Chemical carcinogens induce varying patterns of LOH in mouse T-lymphocytes

Susan W.P.Wijnhoven, Edwin Sonneveld, Hanneke J.M.Kool, Corrie M.M.van Teijlingen and Harry Vrieling

Department of Radiation Genetics and Chemical Mutagenesis – MGC, Leiden University Medical Center, PO Box 9503, 2300 RA Leiden, The Netherlands

To whom correspondence should be addressed
Email: h.vrieling@lumc.nl

We have shown previously that a wide range of mutagenic carcinogens are capable of inducing loss of heterozygosity (LOH) at the endogenous Aprt locus in mouse splenic lymphocytes. To investigate whether LOH might be caused by a single common mechanism, we set out to determine the extent of LOH by microsatellite analysis along (the Aprt gene containing) mouse chromosome 8. Aprt +/− hybrid B6C3F1 mice were treated with mutagens that induce different classes of DNA lesions, i.e. bulky DNA adducts, DNA methylation, DNA inter-strand crosslinks or DNA strand breaks. Aprt mutant frequencies (MF) in this C57B/6-C3H hybrid background were significantly reduced for mitomycin C (MMC) and methylmethanesulfonate (MMS) in comparison with MF in C57B/6 background, suggesting either enhanced repair or reduced formation of MMC- or MMS-induced mutagenic lesions in a hybrid B6C3F1 background. In contrast, Aprt MF after dimethylbenz[a]anthracene (DMBA), methylinitrosurea (MNU) and etoposide treatment were similar in both genetic backgrounds. Microsatellite analysis of Aprt mutant clones indicated a dominant role for mitotic recombination (MR) in generating spontaneous, DMBA- and etoposide-induced LOH at Aprt. However, over 80% of the MNC-induced Aprt LOH mutants had lost heterozygosity for all markers tested, suggesting that either the crossover points were located close to the centromere or that these mutants arose by chromosome loss and duplication of the remaining chromosome 8. A substantial fraction (40%) of MNU-induced Aprt mutants had lost the wild-type Aprt allele, but had retained heterozygosity at all polymorphic markers tested at chromosome 8 indicating an important role for deletions in LOH formation by MNU. Patterns of MR differed quite dramatically for the various chemical mutagens tested, suggesting different mechanisms to be involved in inducing recombination between homologous chromosomes. In addition, non-random adduct formation and repair between chromosomal regions, i.e. heterochromatin versus euchromatin, may contribute to a non-random distribution of recombinational crossover points.

Introduction

Loss of heterozygosity (LOH) is the most common molecular genetic alteration in human cancer. It can result in the complete loss of function of tumour suppressor genes, leading to abnormal growth control of cells. For example, LOH at loci such as RB1, WT1 or TP53 has been demonstrated to be a key event in the hereditary syndromes retinoblastoma, Wilms tumour or Li Fraumeni, respectively (1). In sporadic tumours, up to 50% of all chromosomes may have undergone LOH events. Apart from LOH of tumour suppressor genes, secondary regions of LOH exist that do not bear a tumour suppressor gene and mostly arise as a consequence of general chromosome instability in advanced tumours (2). Losses of chromosomal regions can alter patterns of gene expression, adapting the cells to optimal growth within the tumour environment (3).

Several genetic events, including mitotic recombination, multi-locus deletion or chromosome loss with or without duplication of the remaining homologous chromosome, have been proposed to generate LOH of extensive genomic regions. The term mitotic recombination (MR) refers to recombinational events between homologous chromosomes that, following chromosome segregation and cell division, can result in LOH of numerous genetic loci on the involved chromosome. MR has been observed in several human tumour types and has been implicated as a major contributor to the tumorigenic process. However, LOH and MR are not restricted to tumour cells. LOH events can occur in somatic cells of man and mice (4–7), are compatible with cell survival and are suggested to represent an initiating event in tumorigenesis (8,9). However, the molecular mechanisms triggering and controlling formation of LOH in somatic and genetically stable cells are still largely unknown.

To further unravel underlying mechanisms and events that generate LOH in somatic cells, we and others have generated via gene targeting a mouse model that is heterozygous at the selectable marker encoding the purine salvage enzyme adenine phosphoribosyltransferase (APRT) (10–12). In this mouse model, spontaneous and chemically induced events leading to loss of function of the autosomal Aprt locus, can be detected in T-lymphocytes and skin fibroblasts. About 70–80% of spontaneous Aprt mutants in these somatic cell types exhibited loss of the non-targeted Aprt allele (6,12–14). Data gained in two subsequent studies have shown that also carcinogen treatment of Aprt +/− mice predominantly led to LOH events at the autosomal Aprt locus (8,9). In the majority of the chemically induced Aprt mutants, a duplication of the targeted allele was observed, pointing to MR as the cause for LOH. The observation that one mechanism appeared to be responsible for the LOH events induced by a wide spectrum of carcinogens, suggested the existence of a common trigger of MR. A possibility for such a mutual trigger of recombinational events might be the blockage of DNA replication (9). This notion was strengthened by the proposal of a new replication-based recombination model in which recombination is visualized to

Abbreviations: 8-AA, 8-azaadenine; APRT, adenine phosphoribosyltransferase; DMBA, dimethylbenz[a]anthracene; DSB, double stranded breaks; ICL, inter-strand cross-links; LOH, loss of heterozygosity; MF, mutant frequency; MMC, mitomycin C; MMS, methylmethanesulfonate; MNU, methylinitrosurea; MR, mitotic recombination.

© The Article Author(s). Published by Oxford University Press 2003

139

Carcinogenesis vol.24 no.1 pp.139–144, 2003
be intimately coupled with replication. It has been reported that homologous recombination is thought to be essential for restart of stalled replication forks in *Escherichia coli*, yeast and in mammalian cells (15–17).

In the present study, a more detailed analysis of the extent of LOH and its possible causes in carcinogen-induced *Aprt* mutant T-lymphocytes is described. *Aprt*<sup>+/−</sup> mice in a C57Bl/6 background were crossed with wild-type C3H/HeJBr mice in order to obtain *Aprt*<sup>+/−</sup> hybrid B6C3F1 mice. These mice were treated with a variety of chemical carcinogens, including dimethylnitrosurea (DMBA), methylNitosourea (MNU), etoposide, methylmethanesulfonate (MMS) and mitomycin C (MMC), that induce structurally different types of DNA adducts. Carcinogen-induced *Aprt*-deficient mutants were isolated and analysed in detail using polymorphic microsatellite markers along the whole length of chromosome 8.

**Materials and methods**

**Mice**

The generation of *Aprt*<sup>+/−</sup> mice in which one copy of the *Aprt* gene was inactivated by gene targeting has been described elsewhere (12). *Aprt*<sup>+/−</sup> mice in a C57Bl/6 background (backcross generation F<sub>6</sub>) were crossed with C3H/HeJBr mice (Charles River, Maastricht, The Netherlands) to obtain F<sub>1</sub>-hybrid *Aprt*<sup>+/−</sup> B6C3F1 mice that were subsequently used in the described mutational studies. Genotypes were determined by allele-specific PCR analysis of lysates from tail tips. Lysis of tail tips was done overnight at 60°C in a lysis buffer containing 50 mM Tris–HCl (pH 8.9) and 12.5 mM MgCl<sub>2</sub>, in the presence of 0.2 mg proteinase K. After deactivating the proteinase K for 10 min at 95°C, PCR analysis was performed. The PCR protocol and the sequences of the *Aprt* primers have been described previously (8).

**Chemicals and exposure**

Both male and female *Aprt*<sup>+/−</sup> mice were treated with a single i.p. dose of chemical agent at the age of 8–12 weeks. MNU (Pfaltz and Bauer, Waterbury, USA) was dissolved in phosphate-citrate buffer (18) and administered at a dose of 60 mg/kg. MMC (Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in PBS pH 7.4 and mice were treated with a dose of 2 mg/kg MMC. DMBA (Sigma-Aldrich, Zwijndrecht, The Netherlands), was given at a dose of 40 mg/kg dissolved in Tricaprilyn (Sigma-Aldrich, Zwijndrecht, The Netherlands). Etoposide (Bristol-Myers, NY) was diluted with 0.9% saline and mice were exposed to a dose of 1 mg/kg. MMS (Sigma-Aldrich, Zwijndrecht, The Netherlands) was diluted with PBS and administered at a dose of 60 mg/kg.

The number of animals per treatment group varied between 6 and 15 animals. The group of untreated control mice consisted of 14 hybrid B6C3F1 and 55 C57Bl/6 mice. Seven weeks after treatment, mice were killed and spleens were isolated.

**Isolation and culture of splenic T-lymphocytes**

Priming and cloning of T-lymphocytes was performed in RPMI culture medium as described by Tates *et al.* (19). The detailed protocol for isolation, freezing and thawing of mouse splenocytes as well as priming of the cells with concanavalin A has been described in detail previously (8). Mutant clones were selected by adding 8-azadenedine (8-AA) to the culture medium for the recovery of *Aprt*-deficient mutants. Calculation of the cloning efficiencies and mutant frequency (MF) was performed as described (19).

**Isolation and allele-specific PCR of *Aprt* mutant clones**

8-AA resistant clones were selected and diluted 1:3 in culture medium containing 50 μg/ml 8-AA. After 3–4 days of culturing, cells were collected and centrifuged. Cell pellets were washed with phosphate-buffered saline and processed to give crude cell lysates. Subsequently, an allele-specific PCR with the following primers (*Eurorgenet*, Seraing, Belgium) was performed to determine whether mutants had lost the wild-type allele. 533: 5′-CCC-AGGTCCAGAAGACTAGC-3′; 854: 5′-GGCAGGGAGGAGTAGTAT-3′; ums-1: 5′-GGGTTTGTATCGGTGGACAG-3′.

**PCR of simple sequence repeat markers (SSR) flanking *Aprt***

*Mutant clones* that had lost the wild-type allele (LOH) were further characterized by PCR amplification of SSR markers along the length of chromosome 8. The sequence of the markers and the location at chromosome 8 are according to the Mouse Genome Database (The Jackson Laboratory; http://www.informatics.jax.org). PCR primers for SSR markers were purchased from Eurogentec. A multiplex PCR with Hotgoldstar DNA-Polymerase (Eurogentec) was performed according to manufacturers procedures.

**Fragment analysis**

DNA-fragments were analysed on an ALF Express II machine using the ALfwin® Fragment Analyzer 1.00 software (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Results**

**Aprt MF in C57Bl/6 and hybrid B6C3F1 mice**

Young adult mice of pure C57Bl/6 or hybrid B6C3F1 background were treated with various carcinogens in order to determine mutation induction at the autosomal *Aprt* gene (Table I). All tested agents were administered by i.p. injection at a subtoxic dose, while for all chemicals, a group of control mice was treated with the relevant solvent. Seven weeks after i.p. exposure, *Aprt* MF were determined in isolated splenic T-lymphocytes. The data set concerning C57Bl/6 mice was partly published previously (9).

Although we have used four different solvents in this and the previous study, *Aprt* MF of all of the control animals were in the same range (separate data not shown). Levels of background MF were therefore combined into one unexposed

---

**Table I. *Aprt* mutant frequencies and LOH frequencies in *Aprt*<sup>+/−</sup> mice in a C57Bl/6 inbred or B6C3F1 hybrid background**

<table>
<thead>
<tr>
<th></th>
<th>C57Bl/6 inbred background&lt;sup&gt;a&lt;/sup&gt;</th>
<th>B6C3F1 hybrid background</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>% induced&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.6 ± 1.1</td>
<td>135/188</td>
<td>72</td>
</tr>
<tr>
<td>185.0 ± 38.0</td>
<td>28/109</td>
<td>26</td>
</tr>
<tr>
<td>72.2 ± 14.8</td>
<td>61/74</td>
<td>82</td>
</tr>
<tr>
<td>34.9 ± 10.1</td>
<td>64/92</td>
<td>70</td>
</tr>
<tr>
<td>25.0 ± 3.9</td>
<td>44/54</td>
<td>81</td>
</tr>
<tr>
<td>20.8 ± 3.8</td>
<td>108/124</td>
<td>87</td>
</tr>
<tr>
<td>Background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MNU (60 mg/kg)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.4 ± 1.1</td>
<td>89/109</td>
<td>82</td>
</tr>
<tr>
<td>314.0 ± 81.0</td>
<td>23/70</td>
<td>33</td>
</tr>
<tr>
<td>15.9 ± 3.8</td>
<td>95/110</td>
<td>86</td>
</tr>
<tr>
<td>47.2 ± 11.3</td>
<td>29/34</td>
<td>85</td>
</tr>
<tr>
<td>18.3 ± 5.9</td>
<td>82/82</td>
<td>82</td>
</tr>
<tr>
<td>4.7 ± 0.7</td>
<td>44/55</td>
<td>80</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data on C57Bl/6 mice were partly published in Wijnhoven *et al.* (9).

<sup>b</sup>Values represent mean *Aprt* mutant frequency ± SEM.

<sup>c</sup>Percentage of *Aprt* mutants with LOH induced by the chemical treatment. Percentages were calculated by correcting for the contribution of spontaneous LOH mutants.

<sup>d</sup>Total number of mice analysed.

<sup>e</sup>All treatments were performed by exposing the mice to a single i.p. dose of mutagen.

<sup>f</sup>ND, not determined.
Table II. Markers lost in carcinogen-induced Aprt-deficient LOH mutants of hybrid B6C3F1 mice

<table>
<thead>
<tr>
<th>Exposure</th>
<th>DMB</th>
<th>MNU</th>
<th>Etoposide</th>
<th>MMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>47.2</td>
<td>314</td>
<td>6.4</td>
<td>15.9</td>
</tr>
<tr>
<td>MF</td>
<td>Total no. of clones</td>
<td>% induced</td>
<td>Total no. of clones</td>
<td>% induced</td>
</tr>
<tr>
<td>0</td>
<td>10/87</td>
<td>11</td>
<td>0.6</td>
<td>8.6</td>
</tr>
<tr>
<td>1</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3/92</td>
<td>3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>7/92</td>
<td>8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>6/87</td>
<td>7</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>1/87</td>
<td>1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>2/87</td>
<td>2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>2/87</td>
<td>2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>8</td>
<td>27/87</td>
<td>31</td>
<td>3.8</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*Mutant frequency (×10^-4) as determined spontaneously and after chemical treatment at the Aprt locus in hybrid B6C3F1 mice.*

*Injection sites of different classes of genetic events leading to LOH such as MR, multilocus deletion or chromosome loss and reduplication.*

*The fraction of MMS-induced mutants with LOH is thus (18.1 - 6.2) × 10^-6/(20.8 - 8.6) × 10^-6 = 0.97. It is clear that background LOH levels can strongly influence mutagen-induced LOH percentages, especially when the induced MF is only slightly above the background.*

*Analysis of the mechanisms underlying LOH in carcinogen-induced Aprt-deficient mutants*

Microsatellite analysis using polymorphic microsatellite markers that cover the total length of chromosome 8, was performed on the isolated Aprt mutants from hybrid B6C3F1 background to obtain more information concerning the mechanisms underlying LOH formation induced by the various mutagens. We used eight different markers that can distinguish between the chromosomes 8 of C57Bl/6 (with the targeted Aprt allele) and C3H (with the wild-type Aprt allele) mouse strain. It is possible in this way to discriminate between the different classes of genetic events leading to LOH such as MR, multilocus deletion or chromosome loss and reduplication. Table II describes the number of markers that were lost in individual 8-AA clones obtained from untreated as well as control group. In general, spontaneous Aprt MF in hybrid B6C3F1 mice seem to be somewhat lower than in C57Bl/6 mice (Table I), although the differences are not significant. No effect of genetic background on Aprt MF was observed after DMBA, MNU or etoposide treatment (Table I).

In contrast, the induced Aprt MF was significantly lower in hybrid B6C3F1 mice than in C57Bl/6 mice after MMC- or MMS-treatment. The mean Aprt MF in MMC-treated hybrid B6C3F1 animals was 5-fold lower than in C57Bl/6 mice treated with the same dose of 2 mg/kg MMC (15.9 ± 3.8 × 10^-6 versus 72.2 ± 14.8 × 10^-6 respectively, Table I). A dose of 60 mg/kg MMS appeared not to be mutagenic at all in hybrid B6C3F1 mice (the MF did not exceed the background frequency) while in C57Bl/6 mice a 2.4-fold induction in Aprt MF could be observed (Table I).

**LOH analysis of spontaneous and chemical-induced Aprt mutants**

An allele-specific PCR analysis was performed to detect the loss of the normal, non-targeted Aprt allele in isolated mutants obtained from untreated and chemical-exposed Aprt+/- mice. In Table I, the numbers of Aprt mutants analysed as well as the percentage of mutants showing LOH at Aprt in C57Bl/6 and hybrid B6C3F1 mice are presented. Additionally, the percentage of LOH induced by the mutagen was calculated by correcting the Aprt MF after treatment for the background frequency in untreated mice, and the contribution of LOH mutants therein. For example, the frequency of LOH mutants after MMS treatment in C57Bl/6 mice is 0.87 × 20.8 × 10^-6 = 18.1 × 10^-6. The contribution of background mutants herein is 0.72 × 8.6 × 10^-6 = 6.2 × 10^-6. The fraction of MMS-induced mutants with LOH is thus (18.1 - 6.2) × 10^-6/(20.8 - 8.6) × 10^-6 = 0.97. It is clear that background LOH levels can strongly influence mutagen-induced LOH percentages, especially when the induced MF is only slightly above the background.

Although the absolute level of spontaneous mutants was lower in hybrid B6C3F1 background (Table I), the relative frequency of LOH mutants seemed to be slightly higher (72% in C57Bl/6 mice and 82% in hybrid B6C3F1 mice, respectively). After chemical treatment, only in DMBA-treated animals a difference in LOH percentage could be observed between the two mouse strains being 86% in hybrid B6C3F1 and 69% in C57Bl/6 inbred mice. Even in MMC- and MMS-treated mice, where absolute Aprt MF were substantially different between the two mouse strains tested, the relative LOH frequencies in inbred and hybrid mouse strains were comparable (Table I).
polymorphic markers tested, indicating that the loss of Aprt subsequent duplication, or MR between could either be due to whole chromosomal loss with or without 142 after MMC treatment that had lost eight markers was ground clones. For example, the frequency of LOH mutants induction over the background was substantially different analysed as presented in Table II. Because the level of mutation observed for spontaneous, etoposide- and DMBA-induced Fig. 2. Distribution of LOH in background and carcinogen-induced Aprt LOH mutants. Loss of a particular marker always coincided with the loss of all markers distal. Lines connect for every marker the percentage of mutants in which that particular marker was lost.
carcinogen-treated Aprt+/– hybrid B6C3F1 mice. For every treatment, the absolute number as well as the percentage of clones that had lost 0, 1, 2, etc., markers was calculated. The location of the markers at mouse chromosome 8, i.e. D8Mit143, D8Mit4, D8Mit129, D8Mit208, D8Mit242, D8Mit321, D8Mit14 and D8Mit56, is depicted in Figure 1. The distribution of crossover points based on the analysis of independent mutant clones (isolated from different mice, data not shown) was very similar to those based on the total numbers of clones analysed as presented in Table II. Because the level of mutation induction over the background was substantially different between the various chemical-treatments, each class of LOH patterns was corrected for the relative contribution of background clones. For example, the frequency of LOH mutants after MMC treatment that had lost eight markers was 0.62 × 15.9 × 10−6 = 9.9 × 10−6. The contribution of spontaneous mutants herein was 0.31 × 6.4 × 10−6 = 2.0 × 10−6. The fraction of mutants with LOH induced by MMC is thus (9.9 – 2.0) × 10−6/(15.9 – 6.4) × 10−6 = 0.83. For all mutants it was observed that loss of a particular microsatellite marker always coincided with the loss of all other more distally located markers.

Figure 2 gives an overview of the LOH intervals in Aprt mutants of different origin. From this diagram, three different patterns of LOH can be distinguished. A more or less random distribution of the crossover points along chromosome 8 is observed for spontaneous, etoposide- and DMBA-induced Aprt mutants, pointing to MR as principal mechanism underlying LOH. The majority of crossover events in the spontaneous and DMBA-induced mutants occurred between marker D8Mit321 and D8Mit14 (59–67 cM), although DMBA-induced mutants also frequently recombine between marker D8Mit208 and D8Mit242 (42–48 cM). Etoposide has the largest frequency of crossover events between polymorphic markers D8Mit242 and D8Mit321 (48–59 cM). In contrast, >80% of MMC-induced Aprt mutant clones had lost heterozygosity at all eight polymorphic markers tested, indicating that the loss of Aprt could either be due to whole chromosomal loss with or without subsequent duplication, or MR between D8Mit143 and the centromere. Crossover points in MNU-induced mutants occurred predominantly between markers D8Mit242 and D8Mit14. Surprisingly, ~40% of the MNU-induced (and 10% of the spontaneous) mutants that had lost the wild-type Aprt allele had retained heterozygosity at all other markers, including marker D8Mit14 that is located on the same cM position in the meiotic map of chromosome 8 than Aprt. FISH analysis of MNU-induced mutants showed that in one out of three MNU-induced Aprt LOH mutants, all signal from the wild-type Aprt allele was absent (data not shown). This suggests that interstitial deletion may cause LOH at Aprt in a significant fraction of MNU-induced and spontaneous mutants.

Discussion

Although genetic events that lead to LOH contribute eminently to the process of carcinogenesis, remarkably little is still known on the molecular triggers for LOH formation. We have shown previously that various types of DNA damage, such as interstrand crosslinks, methylation of DNA bases, DNA strand breaks and bulky DNA adducts can induce LOH at Aprt in the mouse (8,9). The vast structural differences of the induced DNA lesions suggest that the mechanisms by which LOH is induced, may vary among mutagens. DNA double stranded breaks (DSB) may play an important role both in the formation of deletions as well as in the generation of LOH by MR. However, gene conversion unassociated with crossing over appears to be the predominant route of repair of a DSB in mammalian cells, resulting in LOH of only a short stretch of DNA (20). In line with this result, the DSB induced by ionizing radiation were found to be poor inducers of MR (8,21). We have here performed detailed analysis of LOH patterns induced by various chemical carcinogens to investigate if LOH formation might occur through a common mechanism independent of the type of DNA lesion triggering LOH.

Three patterns of LOH could be distinguished after analysis of all spontaneous and chemical-induced Aprt LOH mutants. The majority of spontaneous, etoposide- and DMBA-induced LOH appears to be due to MR as has been reported previously by others for spontaneous Aprt mutants from adult mouse primary skin fibroblasts and splenic T-lymphocytes (6,12–14). Large contiguous segments of chromosome 8 are lost in the majority of these mutants, including the most distally located marker D8 Mit56, which excludes the mutants to be caused by gene conversion or interstitial deletion. LOH mutants only contained a single crossover point, with the majority (>50%)
of the crossing over events located in the 25 cM interval between marker D8Mit208 and D8Mit14 (42-67 cM). Similarly, in spontaneous Aprt mutant fibroblasts, nearly one-third of the breakpoints at chromosome 8 was located between 59 and 67 cM (13), which suggests that this region contains a hotspot for recombination. LOH patterns for spontaneous, etoposide- and DMBA-induced Aprt mutants are highly similar, suggesting that in these cases MR is triggered by a common mechanism, such as stalled replication forks at DNA lesions. Replication forks may be converted into recombination intermediates via different mechanisms. Single stranded nicks induced by the topoisomerase II inhibiting activity of etoposide can be converted into a DSB when the replication fork runs through it. Arrested replication forks at spontaneously arisen or DMBA-induced DNA adducts may be target for nucleases, providing a substrate for DSB repair enzymes (22). The sister chromatid is the preferred substrate for repair of DSB at replication forks, since this will guarantee error-free repair. However, our studies indicate that also the homologous chromosome is frequently used as substrate for recombinational repair, leading to crossing over and LOH.

About 30% of the spontaneous, DMBA- and etoposide-induced Aprt mutants had lost all eight polymorphic markers tested in agreement with previous findings on the nature of spontaneous Aprt T-lymphocyte clones (14). Different markers were tested in this latter study, but even marker Polb, located only 8 cM from the centromere, was lost in 46% of the Aprt mutants analysed (14). Because of the absence of a suitable microsatellite marker that is located (even more) close to the centromere, chromosome loss/reduplication cannot be excluded as the underlying mechanism causing LOH. However, we assume that MR with the crossover point located between the centromere and marker D8Mit143 (and not chromosome loss) is the underlying mechanism causing LOH. It was shown previously that (i) in Aprt mutant fibroblasts of the same hybrid B6C3F1 mice, the proximal centromeric segment of chromosome 8 of strain C3H was retained (13) and (ii) in contrast to mouse embryonic stem cells where it appears to be the most predominant cause for LOH, the incidence of chromosome loss/reduplication in most somatic cell types was shown to be very low (23).

The Aprt MF after MMC treatment was 4.5-fold lower in hybrid B6C3F1 mice than in inbred C57Bl/6 mice. Recent reports have shown that recombinational exchanges between genetically divergent homologous chromosomes are suppressed by chromosomal divergence (24,25). However, the reduced homology between the two chromosomes 8 in a hybrid background is unlikely to be the cause for this reduction in MF, since the fraction of MMC-induced LOH mutants was similar in both genetic backgrounds. MMC induces inter-strand cross-links (ICL) in the DNA, which when unrepaird form impenetrable blocks for DNA replication and are highly cytotoxic. Repair of ICL in mammalian cells involves the coordinated action of excision repair reactions and homologous recombination (26) while DSB formed at stalled replicating forks appear to be an intermediary step in ICL repair.

Extensive LOH was observed in >80% of the MMC-induced mutants, converting all investigated markers into a homozygous configuration, while these events represented only a minor fraction of the spontaneous, DMBA-induced and etoposide-induced mutants. A high frequency of crossover points between marker D8Mit143 and the centromere might occur if either induction or repair of inter-strand crossovers by MMC is not uniform over the genome. The observation that MMC-induced sister chromatid exchanges had a higher frequency of occurrence in the heterochromatic regions of chromosomes in human lymphocytes than in euchromatic DNA (27) is in support of a non-random distribution of recombinational crossover points by MMC.

Differential induction of LOH was observed for the two methylating agents investigated. We have shown previously using Aprt+/- mice in a C57Bl/6 background that MMS was not mutagenic at the X-chromosomal Hprt gene, while it selectively induced LOH type of mutations at Aprt (9). Surprisingly, in hybrid B6C3F1 mice MMS was found also not to be mutagenic at Aprt, although parallel exposure of Aprt+/- C57Bl/6 mice to the same dose of MMS resulted in Aprt MF that were 4-fold increased above the background. We speculate that differences in the repair capacity of MMS-induced DNA adducts account for the difference in mutability of MMS between the two genetic backgrounds.

MNU was equally mutagenic in both genetic backgrounds with a similar contribution of LOH mutants to the mutational spectrum. The different capacity in LOH induction between MMS and MNU in hybrid B6C3F1 mice indicates that the DNA adducts leading to LOH for these two methylating agents are probably different in nature. MMS reacts preferentially with N-atoms of the DNA bases with N3-Me-A and N7-Me-G being the major types of adducts induced. However, N-methylated DNA adducts hardly have replication blocking properties. Therefore, recombination between homologous chromosomes may be initiated by MMS at replication forks when single stranded nicks in the DNA, that occur as intermediates during repair of N-methyl adducts or secondary lesions such as AP-sites by base excision repair, are converted into double stranded breaks (DSB).

MNU has a much higher affinity for O-atoms in DNA than MMS. It may exert its LOH-inducing capacity via futile mismatch repair (MMR) of T and C bases opposite O6-Me-G adducts, giving rise to persisting regions of single stranded DNA that may be turned into DSB during replication. It has been shown in human fibroblast cell lines heterozygous for p53, that MNU-induced O6-Me-G adducts can stimulate homologous MR resulting in LOH of the wild type p53 allele (28). Interestingly, ~40% of the MNU-induced LOH events (and about 10% of the spontaneous) affected only Aprt while heterozygosity was retained for all other markers investigated, including marker D8Mit14 that occurs in the meiotic map at the same location (68.0) as Aprt. This class of localized LOH might be due to relatively small deletions. In this respect, it is remarkable that 30% of FISH-analysed MNU-induced Aprt LOH mutants also had lost the wild-type Aprt allele by deletion.

In conclusion, MR appears to be the predominant mechanism for LOH in somatic cells from the mouse, both spontaneously as well as by chemical carcinogens, while DSB formed at stalled replication forks may well be the common trigger for the induction of MR by the various types of DNA lesions induced. It remains however uncertain to what extent the structure of ‘collapsed’ replication forks are similar after encountering structurally very different replication blocking lesions such as single stranded nicks, bulky DNA adducts or inter-strand crosslinks.
Acknowledgements

We would like to thank Dr. J.Jansen for critical reading of the manuscript. This work was supported by Dutch Cancer Society grant EUR-98-1800 and the J.A. Cohen Institute, Inter-University Institute for Radiopathology and Radiation Protection, Leiden, The Netherlands.

References


Received July 24, 2002; revised September 5, 2002; accepted September 7, 2002