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Msh2 deficiency does not contribute to cisplatin resistance in mouse embryonic stem cells

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Several reports have suggested that a defect in the DNA mismatch repair (MMR) system not only causes resistance to methylating agents but also confers low-level resistance to the chemotherapeutic drug cisplatin. Here we report that in a clonogenic assay, mouse embryonic stem (ES) cells deficient for the MMR protein MSH2 respond similarly as wild-type cells to cisplatin. Furthermore, restoring MSH2 expression in a cisplatin-resistant subclone selected from an *Msh2*$^{-/-}$ cell population did not sensitize cells to cisplatin. To ascertain that our observations were not the result of a mutation in the *Msh2*$^{-/-}$ cells that obscured the contribution of a defective MMR machinery to cisplatin resistance, we made use of the Cre-lox system to create a cell line in which the *Msh2* gene can be conditionally inactivated. However, while *de novo* inactivation of *Msh2* rendered cells tolerant to the methylating drug *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine as expected, it did not alter the sensitivity to cisplatin. In addition, we were not able to derive cisplatin-resistant subclones from this freshly generated MMR-deficient cell line. Thus, in ES cells we did not find evidence for direct involvement of MMR deficiency in cisplatin resistance.
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Introduction

The primary function of DNA mismatch repair (MMR) is restoring mismatched and unpaired nucleotides that occur during DNA replication. In addition, the MMR system mediates the cytotoxicity of several DNA-damaging drugs. Thus, cells deficient for the central MMR repair proteins MSH2 or MLH1 are highly tolerant to methylating agents and the base analogue 6-thioguanine (Koi *et al*., 1994; Hawn *et al*., 1995; Risinger *et al*., 1995; Buermeyer *et al*., 1999; Claij and Te Riele, 2002). These drugs derive their cytotoxicity from the formation of O^6-methylguanine or S^6-methylguanine lesion in DNA, respectively, causing mismatched base pairs (Zhukovskaya *et al*., 1994; Swann *et al*., 1996). Recognition of such lesions by MMR proteins may directly activate the apoptotic machinery. Alternatively, it was suggested that MMR only recognizes mispairs formed during replication of these lesions, thereby inhibiting translesion synthesis and causing cell death. Furthermore, MMR deficiency was reported to confer low-level resistance to the chemotherapeutic agent cisplatin (*cis*-diamminedichloroplatinum). The observation that MMR proteins can bind to oligonucleotides containing a 1,2-diguanyl intrastrand crosslink preferably when a T is present opposite the 3′-guanine in the crosslink, has suggested that here also MMR counteracts translesion synthesis thereby contributing to the cytotoxicity of cisplatin (Yamada *et al*., 1997). Cisplatin is widely used for the treatment of human malignancies; however, its clinical effectiveness is limited by the frequent development of drug-resistant tumor cell populations during treatment. The mechanism of resistance is often found to be multifactorial (Kartalou and Essigmann, 2001); for example, cisplatin-resistant derivatives of the ovarian tumor cell line A2780 have increased levels of glutathione, reduced drug accumulation, reduced adduct formation and increased DNA repair (Siddik *et al*., 1998). Furthermore, in these derivatives, cisplatin resistance is often accompanied by loss of expression of the MMR protein MLH1 (Anthoney *et al*., 1996; Drummond *et al*., 1996), suggesting that the MMR system contributes to the cellular response to cisplatin. The hMLH1-deficient colorectal carcinoma cell line HCT116 and the hMSH2-deficient endometrial carcinoma cell line HEC59 provided additional indications for the involvement of MMR proteins in response to cisplatin treatment. Restoration of the repair capacity by complementation with chromosome 3 or 2, respectively, sensitized the cells approximately twofold to cisplatin (Aebi *et al*., 1996; Fink *et al*., 1996). By using the transfer of whole chromosomes, however, there remains the possibility that other genes carried on the chromosome influence the sensitivity; for example, in a cisplatin-resistant, MLH1-deficient derivative of A2780 cells, correction of MMR by chromosome 3 transfer sensitized the cells fivefold (Durant *et al*., 1999). In contrast, complementation of hMLH1 by expression of the cDNA resulted in only a 1.3-fold sensitization (Branch *et al*., 2000), making the contribution of MMR deficiency to cisplatin resistance questionable.

We have previously generated mouse embryonic stem (ES) cells in which both *Msh2* alleles were disrupted

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(de Wind *et al*., 1995). This ES cell line could provide a more precise system to test whether loss of function of just the MMR machinery is sufficient to cause low-level cisplatin resistance. Fink *et al*. (1997) observed a 2.1-fold resistance to cisplatin in these *Msh2*$^{-/-}$ ES cells, which is comparable to the level of resistance observed in the HCT116 and HEC59 cell lines. However, here we report that by using the same ES cells we did not observe any differential response to the cytotoxic effects of cisplatin between *Msh2*$^{-/-}$ and wild-type cells in a clonogenic assay. Furthermore, restoring MSH2 expression in a cisplatin-resistant derivative of *Msh2*$^{-/-}$ cells did not sensitize the cells to drug treatment. A potential problem associated with the extensive culturing of MMR-deficient cells may be the accumulation of inadvertent genetic alterations that may preclude reliable assessment of the MMR defect only. To circumvent this problem, we have made use of the Cre-lox system to generate a cell line in which MSH2 expression can be *de novo* inactivated and reactivated. This provided us with a robust system to test the contribution of MMR to the cytotoxicity of cisplatin.

Results

Msh2-proficient and -deficient ES cells have a similar response to cisplatin

We have previously generated an ES cell line with a targeted disruption of the *Msh2* gene (de Wind *et al*., 1995). In a clonogenic assay, the response of these *Msh2*$^{-/-}$ cells to cisplatin was compared to that of wild-type ES cells. Briefly, we allowed 500 cells/1.8 cm^2 to attach on a feeder layer. The next day, we treated the cells for 1 h with different concentrations of cisplatin and after 4 days we counted the number of surviving colonies. By this method, we were not able to detect a significant difference in the survival of *Msh2*$^{-/-}$ and wild-type ES cells (Figure 1).

Ectopic expression of Msh2 does not sensitize cisplatin-resistant Msh2$^{-/-}$ *cells to cisplatin*

As judged from the clonogenic assay in Figure 1, a defective MMR system was not sufficient to increase cell survival in response to cisplatin. However, after exposure of a large number (1.5×10^6) of cells for 24 h to 10 μM cisplatin, a difference in the number of surviving *Msh2*$^{-/-}$ and wild-type colonies manifested. In one of the experiments in which sixfold more *Msh2*$^{-/-}$ colonies appeared (300 versus 50 for wild-type cells), individual colonies were picked, expanded and their response to cisplatin was determined in a clonogenic assay. Of the 32 *Msh2*$^{-/-}$ colonies tested, 13 (which would correspond to 122 out of 300) showed enhanced resistance to cisplatin, while six of the 32 (i.e. nine out of 50) wild-type colonies were more resistant. All the other clones were as sensitive as the original *Msh2*$^{-/-}$ and wild-type cultures in a clonogenic assay. The level of resistance was low, that is, not more than two- to threefold as shown for clone Pt7 in Figure 1. Since

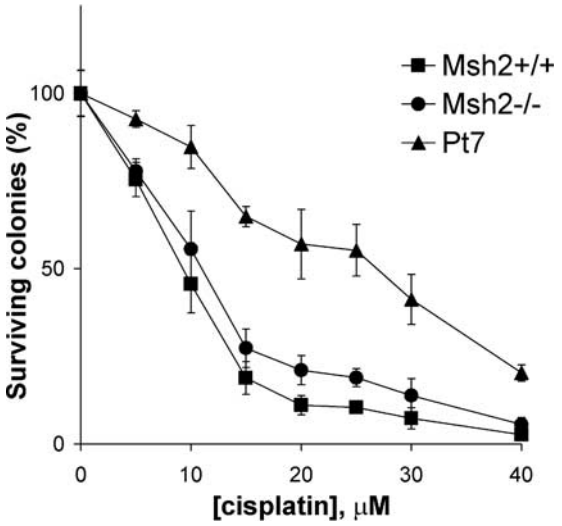

Figure 1 Response of *Msh2*-proficient and -deficient ES cells to cisplatin. *Msh2*$^{+/+}$ (squares), *Msh2*$^{-/-}$ (circles) and Pt7 (triangles) cells were exposed for 1 h to 0–40 μM cisplatin. After 4 days, the number of surviving colonies was counted. These experiments were performed in triplicate

Msh2$^{-/-}$ cells have a mutator phenotype and have been cultured for many passages since they were generated, it is not unlikely that the *Msh2*$^{-/-}$ culture contained a small subpopulation of cells that had additional mutations affecting the response to cisplatin. A low-level resistance may be sufficient to provide a growth advantage in the presence of platinating agents, which may at least partly account for the extra colonies found in the *Msh2*$^{-/-}$ population. The resistant clone Pt7 was randomly chosen to explore whether the MMR defect was directly involved in cisplatin resistance by collaborating with an additional mutation. By introduction of the full-length cDNA of the *Msh2* gene, MSH2 protein expression was restored in the Pt7 cell line (Figure 2a). This strongly sensitized the cells to 6-thioguanine as shown for clones B and D in Figure 2b. However, re-expression of MSH2 did not affect the cisplatin-resistance phenotype of the Pt7 cells (Figure 2c) suggesting that, at least in this Pt7 cell line, MMR deficiency is not directly involved in cisplatin resistance.

Generation of an ES cell line in which Msh2 can be de novo *inactivated*

As cells with a defective MMR system have a mutator phenotype, they may more easily accumulate mutations that interfere with the cellular response to cisplatin. Such mutations could obscure the contribution of MMR deficiency to resistance, thereby causing the discrepancy between our observations and those of others (Drummond *et al*., 1996). To avoid problems of additional genetic changes, we made use of the Cre-lox system to generate an ES cell line in which MSH2 expression can be switched off and on again. The previously generated *Msh2*-heterozygous ES cell line Msh2-55 (de Wind *et al*., 1995) was used to modify the wild-type allele with the targeting construct shown

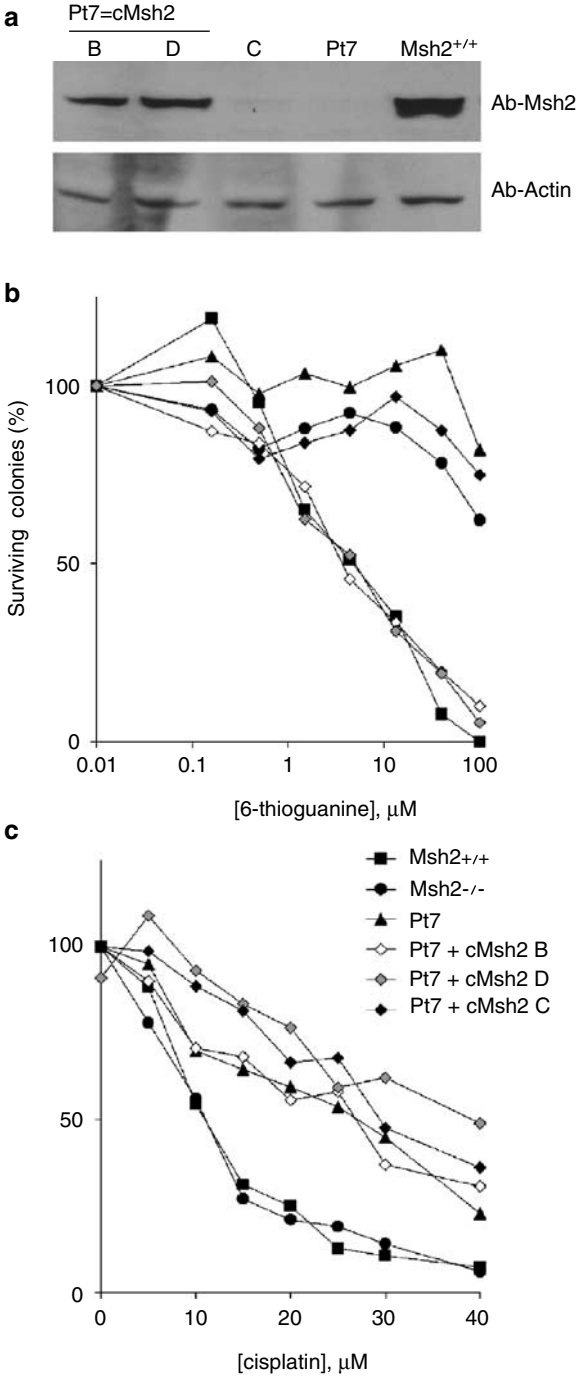

Figure 2 The expression of MSH2 in Pt7 cells restores sensitivity to 6-thioguanine but not to cisplatin. (**a**) Western blot analysis of Pt7 cells transfected with the cDNA of *Msh2* shows MSH2 protein expression in clones B and D. Actin was used as a loading control. (**b**) $Msh2^{+/+}$ (squares), Pt7 (triangles) and Pt7 + c*Msh2 clone* B (open diamond), clone D (gray diamond) and clone C (closed diamond) ES cell lines were exposed to increasing amounts of 6-thioguanine for 1 h. After 4 days, the number of surviving colonies was counted. $Msh2^{-/-}$ cells (circles) were included as a 6-thioguanine-resistant control. (**c**) The same clones were treated with different concentrations of cisplatin. Survival curves were simultaneously determined for all cell lines in two independent experiments giving similar results. One is shown in (**b**) and (**c**)

in Figure 3a. Successful gene targeting produced an $Msh2^{lox}$ allele in which a neomycin-resistance marker flanked by two *loxP* sites (floxed *neo*) was inserted 2 kb upstream of exon 12 and a single *loxP* site was introduced 53 bp downstream of exon 13. One of 480 G418-resistant clones contained both the floxed *neo* gene and the single *loxP* site targeted at the wild-type *Msh2* locus, while the knockout allele remained unchanged.

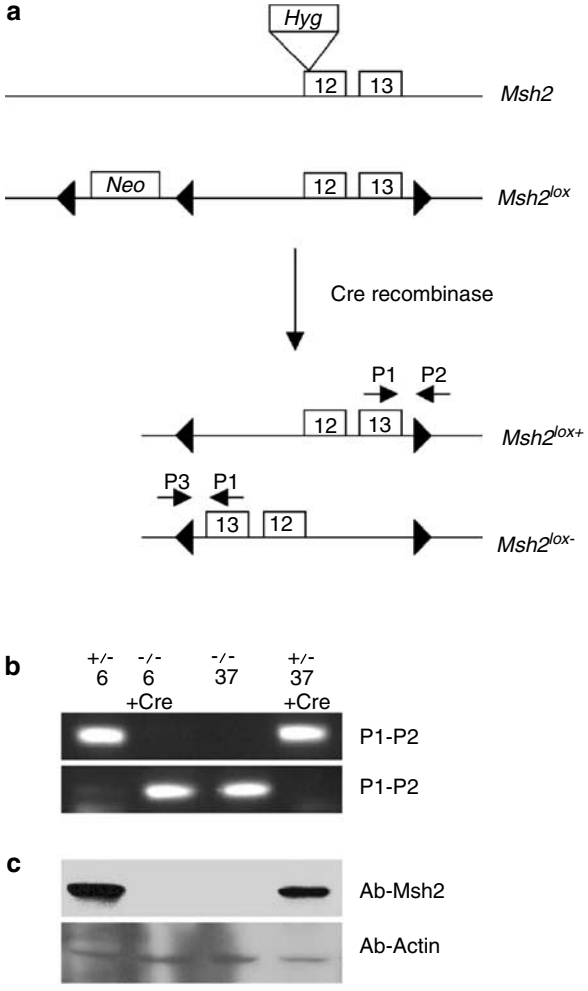

Figure 3 *De novo* inactivation and reactivation of *Msh2* by Cre-mediated recombination. (**a**) Previously, the $Msh2^{-}$ allele was generated by insertion of a hygromycin-resistance marker (*hyg*) into a unique *Sna*BI site within exon 12 (de Wind *et al*., 1995). At the other allele indicated as $Msh2^{lox}$, a single *loxP* site (triangle) was introduced 53 bp downstream of exon 13. Furthermore, a floxed *Neo* gene driven by the MC1 promoter was inserted 2 kb upstream of the exon 12 with both *loxP* sites in the opposite orientation of the single *loxP* site. Cre-mediated recombination of the *loxP* sites results in deletion of the *Neo* gene and can cause inversion of the exons 12 and 13 containing fragment. The exons 12–13 orientation results in MMR-proficient $Msh2^{lox+/-}$ cells, the exons 13–12 orientation in MMR-deficient $Msh2^{lox-/-}$ cells. (**b**) Confirmation of the orientation of the exons 12 and 13 containing fragment by PCR. Primer pair P1, P2 amplifies the $Msh2^{lox+/-}$ orientation in clone 6 and clone 37 + Cre; primers P1 and P3 amplify the $Msh2^{lox-/-}$ orientation in clone 37 and clone 6 + Cre. (**c**) Western blot analysis of MSH2 protein expression in $Msh2^{lox+/-}$ and $Msh2^{lox-/-}$ cells. The blot was probed with an antibody against MSH2, and an antibody against actin was used as a loading control

The correctly targeted cell line was subsequently electroporated with a Cre-encoding plasmid to express transiently the Cre enzyme that mediates recombination between the *loxP* sites. The *loxP* sites flanking the *neo* gene were oriented in the same direction allowing deletion of the *neo* gene upon Cre-mediated recombination. Indeed, 45 of 192 clones (23%) acquired G418 sensitivity after Cre expression. Since the remaining *loxP* site and the single *loxP* site downstream of exon 13 had the opposite orientation, Cre-mediated recombination could also result in inversion of the fragment containing exons 12 and 13. This would, in combination with the knockout allele, result in a completely *Msh2*-deficient cell line, tolerant to 6-thioguanine and methylating drugs. Indeed, screening of 192 Cre-exposed clones for inversion of exons 12 and 13 by testing their sensitivity to 6-thioguanine revealed 10 tolerant clones (5%), of which nine were also G418 sensitive. Subsequently, the orientation of exons 12 and 13 was determined in cell lines either sensitive or tolerant to 6-thioguanine by a PCR specific for either orientation (Figure 3b). In all cases, the PCR confirmed the orientation we expected based on the response to 6-thioguanine. MMR-proficient cells with the normal orientation of exons 12–13 will further be designated as $Msh2^{lox+/-}$; MMR-deficient cells with exons 12–13 in the reversed orientation will be designated as $Msh2^{lox-/-}$. By Western blot analysis, we were able to show that MSH2 was expressed at high levels in $Msh2^{lox+/-}$ cells, whereas the expression of MSH2 protein was completely abolished in $Msh2^{lox-/-}$ cells (Figure 3c).

Cre-recombinase was transiently expressed for a second time in the $Msh2^{lox+/-}$ clone 6 and $Msh2^{lox-/-}$ clone 37 to invert exons 12–13 and to reverse the MMR status. In both cell lines, the frequency of inversion was 13% (six of 48 and 12 of 90 tested clones, respectively) as determined by PCR. Indeed, after inversion of exons 12 and 13, clone 37 (37 + Cre) cells regained MSH2 expression, while clone 6 (6 + Cre) cells stained negative for MSH2 (Figure 3c).

$Msh2^{lox-/-}$ cells are tolerant to MNNG

6-Thioguanine was used for a quick screen to identify $Msh2^{lox-/-}$ clones after Cre-mediated recombination. In a more accurate clonogenic assay, we determined the response of $Msh2^{lox+/-}$ (clone 6) and $Msh2^{lox-/-}$(clone 37) ES cells to the methylating drug *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). As shown in Figure 4a, MSH2-deficient $Msh2^{lox-/-}$ (37) cells were tolerant to MNNG when compared to the MSH2-proficient $Msh2^{lox+/-}$ (6) cells. Inversion of exons 12–13 in $Msh2^{lox+/-}$(6) cells by expression of Cre-recombinase, giving $Msh2^{lox-/-}$ (6 + Cre) cells, greatly enhanced the tolerance to MNNG. Likewise, by changing the $Msh2^{lox-/-}$ genotype of clone 37 cells to $Msh2^{lox+/-}$ (37 + Cre), the methylation-damage-tolerance phenotype was reversed, making the cells as sensitive as $Msh2^{lox+/-}$ (6) cells. These data show that by using the Cre-lox system, we can easily switch between MMR proficiency and deficiency, thereby creating a valuable tool to evaluate the involvement of the MMR system in the response of cells to treatment with DNA-damaging agents.

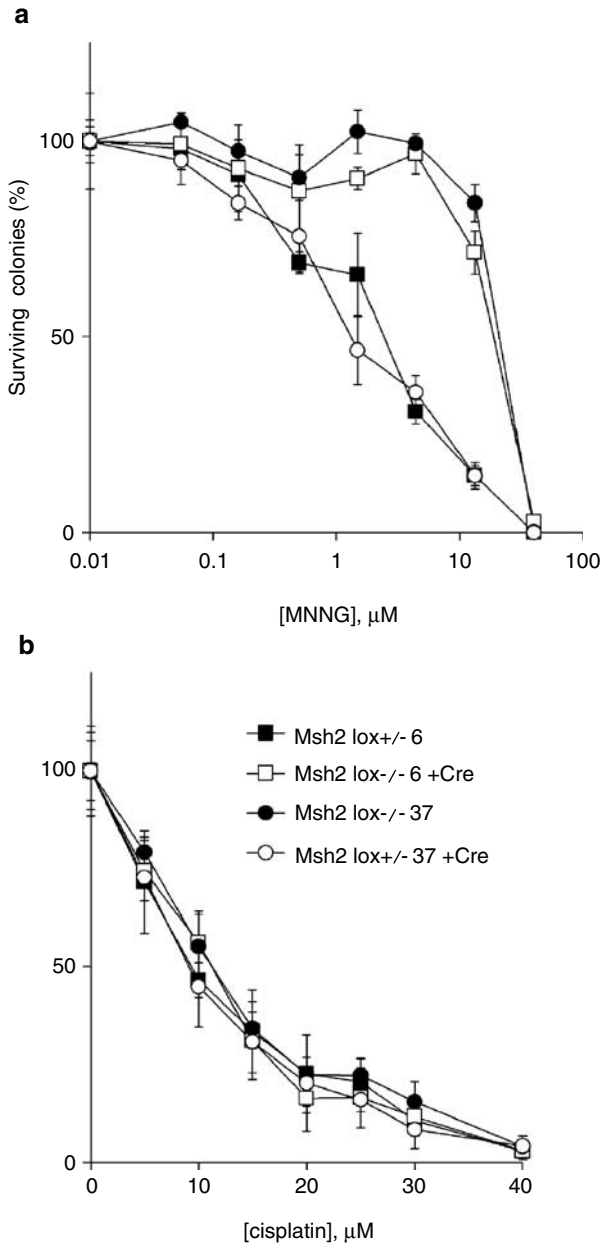

Figure 4 Response of $Msh2^{lox+/-}$ and $Msh2^{lox-/-}$ cells to DNA-damaging agents. $Msh2^{lox+/-}$(6) (closed square), $Msh2^{lox-/-}$(37) (closed circle), $Msh2^{lox-/-}$(6 + Cre) (open square) and $Msh2^{lox+/-}$(37 + Cre) (open circle) ES cell lines were exposed to increasing amounts of the methylating drug MNNG (**a**) or cisplatin (**b**) for 1 h. After 4 days, the number of surviving colonies was counted. Experiments were performed in triplicate

Similar response of $Msh2^{lox+/-}$ and $Msh2^{lox-/-}$ cells to cisplatin

Next, we tested the sensitivity of *de novo* generated $Msh2^{lox-/-}$ and $Msh2^{lox+/-}$ cells to the cytotoxic effects of cisplatin. The number of colonies that survived cisplatin

treatment was equal both in a clonogenic assay (Figure 4b) and after a 24 h exposure of 1.5×10^6 cells (data not shown). Furthermore, we were not able to identify cisplatin-resistant variants in the *Msh2*$^{lox-/-}$ cell population. These data demonstrate that in ES cells, MMR deficiency is not directly involved in cisplatin resistance.

Discussion

Several observations have suggested that MMR deficiency is involved in low-level cisplatin resistance. In human ovarian tumor cell lines selected for cisplatin resistance, the resistance phenotype was often found to be accompanied by loss of MLH1 activity (Anthoney *et al*., 1996; Drummond *et al*., 1996), and in ovarian cancer, poor response to cisplatin has been correlated with a reduction or loss of expression of MSH2 or more frequently MLH1 (Samimi *et al*., 2000; Strathdee *et al*., 2001; Watanabe *et al*., 2001). Cell lines defective for MLH1 or MSH2 appeared to be twofold resistant to cisplatin in comparison with corresponding MMR-proficient cell lines (Aebi *et al*., 1996; Fink *et al*., 1997). This low-level resistance was sufficient for enrichment of an MMR-deficient cell population *in vitro* (Fink *et al*., 1997, 1998) and a reduced response of tumor cells in an *in vivo* model (Fink *et al*., 1997). Furthermore, purified MSH2 protein, either alone or in complex with its dimerization partner MSH6, can bind to oligonucleotides containing a cisplatin adduct (Duckett *et al*., 1996; Mello *et al*., 1996; Mu *et al*., 1997). Recently, the MMR proteins have been suggested to have a function as general sensors of DNA damage with the ability to signal directly to cell cycle checkpoints and apoptosis. Cisplatin-induced initiation of a G1 arrest by cyclin D1 degradation was found to be dependent on the presence of MMR proteins (Lan *et al*., 2002), although we did not find a differential reduction of cyclin D1 levels in cisplatin-treated *Msh2*-deficient and wild-type mouse embryonic fibroblasts (MEFs) (unpublished observation). Also, the cisplatin-induced activation of c-Abl, one of the proteins of the p73-dependent apoptosis pathway, was shown to be dependent on functional MSH2 and MLH1 (Nehme *et al*., 1997; Gong *et al*., 1999). However, the number of reports disputing the involvement of the MMR system in cisplatin resistance is increasing; for example, evidence was obtained that the MMR-proficient ovarian carcinoma cell line A2780, which was widely used to study cisplatin responsiveness (Anthoney *et al*., 1996; Drummond *et al*., 1996) contains a small pre-existing subpopulation of MLH1-deficient cells that are significantly more resistant to cisplatin than the parental cells (Aquilina *et al*., 2000). It seems that most cisplatin-resistant variants that have been isolated over the years were derived from this same subpopulation that contains, in addition to MLH1 deficiency, a mutation in the p53 gene (Branch *et al*., 2000). Careful examination of both genetic changes suggested that defective MMR was only a minor contributor to cisplatin resistance, accounting for less than 1.3-fold resistance and that the abrogated p53 response was the main determinant of resistance (Branch *et al*., 2000). When cisplatin-resistant clones were selected from a pure population of cisplatin-sensitive, MMR-proficient A2780 cells, in all cases they had retained normal MMR repair capacity (Massey *et al*., 2003). Similarly, colon tumor cell lines selected for cisplatin resistance did not present with defects in DNA MMR proteins (Sergent *et al*., 2002). Finally, in *Msh2*-deficient murine intestinal enterocytes, only a slightly reduced apoptotic response to cisplatin could be observed that did not influence the overall survival of crypt cells (Sansom and Clarke, 2002), and indications for the involvement of the p73 pathway were not found.

We have previously generated an *Msh2*-deficient ES cell line (de Wind *et al*., 1995). In contrast to Fink *et al*. (1997), who previously reported a twofold resistance of these *Msh2*$^{-/-}$ cells, we show here that the cells respond similarly to the cytotoxic effects of cisplatin as wild-type cells. However, a 24 h exposure of 1.5×10^6 *Msh2*$^{-/-}$ and wild-type cells to a low dose of cisplatin revealed an increased number of surviving colonies in the *Msh2*$^{-/-}$ cell population. The majority of surviving *Msh2*$^{-/-}$ colonies showed a twofold resistance to cisplatin in a clonogenic assay, suggesting that the Msh2-deficient cell line contained a small subpopulation of cells with an altered response to cisplatin. To explore whether in one of the cisplatin-resistant ES cell clones, *Msh2* deficiency contributed directly to the resistance phenotype by collaboration with an additional mutation, we restored MSH2 expression by introducing the cDNA of the *Msh2* gene. Although the MSH2 protein level was sufficiently high to restore the sensitivity to 6-thioguanine, the cellular response to cisplatin remained unchanged. This indicates that the cells had acquired a genetic alteration that confers resistance to cisplatin irrespective of the MMR status. The nature of this genetic change remains to be identified.

By using a Cre-lox-based system in which *Msh2* can be *de novo* inactivated and reactivated, we generated a set of ES cell lines in which the consequences of uniquely *Msh2* inactivation could be assessed. Again, we found no relation between the MMR capacity of cells and their response to cisplatin in a clonogenic assay. Furthermore, long-term exposure to cisplatin of *Msh2*$^{lox+/-}$ and *Msh2*$^{lox-/-}$ cells directly after inactivation of the *Msh2* gene resulted in a similar number of surviving colonies for the MMR-proficient and -deficient cell lines. Testing these surviving colonies for acquired cisplatin resistance in a clonogenic assay did not reveal resistant variants. It remains possible that upon prolonged culturing, and in the presence of low levels of cisplatin, resistant variants arise more frequently in MMR-deficient cells than in wild-type cells.

An explanation for the discrepancy between our results and those of others who did find cisplatin resistance in MMR-deficient cell lines, may be the requirement for specific genetic changes that can *collaborate* with MMR deficiency in affecting the cytotoxicity of cisplatin; for example, mutated p53 (Branch *et al*., 2000), increased recombinational repair

or increased replicative bypass may be candidates. In yeast, data were obtained suggesting that drug resistance mediated by loss of MMR is dependent on *RAD52/RAD1* activity, implicating the involvement of a recombination-dependent process (Durant *et al*., 1999). Enhanced replicative bypass has been observed in human tumor cell lines with a defective MMR system (Vaisman *et al*., 1998). Furthermore, enhanced expression and activity of DNA polymerase β was found to correlate with cisplatin resistance (Mamenta *et al*., 1994; Canitrot *et al*., 1998).

In conclusion, we did not find any evidence for a direct contribution of MMR to the cytotoxicity of cisplatin in ES cells. This observation is particularly important, as previous work has suggested that the MMR status of tumor cells may be an important determinant of the outcome of platinum-based chemotherapy. In view of our data, such a conclusion should be considered with caution.

Materials and methods

Clonogenic assays

ES cell lines were seeded onto feeder layers of MEFs, mitotically inactivated by 25 Gy of irradiation, at a density of 500 cells/1.8 cm^2. The next day, the cells were treated for 1 h with 0–40 μM MNNG (Sigma), 0–100 μM 6-thioguanine (Sigma) or 0–60 μM cisplatin (Platosin, Pharmachemie, The Netherlands). After 4 days, densely packed surviving colonies were counted under a microscope. For MNNG, during the whole procedure from 1 h prior to exposure, 20 μM O^6-benzylguanine (kindly provided by R Moschel) was present in the medium to inhibit the removal of methyl groups from the O^6-position of guanine by endogenous methyltransferase activity.

Selection for cisplatin-resistant clones

ES cell lines were seeded onto gelatin-coated tissue culture plates at a density of 1.5×10^6 cells/60 cm^2. The following day, the cells were exposed for 24 h to 10 μM cisplatin. After 2 weeks, the number of surviving colonies was counted. A number of these colonies were picked, expanded and their cisplatin sensitivity was determined in a clonogenic assay.

Msh2 cDNA expression

The full-length cDNA of *Msh2* was cloned behind the EF1α promoter. A puromycin-resistance marker driven by the PGK promoter was inserted into the same vector upstream of EF1α-c*Msh2*. The vector was linearized before electroporation into the cisplatin-resistant *Msh2*-deficient cell line Pt7. Puromycin (2.2 μg/ml)-resistant colonies were picked and expanded. As cells expressing a high level of MSH2 are sensitive to 6-thioguanine, individual clones were plated on a 96-well plate at a density of 500 cells per well and treated with 60 μM 6-thioguanine for 1 h the next day. After 7 days, in 35% of the wells, all cells had died, identifying clones possibly expressing a high level of MSH2.

Western blot analysis

The MSH2 protein level was determined by Western blot analysis, using an antibody against MSH2 (de Wind *et al*., 1998). An antibody against actin (I-19, Santa Cruz) was used as a loading control.

Generation of an ES cell line in which Msh2 can be de novo *inactivated*

In a 12.5 kb *Bam*HI genomic fragment of the mouse *Msh2* locus containing exons 12 and 13 (de Wind *et al*., 1995), a single *loxP* site was inserted into the *Nsi*I site located 53 bp downstream of exon 13. Subsequently, the G418-resistance marker neomycin (MC1*neo*) flanked by *loxP* sites was introduced into the *Hind*III site 2 kb upstream of exon 12, with the *loxP* sites in the opposite orientation of the single *loxP* site. This targeting vector was introduced into the *Msh2*-heterozygous ES cell line Msh2-55 as described (de Wind *et al*., 1995). Southern blot analysis of *Eco*RI-digested DNA from G418 (250 μg/ml)-resistant colonies, using probes flanking the targeting construct, identified a modified wild-type allele containing both the floxed MC1*neo* gene and the single *loxP* site in one of 480 clones.

Cre-recombinase-mediated deletion of MC1neo and inversion of exons 12 and 13

Into the correctly targeted cells, a plasmid containing Cre-recombinase driven by the CMV promoter was electroporated, together with a plasmid containing a puromycin-resistance marker (PGK*pur*) in the ratio of 10 : 1. Cells were selected for uptake of DNA with 1.8 μg/ml puromycin from 20 to 72 h after electroporation (Taniguchi *et al*., 1998). Puromycin-resistant clones were screened for deletion of the *neo* gene by testing for G418 sensitivity (250 μg/ml) and for inversion of the fragment containing exons 12 and 13 by selecting 6-thioguanine (10 μg/ml) resistance. The orientation of the exons 12 and 13 containing fragment was confirmed by PCR. The $Msh2^{lox+/-}$ orientation was detectable in a reaction with primer P1 in exon 13 (5′-GCATCCTTGCTCGAGTCG-3′) and primer P2 specific for the polylinker sequence downstream of the 3′ *loxP* site (5′-CTGCAAAATCAGATCCCCG-3′). The inverted $Msh2^{lox-/-}$ orientation was determined by PCR with the same P1 primer and a primer P3 specific for the upstream polylinker sequence of the remaining 5′ *loxP* site (5′-GCAGGAATTC-GATATCAAGC-3′).

An $Msh2^{lox+/-}$clone (6) and an $Msh2^{lox-/-}$ clone (37) were electroporated for a second time with Cre-recombinase and PGK*pur* to invert exons 12 and 13, thereby changing the MMR status.

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