether methylation tolerance with associated mismatch repair defects might occur in colorectal tumors. Using a bandshift assay (26), we have identified two additional colorectal tumor cell lines, DLD1 and HCT15, that are unable to bind to G-T mismatches. These defective cells are mutators at the HPRT locus, consistent with a defect in mismatch correction in an in vitro assay (14). A high level of MGMT expression in DLD1 and HCT15 precluded an assessment of their methylation tolerance.

A third cell line, SW48, provided a direct example of a colorectal carcinoma line with a methylation-tolerant phenotype, microsatellite instability, and an increased rate of spontaneous mutation, indicative of a compromised mismatch repair pathway. DNA mismatch binding in vitro was normal in SW48, suggesting that mutations in one of the human MutL homologues underlies its mismatch correction defect. The demonstration of methylation tolerance in SW48 suggests that this type of methylation damage resistance may be a property of some colorectal carcinomas. Endogenous formation and persistence of DNA methylation damage might provide a selective pressure for the emergence of tumor cells with a mutator phenotype.

MATERIALS AND METHODS

Cell Lines. Colorectal adenocarcinoma cell lines LS174T, HCT15, HCT116, DLD1, LS1034, SW620, LS411, HCA7, and SW48 were kindly
provided by C. Dixon, Cancer Genetics Laboratory, Imperial Cancer Research Fund. LoVo, HT29, and Raji were originally obtained from the American Type Tissue Collection and have been maintained at Imperial Cancer Research Fund, Clare Hall. All cell lines were cultured in appropriate growth medium in humidified CO₂ incubators. Genetic fingerprinting indicated that LoVo could be distinguished from SW48 by the same method.

Chemicals. All chemicals were from Sigma Chemical Co. (Poole, UK) unless otherwise indicated. Methyl methanesulfonate was obtained from Aldrich (Gillingham, UK) and diluted in PBS immediately prior to use. Recrystallized MNU, a gift from Professor Peter Swann (University College, University of London, London, England) was dissolved in 10 mM potassium acetate (pH 5.0), stored in aliquots at -20°C, and thawed immediately before use. O6-bzGua was kindly provided by Professor Manfred Rajewsky (University of Essen Medical School, Essen, Germany). Radiolabeled compounds were obtained from Amersham International (Buckinghamshire, United Kingdom).

Bandshift Assay. Bandshift assays using duplex 34-mer oligonucleotides containing a single G-T mismatch were performed as described previously (26, 27). Briefly, cell extracts (15 μg) were combined with 20 fmol 32P end-labeled heteroduplex in the presence or absence of a 4-fold excess of nonradioactive homoduplex. After 20 min at 20°C, free and bound heteroduplexes were separated by electrophoresis through 6% nondenaturing polyacrylamide gels. Reaction products were detected by autoradiography.

Methyltransferase Assay. MGMT was assayed as described previously (28). Cell extracts were prepared by suspending 2 × 10⁶ cells in 200 μl ice-cold extraction buffer composed of 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 10 mM DTT, and 0.2% Triton X·100, followed by centrifugation (5 min at 105 × g). The supernatant was retained, and 0–150 μg protein was added to a 100-μl reaction mixture composed of 50 mM HEPES·KOH (pH 7.8), 1 mM EDTA, and 10 mM DTT containing [3H] methylated DNA enriched for O6-methylguanine (Ref. 29; specific activity, 10⁶ dpm/pmol O6-methylguanine, approximately 0.1 pmol/assay). After 20 min at 37°C, DNA and protein were precipitated by the addition of 10% trichloroacetic acid and recovered by centrifugation. The precipitated DNA was hydrolyzed with 0.1 N HCl for 30 min at 70°C, and the radioactive O6-methylguanine liberated was quantitated by scintillation counting. One unit of MGMT activity removes 1 pmol O6-methylguanine from DNA.

Cell Survival. Exponentially growing cells were seeded in full growth medium in 10-cm Petri dishes and allowed to attach for 2 h at 37°C. MNU was added, and the cells were returned to 37°C. Surviving colonies were scored 10–14 days later. Treatment with MMS was identical with the exception that the MMS-containing medium was replaced by fresh growth medium after 60 min. Pretreatment of cells with O6-bzGua at a final concentration of 12 μM was carried out for 60 min prior to treatment with MNU.

Mutation Rates. Cells were cloned by growth in 96-well microtiter plates. The generation time of each individual clone was determined by daily cell counting. After a known number of population doublings, cells were plated in 10-cm dishes in medium containing 6-thioguanine (5 μg/ml). Surviving colonies were scored after 10–14 days, and the frequency of 6-thioguanine-resistant clones was calculated. The mutation rate was approximated by dividing the HPRT₁ mutation frequency by the estimated number of cell doublings undergone by the population. Data from determinations on at least two individual clones for each cell line were averaged.

Microsatellite Instability. Subclones of SW48 and SW620 were isolated in 96-well microtiter plates. A single clone of each cell line was maintained for a known number of cell doublings. The subcloning of SW48 was carried out some time prior to that of SW48, and these values were estimated to be 20 and 40 for SW48 and SW620, respectively. Each population was plated in 96-well plates at single-cell density. When colonies became visible by the naked eye (15–25 days), the medium was removed, and colonies were washed with PBS. Cells were lysed by suspension for 2 h at 37°C in PCR-compatible lysis buffer [10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 100 μg/ml BSA, and 50% Tween 20] containing 100 μg/ml Proteinase K. After inactivation of the Proteinase K by heating at 95°C for 3 min, the lysate was used as a template for PCR. Thirty-five cycles at 90°C for 1 min, 55°C for 0.5 min, and 72°C for 0.5 min were performed with fluorescently labeled primers for microsatellite loci D2S123, D2S378, S11S904, and D10S197 (30). Products were analyzed by PAGE on an Applied Biosystems 373A DNA Sequencer.

RESULTS

Mismatch Binding by Cell Extracts. Bandshift assays with oligonucleotides containing a G-T mismatch were performed with extracts of the established colon carcinoma cell lines. Specific mismatch
binding is detected as a complex (Fig. 1, band B) that migrates more slowly than a nonspecific complex. The latter (Fig. 1, band C) is also formed with homoduplexes (27), and its formation is suppressed by the inclusion of a homoduplex competitor in the assay. Detectable band B (Fig. 1) was not formed by extracts of HCT15 and DLD1. These two lines resembled LoVo cells in which a similar G-T binding defect has been described (17). Mixing extracts of LoVo and either DLD1 or HCT15 did not restore G-T binding activity (data not shown), indicating that LoVo and DLD1/HCT15 may share a common defect. G-T mismatch binding by extracts of HCA7, HCT116, LS174T, LS411, LS1034, SW48, and SW620 was apparently normal and was comparable to that of the Raji and HT29 control cells. Formation of the non-specific complex (Fig. 1, band C) was similar with extracts of HCT15, DLD1, the other nine colorectal carcinoma cells, and Raji. The formation of this complex serves as an internal control for the quality of the extracts.

SW620, HCT116, and DLD1 were investigated further. HCT15 was isolated from the same tumor as DLD1. It shares the same binding defect and is apparently identical by genetic fingerprinting; it was, therefore, excluded from further study. HCT116 was included as an example of a line that is deficient in mismatch repair and carries a defect and is apparently identical by genetic fingerprinting; it was, isolated from the same tumor as DLD1. It shares the same binding control for the quality of the extracts.

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The Mex+ Phenotype and Sensitivity to DNA Methylation Damage. MGMT activity was determined in extracts of all cell lines. With the exception of SW620 and SW48, all cell lines expressed MGMT levels in the normal range. The specific MGMT activities of DLD1, HT29, SW620, HCT116, and SW48 are presented in Table 1. Extracts of SW620 and SW48 cells contained <0.05 enzyme units/mg protein, indicating that these cells have a Mex− phenotype. DLD1 and HT29 were Mex+, and extracts contained 0.7 and 1 enzyme unit/mg protein, respectively. HCT116 cells were also Mex+, but extracts of these cells contained somewhat less MGMT activity (0.4 units/mg protein).

Among these cell lines, the Mex− SW48 cells were anomalously resistant to killing by MNU. The other Mex− line, SW620, exhibited the characteristic sensitivity of Mex− cells that are not methylation tolerant (Fig. 2a). The high MNU resistance of HT29 and DLD1 reflected their high levels of MGMT. The more moderate MGMT level in HCT116 was associated with an intermediate MNU sensitivity (Fig. 2; Table 1). The survival of the Mex− SW48 closely resembled that of the Mex+ HCT116 and DLD1 cell lines, suggesting that SW48 is methylation tolerant.

We determined the sensitivity of SW48 and SW620 to MMS. O6-meGua comprises ≤0.2% of the total DNA methylation products of MMS. Other abundant and potentially lethal methylation products, 3-methyladenine, 7-methylguanine, and 3-methylguanine are produced to similar extents by MMS, MNU, and MNNG (31). SW48 exhibited the slight (approximately 2-fold) resistance to MMS that is characteristic of methylation-tolerant cells (32). This observation is consistent with a selective resistance to DNA O6-meG in SW48 and confirms that these cells are methylation tolerant.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MGMT activity (units/mg protein)</th>
<th>D37, MNU (mm)</th>
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<tbody>
<tr>
<td>SW48</td>
<td>≥0.05</td>
<td>0.8</td>
</tr>
<tr>
<td>DLD1</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>HCT116</td>
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<td>0.6</td>
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<tr>
<td>HT29</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>SW620</td>
<td>≥0.05</td>
<td>0.05</td>
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Table 1 MGMT activity and MNU sensitivity of colorectal carcinoma cells

In attempts to assess whether any of the Mex+ colon cell lines were methylation tolerant, MGMT activity of HCT116, DLD1, and control HT29 cells was inactivated by growth in the presence of the pseudosubstrate O6-bzGua, which causes a rapid and irreversible inactivation of MGMT (33). This treatment has been reported to sensitize HT29 cells somewhat to the lethal effects of monofunctional methylation agents 5-(3-methyl-1-triazeno)imidazol-4-carboxamide and streptozotocin (34). After preincubation for 1 h in growth medium containing 12 μM O6-bzGua, active MGMT levels were reduced by ≥85% in extracts of DLD1, HT29, or HCT116. When the MNU resistance of these MGMT-depleted cells was examined, it was found that the reduction of active MGMT in DLD1, HCT116, or the control HT29 cells did not detectably affect their sensitivity to MNU killing, and the D37 values remained essentially unchanged (data not shown). The residual MGMT activity remaining after O6-bzGua treatment is >85% in extracts of DLD1, HT29, or HCT116. When the MNU resistance of these MGMT-depleted cells was examined, it was found that the reduction of active MGMT in DLD1, HCT116, or the control HT29 cells did not detectably affect their sensitivity to MNU killing, and the D37 values remained essentially unchanged (data not shown).
The increase in mutation rate in SW48 was more modest than in DLD1 very close to published values for other human cell lines without a mismatch repair defect. The mismatch binding defect in DLD1 and LoVo conferred a mutator phenotype after its isolation. These values were used to derive the mutation rate to HPRT\(^{-}\). Table 2 shows the estimated rate for each line. This method of approximating the mutation rate is less rigorous than fluctuation analysis, but our estimated values for the mutation rate in SW48 cells, a total of seven determinations were performed. The mutation rates estimated from these values were closely similar for all the clones, and the data have been pooled. They are presented with SDs in Table 2. The increase in mutation rate in SW48 was more modest than in DLD1 or HCT116 and was about 5-fold higher in DLD1 cells compared to the control HT29, which exhibited a rate very close to published values for other human cell lines without mismatch repair defects. For the methylation-tolerant SW48 cells, a total of seven determinations were performed. The mutation rates estimated from these values were closely similar for all the clones, and the data have been pooled. They are presented with SDs in Table 2. The increase in mutation rate in SW48 was more modest than in DLD1 or HCT116 and was about 5-fold higher than HT29. The rate of mutation to HPRT\(^{-}\) in SW620 cells was very low \((<1 \times 10^{-9}/\text{cell/generation})\), in agreement with the reported presence of two active HPRT genes in this line. The mismatch binding defects in DLD1 and LoVo, the hMLH1 defect in HCT116, and methylation tolerance in SW48 are, therefore, all associated with a mutator phenotype. Both DLD1 and LoVo are defective in mismatch repair in an in vitro assay. It seems highly probable, therefore, that SW48 cells are mismatch repair deficient.

### Spontaneous Mutation Rates

Clonal isolates of DLD1, HCT116, HT29, SW48, SW620, and LoVo were obtained by growth in microwell plates. The growth rates of the clones were similar, and doubling times ranged from 24–30 h. The frequency of HPRT\(^{-}\) mutants was determined for each clonal population 30–50 doublings after its isolation. These values were used to derive the mutation rate to HPRT\(^{-}\). Table 2 shows the estimated rate for each line. This method of approximating the mutation rate is less rigorous than fluctuation analysis, but our estimated values for the mutation rate in DLD1 and HCT116 are, nevertheless, close to those reported (13, 16). The mismatch binding defect in DLD1 and LoVo conferred a mutator phenotype of comparable magnitude to that in HCT116. The rate of spontaneous mutation to HPRT\(^{-}\) was close to 100-fold higher in DLD1 and HCT15 compared to the control HT29, which exhibited a rate very close to published values for other human cell lines without mismatch repair defects (13, 16). For the methylation-tolerant SW48 cells, a total of seven determinations were performed. The mutation rates estimated from these values were closely similar for all the clones, and the data have been pooled. They are presented with SDs in Table 2. The increase in mutation rate in SW48 was more modest than in DLD1 or HCT116 and was about 5-fold higher than HT29. The rate of mutation to HPRT\(^{-}\) in SW620 cells was very low \((<1 \times 10^{-9}/\text{cell/generation})\), in agreement with the reported presence of two active HPRT genes in this line. The mismatch binding defects in DLD1 and LoVo, the hMLH1 defect in HCT116, and methylation tolerance in SW48 are, therefore, all associated with a mutator phenotype. Both DLD1 and LoVo are defective in mismatch repair in an in vitro assay (14). It seems highly probable, therefore, that SW48 cells are mismatch repair deficient.

### Microsatellite Instability

Microsatellite instability is a characteristic feature of mismatch repair-deficient cells. The methylation-tolerant SW48 cells exhibited microsatellite instability. Five of a total of 75 SW48 subclones had mutated alleles at the D2S123 locus, 3 of 30 at the D11S9043 locus, 5 of 30 at the D10S197 locus, and 1 of 42 had mutations at the D2S177 locus. Altered alleles invariably differed by the loss or gain of a single dinucleotide repeat, and there were no instances of larger deletions or insertions (Fig. 3). No mutations were observed at any locus among 36 clones of SW620, a total of 144 determinations. The estimated number of cell doublings for the respective clones were 20 (SW48) and 40 (SW620). The combined values indicate a rate of mutation for SW48 of \(4 \times 10^{-3}\) and for SW620 of \(<1.7 \times 10^{-4}/\text{cell/generation/locus}\), an increased mutation rate of >20-fold \((P < 0.05)\) in SW48.

### DISCUSSION

To date, HNPCC mutations have been detected in hMSH2 and three mutL\(^{+}\) homologues (5–8), and these genes are probably also involved in some of the 15–20% of sporadic colorectal cancers that exhibit microsatellite instability (35). We have previously reported a mismatch binding defect in the colorectal adenocarcinoma line LoVo (17). LoVo cells exhibit microsatellite instability, cannot correct mismatches in a cell-free assay, and are homozygous for a partially deleted gene that encodes a truncated hMSH2 protein (14). Our observation of a mutator phenotype at the HPRT locus of LoVo is, therefore, consistent with its known genotype. We have shown here that DLD1 and its relative HCT15 are defective in the same mismatch binding function as LoVo. These lines have been reported to be mutators (13, 16), and our data confirm this and suggest that the magnitude of the mutator effect is similar in LoVo. DLD1 and HCT15 are also unable to complement the G-T mismatch binding defect of LoVo cell extracts. It is possible, therefore, that DLD1 and HCT15 also carry mutations in the hMSH2 mismatch binding activity.

LoVo and DLD1/HCT15 represent 2 (of 11 tested) independent colorectal lines with defective mismatch binding. The bandshift assay is the only one presently available that investigates an individual step in human mismatch repair. Observed binding is not necessarily indic-
ative of functional activity, however. The assay would not distinguish a normal binding protein from an altered protein able to bind but not to interact with one or more MutL homologues. Since mismatch binding is normal in HCT116 in which hMLH1 is mutated (6), functional hMLH1 protein is clearly not required for formation of the complex detected by this bandshift assay.

There is considerable overlap between the phenotypes of mismatch repair-defective tumor cells and cells tolerant to methylation damage. Acquired methylation tolerance is normally studied in Mex− cells in which the constitutive defense against \( O^6 \)-meGua, the MGMT protein, is inactive. The presence of active MGMT in all the mismatch binding-defective colorectal carcinoma lines prevented us from establishing unequivocally whether they were methylation tolerant. Transfer of a single normal human chromosome 3 into HCT116 has been reported to restore efficient mismatch repair, reduce microsatellite instability, and greatly sensitize the cells to MNGN (25). Although these observations are consistent with a methylation-tolerant phenotype in HCT116, there are possible confounding factors. The reported degree of MNNG sensitization is more pronounced than might be expected in a cell line with a Mex− phenotype such as HCT116. It is probable that sensitization to that extent requires at least a partial loss of MGMT expression. The MGMT gene is located on chromosome 10 (36). Expression of MGMT from a human chromosome 10 present in hamster-human hybrid cells is reduced if human chromosome 3 is present simultaneously (37). The presence of an extra chromosome 3 may contribute to the sensitization of HCT116 cells by interfering with the expression of MGMT. This interference would be independent of any possible effects on methylation tolerance, and the methylation tolerant status of HCT116 cells should be regarded as undefined.

SW48 cells provides the first unequivocal example of methylation tolerance in a colon carcinoma line. These cells are Mex− but resistant to MNU and MMS to an extent that is compatible with methylation damage, providing the selective pressure to inactivate the mismatch repair pathway.

ACKNOWLEDGMENTS

We thank Cynthia Dixon for providing the cells line and advice on their culture. The skilled assistance of the Clare Hall cell production staff is also gratefully acknowledged.

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