Enhanced DDB2 Expression Protects Mice from Carcinogenic Effects of Chronic UV-B Irradiation

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Abstract
UV-damaged DNA-binding protein (UV-DDB) is essential for global genome repair (GGR) of UV-induced cyclobutane pyrimidine dimers (CPD). Unlike human cells, rodent epidermal cells are deficient in GGR of CPDs and express a subunit of UV-DDB, DDB2, at a low level. In this study, we generated mice (K14-DDB2) ectopically expressing mouse DDB2 at elevated levels. Enhanced expression of DDB2 both delayed the onset of squamous cell carcinoma and decreased the number of tumors per mouse in chronically UV-B light-exposed hairless mice. Enhanced expression of DDB2 improved repair of both CPDs and pyrimidine(6-4)pyrimidone photoproducts (6-4PP) in dermal fibroblasts. However, GGR of CPDs in K14-DDB2 mice did not reach the level of efficiency of human cells, suggesting that another repair protein may become rate limiting when DDB2 is abundantly present. To complement these studies, we generated mice in which the DDB2 gene was disrupted. DDB2−/− and DDB2+/− mice were found to be hypersensitive to UV-induced skin carcinogenesis. On the cellular level, we detected a delay in the repair of 6-4PPs in DDB2−/− dermal fibroblasts. Neither the absence nor the enhanced expression of DDB2 affected the levels of UV-induced apoptosis in epidermal keratinocytes or cultured dermal fibroblasts. Our results show an important role for DDB2 in the protection against UV-induced cancer and indicate that this protection is most likely mediated by accelerating the repair of photolesions. (Cancer Res 2005; 65(22): 10298-306)

Introduction
DDB1 and DDB2 are subunits of the UV-damaged DNA-binding protein (UV-DDB), a heterodimer that binds to the two major types of UV-induced DNA lesions [i.e., pyrimidine(6-4)pyrimidone photoproducts (6-4PP) and cyclobutane pyrimidine dimers (CPD); ref. 1]. In nuclear extracts of human cells, the ability of UV-DDB to bind UV-irradiated DNA (UV-DDB activity) is shown by an electrophoretic mobility shift assay (EMSA; ref. 2). No UV-DDB activity was detected in nuclear extracts derived from xeroderma pigmentosum group E (XPE) patients carrying mutations in the DDB2 gene (2, 3). XPE patients display the mildest clinical symptoms among xeroderma pigmentosus patients and their cells retain ~50% of unscheduled DNA synthesis (for review, see ref. 4). Cells of XPE patients are completely deficient in global genome repair (GGR) of CPDs (5), whereas repair of 6-4PPs is delayed (6, 7). In contrast, all XPE cells are proficient in transcription-coupled repair (TCR; ref. 5).

Several findings suggest that UV-DDB acts in GGR as a damage recognition factor. The rate of CPD and 6-4PP repair in a reconstituted repair system using purified factors was enhanced by the addition of UV-DDB, particularly in CPDs (8, 9), although other cell-free systems showed no effect on excision of CPDs (10) or cisplatin adducts (11) or even an inhibitory effect on 6-4PP excision (10, 12). Microinjection of purified UV-DDB protein corrected nucleotide excision repair (NER) deficiency in XPE cells (11, 13). Wakasugi et al. (14) also showed accumulation of green fluorescent protein–tagged DDB2 at sites of UV DNA damage. Recruitment of XPC to UV damage in human cells was significantly decreased in absence of functional DDB2, suggesting that DDB2 is the first recognition factor in NER of UV-induced DNA lesions (7, 15).

In addition, functions other than damage recognition in NER have been proposed for DDB2. Datta et al. (16) observed in vivo and in vitro interaction of UV-DDB with histone acetyltransferase cyclic AMP–responsive element–binding protein–binding protein/p300 and suggested that UV-DDB might be involved in chromatin remodeling during repair. Furthermore, UV-DDB was shown to be the part of an ubiquitin ligase complex together with cullin4A, Roc1, and the COP9 signalosome that becomes active after UV irradiation of cells (17). This finding and the recently reported ability of UV-DDB to ubiquitinate the XPC protein (9) point to a role for UV-DDB in modification or proteolysis of DNA-binding and repair proteins.

Rodent cells display an extensive deficiency in GGR of CPDs but are fully capable in GGR of 6-4PPs as well as TCR as shown for cultured hamster and mouse fibroblasts (18–20) and for epidermal keratinocytes of hairless mice (21). Deficient GGR of CPDs in rodents correlates with a lack of UV-DDB activity in rodent skin cells using common protocols for EMSA (22, 23). Expression of human DDB2 in hamster cells restored both UV-DDB activity and GGR of CPDs and suppressed UV-induced mutagenesis (22). In mouse epidermis, the DDB2 gene is expressed at a very low level (23) and UV-DDB activity in mouse cells could be detected only after concentration of proteins in nuclear extracts (24). Two groups have recently produced DDB2−/− mice (24, 25). DDB2+/− mice are viable and fertile, but both homozygous and heterozygous DDB2 knockout mice exhibited enhanced skin carcinogenesis in response to chronic UV irradiation (24, 25). Mouse embryonic fibroblasts (MEF) of DDB2−/− animals were more resistant to UV irradiation and displayed a reduction in caspase-3 activity in comparison with wild-type (WT) MEFs (24). Based on these observations and similar results in human XPE cell lines, Itoh et al. (24, 26) proposed that DDB2 protects against cancer by increasing apoptosis in UV-damaged cells rather than by enhancing the repair of UV-induced photoproducts. To investigate the protective role of DDB2 in UV-induced skin carcinogenesis, we generated a transgenic mouse line ectopically expressing mouse DDB2 in...
epidermal cells at a much higher level than endogenous DDB2. Enhanced expression of DDB2 made mice more resistant to UV-induced skin cancer. In contrast to a role for DDB2 in UV-induced apoptosis as suggested by Itoh et al. (24, 26), we found no indications that the extent of UV-induced apoptosis was affected by the level of expression of DDB2. In contrast, our findings indicate that the protective role of DDB2 against UV-induced skin cancer is primarily mediated by its ability to enhance NER.

Materials and Methods

Generation of mice ectopically expressing FLAG-DDB2 (K14-DDB2 mice). A PCR fragment containing mouse DDB2 cDNA was amplified using primers p48mus16HindIII and p48mus17NotI (5′-AAGAGAAAAAAGGCGGGCGTCCACTCTCCAC-GAAT) using mouse splenocyte cDNA as a template. Amplified mouse DDB2 cDNA was cloned into p3XFLAG-cytomegalovirus-10 (Sigma, St. Louis, MO) using restriction sites in the primers (HindIII and NotI, respectively), with the FLAG tag located at the NH2 terminus of the DDB2 protein. From the resulting plasmid, the SacI/NotI fragment containing the DDB2 coding region at the 5′ end fused to the sequence encoding a FLAG epitope was recloned into the pC9648 V vector (using restriction sites in the primers, shown in bold) at the 5′ end of the puromycin resistance gene. The herpes simplex virus type 1 puromycin resistance gene expressed primarily mediated by its ability to enhance NER.

UV irradiation of animals. For determination of the minimum edema/erythema dose (MED), hairless mice were exposed to UV-B radiation using a Kromayer lamp (Hanovia, Slough, United Kingdom) equipped with a Schott-WG305 filter (Schott, Tiel, the Netherlands) (ref. 29; exposure rate was 100 J/m²/200–400 nm). For both genotypes, two male mice were exposed to 15, 20, 25, 30, and 35 seconds of UV radiation from the Kromayer lamp. All exposures were given in duplicate on separate mice. The mice were checked for erythema and/or edema at 24 and 48 hours after the exposures. For in vivo apoptosis experiments, mice were irradiated for 80 seconds corresponding to 4 MED. For the UV carcinogenesis experiment, Philips TL-12/40W fluorescent tubes (Philips, Eindhoven, the Netherlands) were employed. Hairless mice were daily dorsally exposed to a dose 500 J/m² UV approximating 1 MED for SKH-1 mice (30).

Isolation of RNA from mouse keratinocytes and mouse dermal fibroblasts. For keratinocyte isolation, a strip of dorsal skin was excised. Epidermis and dermis were separated after overnight incubation with thermolysin (Sigma; 200 μg/ml in PBS, 2 mmol/L CaCl₂) at 4°C. RNA from the epidermal layer was isolated using an Ultra-Turrax T8 (IKA-Werke, Staufen, Germany) rotor-stator homogenizer and an RNasey Mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturers. RNA isolation from mouse dermal fibroblasts (MDF) was done with a RNasey Mini kit.

Mouse dermal fibroblast cell cultures. Primary MDFs were isolated from 1-day-old mice as described (20) and grown in DMEM supplemented with 15% FCS.

Quantitative real-time PCR. Total cDNA was produced using the SuperScript II RT Polymerase kit (Invitrogen, Carlsbad, CA). Keratinocyte or MDF cDNA was used as template for quantitative real-time PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and SYBR Green PCR Master Mix (Applied Biosystems) according to the instructions of the manufacturer. We used primers ENDR2F2W (5′-GGTTGCGGCAAATGTTCTGTC and ENDR2R (5′-CAGAGAGAGATCGTAT-GGACC) for amplification of endogenous DDB2 mRNA and primers BGLNF (5′-GCCCATCATTGTGCAAGAA and BGLNR (5′-CCACAC- CAGCCACACTTTC) for amplification of FLAG-DDB2 mRNA. As a reference housekeeping gene, we used the β-actin gene primers BACTFW (5′-AAGCTTCTGAGGATCC) and BACTR (5′-CAAGAGCTGAGTACCAGT). Results were calculated as described elsewhere (31).

Electrophoretic mobility shift assay. To create the DNA probe, an oligonucleotide (GATACAGTGCTGCAGGATTAACTCCTCTGCATG) was labeled with 32P using T4 kinase (Invitrogen) and annealed to the complementary oligonucleotide. The DNA fragment was irradiated with 5 kJ/m² using a Philips TUV lamp (200–400 nm). For the binding reaction, 2.5 μg nuclear extract, prepared as described (32), was added to the reaction mix (0.5 μg poly(adenylic acid-adenylic acid) and 200 fmol specific competitor (unlabeled and unirradiated probe) in band-shift buffer [final concentration 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 0.2 mmol/L EDTA, 1 mmol/L DTT, 5% glycerol] in a total volume of 8 μL and incubated for 5 minutes at 30°C. Then, 2 μL probe (10 nmol/L) was added to the reaction and incubated for another 20 minutes at 30°C. After incubation, the binding mixture was put on ice, immediately loaded on a 6% polyacrylamide gel (45 mmol/L Tris-borate, 1 mmol/L EDTA; size 83 × 73 × 0.5 mm), and run at 9 mA for 20 minutes.
**Global and local UV irradiation.** Confluent MDFs were washed once with PBS and subsequently irradiated with a Philips TUV lamp (predominantly 254 nm) at a dose rate of 0.2 W/m² as described previously (33). For local UV irradiation, cells grown on coverslips were washed once with PBS, covered with an isopore polycarbonate filter (Millipore, Bedford, MA) with pore sizes of 5 μm, and subsequently UV irradiated (34). After irradiation, the filter was removed and cells were returned to culture conditions or immediately used for immunostaining.

**Antibodies.** Primary antibodies were mouse monoclonal anti-6-4PP and monoclonal anti-CPD (a kind gift from Dr. 0. Nikaido, Kanazawa University, Kanazawa, Japan), anti-FLAG rabbit polyclonal (Sigma), anti-FLAG mouse monoclonal M2 (Sigma), rabbit anti-active caspase-3 antibody (PharMingen, San Jose, CA). Secondary antibodies used in this study were FITC-conjugated donkey anti-rabbit IgG (The Jackson Laboratory, Westgrove, PA), horseradish peroxidase–conjugated goat polyclonal anti-mouse IgG (DakoCytomation, Copenhagen, Denmark), and biotin-conjugated goat anti-rabbit IgG antibody (Vector, Burlingame, CA). All secondary antibodies were used according to the manufacturer’s instructions.

**Fluorescent immunolabeling, microscopy, and quantification of fluorescent signal.** Fluorescent immunolabeling was done as described (35). Fluorescence images were obtained with a Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) and a Hamamatsu C5935 cooled CCD camera (Hamamatsu, Shizuoka, Japan). Pictures were captured and processed with Metasystems ISIS software (Altlussheim, Germany). The total fluorescence intensity of the nucleus area was quantified using the ISIS software package and divided by the surface area, resulting in a specific fluorescence intensity expressed in arbitrary units.

**Determination of apoptotic keratinocytes in skin biopsies.** Mice were irradiated with a Hanovia Kromayer lamp as described above and sacrificed 24 hours later. A section (0.5 × 0.5 cm) from the middle of the irradiated zone was isolated and snap frozen in liquid nitrogen. Determination of apoptotic keratinocytes in skin biopsies using anti-active caspase-3 antibody was done as described previously, and the level of apoptosis was expressed as the number of active caspase-3–positive cells per arbitrary unit (36).

**Results**

**K14-DDB2 and DDB2−/− mice.** DDB2 is expressed in mouse skin cells at a very low level resulting in extremely poor UV-DDB activity (23). Unlike humans, mice are deficient in GGR of CPDs and much more prone to UV-induced skin cancer than man (20, 21, 37). To gain more insight into the role of DDB2 in DNA repair and in UV-induced skin cancer, we generated a DDB2 KO mouse model (called DDB2−/−) and a mouse model ectopically expressing FLAG-tagged mouse DDB2 from the K14 promoter (called K14-DDB2).

To examine whether the low amount of DDB2 in mouse epidermal cells is still sufficient to facilitate repair of UV-induced DNA lesions and to counteract UV-induced skin cancer, we generated DDB2−/− mice containing a genomic deletion from exon 3 (partially) to exon 7 (Fig. 1A–C). By semiquantitative real-time PCR, we determined that the amount of DDB2 mRNA was significantly reduced in DDB2+/+ MDFs, whereas no PCR product was obtained in DDB2−/− cells (Fig. 1C). DDB2+/+ and DDB2−/− mice were fertile and did not show any developmental abnormalities or growth retardation, in agreement with previous report (24, 25).

K14-DDB2 mice are viable and fertile and do not show any signs of growth retardation or developmental abnormalities. Using real-time PCR, we checked expression of FLAG-tagged DDB2 mRNA in...
different tissues and found transcripts not only in keratinocytes but also in dermal fibroblasts, brain, liver, and lung (data not shown). Levels of FLAG-DDB2 and endogenous DDB2 mRNA were quantified by real-time PCR in both keratinocytes and dermal fibroblasts. In keratinocytes, ~6,000 times more FLAG-DDB2 mRNA was detected compared with endogenous DDB2 mRNA. The difference in expression between ectopic and endogenous DDB2 mRNA in MDFs was substantially smaller than in keratinocytes but still approximated 500-fold. Because no antibody for endogenous mouse DDB2 protein was available to us, we only checked by Western blot analysis the concentration of FLAG-DDB2 protein in whole-cell extracts of MDFs (Fig. 1D).

**FLAG-DDB2 recognizes UV photolesions in vitro and in vivo.**

To check if FLAG-DDB2 was able to recognize and bind to UV-induced DNA damage, we used two different approaches. First, we employed EMSA to examine UV-DDB activity in nuclear extracts of MDFs of K14-DDB2 mice. In these experiments, UV-DDB activity was evident in K14-DDB2 MDFs, whereas no UV-DDB activity was detected in WT MDFs (Fig. 2A). The level of UV-DDB activity in K14-DDB2 MDFs was comparable with that in primary human fibroblasts (data not shown). Next, we investigated whether FLAG-DDB2 was recruited to the site of local UV-damage. Thirty minutes after local UV irradiation, FLAG-DDB2 accumulated at spots corresponding to UV-damaged parts of the nucleus (Fig. 2B). Thus, ectopically expressed FLAG-DDB2 is able to recognize and bind to UV lesions.

**FLAG-DDB2 becomes degraded after UV irradiation.** In human cells, the concentration of DDB2 protein significantly drops during the first hour after UV irradiation. Subsequently, the amount of DDB2 is restored up to its initial level at 24 hours and reaches a maximum at 48 hours after UV irradiation, when its level is 10- to 12-fold higher than its initial concentration (38). To examine the effect of UV irradiation on the concentration of ectopically expressed FLAG-DDB2, we monitored the amount of FLAG-DDB2 in soluble cell extracts of K14-DDB2 MDFs and in chromatin at time points from 15 minutes up to 24 hours following UV irradiation (Fig. 2C). The concentration of soluble FLAG-DDB2 dropped significantly in the first 15 minutes after UV irradiation, became undetectable at 1 and 3 hours after UV irradiation, and was almost completely restored at 24 hours after UV. The concentration of chromatin-bound FLAG-DDB2 (Fig. 2C) increased at 15 minutes after UV irradiation but significantly dropped at 1 and 3 hours and was restored up to its initial level at 24 hours after UV. Addition of the proteasome inhibitor MG-132 (Calbiochem, San Diego, CA) prevented UV-induced degradation of FLAG-DDB2 in mouse MDFs (Fig. 2C). Thus, the kinetics of ectopically expressed FLAG-DDB2 in UV-irradiated mouse cells closely resembles the pattern of endogenous DDB2 in human cells.

**Figure 2.** Ectopically expressed FLAG-DDB2 is functional. A, ectopic expression of FLAG-DDB2 restores specific UV-damaged DNA-binding activity in nuclear extracts of K14-DDB2 MDFs. B, FLAG-DDB2 accumulates at the site of UV damage in the nucleus. MDFs of transgenic mice were irradiated with 20 J/m² UV-C through isopore polycarbonate filter with pore sizes of 5 µm, fixed 30 minutes after UV, and stained with anti-FLAG antibodies. Spots of FLAG-DDB2 (green) are indicated with arrowheads. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (blue). C, the cellular concentration of FLAG-DDB2 transiently drops after UV irradiation. K14-DDB2 MDFs were either mock treated or irradiated with 10 J/m² UV-C, and whole-cell extracts were prepared as described in ref. 24 at 15 minutes and 1, 3, 16, and 24 hours after irradiation and used for Western blot analysis with anti-FLAG antibodies. To release the remaining chromatin-bound proteins, the pellet produced during cell extract preparation was resuspended in 1× DNase I buffer and digested with 100 units/mL DNase I (Promega, Madison, WI) at 37°C for 30 minutes in the presence of 10 µg/mL protease inhibitors. No degradation of DDB1 was observed after immunostaining with anti-DDB1 antibody, which subsequently was used as a loading control. To study the effect of inhibition of protein degradation on DDB2 stability, K14-DDB2 MDFs were treated with the proteasome inhibitor MG-132 (20 µmol/L) 3 to 4 hours before UV irradiation and whole-cell extracts were prepared at the indicated post-UV time points.
The cellular level of DDB2 affects the repair kinetics of both cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidine photoproducts. To investigate to what extent the concentration of DDB2 influences repair of photolesions in DNA, MDFs of K14-DDB2, DDB2+/−, and WT mice were irradiated with UV-C and the amount of remaining photolesions was determined after different time periods by immunofluorescent labeling with specific antibodies (7). In K14-DDB2 cells, the intensity of CPD-specific fluorescent signal was reduced by 35% at 24 hours after UV, whereas in WT cells ~87% of the CPD signal were retained, showing a 2.7-fold enhancement of CPD repair in FLAG-DDB2-expressing cells (Fig. 3A). We note here that the level of CPD repair in WT cells is so low that a significant and reproducible reduction of CPD repair in DDB2+/− cells could not be detected. Furthermore, ectopic expression of DDB2 increased the rate of the 6-4PP repair during the first hour after UV irradiation but not at later time points (Fig. 3B). In contrast, repair of 6-4PPs was delayed in DDB2+/− MDFs (Fig. 3C); during the first hour after UV, no reduction in 6-4PP-specific immune fluorescence signal was observed in DDB2+/− MDFs. As a negative control, 6-4PP repair was determined in completely NER-deficient XPA MDFs (39) and was shown to be virtually absent. These results indicate that the concentration of DDB2 influences the repair kinetics of CPDs and, during the first hour after UV irradiation, 6-4PPs.

Enhanced expression of DDB2 in mouse skin does not change the sensitivity to acute effects of UV irradiation. Chronic UV exposure of hairless mice is a well-established model for squamous cell carcinomas (SCC) in man. The use of hairless mice for UV carcinogenesis prevents the need of shaving before UV irradiation, allowing an accurate dosimetry (29) and excluding artifacts introduced by damaging of the skin during shaving. K14-DDB2 and DDB2+/− mice were therefore crossed into a hairless Crl:SKH1-HR background to assess the acute effects of UV irradiation (edema/erythema) as well as chronic UV-induced skin cancer. The MED for all three genotypes was 20 seconds corresponding to 2,000 J/m², which is in close agreement with previous studies using albino hairless mice (29). Thus, expression of DDB2 in mouse skin did not change the sensitivity of the mice to UV-induced acute effects.

Levels of DDB2 expression strongly influence the susceptibility of hairless mice to UV-induced skin cancer. To investigate the influence of DDB2 expression on UV-induced skin carcinogenesis, 12 K14-DDB2 and 12 WT littermates were exposed to a daily dose of 500 J/m² representing the MED for WT Crl:SKH1-HR mice (30). UV-induced skin cancer susceptibility of K14-DDB2 mice was reduced in comparison with WT mice. Fifty percent of the K14-DDB2 mice had developed SCCs larger than 1 mm by week 13, whereas this tumor-bearing percentage (t1/2) was already reached in WT mice by week 11. In addition, the time point by which all mice had developed SCCs (t100) was different for WT and K14-DDB2 mice, being weeks 13 and 16, respectively (Fig. 4A). The difference between WT and transgenic mice for larger-sized SCCs (>2 mm) was even more pronounced (Fig. 4B). Ectopic expression of DDB2 also significantly reduced the yield (i.e., average number of tumors per mouse) of UV-induced SCCs (Fig. 4C and D). Thus, enhanced expression of DDB2 in the mouse epidermis reduced UV-induced carcinogenesis both by delaying the onset of tumors and by reducing the number of tumors per mouse. DDB2+/− and DDB2+/− mice and WT littermates were exposed to a daily dose of 250 J/m² (i.e., half of their MED dose) to measure tumor induction. In contrast to mice ectopically expressing FLAG-DDB2, DDB2+/− and DDB2+/− mice were more susceptible to UV-induced skin cancer than WT mice: >50% of DDB2−/− mice had developed SCCs larger than 1 mm by week 10, whereas all DDB2−/− mice exhibited SCCs by week 12. Of their DDB2+/− littermates, 50% and 100% had developed SCCs by weeks 11 and 15, respectively, whereas the WT littermates reached these percentages by weeks 16 and 20, respectively (Fig. 5A). This difference in tumor prevalence between the three genotypes was also observed for tumors larger than 2 mm (Fig. 5B). These results are in good agreement with previous reports where also accelerated development of UV-induced SCCs in homozygous and heterozygous DDB2 knockout mice was detected (24, 25). A significant difference between DDB2−/−, DDB2+/−, and WT mice was also observed in the total yield of SCCs (Fig. 5C and D). The results of the UV carcinogenesis experiments indicate that the level of DDB2 in skin cells dramatically influences the UV cancer susceptibility of mice. The DDB2 level did not...
influence the tumor type as all genotypes developed mainly SCCs and a minority of papillomas.

Expression of DDB2 does not influence apoptosis in cultured mouse dermal fibroblasts or in vivo. To check the effect of different levels of DDB2 protein on UV-induced apoptosis, MDFs of WT, DDB2−/−, and K14-DDB2 mice were irradiated with 8 J/m² UV-C and the fraction of sub-G₁ cells (representing cells undergoing apoptosis) was determined at 8, 24, and 48 hours after UV irradiation in both confluent (Fig. 6A) and growing (Fig. 6B) cells. Although a substantial fraction of the MDFs underwent apoptosis at 48 hours after UV, no differences in apoptotic activity were found between the three genotypes. Analogous results were obtained with an independent apoptosis assay in which the activity of caspase-3, an effector of the apoptosis pathway, was measured (data not shown).

To ascertain that our apoptosis data obtained in MDFs were representative for keratinocytes in the mouse skin, we also determined the apoptotic response to UV-B irradiation directly in the mouse epidermis. Mice were irradiated with a dose of 5000 J/m² UV-B (corresponding to 1 MED for both genotypes). The number and size of SCCs were monitored weekly. A, prevalence of SCCs larger than 1 mm (P = 0.001). B, prevalence of SCCs larger than 2 mm (P < 0.0005). The Mann-Whitney U rank test was used for estimation of P in (A and B). C, yield of SCCs larger than 1 mm. D, yield of SCCs larger than 2 mm. Bars, SE (C and D).

Discussion

The inability of mouse skin cells to perform GGR of CPDs (21) has been postulated to explain the huge difference in UV skin cancer susceptibility between mouse and man. In the present study, we have generated K14-DDB2 mice ectopically expressing FLAG-tagged DDB2 protein to assess the effect of enhanced expression of DDB2 on cellular end points (repair and apoptosis) and on UV-induced skin cancer in mice. To complement these experiments, we also generated DDB2−/− mice to investigate the biological consequences of the complete absence of the DDB2 protein in mice and derived cells.

Keratinocytes and MDFs of transgenic mice express, respectively, 6000- and 500-fold more FLAG-DDB2 mRNA than endogenous DDB2 mRNA. This indicates that the human K14 promoter was highly active in mouse keratinocytes although not strictly tissue specific. The high levels of FLAG-DDB2 mRNA suggest that also FLAG-DDB2 protein was present in these cells in much higher concentrations than the endogenous DDB2 protein. Indeed, a nuclear extract of K14-DDB2 MDFs (unlike WT MDFs) possessed substantial UV-DDB activity. Moreover, ectopically expressed FLAG-DDB2 was efficiently recruited to sites of local UV damage in cultured cells, indicating that it was able to fulfill its role in DNA damage recognition. In addition, we observed similar kinetics of degradation and recovery of FLAG-DDB2 protein after UV as has been described for DDB2 in human cells (38). All these findings indicate that skin cells of K14-DDB2 mice express functional DDB2 protein.

The rate of skin tumor development induced by chronic exposure to UV-B-irradiation and the yield of tumors per mouse in both K14-DDB2 and DDB2−/− mice were clearly different from those observed for WT hairless mice. Mice ectopically expressing FLAG-DDB2 developed UV-induced SCCs more slowly than WT mice, and the yield of tumors per animal was drastically reduced. In contrast, DDB2−/− and DDB2−/− mice were hypersensitive to the carcinogenic effect of UV irradiation, in accordance with results of other studies (24, 25). In MDFs of DDB2−/− mice, which show a sensitivity to UV-induced skin cancer intermediate between DDB2−/− and WT mice, the amount of DDB2 mRNA was ~2-fold reduced in comparison with WT mice, suggesting that DDB2...
protein concentrations might be similarly reduced in DDB2<sup>+</sup>/<sup>-</sup> cells. Combined these results indicate that the cellular concentration of the DDB2 protein is an important determinant of UV skin cancer susceptibility in mice. As human XPE is thought to be a recessive disease, DDB2 gene dosage effects in human cells presumably do not occur possibly because of higher basal levels of DDB2.

Itoh et al. (24) reported a reduced UV sensitivity and reduced levels of apoptosis in MEFs derived from DDB2<sup>-/-</sup> mice. Previously, they had already reported similar results for human XPE cells (26). Based on these observations, they suggested that DDB2 protects against UV-induced skin cancer by increasing p53-mediated apoptosis in UV-damaged cells rather than by enhancing repair of UV-induced photolesions. In contrast, Yoon et al. (25) did not observe a difference in UV sensitivity between DDB2<sup>-/-</sup> and WT MEFs, whereas other studies indicated human XPE cells to have an increased sensitivity for UV (40, 41). Furthermore, a reduction in UV-induced apoptosis was reported for DDB2-overexpressing human cells (42). In the current study, no difference in the level of UV-induced apoptosis was detected in skin sections of DDB2<sup>-/-</sup> mice in comparison with WT mice. In addition, no increase was found in UV-induced apoptotic activity in epidermal cells of K14-DDB2 mice that contain increased levels of DDB2. The methodology for determining UV-induced apoptosis in vivo (i.e., immunostaining for active caspase-3 in mouse epidermal sections) has successfully been used previously to show increases in UV-induced apoptosis in NER-defective XPA, XPC, and Cockayne syndrome B mice (36) as well as a reduction of UV-dependent apoptotic activity in p53<sup>-/-</sup> mice (43). Consistent with the in vivo apoptosis data, we also did not observe any differences in the extent of UV-C-induced apoptosis in either dividing or confluent MDFs derived from K14-DDB2, DDB2<sup>-/-</sup>, and WT mice. The apparent discrepancy between our apoptosis data and the data of Itoh et al. (24) is not easy to explain. A difference exists in the size of the deletion of the DDB2 gene between the two types of DDB2<sup>-/-</sup> strains, as Itoh et al. (24) created a genomic deletion of exons 4 to 7, whereas in our DDB2 knockout model also part of exon 3 of DDB2 gene was deleted. In addition, we cannot exclude that differences in apoptotic activity between MEFs and MDFs might exist. However, the fact that we detected similar levels of UV-induced apoptosis in the epidermis as well as in MDFs of the various genotypes excludes, the possibility that the observed differences in UV-induced skin cancer susceptibility of these mice are due to differences in apoptosis.

Therefore, the most likely explanation for the role of DDB2 in protection against UV-induced skin cancer lies in its ability to stimulate GGR of UV-induced photolesions. In MDFs derived from K14-DDB2 mice, we found that enhanced expression of DDB2 accelerated repair of 6-4PPs and led to increased CPD repair, whereas repair of 6-4PPs was delayed in DDB2<sup>-/-</sup> MDFs. The very low level (13% after 24 hours) of CPD repair found in WT cells as determined by immunofluorescence corresponds very well with previous measurements using a biochemical method (21). Although a possible additional deficiency in CPD repair in DDB2<sup>-/-</sup> cells might exist, this cannot be determined with the methodologies employed and, if present, will anyhow be very small. The finding that DDB2 influenced kinetics of 6-4PP repair only during the first hour after UV irradiation is highly consistent with the rate and extent of DDB2 degradation after UV (ref. 38; Fig. 3C). Thus, the susceptibility to UV-induced skin cancer seems to correlate with DDB2-dependent modulation of GGR. Recent work of Sugawara et al. (9) showed that the DDB2 protein is indispensable for UV-induced polyubiquitination of XPC to which it physically interacts and that such ubiquitination increases the DNA-binding capacity of the XPC protein. These
cells per arbitrary unit (as described in Materials and Methods) was used. As a measure of apoptosis, the number of active caspase-3-positive keratinocytes in the epidermis of WT, K14-DDB2, and DDB2+/- mice. Twelve WT, DDB2+/-, and 6 K14-DDB2 mice were irradiated with 8 J/m² UV-C. The percentage of sub-G1 cells was calculated at different time points after UV using fluorescence-activated cell sorting analysis. Columns, results of two independent experiments; bars, SE.

Figure 6. Ectopic expression of DDB2 does not influence UV-induced apoptosis in cultured cells or mouse epidermis. Percentage of sub-G1 cells in confluent (A) and growing (B) MDFs after UV irradiation. WT, DDB2+/-, and K14-DDB2 MDFs were irradiated with 8 J/m² UV-C. The percentage of sub-G1 cells was calculated at different time points after UV using fluorescence-activated cell sorting analysis. Columns, results of two independent experiments; bars, SE. C, number of apoptotic keratinocytes in the epidermis of WT, K14-DDB2, and DDB2+/- mice. Twelve WT, 6 DDB2+/-, and 6 K14-DDB2 mice were irradiated with a dose of UV-B corresponding to 4 MED. Twenty-four hours after UV irradiation, skin sections were collected and stained with anti-active caspase-3 antibodies. As a measure of apoptosis, the number of active caspase-3-positive cells per arbitrary unit (as described in Materials and Methods) was used.

finding strongly indicate a direct involvement of DDB2 in the recruitment of XPC to the site of UV damage and, therefore, in the stimulation of NER.

Which of the two types of UV-photolesions (i.e., 6-4PPs or CPDs) is mainly responsible for the induction of skin cancer in mice? Several lines of evidence suggest a major role for the poorly repaired CPDs in UV skin carcinogenesis: (a) p53 mutations in UV-induced skin tumors were predominantly found at sites of potential photoproducts in the nontranscribed strand (44), because repair of 6-4PPs in the transcribed and nontranscribed strand in mouse cells is equally efficient (20), such strand bias suggests defective GGR of CPDs to be the main cause for UV-induced mutations that leads to development of cancer in WT mice. (b) Recent work by Jans et al. (45) using mouse strains expressing CPD or 6-4PP photolyase in epidermal keratinocytes unequivocally showed that photoreactivation of CPDs dramatically increased the resistance to UV-induced skin cancer, whereas enhancement of 6-4PP repair had only a minor effect on UV carcinogenesis. In line with these findings, we suggest that the reduction in skin cancer susceptibility of K14-DDB2 mice exposed to UV-B light is most likely due to increased GGR of CPDs in their epidermal cells.

In contrast, reduced repair of 6-4PPs most likely underlies the increased UV skin cancer proneness of DDB2+/- and DDB2+/- mice. In the present study, we showed a delay in the repair of 6-4PPs in DDB2+/- MDFs during the first hour after UV, resembling the observed delay in repair of 6-4PPs in human XPE cells (7). The UV skin cancer data suggest 6-4PP repair kinetics in DDB2+/- cells to be intermediate between WT and DDB2+/- cells due to a gene dosage effect. In the presence of functional TCR, epidermal keratinocytes rapidly restore DNA synthesis after UV irradiation (36); hence, it is likely that delayed repair of 6-4PPs contributes to mutagenesis and to the development of cancer. Although the role of 6-4PPs in UV-induced carcinogenesis seems to be minor in WT mice (45), it is conceivably that in GGR-deprived mice 6-4PPs might play a more pronounced role in cancer induction. Most notably, an elevated UV-induced skin cancer proneness has been found for XPC+/- mice (29), which are TCR proficient and differ from the WT mice by the absence of 6-4PP GGR (20). In this respect, it is worth noting that mutagenic bypass of 6-4PPs in mammalian cells has been reported to be >10 times higher than for CPDs (46). The similar delay in repair of 6-4PPs in both human and mouse cells deficient for DDB2 suggests that the accelerating role of DDB2 in 6-4PP repair might also be important in the protection of humans against UV-induced skin cancer.

In spite of the enhanced expression of DDB2 in K14-DDB2 mice, GGR of CPDs did not reach the level of efficiency of human cells in which ~80% of CPDs are repaired at 24 hours after 5 J/m² UV-C (47). A possible explanation for the incomplete correction of CPD repair is that another repair protein might become rate limiting when DDB2 is abundantly present. The most probable candidate for the role of such rate-limiting factor is the XPC protein, which was found to be the rate-limiting factor for the GGR of UV-induced DNA lesions in TCR-deficient mice (48).

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Possibly, simultaneous increased expression of XPC and DDB2 in mouse cells will lead to an efficiency of CPD repair comparable to UV skin carcinogenesis. In line with these findings, we suggest that the accelerating role of DDB2 in 6-4PP repair might also be important in the protection of humans against UV-induced skin cancer.

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