5 mM $MgCl_2$, 20% Glycerol, 1 mM DTT, 1 mM PMSF) and incubated for 30 min. at 4°C while rotating. The samples were then centrifuged for 30 min. at 15000 rpm. and the supernatant was used as nuclear extract. The protein concentration was quantitated by a Bradford protein assay (Bio-Rad). Proteins were separated by 7.5% SDS-PAGE and transferred to Immobilon-P membrane (Millipore). The blots were blocked with 10% (w/v) low fat milk in PBS-T (PBS+0.1% Tween20) for 1 hr. at room temprature, and then incubated with the following primary antibodies in PBS-T-10% milk for 1hr. at room temprature: anti-hMSH2 (Oncogene Science), anti-hMSH3 (supplied by M. Ikejima et al.), anti-hMSH6 (supplied by J. Jiricny et al. and designated 2D4), anti-hMLH1 (Pharmingen) and anti-PMS2 (Pharmingen). After being washed three times with PBS-T, the blots were incubated in the horseradish peroxidase-conjugated secondary antibodies (Amersham) and visualized using the ECL+plus western blotting detection system (Amersham).

2.6. Gel shift assay

^{32}P-labeled DNA duplexes containing the G/T mismatch and G/C match were prepared as described previously [11]. They were incubated with 30 μg of the nuclear extracts in DNA binding buffer (25 mM Hepes-KOH pH7.9 , 0.5 mM EDTA , 0.01 mM $ZnCl_2$, 20% Glycerol , 1mM DTT , 0.25 mg/ml Calf Thymus DNA , 0.1 mg/ml BSA) and preincubated with 150-fold excess of G/C and G/T non-labelled competitors at 20°C. After 20 min. of preincubation, the mixtures were supplemented with 40 fmol of ^{32}P-labelled substrate and

incubated for 30 min. at 20°C. The products of the reaction were separated on 6 % non-denaturing polyacrylamide gels. In all assays, a 150-fold excess of unlabelled duplex competitor oligonucleotide was included. Gel electrophoresis was performed in 0.5 x TBE buffer and detection was by autoradiography.

2.7. In vitro Mismatch Repair Assay

The *in vitro* mismatch repair (MMR) activity of the skin cancer cell lines was measured using an M13mp2 *lacZ*α-complementation method as described [12,13]. M13mp2 DNA substrate, containing a covalently closed (+) strand and a nicked (-) strand with either a G/T mispair or a loop of two extrahelical nucleotides in the *lacZ*α gene coding sequence, were incubated with the skin cancer cell extracts. The DNA was then purified, electroporated into a *mutS* strain of *E. coli* and plated along with the α-complementation strain CSH50, isopropyl β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside. If no repair occurred, a high percentage of mixed plaques, containing both blue and colourless progeny, was observed. A reduction in the percentage of mixed plaques and a concomitant increase in single-colour plaques were indicative of repair. Repair efficiency is expressed as 100 x [1 - (% mixed plaques in extract-treated sample) / (% mixed plaques in extract-untreated sample)].

2.8.Cell Cycle Analysis

Cells were collected by trypsinization, washed and then fixed with 70% ethanol at 4°C. Fixed cells were centrifuged and resuspended in 1ml of 5 μg/ml RNaseA and incubated for 45 min. at 37°C. The DNA was then labelled using 50 μg/ml propidium iodide (Sigma). Cell cycle analysis was performed using a FACSCalibur (Becton Dickinson) and data were analyzed with the software ModFit LT.

3. Results

3.1. Loss of sensitivity to UV in XPA (-/-) skin cancer cell lines

Five cell lines derived from UVB-induced skin cancers in XPA-deficient mice were used in this study. As shown in Table, the cancer cell lines 18, 26, 108 and 174 were established from each squamous cell carcinoma, while 161 was established from a fibrosarcoma. All five cell lines retained the XPA(-/-) genotype (data not shown). Three cell lines, 26, 161, and 174, had different p53 mutations, while 18 and 108 had wild type p53 (Table). We first examined the colony-forming ability of these lines after various doses of UV-irradiation. Unexpectedly, all the skin cancer cell lines were less sensitive to killing by UV-irradiation than the control XPA (-/-) fibroblast cell line, MI-X (Fig. 1). The other spontaneously transformed XPA (-/-) control fibroblast cell line 207 showed the same UV-sensitivity as MI-X, and two cell lines derived from skin cancers of the UVB-irradiated normal mice showed the same UV-sensitivity as normal

control fibroblast cell line 202 (data not shown). Thus, no variations of UV-survivals were detected among the spontaneously transformed control XPA (-/-) fibroblast cell lines and among the skin cancer cell lines derived from UVB-irradiated wild type mice.

To exclude the possibility that the less sensitivity to UV in the cancer cell lines derived from XPA (-/-) mice was due to a restored NER activity, we examined the strand specific repair. As expected, in the XPA(+/+) fibroblast cell line 202, CPDs were removed faster on the transcribed than on the non-transcribed strand (Fig. 2). In contrast, in the XPA(-/-) fibroblast cell line MI-X, no removal of CPDs on either strand was detected. Consistent with the XPA(-/-) genotype of the cancer cell lines, CPD on both the transcribed and non-transcribed strands of the DHFR genes was not removed in all the skin cancer cell lines, 18, 26,108, 161 and 174 (Fig. 2). Furthermore, all the skin cancer cell lines were defective in global genomic repair of both CPDs and 6-4 photoproducts as measured by the immunoblot assay (data not shown). UV-induced unscheduled DNA synthesis was also absent in all the skin cancer cell lines derived from XPA (-/-) mice and XPA (-/-) fibroblast cell line MI-X, whereas the XPA (+/+) fibroblast cell line 202 was proficient (data not shown).

3.2. Resistance to 6-TG

We originally intended to measure the UV-induced mutation frequency at the HPRT (hypoxanthine-guanine phosphoribosyl transferase) locus of these cells, using 6-thioguanine (6-TG) selection. Surprisingly, all the cancer

lines derived from XPA (-/-) mice displayed resistance to 6-TG treatment, while the 202 and MI-X control fobroblast cell lines remained sensitive to killing by this agent (Fig. 3). Because HPRT-deficient cells were eliminated by selection in HAT (hypoxanthine, aminopterin and thymidine) medium prior to 6-TG exposure, the 6-TG resistance of the cancer lines was not due to the presence of mutations in the HPRT locus. We therefore decided to test whether the observed 6-TG resistance might be linked with a defect in postreplicative MMR, since MMR-deficient cells have been shown to be more resistant to killing by this agent [14]. When we examined the expression of MMR proteins in whole cell extracts from these lines, we observed that the steady-state levels of the heterodimeric mMSH2/mMSH6 mismatch recognition factor were lower in four lines (18, 108,161, and 174) as compared to the control 202 and MI-X fibroblast cells (Fig. 4). The level of mMSH3, which forms an alternative heterodimer with mMSH2 that acts in the recognition of small insertion/deletion loops during DNA replication, was also lower in the cancer cell lines than in the control cells. The levels of mMLH1 and mPMS2 proteins, which also exist as a heterodimer that plays an indispensable role in MMR, were decreased in 18, 108 and 174 cells, when compared to 202 and MI-X cells. Western blot analysis of nuclear extracts yielded similar results, the exception being cell line 174, which showed normal steady-state levels of MMR proteins (data not shown). Since extracts form this line were able to bind mismatch-containing oligonucleotides in an *in vitro* assay (see below), we think that the low amount of MMR proteins in the whole cell extracts of these

cells was caused by an increased cytoplasmic protein contents. The expression level of MMR proteins in whole cell extracts of the two skin cancer cell lines derived from wild type mice was the same as that in control 202, 207 and MI-X cells (data not shown).

3.3. G/T mismatch binding and in vitro mismatch repair activities of the skin cancer cell lines

Since the levels of mMSH2, mMSH3 and mMSH6 proteins were decreased in the skin cancer cell lines, we examined whether the DNA mismatch binding activity of these cancer cell lines was decreased using gel shift assays. As shown in Fig. 5, a band shift was observed in the nuclear extracts from control fibroblast cell lines 202 and MI-X using a G/T mismatch containing oligonucleotide as a substrate. The band shift was abrogated when excess unlabelled G/T oligonucleotide was used as competitors. On the contrary, presence of excess G/C probe did not affect the band intensity, indicating that we detected specific binding of the heterodimer mMSH2/mMSH6 to the G/T mismatch. The G/T mismatch binding activity was significantly decreased in the extracts of 18, 108, and 161 cell lines, while extracts from 26 and 174 cell lines were able to recognise G/T mismatches.

We then prepared protein extracts competent for an *in vitro* mismatch repair assay (see Methods) from cell lines 202, 18, 26, and 161. As expected, the G/T mismatch and loop repair activities were significantly decreased in cell lines 18 and 161, while they were normal in cell line 26 (Fig. 6). This latter cell

line showed repair efficiency similar to that of HeLa, a human cell line used as positive control in our experiments. The normal fibroblasts 202 showed intermediate levels of repair as usually observed in cells with lower proliferation rate. The results were consistent with the western blot (Fig. 4) and band shift (Fig. 5) analyses and indicate that cell lines 18 and 161 were partially deficient in mismatch recognition.

3.4. Derangement of the cell cycle checkpoints in skin cancer cell lines

We evaluated the cell cycle checkpoints of the skin cancer cell lines after UV-irradiation or 6-TG treatment by FACS analyses. Accumulation of cells in G1 was observed at 24 hrs after 5 J/m^2 of UV-irradiation in the exponentially growing XPA (-/-) NER-deficient fibroblast cell line MI-X (Fig. 7), whereas a high percentage of S phase cells was observed at this timepoint in the XPA (+/+) NER-proficient fibroblast cell line 202. These results suggest that unrepaired UV damage in MI-X cells evoked a G1 checkpoint, while a functional NER in 202 cells evoked an S phase checkpoint or a shorter arrest in G1 which was already released at 24 hours. In spite of the NER deficiency, none of the cancer cell lines showed G1 arrest 24 hrs after UV-irradiation, when a high percentage of cells were in S-phase (Fig. 7 and Table 1). This finding was observed in skin cancer cell lines irrespective of the p53 status.

When the cells were treated continuously with 0.5 μg/ml of 6-TG for 72 hrs, a G2/M checkpoint was evoked in the fibroblast cell lines (Fig. 7 and Table). This checkpoint was not observed in any skin cancer cell line, which

instead showed a slight accumulation of cells in different phases of the cell cycle. Abrogation of a G2/M checkpoint in MMR-deficient cells after 6-TG exposure was described [14], and might have been anticipated in the partially MMR deficient clones 18 and 161. The finding that also the MMR-proficient skin cancer cell lines 26 and 174 failed to display this checkpoint in spite of their MMR proficiency suggests that they are mutated in another pathway controlling response to 6-TG.

4. Discussion

The five cell lines described in this study were established from UVB-induced skin cancers in XPA-deficient mice. Analysis of several features of their phenotypes showed that they all became markedly tolerant to UV and 6-TG and that they showed abnormal cell cycle checkpoints following treatment with these agents. However, the finding that three of the lines, 26, 161 and 174 acquired mutations in the p53 tumour suppressor gene, while lines 18, 108 and 161 displayed a partial MMR defect, suggests that the UV-induced tumorigenic transformation process in XP-A cells can follow several distinct pathways. The fact that CPDs were not removed from the DNA of these cells following UV-irradiation implies that the cells became tolerant to UV damage, similarly to the case of MMR-deficient cells treated with DNA methylating agents [15]. Because UV-induced photoproducts are DNA polymerase blocking lesions, cells can only go through DNA replication if they are given

enough time to bypass the unrepaired photodimers. This can happen either through the inactivation of genes that are directly involved in apoptotic response to DNA damage, such as p53, which was found to be the case in three of our lines. Alternatively, CPDs may be recognised (but not repaired) by the MMR system, which is thought to signal the presence of the DNA damage to the apoptotic machinery [16-18]. Thus, if the MMR system is not fully functional, such as in lines 18, 108 and 161, UV-DNA lesions may not bring about cell death, and the cells will gain more time to bypass the UV damage. The third way to escape UV-induced killing in the absence of the NER system is to increase the efficiency of error-free lesion bypass. In this scenario, the UV lesions would remain in DNA, but would stay undetected by the MMR system, because they would not contain mismatches [19, 20]. We have no evidence at this time that this mechanism contributes to the survival of our clones, but recent identification of mammalian lesion bypass DNA polymerases [21] will permit the testing of this hypothesis.

The finding that all the skin cancer cell lines were resistant to 6-thioguanine (6-TG) is puzzling. This phenotype was described in MMR deficient cells [14], and the mechanism underlying this phenomenon was suggested to be similar to that hypothesised for the tolerance of MMR deficient cells to alkylating agents [22]. However, 6-TG resistance in the absence of a MMR deficient phenotype suggests that there might be other, as yet uncharacterised, tolerance mechanisms. Should these involve the p53 protein in the absence of the MMR system, then all our cell lines are accounted for,

inasmuch that they carry either a mutated p53 gene or a partially defective MMR system. The tolerance of MMR-deficient cells to methylating agents results from their not entering futile MMR cycles. This is independent of p53, and apoptosis is not really relevant. [17, 23].

Treatment of several tumor cell lines with chemotherapeutics ranging from simple methylating agents to cisplatin has been reported to select for cells with MMR defects [15,24] and there is mounting evidence that a similar situation may also occur in tumors treated with cisplatin [25, 26]. The mechanism of MMR inactivation appears to involve predominantly the epigenetic silencing of the hMLH1 gene [27], although other MMR genes may be similarly inactivated [28]. Inactivation of the MMR genes by mutation has to date been described in only one case, that of the MT1 lymphoblastoid line where both alleles of the hMSH6 gene were mutated following treatment with ICR191 and selection for methylating agent resistance [29]. Although it is conceivable that the UV-induced mutagenic load in cell lines devoid of NER might have been sufficient to alter both alleles of a given MMR gene, we consider this unlikely, as none of the lines were devoid of MMR proteins; rather, they contained reduced steady state levels, which would be more consistent with an epigenetic effect on transcriptional activity of these genes.

At the beginning of this study, we set out to characterise the phenotypic traits of skin cancer cell lines established from UV irradiated NER deficient mice. Our finding imply that cell cycle checkpoints and damage signalling must be inactivated in order for cells to be transformed to tumors. It

would also appear that there are several distinct ways of achieving this goal. Some of these, such as the loss of the p53 function, might have been anticipated based on data from similar studies carried out in other laboratories [30]. However, to our knowledge, this is the first report that implicates the MMR system in UV induced killing. Mellon et al. [31] reported that MMR-deficient colon cancer cell lines also lacked TCR subpathway of NER and were hypersensitive to killing by UV. However, their results are not relevant to our current situation. Because both GGR and TCR subpathways have been deficient in the skin cancer cell lines derived from XPA(-/-) mice and the control XPA (-/-) fibroblast cell lines. Therefore, the differences in UV sensitivity between skin cancer cell lines derived from XPA (-/-) mice and control XPA (-/-) fibroblast cell lines do not reflect the differences in the TCR activity. UV damage thus joins other DNA modifications, ranging from simple methylating lesions to cisplatin induced cross-links, the presence of which is signalled to the apoptotic machinery through the MMR system. Downregulation of MMR may thus play one of the key roles in a pathway towards UV induced cell transformation and cancer.

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Table I. Characteristics of skin cancer cell lines and control fibroblast cell lines derived from XPA-deficient mice. p53 mutations were determined by

direct sequencing of PCR products of the p53 gene as described [7].

Figure Legends

Fig. 1. UV-survival curves of cell lines (18, 26, 108, 161 and 174) derived from skin cancers of UVB-irradiated XPA-deficient mice and control fibroblast cell lines 202 and MI-X, derived from XPA (+/+) and (-/-) mice, respectively. Each point represents the mean of three independent experiments. Bars represent standard errors.

Fig. 2. Strand specific repair within the mouse *DHFR* gene in skin cancer lines (18, 26, 108, 161 and 174) and in control fibroblasts (202 and MI-X). Exponentially growing cells were irradiated with 10 J/m^2 of UV, allowed to grow for 0 to 18 hr, and were then lysed and the DNA was collected. CPD repair efficiency on the transcribed (●) and non-transcribed (■) strands is shown [9]. The values are the averages of two independent experiments for each cell line.

Fig. 3. 6-thioguanine-survival curves of skin cancer lines 18, 26, 108, 161 and 174, and of control fibroblast (202 and MI-X) cell lines. Each point represents the mean of three independent experiments. Bars represent standard errors.

Fig. 4. Expressions of mismatch repair proteins in the skin cancer lines 18, 26, 108, 161 and 174, and in control fibroblast (202 and MI-X) cell lines. 40 μg aliquots of whole cell extracts were loaded in each lane. The data are representative of at least three independent experiments.

Fig. 5. Mismatch binding activities of nuclear extracts from skin cancer lines 18, 26, 108, 161 and 174 and in control fibroblast (202 and MI-X) cell lines. Nuclear extracts (30 μg) were incubated with radioactively labelled oligonucleotides containing a single G/T mispair (G/T oligonucleotides) in the presence or absence of unlabelled competitor oligonucleotides G/T or G/C. Arrowheads indicates specific complexes of mMutSα bound to the labelled G/T oligonucleotide.

Fig. 6. Efficiency of mismatch correction in nuclear extracts of skin cancer cell lines 18, 26 and 161, and in control HeLa cells. (A) Efficiency of G/T mismatch repair in nuclear extracts of skin cancer cell lines 18, 26, 161 and HeLa. (B) Efficiency of 2 base loop repair in nuclear extracts of skin cancer cell lines 18 , 26 and 161, and HeLa cells. In the mock reaction the mismatch substrates were not incubated with nuclear extracts. The values are the averages of two independent experiments for each cell line. Bars represent standard errors.

Fig. 7. Cell cycle regulation in cancer cell lines derived from XPA-deficient mice after UV-irradiation (5 J/m^2) or in the presence of 6-TG (0.5 μg/ml). After

24 hrs (UV-irradiation) or 72 hrs (6-TG treatment) incubation, the cells were stained with propidium iodide and sorted using FACSCalibur. The populations of cells in G1, G2-M, and S phases are indicated as percentages.

Cell line	Genotype XPA	NER activity	Tissue type	Mutation in p53 gene	UV sensitivity	UV-induced cell cycle checkpoints	6-TG sensitivity	6-TG-induced cell cycle checkpoints	mMSH2/mMSH6 level	mMLH1/mPMS2 level
Cancer cell lines										
18	-/-	-	SCC	none	r	S-phase/G2	R	(G2/G1-phase)##	↓↓	↓
26	-/-	-	SCC	C→T at codon 275[a]	R	S-phase	R	(S-phase)##	Normal	Normal
108	-/-	-	SCC	none	r	S-phase	R	(G1-phase)##	↓	↓
161	-/-	-	Fibrosarcoma	CC→TT at codon 238[b]	R	S-phase	R	G1-phase	↓↓↓	Normal
174	-/-	-	SCC	C→T at codon 188[c] G→A at codon 255[c]	r	S-phase	R	S-phase	Normal#	Normal#
Control cell lines										
202	+/+	+	Fibroblast	none	R	S-phase	S	S/G2-phase	Normal	Normal
MI-X	-/-	-	Fibroblast	none	S	G1-arrest	S	S/G2-phase	Normal	Normal

SCC : Squamous cell carcinoma
a : heterozygous mutation
b: heterozygous mutation plus LOH
c : two heterozygous mutations in one allele
r : slightly more resistant when compared with XPA (-/-) control fibroblast cell line MI-X
R : resistant when compared with XPA (-/-) control fibroblast cell line MI-X
S : sensitive as XPA (-/-) control fibioblast cell line MI-X
: Based on nuclear extracts
: Parenthesis in the column of 6-TG-induced cell cycle checkpoints indicates that the delays in these phases were not very pronounced.