Evidence for Msh2 haploinsufficiency in mice revealed by MNU-induced sister-chromatid exchange analysis

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Summary The role of Msh2 in chromosome stability has been investigated in a targeted mouse model for HNPCC, Msh2Δ7N. Chromosome aberration frequencies were similar in bone marrow of Msh2⁻/⁻, Msh2⁺/⁻ and Msh2⁺/+ mice and no differential effects of in vivo X-irradiation were noted. By contrast, the induction of sister-chromatid exchanges (SCEs) by methyl nitrosourea (MNU) was reduced in Msh2⁻/⁻ and Msh2⁺/⁻ cells to ~20% and ~45% wild-type levels respectively indicating a phenotypic effect of haploinsufficiency of the mouse Msh2 gene.

Keywords Msh2; mismatch repair; alkylating agent; ionizing radiation; sister-chromatid exchange

Human individuals carrying a mutation in the mismatch repair gene MSH2 are at increased risk of developing tumours in the colon and endometrium (Leach et al, 1993; Kolodner et al, 1994). Tumours which develop in these patients display microsatellite instability (MSI) characterized by mutations at microsatellite and other simple sequence repeat loci (Aaltonen et al, 1993; Ionov et al, 1993 Boland et al, 1998). The majority of these tumours have no active MSH2 protein and the elevated mutation frequency is a consequence of non-functional mismatch repair (Leach et al, 1993; Parsons et al, 1993). Mutations in a number of other mismatch repair genes give rise to similar phenotypes. Germline mutations in such mismatch repair genes are found in patients affected by HNPCC (hereditary non-polyposis colorectal cancer – reviewed by Modrich and Lahue, 1996; Kolodner, 1996). The underlying cause of the increased tumour risk in HNPCC patients remains a point of some controversy, in most cases mutation or loss of the remaining wild type (WT) mismatch repair gene allele appears to be required. The more difficult issue is whether somatic inactivation of the WT allele occurs at a ‘normal’ frequency or whether mutation/loss frequencies are elevated due to Msh2 (or other mismatch repair gene) haploinsufficiency. In the first case, the excess risk is simply a consequence of the greater probability of mutation of one functional allele rather than two; in the second case a further enhancement due to cells with a single functional allele being somewhat genetically unstable (albeit to a lesser extent than in doubly mutant cells) is envisaged. Haploinsufficiency of a number of cancer-related genes has now been reported including Atm (Barlow et al, 1999), Trp53 (Bouffler et al, 1995; Venkatachalam et al, 1998), TGFβ (Tang et al, 1998) and p27 (Fero et al, 1998).

In order to address this and related questions, a series of experiments employing Msh2-deficient mice was carried out. Msh2⁻/⁻ mice develop lymphomas at high frequency and also tumours of the small bowel at lower incidence (deWind et al, 1995, 1998; Reitmair et al, 1995, 1996). Survival of Msh2⁺/+ mice is comparable to that of wild type animals although some evidence suggests that tumours contribute disproportionately to the mortality of heterozygotes (deWind et al, 1998). However, loss/mutation of the wild type Msh2 allele and MSI, as seen in HNPCC tumours, is relatively rare in tumours developing in heterozygous mice (deWind et al, 1998). The Msh2Δ7N mouse model employed in this study carries a targeted deletion of Msh2 exon 7 resulting in a null allele (Smits et al, in press). The incidence of lymphomas and small bowel tumours observed in homozygous and heterozygous mice closely resembles those reported for previously described models (de Wind et al, 1995, 1998).

MATERIALS AND METHODS

Animals were bred and housed in Leiden University Medical Center. All animal procedures were conducted according to appropriate local Leiden University and Dutch national ethics guidelines and legislation. Spontaneous and X-ray induced chromosomal aberrations were assessed in direct bone marrow metaphase preparations of mutants and wild types. Age matched mice of each genotype were irradiated with an acute dose of 3 Gy X-rays. Direct bone marrow metaphases were made as described (Bouffler et al, 1995) from irradiated mice 1 month after exposure and age matched control, untreated mice. All samples were scored blind, the code being kept in Leiden while scoring was carried out at NRPB. Preparations were examined after block staining, G-banding or chromosome painting as described (Bouffler et al, 1996, 1997). G-banded slides were analysed using Chantel software (Leica Microsystems Imaging Solutions Ltd). Sister-chromatid exchange frequencies were determined in mitogen-stimulated spleen cultures, prepared as described (Bouffler et al, 1995). Methyl nitrosourea (Sigma) was initially diluted in dimethyl sulfoxide and further diluted in PBS immediately prior to adding to cultures 24 hours prior to harvesting.

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RESULTS AND DISCUSSION

The gross morphology and number of chromosomes, assessed in block stained preparations, was normal in all three genotypes. Some indications of elevated frequencies of minute chromosome fragments in bone marrow cells were obtained. However, given the difficulty in scoring such chromosomal events, further analysis was not carried out. Painting of chromosomes 1 and 2 did not reveal any evidence of spontaneous chromosomal instability in any genotype (Table 1). Thus, the MSI phenotype does not appear to lead to elevated frequencies of spontaneous translocations. Some evidence in the literature suggests that Msh2-deficient cells have a somewhat elevated sensitivity to ionizing radiation (Fritzell et al, 1997; deWeese et al, 1998) and this sensitivity extends to elevated mutability at the HPRT locus after low dose rate exposure (deWeese et al, 1998). Thus, the in vivo induction of chromosome aberrations by X-irradiation was examined. Sampling at one month post-irradiation provides a reasonably accurate picture of induced stable, persistent aberrations (Bouffler et al, 1995, 1996). Chromosome painting indicated that the yield of exchange-type aberrations giving rise to colour junctions (e.g. translocations and insertions) was comparable in the three Msh2 genotypes (Table 1). Chi-squared tests revealed no significant differences other than the effect of radiation elevating aberration yields. As chromosome painting analysis is not well suited to the detection of aberrations such as deletions and inversions, metaphases were also examined after G-banding. G-banding has the additional advantage of examining the entire genome. These data (Table 2) also indicated that all genotypes reacted similarly to X-irradiation, no statistically significant differences between genotypes were observed. Note that the frequency of breakpoints per cell assessed by G-banding is higher than the junctions per 100 cells scored by painting as G-banding scores over the entire genome while painting scored only chromosomes 1 and 2 (approximately 14% of the genome). Bearing this proportion in mind, the frequencies of aberrations scored by the two methods are broadly comparable.

These findings of no excessive spontaneous or induced chromosomal aberration formation are reflected in the observations of others that tumours arising in a background of mismatch repair deficiency infrequently show gross chromosomal changes (Aaltonen et al, 1993; Lengauer et al, 1997; Eshleman et al, 1998). The data presented here demonstrate clearly, however, that aberrations of the types associated with tumorigenesis (translocations, deletions, etc.) can be formed in the absence of mismatch repair, i.e. intact mismatch repair systems are not required for aberration formation. This suggests that in mismatch repair deficient cells, mutations in genes promoting proliferation and neoplastic development tend to take the form of sub-microscopic events principally, perhaps, DNA base pair changes.

Sister chromatid exchanges (SCEs) are a highly sensitive indicator of DNA damage induced by specific agents. Defects in mismatch repair are associated with reduced cellular sensitivity to and SCE induction by O6-guamine-methylating agents such as methyl nitrosourea (MNU) (reviewed by Karran and Bignami, 1994). Assessment of SCE in splenocytes from Msh2+/–(+D7N), Msh2–/–(D7N) and wild type littermates revealed no effect of the mutation on baseline frequencies (Fig. 1). SCE induction was strongly reduced in Msh2–/– splenocytes (Fig. 1), and to a lesser extent in Msh2 heterozygotes. Thus, Msh2 shows haploinsufficiency in this assay. Western analysis does not detect a difference in Msh2 protein level between wild type and heterozygote ES cells (RF, unpublished observation) most probably due to limitations of sensitivity. MNU-induced SCEs are generally attributed to persistent DNA breaks caused by ‘futile repair’ opposite O6-methylguanine residues by mismatch repair enzymes (see Karran and Bignami, 1994; Kaina et al, 1997). These persistent lesions contribute to cytotoxicity and SCE formation. In the absence of mismatch repair (Msh2–/– cells) few persistent lesions are produced. Hence, mismatch repair defective cells are resistant to the cell killing effects of O6-guanine methylating agents (Karran and Bignami, 1994; deWind et al, 1995). The remaining alkyl lesions promote mutation (Andrew et al, 1998; Toft et al, 1999; Reitmair et al, 1997). However, elevated mutation frequencies have not been observed in Msh2–/– cells (Reitmair et al, 1997; Andrew et al, 1998; Toft et al, 1999). By contrast, the reduction in MNU-induced SCE observed here in Msh2–/– cells (Fig. 1), suggests that mismatch repair is compromised by Msh2 haploinsufficiency. The apparent discrepancy between these data and the mutation studies may be reconciled by the observation that Msh2 also plays a role in the signalling of apoptosis (Humbert et al, 1999; Toft et al, 1999). Haploinsufficiency of Msh2 may exert a negative effect on its repair function but not on its capacity to signal apoptosis. Therefore, Msh2–/– cells with low induced SCE frequencies (and so, by implication, elevated O6-methyl guanine burdens) are successfully channelled into apoptosis prior to mutations being fixed. Msh2–/– ES cells appear as sensitive to MNNG as wild-type cells (deWind et al, 1995) indicating that apoptosis is functional in heterozygotes. Thus, while Msh2

### Table 1: Spontaneous and X-ray induced stable chromosome aberrations assessed by painting of chromosomes 1 and 2

| Genotype | X-ray dose/ sample time | Cells scored | Junctions/ 100 cells |
|----------|-------------------------|--------------|---------------------|
| + +      | 0 Gy                    | 159          | 0                   |
|          | 3 Gy/1 month            | 250          | 18                  |
| + –      | 0 Gy                    | 102          | 0.98                |
|          | 3 Gy/1 month            | 297          | 14.5                |
| – –      | 0 Gy                    | 41           | 0                   |
|          | 3 Gy/1 month            | 219          | 16.9                |

*Frequency of junctions between painted and unpainted chromosome fragments per 100 cells. In this experiment the majority of junctions are due to translocations, some insertions, dicentrics, Robertsonian translocations and two coloured fragments were also seen.

### Table 2: G-band analysis of chromosomal aberrations in wild type and Msh2 deficient mouse bone marrow cells 1 month following 3 Gy X-irradiation in vivo

| Genotype | Cells scored | Aberrations | Breakpoints per cell |
|----------|--------------|-------------|----------------------|
| RT       | TT           | TD          | ID                   | INS       | Rb         |
| ++       | 18           | 8           | 0                    | 4         | 3          | 0          | 1.8          |
| – –      | 18           | 2           | 1                    | 1         | 9          | 3          | 1.8          |
| – +      | 24           | 4           | 1                    | 2         | 7          | 1          | 1.3          |

*RT – reciprocal translocation, TT – terminal translocation, TD – terminal deletion, ID – interstitial deletion, INS – insertion, Rb – Robertsonian translocation.

**Calculated scoring 1 breakpoint for TT, TD and Rb, 2 breakpoints for RT and ID, 3 breakpoints for INS.**
haploinsufficiency can be effectively measured by the MNU-induced SCE assay, there would appear to be no reason to expect this to contribute to mutagenesis or carcinogenesis in the mouse.

Effects of Msh2 in the heterozygous state have been observed following low dose rate irradiation exposure. deWeese et al. (1998) found Msh2<sup>–/–</sup> cells to have a greater resistance to low dose rate radiation, and showed indications of elevated mutation frequencies. As with X-ray-induced chromosomal aberrations (Tables 1 and 2), in the present studies, no effect of Msh2 deficiency was observed using SCEs as an endpoint (Fig. 1). The high dose rate employed here may be masking effects which would otherwise be seen with low dose rates. Furthermore, X-rays are only a weak inducer of SCEs despite being a potent carcinogen. The latter also underscores the lack of correlation between SCE induction and cancer.

In summary, it is reported here that haploinsufficiency of Msh2 in mice can be revealed by MNU-induced SCE analysis. No phenotypic effect of loss of one or two Msh2 alleles was detected with regard to spontaneous or X-ray induced structural chromosome aberrations. The data presented here add to the evidence accumulating which indicates that haploinsufficiency for some cancer related genes, including Msh2, has detectable phenotypic effects.

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Figure 1 Sister chromatid exchange in wild type (diamonds) and Msh2<sup>–/–</sup> (solid circles) and Msh2<sup>+/–</sup> (open circles) splenocytes induced by MNU (A) and X-rays (B)
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