DNA mismatch repair (MMR) proteins promote apoptosis and suppress tumorigenesis in response to UVB irradiation: an \textit{in vivo} study

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Abstract: MMR-deficiency was associated with reduced levels of apoptosis and increased residual UVB-induced DNA adducts in the epidermis 24-h following acute UVB exposure. Moreover, Msh2-null mice developed UVB-induced skin tumors at a lower level of cumulative UVB exposure and with a greater severity of onset than wild-type mice. The Msh2-null skin tumors did not display microsatellite instability, suggesting that these tumors develop via a different tumorigenic pathway than tumors that develop spontaneously. Therefore, we propose that dysfunctional MMR promotes UVB-induced tumorigenesis through reduced apoptotic elimination of damaged epidermal cells.

Introduction

DNA mismatch repair (MMR) proteins mediate the removal of single base mismatches and small loops acquired during DNA replication. The importance of MMR in maintaining genomic integrity is illustrated by hereditary non-polyposis colorectal cancer (HNPPC) syndrome, which is caused by a deficiency in a single MMR protein and a resultant mutator phenotype.

In addition to its established role in repair of post-replicative DNA errors, MMR proteins also are involved in mediating the removal of some forms of DNA damage induced by exogenous agents (e.g., SN1 alkylating agent MNNG). More recently, a role for MMR proteins has been demonstrated in promoting cytotoxicity, apoptosis, p53 phosphorylation and cell-cycle arrest following exposure to exogenous DNA damaging agents (e.g., MNNG) (reviewed in ref. 1), including those agents that form DNA adducts not removed by MMR [e.g. cisplatin (2)]. For example, cancer cell lines deficient in MMR display reduced apoptosis levels in response to some forms of chemical carcinogens and oxidative stress (3–9), suggesting that apoptosis protects mammals against tumorigenesis by eliminating mutated cells. Therefore, loss of a single MMR protein contributes to a cellular environment that is primed for malignant transformation due to increased mutation levels and decreased elimination of these cells.

Ultraviolet (UV)-induced DNA damage is primarily removed by nucleotide excision repair (NER) proteins, and adducts within coding sequences are removed specifically by transcription coupled repair (TCR) (10). Although alterations in MMR protein levels are not thought to alter the TCR of UV-induced adducts, we have demonstrated previously that loss of MMR does decrease the cellular response to UV-induced damage (11–13). MMR proteins interact with NER proteins (14) and, in reconstituted assays, human MSH2/MSH6 heterodimers bind oligoduplexes engineered to contain the types of lesions formed by UV radiation (15,16), thus providing evidence that MMR proteins are recruited to sites of UV-induced DNA damage. Studies comparing the cytotoxic effects of UV radiation in MMR-proficient and -deficient cell lines have generated conflicting results (11); however, the method used to transform mammalian cells modulates UV-induced cytotoxicity (11,17–20), and could partially account for these conflicting results. Much of the published research into the role of MMR in UV-induced DNA damage has been performed using UVC (germicidal, 200–280 nm) radiation. However, UVB (280–320 nm) is considered the most important type of UV radiation in skin carcinogenesis because it carries sufficient energy to damage human cells and is ubiquitous in the environment. These findings are consistent with the hypothesis that MMR proteins have a role in cellular responses to UV-induced DNA damage that does not include repair/removal of UV photoproducts.

Previously we have shown that primary mouse embryonic fibroblasts (MEFs) generated from Msh2-null and Msh6-null mice are less susceptible to the cytotoxic effects of UVB radiation than MEFs generated from isogenic wild-type control mice (12,13). Specifically, the Msh2-null and Msh6-null MEFs display lower levels of apoptosis following UVB exposure than wild-type controls (12,13). Moreover, preliminary results from our laboratory show that human MSH2-null human lymphoblasts [established from a patient homozygous for mutated MSH2 (21)] and Msh6-null primary MEFs (13) exhibit a reduced G1 cell-cycle arrest following exposure to UVB radiation (Narine et al. and Felton et al., unpublished data). Therefore, we hypothesize that loss of either Msh2 or Msh6 from a population of epidermal cells may promote malignant transformation and predispose mammals to skin cancer.

Abbreviations: MEF, mouse embryonic fibroblast; MMR, DNA mismatch repair; MSI, microsatellite instability; NER, nucleotide excision repair; SBC, sunburn cells or apoptotic epidermal cells; SCC, squamous cell carcinoma; TCR, transcription coupled repair; UV, ultraviolet; UVB, ultraviolet radiation between 280 and 315 nm; XPA, xeroderma pigmentosum A.
In this study we sought to expand our in vitro studies by using whole murine skin in vivo, thus mimicking the normal human physiological state more closely. We exposed $Msh2$-null, $Msh6$-null and wild-type control mice to both acute and chronic UVB radiation. We found that loss of either $Msh2$ or $Msh6$ is sufficient to significantly reduce the induction of apoptosis in the epidermis of these mice. Moreover, the $Msh6$-null epidermis had higher levels of residual thymine dimers than the $Msh6$ wild-type mice. Comparing the rate of tumor formation in the $Msh2$-null and wild-type mice following chronic UVB exposure, $Msh2$-null mice developed malignant skin tumors at an earlier cumulative UVB exposure than the wild-type mice and were more likely to present with multiple skin tumors. We found that, unlike spontaneous tumors arising in $Msh2$-null mice, UVB-induced skin tumors from $Msh2$-null mice did not display microsatellite instability (MSI). Therefore, the data demonstrate that loss of MMR is associated with accelerated onset and a greater severity of skin cancer in response to chronic UVB exposure. This predisposition to UVB-induced skin cancer also is associated with a reduced ability to undergo UVB-induced apoptosis and to eliminate UVB-induced DNA damage.

Materials and methods

**MMR knockout mice**

The $Msh2$-null mouse model was constructed by Reitmair et al. (22) and genotyping was performed using previously described modifications (12). The $Msh6$-null mouse model was generated using `R' version 1.8.0 (www.r-project.org). Microsatellite instability assay DNA from skin tumors and adjacent normal skin was isolated using the DNeasy Tissue System (Qiagen, Mississauga, ON, Canada). Microsatellites were amplified using fluorescently labeled primers and the PCR product was analyzed on the LIFCOR LongReader 4200 (LI-COR Biosciences, Lincoln, NE). The following 10 microsatellites were analyzed: JH101, JH102, JH103, JH104 (23); U12235 (24); D7mit17 (Whitehead Institute: www.genome.wi.mit.edu/cgi-bin/mouse/sts_info); D1mit83 (Whitehead Institute); D7mit91 (Whitehead Institute); Tcrb (25); and Cyp1a2 (25). This panel represents five mononucleotide repeats, three dinucleotide repeats, one trinucleotide repeat and one tetranucleotide repeat.

**UVB light source**

The UVB light source consisted of an arc'd bank of 20 Philips UVB bulbs (TL 20W/12 RS) filtered by Kodacel (TA422 clear cellulose triacetate 0.127 mm, Eastman Kodak, Rochester, NY) to remove residual UV below 290 nm. The output of the filtered UVB source was measured using an IL1700 Research Radiometer with a SED 240/UVB-1/W detector (International Light, Newburyport, MA) at a minimum of once a week. During UVB exposure, each mouse was placed in a close-fitting cage that ensured the backs of the mice remained directed toward the UVB source and that the ears and eyes of the mice were protected. UVB exposure of the mice was approved by the Health Sciences Laboratory Animal Services at the University of Alberta. [Since our previous publications alterations in the irradiation equipment has resulted in a difference in UVB measurement and in the reporting of UVB radiation delivered: 4000 J/m² in the current set-up is equivalent to 2000 J/m² in our previous publications (12,13).]

**Acute UVB exposure and specimen preparation**

The backs of both the UVB-irradiated and non-irradiated mice were shaved to reveal a longitudinal section of bare skin – $\times$ 3 cm. $Msh2$-null and wild-type mice were irradiated with 4000 J/m² of UVB and $Msh6$-null and wild-type mice were irradiated with 8000 J/m² of UVB. Twenty-four hours after UVB exposure the irradiated and non-irradiated mice were killed. The shaved section of skin was excised and divided in half longitudinally. One half was snap frozen and archived. The other half was flattened, pinned onto dental wax, and fixed in 10% buffered formalin. Once fixed, the skin was subdivided into six to eight sections, which were embedded in paraffin blocks vertically to allow for a survey of the entire length of the shaved back (prepared by Laboratory Medicine and Pathology, University of Alberta Hospital).

**Sunburn cell counting**

Two sections (4 μm) from each block containing the acute UVB sections embedded vertically were stained with hematoxylin and eosin. For each field (40 x objective) the number of sunburn cells (SBC, apoptotic cells) were counted. The number of fields counted per mouse ranged from 180 to 300. The variance in the number of fields counted reflects differences in the width and length of skin harvested from each mouse and, therefore, the number of subsections.

**Measurement of thymine dimers**

For each of the paraffin blocks containing $Msh6$-null, $Msh2$-null or wild-type skin harvested 24 h after irradiation, one section (4 μm) was stained with an antibody specific for DNA thymine dimers (clone KTM53, Cedarlane Laboratories Limited, Hornby, ON, Canada) at a 1:8000 dilution for 4°C overnight. Slides were not counterstained. Using either a 16 x or 20 x objective, between 12 and 20 non-adjacent fields were digitally photographed from each slide and the staining intensity of the epidermis was quantified using densitometry (Quantity One, Bio-Rad, Mississauga, ON, Canada). The variance in the number of fields photographed reflect differences in the length of skin harvested from the mouse, and hence the number of subsections that were embedded. Typically two or three non-adjacent fields were photographed from each subsection, depending on the width of the subsection.

**Chronic UVB exposure**

The posterior two-thirds of the back of each mouse was shaved with an electric razor as required. $Msh2$-null, $Msh6$-null and wild-type mice were irradiated with 4000 J/m² of UVB three times per week (Monday, Wednesday and Friday) until the first presentation of a skin lesion or until 37 weeks of UVB irradiation. At the first presentation of a skin lesion, the cumulative UVB exposure of the mouse was noted and the mouse continued to receive chronic UVB radiation until the lesion reached ~0.5 cm in diameter. After death, the tumor was excised, a sample prepared for histological examination (prepared by Laboratory Medicine and Pathology, University of Alberta Hospital), and the remainder of the tumor subdivided and snap frozen. In addition, samples of UVB-exposed non-tumor skin and non-UVB exposed abdominal skin were snap frozen. Kaplan-Meier survival curves and associated statistics were generated using ‘R’ version 1.8.0 (www.r-project.org).

**Microsatellite instability assay**

DNA from skin tumors and adjacent normal skin was isolated using the DNeasy Tissue System (Qiagen, Mississauga, ON, Canada). Microsatellites were amplified using fluorescently labeled primers and the PCR product was analyzed on the LICOR LongReader 4200 (LI-COR Biosciences, Lincoln, NE). The following 10 microsatellites were analyzed: JH101, JH102, JH103, JH104 (23); U12235 (24); D7mit17 (Whitehead Institute: www.genome.wi.mit.edu/cgi-bin/mouse/sts_info); D1mit83 (Whitehead Institute); D7mit91 (Whitehead Institute); Tcrb (25); and Cyp1a2 (25). This panel represents five mononucleotide repeats, three dinucleotide repeats, one trinucleotide repeat and one tetranucleotide repeat.

**Results**

**SBC formation and thymine dimer removal**

$Msh2$-null and $Msh6$-null mice, together with isogenic wild-type controls, were subjected to a single UVB exposure. Twenty-four hours after exposure, the skin of the UVB irradiated mice did not display, by visual inspection, more erythema than non-irradiated control mice. Upon microscopic examination it was observed that the epidermis of both the $Msh2$- and $Msh6$-null mice contained significantly ($P = 0.044$ and $P = 0.007$, respectively) fewer SBCs than the wild-type control mice (Figure 1A). Moreover, staining with an antibody specific for thymine dimers (Figure 2) revealed that the epidermis of the $Msh6$-null mice contained significantly ($P = 0.005$) higher levels of residual thymine dimers than wild-type mice (Figure 1B). The epidermis of $Msh2$-null and wild-type mice did not demonstrate different levels of thymine dimer staining ($P = 0.130$, Figure 1B). However, these mice were irradiated with a lower dose of UVB than the $Msh6$ mice and these results are consistent with the smaller differential observed for SBC formation in the epidermis of the $Msh2$ mice (Figure 1A).

**Tumor formation**

$Msh2$-null and wild-type mice were exposed to chronic UVB irradiation and monitored for the appearance of skin tumors. We found that the $Msh2$-null mice developed malignant skin tumors at a significantly ($P = 0.0276$) lower cumulative UVB dose than wild-type control mice as depicted by the
Kaplan–Meier survival curve (Figure 3, Table I). The majority of tumors were invasive spindle squamous cell carcinomas (SCC) (Table I), which is the type of UV-induced skin tumor commonly generated in mice. No benign tumors were generated in either genotype. Also, a greater number of Msh2-null mice than wild-type mice developed multiple skin tumors (Table I). Therefore, the Msh2-null mice displayed both accelerated UVB-induced tumor formation and increased severity of UVB-induced tumor formation.

Although similar numbers of Msh2-null and wild-type mice entered the chronic UVB study (35 and 33, respectively), 22 Msh2-null mice died of thymic lymphoma before the onset of skin cancer. Msh2-null mice are predisposed to spontaneous thymic lymphomas at an average age of onset of 6 months (26). At 6 months of age the chronically irradiated mice had received ~190 000 J/m² of UVB and this cumulative UVB exposure was lower than required for skin tumor generation in these experiments.

Msh6-null (n = 12) and wild-type (n = 15) mice also were subjected to chronic UVB irradiation. It was observed that the
there may be a trend towards accelerated UVB-induced tumorigenesis in the Msh6-null mice ($P = 0.231$, Figure 4A), this experiment was stopped before sufficient data could be collected and the difference between the two genotypes is not statistically significant. Of the seven UVB-induced skin tumors collected, five were from Msh6-null mice and two from Msh6 wild-type mice; the majority of the tumors were invasive spindle SCCs. The Msh6 mice present with spontaneous thymic lymphomas with an average age of onset of 10 months (23). No mice were removed from the experiment due to health problems associated with other tumor types.

**Microsatellite instability**

Ten microsatellites were used to test for instability in five UVB-induced skin tumors from Msh2-null mice and four UVB-induced skin tumors from wild-type mice. The primers and amplification conditions have been used to demonstrate MSI in spontaneously arising tumors in these Msh2-null mice (M.R. Campbell et al., manuscript in preparation). Using these primers and amplification conditions, we found none of these 10 microsatellites to be unstable in any of the UVB-induced skin tumors tested (Figure 5).

**Discussion**

We have demonstrated previously that Msh2-null and Msh6-null primary MEFs display a modest (2-fold) but significant decrease in UVB-induced apoptosis levels than wild-type control MEFs (12,13). We hypothesize that UVB-induced apoptosis is decreased in the epidermis of MMR-deficient mice in vivo and that this decrease is sufficient to accelerate the onset of UVB-induced skin tumorigenesis.

In order to confirm that the MMR-dependent apoptosis observed using the MEFs is a physiologically relevant observation, we exposed Msh2-null, Msh6-null and wild-type mice to a single UVB exposure and measured the number of SBCs (apoptotic keratinocytes) 24 h after irradiation. As found in the case of the MEFs, UVB irradiation-induced apoptosis in the epidermis of both wild-type and knockout mice. However, significantly fewer epidermal cells of the Msh2-null and Msh6-null mice were apoptotic compared with isogenic wild-type control mice (Figure 1a). This difference was particularly pronounced in the epidermis of the Msh6-null mice, which were irradiated at a higher UVB dose than the Msh2-null mice. Comparing the induction of apoptosis in the Msh2 and Msh6 mouse models, irradiated at 4000 and 8000 J/m², respectively, the epidermis of the wild-type mice displayed a dose-dependent response to UVB radiation (Figure 1A). In contrast, the UVB-induced apoptotic response in the epidermis of the knockout mice was not dose-dependent; the levels of apoptosis in the Msh2- and Msh6-null epidermis were similar despite the 2-fold difference in UVB exposure (Figure 1A). Therefore, our data demonstrate that UVB-induced apoptosis is partially MMR-dependent in murine epidermis.

Upon incurring DNA damage, a mammalian cell can either repair the DNA or eliminate the mutated cell by apoptosis. Our finding that thymine dimer levels were higher in the Msh6-null epidermis than in the wild-type epidermis (Figures 1B and 2) confirms that the observed MMR-dependent decrease in apoptosis is not due to increased adduct removal. Conversely, the lower levels of apoptosis in the Msh6-null epidermis are associated with higher levels of residual DNA damage.
suggesting that UVB-damaged cells are escaping MMR-dependent apoptotic elimination.

The increased levels of residual thymine dimers in the Msh6-null epidermis could instead be interpreted as MMR-dependent removal of these adducts. However, experiments comparing the repair of cyclobutane pyrimidine dimers (CPDs) indicate that mammalian MMR proteins are not required for CPD removal from the transcribed strand (15,27--31), although there are some data to the contrary (32). Bulky UV-induced photoproducts are removed by NER; adducts are removed preferentially from the transcribed strand by the rapid TCR, as opposed to the slower global genomic repair (GGR). Mice engineered to be deficient in TCR [Cockayne syndrome B (Csb)-null] are more susceptible to sunburn (erythema and edema) and SBC formation than wild-type mice (33--35). In comparison, GGR-deficient mice [xeroderma pigmentosum group C (Xpc)-null] are not more susceptible to sunburn and SBC formation than wild-type mice (33--35). Similarly, mice lacking xeroderma pigmentosum A, a more general NER protein involved in both TCR and GGR, demonstrate increased SBC formation after acute UVB exposure (34--36). Comparing the responses of the epidermis of mice deficient in either MMR (Figure 1) or TCR (summarized above), it can be inferred that MMR proteins are not removing thymine dimers and that MMR-dependent apoptosis is not related to increased adduct removal. However, we cannot rule out that MMR proteins are regulating both apoptosis and NER in murine epidermal cells (keratinocytes) in vivo.

These experiments investigating the response of the Msh2 and Msh6 knockout mice to acute UVB exposure demonstrate that UVB-induced apoptosis is partially MMR-dependent. Moreover, we found that failure to apoptose is associated with decreased elimination of epidermal cells displaying UVB-induced DNA damage. The data presented here together with the data of others (summarized above) suggest that loss of either Msh2 or Msh6 promotes UVB-induced tumorigenesis. Accordingly, we observed that Msh2-null mice chronically exposed to UVB radiation develop skin cancer at a lower cumulative UVB dose than wild-type control mice (Figure 3). Moreover, Msh2-null mice appear to have a more severe onset of tumorigenesis, as demonstrated by the increased incidence of mice with multiple tumors (Table 1).

Initially this chronic UVB experiment was performed also with the Msh6 knockout mouse model. However, the Msh6 mice scratched excessively in response to chronic UVB

Fig. 5. Examples of the microsatellite instability analysis performed on UVB-induced tumors in Msh2-null and wild-type mice using the Licor LongReader 4200. (A) Murine Msh2-null hematological tumors showing microsatellite instability (MSI+) using a mononucleotide marker (U12235) and a dinucleotide marker (D7mit17). Dotted lines indicate normal allele size in each mouse (DNA from non-tumor brain tissue) with arrows indicating instable alleles. (B) Microsatellite stable (MSI−) murine Msh2-null haematological tumors using the same markers. Dotted lines indicate the normal allele size as observed in the control tissue (non-tumor brain tissue). (C) Microsatellite instability assayed in UV-induced Msh2-null tumors; non-UV exposed abdomen and UV-exposed non-tumor tissue were included as controls. All tissue including tumor tissue showed stable microsatellites.
irradiation and the experiment was ceased before an informative number of skin tumors could be collected. Interestingly, the Msh6-null mice were significantly (P = 0.06) less affected by this scratching behavior than the isogenic control mice (Figure 4). Despite the small number of tumors collected, there was a trend toward an accelerated tumor onset in the Msh6-null mice (P = 0.231, Figure 4).

During the course of these experiments, two studies comparing the onset of UVB-induced tumorigenesis in MMR-deficient mice were published. Meira et al. (37) and Yoshino et al. (38) observed that Msh2-null mice were more susceptible to UVB-induced skin cancer. The experiments presented here extend and confirm the initial observations of Meira et al. (37). Both Meira et al. (37) and Yoshino et al. (38) used the Msh2 knockout mouse model generated by de Wind et al. (39,40); this mouse model displays low levels of spontaneous sebaceous gland and skin tumors (40) analogous to the phenotypic variant of HNPCC known as Muir-Torre syndrome (MTS). Interestingly, the genetic background and/or housing conditions of the mice used by Meira et al. and Yoshino et al. appear to have modulated the relative frequency of spontaneous skin tumors. Meira et al. did not observe spontaneous skin tumorigenesis, but did observe that Msh2-null mice had an earlier onset of UVB-induced skin tumors compared to wild-type mice (37). Moreover, Meira et al. applied TPA to Msh2-null to induce cellular proliferation. The treatment failed to induce dysplasia or skin tumors on the Msh2-null background, suggesting that increased cellular proliferation alone is not sufficient for tumor formation (37).

Yoshino et al. backcrossed the de Wind Msh2 mouse onto an HR-1 hairless background and found that the incidence of spontaneous skin cancer was increased greatly; over 80% of Msh2-null mice developed spontaneous skin tumors (primarily SCC) by 52 weeks (38). Although UVB irradiation clearly accelerated tumorigenesis in the Msh2-null mice (38), it is not clear if this acceleration is greater than that observed for the wild-type mice. However, this study (38) dramatically illustrates the influence of genetic background on the phenotype of mouse models and demonstrates a role for MMR-deficiency in skin tumorigenesis.

The Msh2 mouse model generated by Reitmair et al. also displays a low level of spontaneous SCC and sebaceous skin tumors (26). For the results presented here, we have used the Reitmair Msh2-null mice backcrossed onto BALB/c. Using these Msh2 mice, and in other studies in our laboratory using this Msh2-null mouse bred onto a Nude background, we have not observed any spontaneous skin neoplasms (M.R.Campbell et al., manuscript in preparation). Therefore, the statistically significant difference in UVB-induced skin tumorigenesis observed between the Msh2-null and wild-type mice in our experiments (Figure 3) represents the increased UVB-induced tumorigenesis conferred by the loss of Msh2. Our data support our hypothesis that decreased MMR-dependent apoptosis is associated with an increased predisposition to skin tumorigenesis.

Spontaneous tumors, but not normal adjacent tissues, from Msh2-null mice display high levels of MSI (22,26,39). Reitmair et al. characterized three SCC and eight sebaceous neoplasms arising spontaneously in Msh2-null mice and found that all of the SCC but none of the sebaceous tumors displayed MSI (26). In the current study we found no indication of MSI at any of 10 microsatellites in a subset of the Msh2-null UVB-induced skin tumors (Figure 5). In comparison, using the same assays, spontaneous tumors (predominantly thymic lymphomas) from equivalently aged Msh2-null mice readily demonstrate MSI (Figure 5) (M.R.Campbell et al., manuscript in preparation).

Our data suggest that dysregulation other than genomic instability, reflected by MSI, contributes to the increased susceptibility of Msh2-null mice to UVB-induced tumorigenesis. Therefore, UVB-induced skin tumorigenesis may develop along different molecular pathways than spontaneous tumor formation associated with lack of repair in MMR-deficient cells. For example, the mutator phenotype (genetic instability) caused by MMR deficiency promotes spontaneous tumorigenesis, whereas our data suggest that a partial impairment in the elimination of UVB-damaged cells via apoptosis promotes UVB-induced tumorigenesis in MMR-deficient mammals. Alternatively, MMR-deficiency may be contributing to tumor progression/growth rather than initiation; loss of MMR has been demonstrated to result in reduced cell-cycle arrest (8,41–46). The data presented here demonstrate that MMR is protective against the onset of UVB-induced skin tumorigenesis, and that MMR-dependent apoptosis may underlie this protection. Further investigation to elucidate the MMR-dependent apoptotic pathway post-UVB is underway.

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