Cytotoxic, Mutagenic and Carcinogenic Properties of Ultraviolet Radiation

Shining light on photolesions

Cytotoxische, mutagene en carcinogene eigenschappen van ultraviolette straling

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Aan Martijn

Contents

Aim and outline of the thesis

Exposure to ultraviolet light (UV light) poses a serious threat to human health. An altered life style (holidays in the sun, tanning devices) has led to increased exposure to UV light in the Western population. UV light damages the DNA, the carrier of genetic information, which can result in permanent alterations in the genome and, ultimately, cancer. The majority of the DNA lesions induced by UV light consists of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidone photoproducts (6-4PPs). The relative contribution of these CPDs and 6-4PPs to the effects of acute and chronic UV exposure, however, is not known (e.g. sunburn, cancer). Although organisms are equipped with the nucleotide excision repair (NER) mechanism for removal of lesions, this mechanism may not offer sufficient protection in cases of excessive exposure to UV light. The aim of this thesis is to further elucidate the mechanisms behind the detrimental effects of UV exposure and to determine the role of individual classes of UV-induced DNA lesions in these processes. To this end, we generated mice transgenically expressing photolyase enzymes. This approach enables light-controlled removal of a single type of lesion, allowing investigation of the contribution of individual lesions to UV responses. In Chapter 1, we review the current knowledge on UV-induced DNA damage and repair, and the consequences of UV exposure on human health. Chapters 2 and 3 describe the generation of ubiquitously expressing CPD and 6-4PP photolyase transgenic mice. We show that these marsupial and plant photolyase gene products are functional in mice, resulting in light-dependent removal of DNA lesions from mouse skin. Furthermore, we provide evidence that CPDs are responsible for adverse UV effects including cell death, sunburn and permanent genomic alterations (mutations). In Chapter 4 we describe the generation of transgenic mice expressing photolyase from the keratinocyte-specific K14 promoter, allowing fast removal of DNA damage by photoreactivation in basal keratinocytes of the epidermis, whereas in other cell types such as fibroblasts and Langerhans cells lesions are repaired in slow fashion (if at all). This gave us the unique opportunity to dissect the cell types involved in UV responses. In Chapter 5 the carcinogenic potential of DNA lesions are studied. Importantly, we show that removal of CPDs alone is sufficient to delay the induction of skin cancer to a great extent. An important role in UV-induced skin carcinogenesis is performed by the immune system. We show that CPD removal not only protects the animal from cancer by lowering the mutation load, but it also abolishes UV-induced immunosuppression, allowing the immune system to respond adequately to malignancies. The results described in this thesis are reviewed in a general discussion in Chapter 6.

Chapter One

Consequences of exposure to ultraviolet light

Sunlight is essential for life on earth, as oxygen production by plants is fully dependent on the energy of visible light (photosynthesis). In mammals and other organisms, even the highly energetic ultraviolet (UV) component of light is required for the synthesis of important substances such as vitamin D. Whereas often appreciated for its cosmetic tanning properties, UV light is better known for its many deleterious effects, one of which is the induction of skin cancer. The consequences of UV exposure have gained more and more attention as a result of the increasing incidence of skin tumors in white populations. This is largely due to prolonged exposure to more energetic UV light caused by alterations in human life-style (e.g. frequent visits to exotic holiday destinations, the use of artificial tanning devices) as well as environmental changes such as the reduced ozone layer. Therefore, it is of great importance to understand the mechanisms behind the detrimental effects of UV light.

Ultraviolet Radiation

Sunlight contains electromagnetic radiation of different wavelengths that can be divided into infrared (>780 nm), visible (400-780 nm) and ultraviolet (UV, <400 nm) light. UV light can be further subdivided into UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (<280 nm, see Figure 1) light. The highly energetic UV-C radiance and part of the UV-B component are effectively absorbed by ozone (O_3) in the atmosphere and do not reach the earth's surface. The ozone layer has gained increased attention by the general public due to the depletion of ozone from the stratosphere and, consequently, increased exposure to potentially detrimental radiation.

It has been estimated that a 1% reduction in the thickness of the ozone layer could already increase the occurrence of squamous and basal cell carcinomas by 3% and 1.7%, respectively (Longstreth, 1988; Slaper et al., 1996; van der Leun et al., 1993). Besides the shielding effect of the ozone layer, the amount of UV that reaches the earth is also dependent on the position of the sun (i.e. geographical position, time of the year) and the climate (de Gruijl et al., 2003; van der Leun and de Gruijl, 2002).

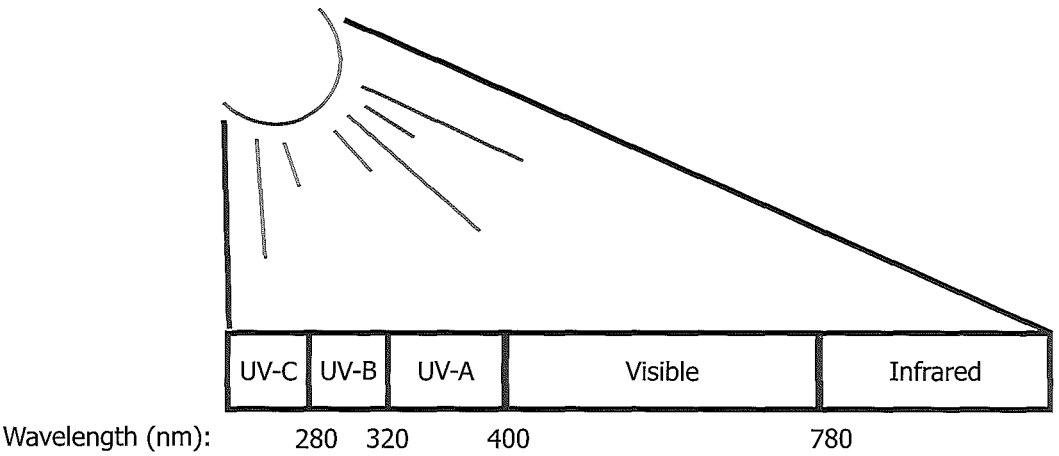

Figure 1
The wavelength spectrum of the sun.

UV light can exert its deleterious effects via different cellular processes. Protein cross-links may occur upon absorption of UV light, affecting the proper function. Furthermore, absorption of UV may alter the DNA, the carrier of genetic information, through formation of pyrimidine dimers or, in the case of UV-A, through oxidation.

UV-induced DNA damage

Ultraviolet radiation can induce a variety of modifications in the DNA. However, extensive studies on isolated DNA have shown that the most significant damage is found at bipyrimidine sites. At such sites, absorption of a photon yields excited thymine (T) and/or cytosine (C) residues, which can react with adjacent pyrimidine bases to form a dimeric reaction product. Dimers can be found in two configurations, either as cyclobutane pyrimidine dimers (CPDs) or as pyrimidine-(6,4)-pyrimidone adducts (6-4 photoproducts, or 6-4PPs). Dependent on the UV dose and wavelength, the latter type of lesion can undergo photoconversion into a so-called Dewar valence isomer upon exposure to UV light (Fig. 2).

Both CPDs and 6-4PPs distort the DNA double helix: a CPD induces a kink of 7-9°, whereas the more distorting 6-4PP induces a kink of approximately 44° (Ciarrocchi and Pedrini, 1982; Kim and Choi, 1995; Wang and Taylor, 1991). The flexibility of DNA determines the likelihood of a specific sequence to be damaged by UV light. Sequences that facilitate bending and unwinding are more favorable sites for damage induction (Thoma, 1999). For example, CPDs are formed at higher frequencies in single-stranded DNA (Becker and Wang, 1989) and at the flexible ends of poly(dA).(dT) tracts, but not in their rigid center (Lyamichev, 1991). DNA flexibility is modified to a great extent by interactions with proteins such as transcription factors. Therefore, it is expected that protein binding affects the yield and distribution of DNA lesions. Indeed, this is the case for several proteins, including the lac repressor protein in *Escherichia coli* and several other sequence-specific DNA binding proteins in yeast and mammalian cells (Becker and Wang, 1984; Becker and Wang, 1989; Pfeifer et al., 1992; Selleck and Majors, 1987a; Selleck and Majors, 1987b; Tornaletti and Pfeifer, 1995). For example, binding of the TATA-box binding protein (TBP) to the TATA-box causes local DNA distortion, allowing 6-4PPs to be formed more easily in the TATA-box sequence. In contrast, CPDs are

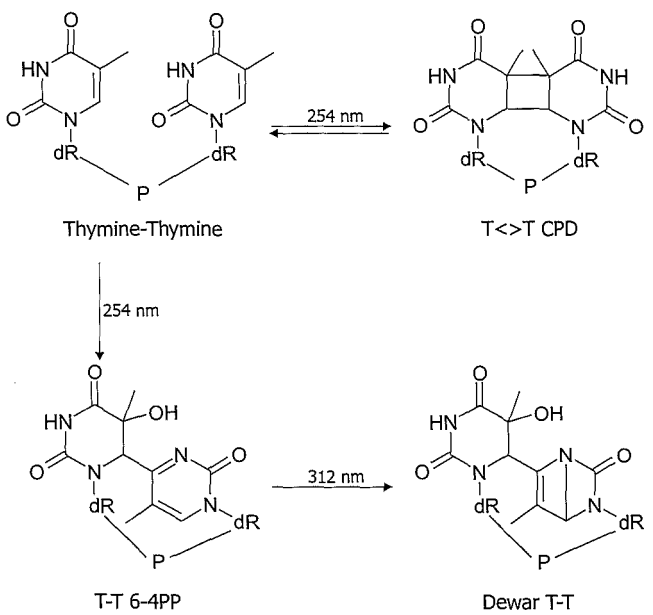

Figure 2
The induction of TT dimers by UV.
Absorption of UV results in the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). 6-4PPs in turn can be converted to the Dewar isomer.

predominantly found at the edge of the TATA-box, where DNA is not or only mildly distorted (Aboussekhra and Thoma, 1999).

The yield and distribution of DNA lesions may also be influenced by the higher order DNA structure in eukaryotes, known as chromatin. Genomic DNA is wrapped around histone octamers into structures called nucleosomes. This nucleosomal DNA has an inner surface facing the histones and an outer surface facing the solvent, resulting in significant distortions of the DNA such as local twist and compression of the grooves facing inside. Interestingly, it has been suggested that bending of DNA around the histone octamer facilitates CPD formation since these lesions are predominantly found in nucleosomal DNA, rather than linker DNA residing between nucleosomes (Brown et al., 1993; Gale et al., 1987; Gale and Smerdon, 1990; Pehrson, 1989; Pehrson, 1995). In strong contrast to CPDs, 6-4PPs are found predominantly in linker DNA (Niggli and Cerutti, 1982). The flexible nature of linker DNA, allowing major distortions such as 6-4PPs to occur, could serve as a possible explanation. Alternatively, it has been suggested that the preferential localization of 6-4PPs in linker DNA could be the result of altered nucleosome positions after damage formation (Thoma, 1999). In other words, major helix distortions such as 6-4PPs in the nucleosome may not be accommodated easily and therefore may cause the nucleosome to slide to another position. Yet, direct evidence to support this hypothesis is lacking.

Despite many attempts, it has proven difficult to quantify the distribution of these three classes of photoproducts (CPDs, 6-4PPs and Dewars) at the four different bipyrimidinic sites in DNA (Douki and Cadet, 2001). Douki and coworkers (2000a; 2000b) developed an HPLC-tandem mass spectrometry (HPLC-MS/MS) method, enabling measurement of photoproducts generated by different wavelengths at all possible bipyrimidinic sites. Both UV-B and UV-C yielded similar distributions of the photoproducts, provided that low fluencies were used. Figure 3 shows the yield of photoproducts expressed as the number of lesions per kilobase per kJ/m² UV-C. In UV-treated DNA, TT CPDs are the most abundant lesions. At TC sites, CPDs and 6-4PPs are formed in equal amounts. The lowest induction of CPD and 6-4PP lesions was found at CC sites, which accounted for less than 5% of all lesions. In contrast, Dewars

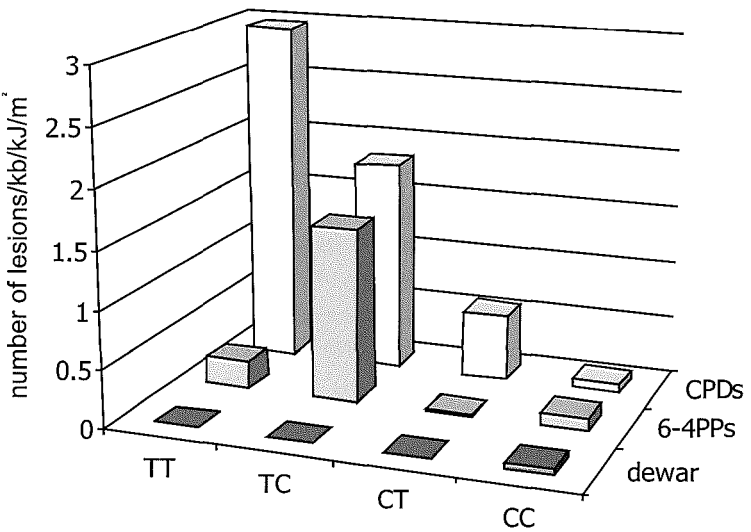

Figure 3
The number of CPDs, 6-4PPs and Dewar isomers per kilobase per J/m² of UV-C at different bipyrimidinic sites. Pattern resembles that observed upon UV-B exposure. Adapted from Douki et al. 2001.

were formed at CC sites more than at any other sequence. In conclusion, apart from influences from chromatin structure or protein binding, lesion induction is highly dependent on the sequence context.

DNA repair

Apart from UV radiation, many other physical and chemical agents continuously challenge the integrity of the genome. DNA damage not only arises from exogenous (environmental) agents but is also inflicted by endogenous sources such as reactive oxygen species (ROS), spontaneous deamination and errors made by the DNA replication machinery. Whereas exposure to ultraviolet light induces mainly CPDs and 6-4PPs, X-rays or treatment with anti-cancer drugs such as *cis*-platinum results in interstrand cross-links or double-strand breaks of the DNA helix. Furthermore, the metabolism of cells will lead to oxidative stress, resulting in more than 100 types of DNA modifications (Cadet et al., 1997). Finally, errors made by the replication machinery may eventuate in base mismatches or even deletion or insertion events. Therefore, organisms have to deal with an extreme diversity of DNA damage.

It is apparent that this plethora of DNA lesions requires highly specialized mechanisms to keep the genome intact. Indeed, many different DNA repair mechanisms have evolved to allow the organism to cope with the occurrence of genomic insults (reviewed by Friedberg (2003) and Hoeijmakers (2001)). DNA double-strand breaks or cross-links may be repaired by recombinational repair, involving either homologous recombination or non-homologous end joining. Repair of DNA damage arising from cellular metabolism, including oxidation, methylation, deamination and hydroxylation is performed by a process called base excision repair (BER). In contrast, mismatch repair (MMR) does not repair lesions, but removes nucleotides that are misincorporated by DNA polymerases as well as insertion- or deletion loops induced by faulty recombination or slippage of the replication machinery at repetitive sequences. For repair of UV-induced lesions, two very distinct processes exist: the multi-step nucleotide excision repair (NER) mechanism and the light-dependent process known as photoreactivation.

Nucleotide Excision Repair

Nucleotide excision repair (NER) is an extremely versatile DNA repair mechanism responsible for removal of numerous types of helix distorting lesions (de Laat et al., 1999), such as UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). Other substrates for NER include bulky adducts (caused by e.g. polycyclic aromatic hydrocarbons found in tobacco smoke, car exhaust fumes and industrial pollution) and 5',8-purine cyclodeoxynucleotides induced by ROS (Kuraoka et al., 2000). NER removes DNA lesions via a "cut and patch" mechanism involving the concerted action of approximately 30 different proteins. NER comprises 4 stages: lesion recognition, local opening of the double helix, excision of the damaged strand and, finally, gap-filling DNA synthesis (Aboussekhra et al., 1995; de Laat et al., 1999; Wood, 1999) (Fig. 4).

Lesion recognition is performed by the protein complex XPC/hHR23B that surveys the genome for helix distortions (Sugasawa et al., 2001). Upon detection of a lesion, DNA repair is initiated and the XPB and XPD helicases of the multi-subunit transcription factor IIH (TFIIH) unwind the DNA around the lesion (Drapkin et al., 1994; Schaeffer et al., 1994; Schaeffer et al., 1993; Winkler et al., 2000). The XPA protein verifies the presence of damage by probing for abnormal backbone structure (Buschta-Hedayat et al., 1999), whereas Replication Protein A (RPA) stabilizes the open complex by binding to the

undamaged strand and the XPA protein (Li et al., 1995; Stigger et al., 1998). Next, the endonucleases ERCC1/XPF and XPG cleave 5′ and 3′ of the damage respectively, excising a 24-32 nucleotide (single stranded) fragment of DNA containing the lesion (Sijbers et al., 1996). Finally, the resulting gap is repaired by the regular replication machinery (Aboussekhra et al., 1995; Budd and Campbell, 1995; Shivji et al., 1995). The efficiency of lesion recognition by the XPC/hHR23B complex depends to a great extent on the type of lesion. The mildly distorting CPDs are only poorly recognized by this so-called global genome NER (GG-NER) mechanism, whereas the severely distorting 6-4PP is readily recognized. As a result, within 24 hours after UV exposure, only 10-20% of the CPDs are repaired by GG-NER while more than 75% of the 64PPs are repaired as fast as 4 hours after UV exposure (Bohr et al., 1985; Mitchell, 1988). In humans, recognition of CPDs is greatly enhanced by p53-dependent upregulation of the p48 subunit of the DNA damage binding protein (DDB) upon UV irradiation, resulting in repair of more than 60% of the CPDs within 24 hours (Bohr et al., 1985; Hwang et al., 1999; Mitchell, 1988). Rodents, however, lack the p53 responsive element of the *p48* gene and, as a consequence, do not upregulate p48, resulting in virtually no repair of CPDs via GG-NER (Hwang et al., 1999).

To allow fast repair of DNA lesions from the transcribed strands of active genes, thereby minimizing interference of these lesions with essential cellular processes (transcription, replication), a specialized pathway of NER exists. This pathway, designated transcription-coupled NER (TC-NER), is initiated when elongating RNA polymerase II (RNApolII) is stalled upon a lesion (Tornaletti and Hanawalt, 1999). In order to make the lesion accessible for repair, the stalled polymerase must be replaced in a reaction involving the TC-NER specific proteins CSB and CSA (Le Page et al., 2000). Since both CPDs and 6-4PPs block RNApolII, these lesions are efficiently repaired from transcribed genes by TC-NER.

NER-related disorders

The vital importance of NER is demonstrated by the occurrence of rare autosomal recessive disorders associated with defective NER: xeroderma pigmentosum (XP, caused by a defect in one of the genes *XPA-XPG*),

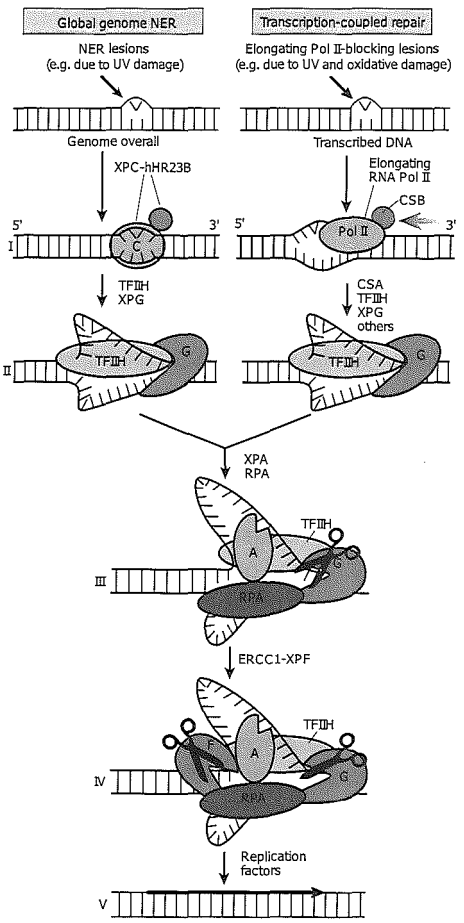

Figure 4
Mechanism of nucleotide excision repair.
In GG-NER, the XPC/hHR23B complex screens the DNA for helix-distorting lesions. In contrast, TC-NER requires the TC-NER specific proteins CSA and CSB and is initiated when RNApolII stalls upon a lesion. After lesion recognition, the XPB and XPD helicases of the TFIIH complex unwind the DNA around the lesion. Next, XPA verifies the presence of a lesion and RPA stabilized the open intermediate by binding to single stranded DNA. The endonucleases ERCC1/XPF and XPG cleave 5′ and 3′ of the lesion, respectively, generating a 24-32 nucleotide fragment containing the lesion. Finally, the resulting gap is filled by the regular replication machinery (adapted from Hoeijmakers (2001).

studies suggest that photolyase can accommodate the DNA in a "flipped-out" conformation. Indeed, photolyase seems to induce a major conformational change in DNA upon binding, as shown by atomic force microscopy studies where a bend of 36° is observed, leading to extrusion of the CPD into the putative lesion-binding site (van Noort et al., 1999; van Noort et al., 1998). Although photolyase can bind to the lesion in the dark, light is required to initiate repair. The second chromophore, MTHF or 8-HDF, acts as a photoantenna and efficiently harvests light energy (350-450 nm) (Kim et al., 1991; Kim et al., 1992) and funnels this energy to the reduced FAD chromophore, which in turn transfers an electron to the CPD. This electron transfer causes the cyclobutane ring to split, generating two pyrimidines, one of which is in a reduced state. The reduced pyrimidine donates an electron back to FAD to regenerate FADH- and the enzyme dissociates from the DNA. The conversion of a CPD to two pyrimidines does not result in a net gain or loss of electrons (reviewed by Carell (2001)).

6-4PP photolyase

Despite the fact that much less is known about 6-4PP photolyases, the strong sequence homology between CPD and 6-4PP photolyases suggests a similar mode of action, involving binding of the DNA lesion, absorption of a photon and reversal of the photoproduct (Hitomi et al., 1997). This is supported by the finding that 6-4PP photolyases contain a FAD chromophore, which in its reduced state is required to perform the photoreactivation reaction (Todo et al., 1997). It has been suggested that reversal of 6-4PPs involves an intermediate oxetane-ring structure (Kim et al., 1994). 6-4PP photolyases may also bind and repair Dewar valence isomers, albeit with lower affinity and lower repair-efficiency (Tamada et al., 1997a).

Photoreactivation in chromatin

Accessibility of DNA lesions to photolyases is largely dependent on the chromatin environment. *In vitro* experiments have shown that photoreactivation is extremely rapid when naked DNA is used as a substrate, whereas the speed of repair is dramatically reduced in reconstituted nucleosomes (Vassylyev et al., 1995). This indicates that folding of DNA into nucleosomes renders the lesions less accessible to photolyases. In contrast, several *in vivo* experiments, utilizing chicken embryo fibroblasts and injection of exogenous photolyase into human fibroblasts, have shown that the majority of lesions are accessible to the enzyme and can be repaired by photoreactivation (van de Merwe and Bronk, 1981; Zwetsloot et al., 1985). A more detailed study by Pendrys (1983) showed that 75% of the DNA is shielded from photolyase immediately after UV exposure, with all sites becoming available only after 12 hours. Taken together, these studies indicate that although the folding of DNA into nucleosomes initially renders lesions inaccessible to photolyases, the dynamic nature of chromatin eventually allows access of the enzyme and repair of almost all lesions. Further evidence for a temporal shielding of lesions by nucleosomes has come from studies in yeast (Suter et al., 1997). Suter and coworkers reported fast repair of UV-induced damage by photolyase in linker DNA and nuclease-sensitive regions (completed in 15-30 min.) but slow repair of DNA lesions mapping to nucleosome sites. Similarly, Gaillard and coworkers (2003) showed in an *in vitro* reconstitution assay that two different chromatin remodeling activities (SWI/SNF and ISW2) act on UV damaged DNA and facilitate repair. In conclusion, many aspects of DNA damage and repair are affected by packaging of DNA into chromatin; not only the

induction of UV lesions but also the accessibility of these lesions to repair proteins is highly dependent on chromatin structure.

Ultraviolet light and health

Obviously, the prime target organ of ultraviolet light is the skin, the largest organ of the human body. Our skin provides us with a protective shield against a diverse set of potentially hazardous environmental factors, ranging from extreme desiccating heat to immersion in hypotonic or hypertonic aqueous solutions. Furthermore, it acts as a barrier that protects against allergenic, infectious and toxic compounds.

Anatomy of the skin

The skin forms an important barrier between the organism and its environment and protects the organism from exogenous threats. The epidermis is the outermost layer and gives the skin much of its protection. The epidermis is connected to the underlying dermis via the interaction of proteins present in the basement membrane. Keratinocytes constitute more than 80% of the epidermal cells. Other cell types present in the epidermis include Langerhans cells, T-lymphocytes (both involved in immune response), the pigment producing melanocytes and Merkel cells (mechanosensors).
In the human skin, the epidermis consists of 4 distinct layers. The basal cell layer, situated at the dermal/epidermal interface is one cell layer thick, and contains the stem cells required for continuous renewal of the post-mitotic pool of keratinocytes within the epidermis. The second layer is 3 to 4 cells thick and contains more differentiated keratinocytes. It is named the stratum spinosum after the numerous intercellular desmosomal structures that provide cohesion between neighboring cells. In the third layer, the stratum granulosum, cells show distinct haematoxylin stained granules in the cytoplasm, easily visualized by light microscopy. The outermost layer of the epidermis is the stratum corneum, represented by cornified keratinocytes that have undergone an apoptotic-like enucleation process. These cells become shielded by crosslinked membranes and extracellular lipid deposits, resulting in a basket-weave appearance. This layer provides the barrier function required to survive environmental stresses. It takes approximately 4 weeks for keratinocytes to complete their differentiation process from stem cell to cornified keratinocyte.
The mouse skin is somewhat different. Although the differentiation process of keratinocytes leads to the appearance of a cornified barrier, similar to the situation observed in man, the dorsal mouse skin does not consist of four distinct layers. Depending on the strain, the mouse skin is only one to a few cell layers thick, while distinct stages of proliferation cannot easily be distinguished.
In the human skin, ultraviolet radiation is partly scattered and reflected in the stratum corneum. In addition, UV radiance is partly absorbed by compounds in the epidermis such as melanin pigment. The depth of penetration is dependent on the wavelength: whereas UV-A photons can reach the dermis of human skin, not much of the UV-B light penetrates beyond the epidermis. In contrast, the relatively thin mouse skin allows UV-B light to reach as far as the upper dermis.

Acute effects of ultraviolet light

Exposure to UV light induces various responses in the skin. Upon excessive UV irradiation, cells will undergo programmed cell death, known as apoptosis (Smith and Fornace, 1997; Tron et al., 1998; Ziegler et al., 1994). Apoptotic keratinocytes are morphologically distinct from non-apoptotic cells in histological sections and are also referred to as sunburn cells. The process of apoptosis avoids that heavily damaged (basal) keratinocytes, which upon replication may acquire mutations that can cause malignant outgrowth, will persist in the skin. UV-induced apoptosis is triggered when RNA polymerase II is stalled upon a lesion, e.g. a CPD or 6-4PP (Conforti et al., 2000; Ljungman and Zhang, 1996; Yamaizumi and Sugano, 1994). Fibroblasts from NER-deficient XP-A and CS-B patients undergo apoptosis at much lower UV doses than cells from healthy individuals, which finds its origin in the inability of these cells to remove photolesions from the transcribed strand of active genes (Conforti et al., 2000). These findings may explain why, despite the DNA repair deficiency, patients with Cockayne syndrome do not experience an elevated risk for skin cancer since potentially pre-mutagenic cells are eliminated by an apoptotic pathway.

Along with the induction of apoptosis, UV exposure provokes an erythemal response, better known as sunburn (Parrish et al., 1982) (Fig. 6A). Erythema represents an inflammatory response and results in a painful, red skin. The redness is caused by an increased vasodilatation of dermal capillary vessels (Diffey, 1987; Logan, 1963). A measure for erythema is the Minimal Erythemal Dose (MED), defined as the minimal dose of UV light required for induction of a clearly demarcated erythema after 24 hours. The MED is highly dependent on the extent of pigmentation of the skin (Olson, 1973). The erythemal response is affected dramatically in mice deficient for TC-NER of UV-induced lesions (Berg et al., 1998; Garssen et al., 2000; van der Horst et al., 1997), which is suggestive for a role for unrepaired DNA damage in transcribed genes in triggering erythema. In addition, photoreactivation

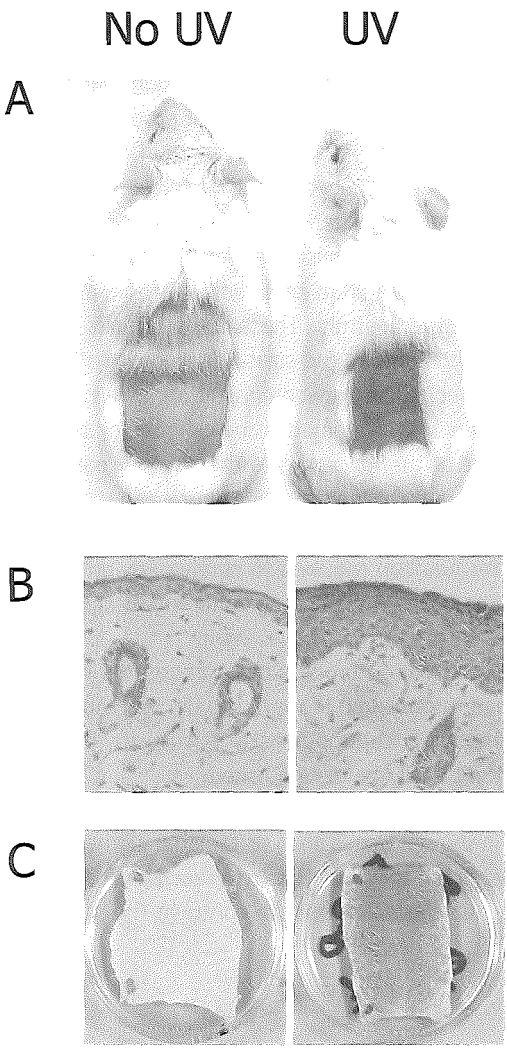

Figure 6
Acute UV responses of the skin.
A. Erythemal response of mouse skin upon exposure to UV. Mice received 4 daily UV treatments (1 MED/day) on the shaved back and were examined for erythema 3 days after the last exposure. B. Histological sections of mice described in panel A. Haematoxylin/eosin staining shows epidermal hyperplasia in UV exposed mice. C. Dorsal skin of hairless mice upon chronic exposure (21 days, 1 MED/day) to UV. UV exposed animals show increased pigmentation.

experiments utilizing the marsupial opossum or treatment of the human skin with liposomes containing CPD-photolyases indicate that CPDs can initiate an erythemal response (Applegate et al., 1989; Yarosh et al., 1994).

Exposure to UV light induces adaptive responses in the skin, including increased pigmentation (tanning) and epidermal hyperplasia. Melanocytes are the pigment (melanin) producing cells of the skin. Exposure to UV light increases not only melanin production, but also the transport of melanin into the keratinocytes (Fig. 6B) (Gilchrest et al., 1996). Melanin may is believed to form an umbrella-like structure on top of the nucleus, shielding the nucleus from UV. It has been shown that a tanned skin filters sunlight twice as efficiently as an untanned skin. Application of T4 endonuclease V (T4N5) to cultured melanocytes, thereby enhancing repair of pyrimidine dimers, results in reduced pigmentation (Gilchrest and Eller, 1999; Gilchrest, 1993). This is consistent with an emerging appreciation for the role of UV-induced DNA lesions in the tanning response.

A second adaptive response of the skin, epidermal hyperplasia, results in an increased number of cell layers in the epidermis and ensures a reduced access of UV light to the basal layer of the skin (Fig. 6C) (Ouhtit, 2000; Pearse, 1987). This adaptive response occurs upon chronic exposure to UV light. As for the tanning and erythema responses, a role for UV-induced DNA damage has been suggested in the induction of hyperplasia, since mice with a defect in nucleotide excision repair already respond at lower UV doses (Berg et al., 1997; Garssen et al., 2000; van der Horst et al., 1997). The adaptive responses such as pigmentation and hyperplasia are required to protect the skin from the possibly devastating effects of UV light, such as cell death, sunburn, mutagenesis, carcinogenesis and suppression of the immune system.

Mutagenesis

Exposure to UV light induces very distinct mutation patterns in the genome, mainly C>T and CC>TT transitions. The latter type of mutation is known as a "UV signature mutation", and is frequently observed in the *p53* tumor suppressor gene in skin carcinomas (Wikonkal and Brash, 1999) (see carcinogenesis section). It is interesting to compare the known mutation spectrum with the distribution of DNA damage, as described earlier in this chapter. The major photoproduct induced by UV is the CPD lesion at TT sites. Strikingly, TT sequences are not mutational hotspots following UV radiation (Drobetsky et al., 1987; Drobetsky and Sage, 1993; Lichtenauer-Kaligis, 1995; Miller, 1985). In contrast, the yield of CC lesions is extremely low, representing less than 5% of all lesions, whereas this sequence forms the main site for UV-induced mutations. This discrepancy may in part be explained by the deamination of cytosines in CPDs to uracil, which further enhances the mutagenicity of CC sites. These remarkable findings emphasize the different properties of photoproducts formed at specific pyrimidines.

It should be noted that if DNA repair is not sufficient and lesions remain unrepaired (as is the case after excessive exposure or when repair machineries are defective), both CPDs and 6-4PPs will block the DNA replication machinery (Friedberg, 1995). To allow the cell to cope with these potential replication blocks, damage tolerance mechanisms exist. One such damage tolerance mechanism, translesion synthesis, involves specialized polymerases that bypass a site of damage, allowing replication of the damaged template to continue (Baynton and Fuchs, 2000; Friedberg and Gerlach, 1999; Hubscher et al., 2000; Woodgate, 1999). Two pathways of translesion synthesis can be identified: an error-prone pathway and an error-free pathway. The error-free pathway inserts the appropriate bases opposite

a lesion, while the error-prone mechanism involves "sloppy" polymerases, resulting in mutations (Masutani et al., 1999a; Masutani et al., 2000; Tissier et al., 2000). Xeroderma pigmentosum-variant (XPV) patients were shown to carry a mutation in one of the translesion synthesis polymerases, pol η (Johnson, 1999; Masutani et al., 1999b).

To study the mutagenic potential of CPDs versus 6-4PPs, several studies have been performed in which photolyase enzymes were injected or transfected into mammalian cells (Asahina et al., 1999; You et al., 2001b). Mutation frequencies were determined utilizing transgenic reporter systems (You et al., 2001b), or by plasmid host-cell reactivation (Asahina et al., 1999). In all studies, photoreactivation of CPDs in UV-treated cells lowers the mutation frequency to a great extent. In contrast to CPD removal, enhanced elimination of 6-4PPs does not alter the mutation frequency, indicating that CPDs are the major cause for UV-induced mutations (You et al., 2001a). The most likely explanation is the fast repair of 6-4PPs by NER. Providing enhanced repair by photoreactivation in the presence of a functional NER system may therefore not be beneficial. However, when measured in a totally NER deficient background, the mutation frequency upon photoreactivation of 6-4PPs is only mildly reduced (Otoshi et al., 2000). In addition, the relative abundance of CPDs versus 6-4PPs (3:1) might explain to some extent the differences in mutagenic potential of these lesions.

Carcinogenesis

Three main types of cancer occur in human skin: basal cell carcinoma (BCC), squamous cell carcinoma (SCC) (both non-melanoma skin carcinomas), and cutaneous malignant melanoma (melanoma). Non-melanoma skin cancers (NMSCs) have a tremendous incidence, in the United States it has been reported to be nearly equal to the incidence of all other human malignancies combined (Scotto, 1983). Fortunately, NMSCs are usually not life threatening and can easily be cured by surgical procedures.

Epidemiological studies demonstrate a correlation between exposure to solar ultraviolet light and the incidence of skin cancer. Various studies in the United States (Muir et al., 1987; Scotto et al., 1983) have shown a relationship of incidence rates of BCC and SCC with the estimated ambient erythemal UV radiation, where the correlation coefficient (R) for both types of cancer is approximately 0.80; an R of −1 indicating a perfect negative correlation whereas an R of +1 implies a perfect positive correlation. Melanoma incidence rates, however, show a much smaller correlation with UV radiation. Contrasting reports have appeared on the incidence of melanoma, in which correlation coefficients may vary from 0.02 to 0.44 (Armstrong and McMichael, 1984; Scotto, 1983), depending on the study and the different ethnic populations under investigation. In general, higher correlation coefficients were obtained when exposure to UV-A was taken into account.

The length of residence in an area of high solar radiation (e.g. Australia) affects the risk on BCC, SCC as well as melanoma, as reviewed by Armstrong and Kricker (2001). For instance, the risk of these three types of skin cancer was strongly reduced in people who migrated to Australia at the age of 10 or older. Although this suggests a correlation between residence time and skin cancer incidence, it might also imply that radiation early in life is of particular importance for the development of skin cancer. Other risk factors involved in the induction of skin cancer are ethnic origin and skin color (English et al., 1998; Holman and Armstrong, 1984; Kricker et al., 1991; Muir, 1987; Parkin and Iscovich, 1997), with fair-skinned populations at the highest risk of developing skin cancer.

In addition to epidemiological evidence for a role of UV radiation in the induction of skin cancer, several other lines of evidence point in the same direction. First of all, the action spectrum of SCC has been determined experimentally in hairless mice (de Gruijl et al., 1993); this action spectrum shows a peak at 293 nm in the UV-B range.
Furthermore, BCCs and SCCs have high proportions of UV signature mutations in the *p53* gene (Wikonkal and Brash, 1999) or, in the case of BCC, in *PTCH*, a gene involved in the hedgehog-signaling pathway (Bodak, 1999; Gailani et al., 1996). *p53* mutations are found in actinic keratosis, one of the pre-carcinogenic stages of SCC, indicating a role for *p53* mutations early in the process of carcinogenesis (Ziegler et al., 1994). Most UV-induced mutations are found in hotspots, clustered around codons 173-179, 235-250 and 273-278 of the *p53* gene (Brash et al., 1991; Ponten et al., 1997; van Kranen and de Gruijl, 1999; Wikonkal and Brash, 1999; Ziegler et al., 1993). Although some signature mutations have also been found in the *INK4A* gene in melanoma patients, whether this type of cancer is mainly caused by exposure to UV-A or UV-B is not yet elucidated (Armstrong and Kricker, 2001).

Immunosuppression

The immune system plays a critical role in the process of UV-induced carcinogenesis. Some thirty years ago, Kripke (1974) investigated the antigenicity of UV-induced skin tumors. Transplantation of these tumors to syngeneic mice resulted in rejection, demonstrating that they are highly immunogenic. In contrast, tumors were transplantable to mice that had received sub-carcinogenic doses of UV, indicating the host had undergone a systemic alteration in its immune response caused by UV radiation. Studies by de Gruijl and coworkers (1982; 1983) showed a decreased tumor induction time when mice were pre-irradiated on a distant site, confirming the presence of a systemic suppression of the immune system upon exposure to UV light. This immunosuppressive effect was transferable to naive animals via T-lymphocytes (Fisher and Kripke, 1982; Fisher, 1977; Spellman and Daynes, 1978), and applies to all UV-induced tumors, but does not affect the resistance to other types of tumors (Fisher and Kripke, 1978; Kripke et al., 1982).
Evidence exists that immunosuppression can also contribute to the progression of skin cancer in humans. In renal transplant patients who receive life-long immunosuppressive therapy, high incidences of NMSC are found (Harteveld, 1990). Furthermore, HIV-infected individuals may show wart-like lesions, which later on develop to cancer (Flegg, 1990; Zmudzka and Beer, 1990).
In addition to its ability to inhibit tumor rejection, UV light can suppress contact hypersensitivity (CHS, antigen is applied on skin) and delayed type hypersensitivity (DTH, antigen is applied into skin) reactions. The suppressive mechanisms for tumor rejection and CHS/DTH are thought to be similar. Therefore, CHS/DTH are frequently used as models to study UV-induced immunosuppression.
The mechanism by which UV light evokes immunosuppression is still not completely clear. In human skin, both epidermal as well as dermal immune systems interact with each other to form an efficient defense mechanism.

Cell types involved in UV-induced immunosuppression
Langerhans cells (LCs) are epidermal dendritic cells that play a crucial role in the skin's immune response (Krueger, 1989; Rowden et al., 1977; Teunissen et al., 1991; Toews et al., 1980). They form a

reticular trap within the epidermis where they take up and process antigens. Upon exposure of skin to UV, a profound depletion of LCs takes place, which may add to the UV-mediated inhibition of CHS. This LC depletion results from migration of these cells to the draining lymphnodes, where they initiate the immune response by presenting antigenic epitopes to T-lymphocytes (Kolgen et al., 2002; Shelley and Juhlin, 1976). IL-1 and TNF-α are important cytokines that stimulate LC migration (Enk et al., 1993; Enk and Katz, 1992). It is believed that these cytokines alter mRNA expression of E-Cadherin in LCs, thereby inducing a loss in adhesion between the cell and neighboring keratinocytes (Jakob and Udey, 1998) and allowing the cells to migrate. In support of this, TNF-α deficient mice show resistance to UV-induced skin carcinogenesis (Moore et al., 1999). In addition, Langerhans cells can digest extracellular matrices by secretion of matrix metalloproteinases (MMP), (Kobayashi, 1997), facilitating their migration through the basement membrane and the dermis. When LCs encounter a lymphatic vessel, they enter the lumen and proceed to the lymph node (Lukas et al., 1996; Weinlich et al., 1998). Once arrived in the lymph node, B7-1 and B7-2 molecules expressed on LCs bind to their counterparts CD28 and CTLA-4, respectively, on T-cells. Overall blockade of all B7-mediated signals inhibits T-cell mediated immune responses (Boussiotis et al., 1994; Lenschow et al., 1992; Tang et al., 1996; Thompson, 1995) and results in a reduction in photocarcinogenesis (Beissert et al., 1999).
Although keratinocytes were previously believed to be immunologically inert, we now know that these cells are extremely important with respect to modulating, or maybe even initiating cutaneous immune reactions. Upon UV exposure, keratinocytes produce and secrete TNF-α and IL-10, cytokines involved in suppression of CHS and DTH respectively.

Chromophores involved in UV-induced immunosuppression
UV-mediated suppression of the immune system implies the presence of a chromophore that absorbs UV radiation and, as a consequence, signals to the immune system. De Fabo and Noonan (1983) proposed that urocanic acid (UCA), a histidine derivative synthesized by keratinocytes, was the chromophore for UV-induced immunosuppression. Upon absorption of light in the UV range, UCA undergoes photoisomerization from the *trans-* to the *cis*-isomer (Noonan and De Fabo, 1992). UCA is present at high concentration in the skin due to a lack of the catabolic enzyme urocanase (Noonan et al., 1981). Apart from the photochemical properties, further evidence for the involvement of UCA in UV-induced immunosuppression is found in the closely matching action spectrum: the optimal wavelength at which immunosuppression occurs, fits well with the absorption spectrum for UCA conversion (De Fabo and Noonan, 1983). Furthermore, application of UV irradiated UCA to the mouse skin suppresses DTH to challenging compounds such as herpes simplex virus (Ross et al., 1986), and increases tumor-yield and malignancy in hairless mice (Reeve et al., 1989). In addition, *cis*-UCA impairs induction of CHS and induces tolerance (Kurimoto, 1992). Cell culture studies show defects in antigen presentation of Langerhans cells upon administration of *cis*-UCA (Beissert, 1995). The immunosuppressive effects of *cis*-UCA can be antagonized by IL-12, a cytokine that is produced by a variety of immunocompetent cells, including dendritic cells, macrophages, B-cells and keratinocytes (Beissert et al., 1999; Beissert, 2001; Boussiotis et al., 1994; Tang et al., 1996; Thompson, 1995).
Treatment with antibodies against *cis*-UCA in some cases can fully restore DTH responses, indicating an important role for UCA in DTH (el Ghorr AA, 1995; Moodycliffe, 1996). However, it was reported that CHS was not or only partially restored upon anti-*cis*-UCA (el Ghorr AA, 1995; Hart, 1997; Moodycliffe,

1996). Furthermore, antibody treatment significantly reduces the tumor development in mice exposed to UV (Beissert, 2001). It is clear that UCA plays an important role in UV-induced immunosuppression of DTH responses and, maybe to a lesser extent, CHS responses, thereby affecting the process of skin carcinogenesis. The exact mechanism by which UCA exerts its effects, however, remains to be elucidated.

Apart from UCA, DNA damage has also been proposed as an important chromophore for the initiation of UV-induced immunosuppression (Kripke, 1992). Several lines of evidence point towards a role of pyrimidine dimers in this process. First of all, the action spectrum of immunosuppression resembles the spectrum of DNA-damage induction (De Fabo and Noonan, 1983). Furthermore, indications were obtained from experiments with *Monodelphis domestica*, a marsupial that contains CPD photolyase activity (Wolf, 1995). Exposure of animals to photoreactivating light was shown to prevent UV-induced immunosuppression (Applegate et al., 1989). In addition, Langerhans cells with DNA damage can be detected in the draining lymphnode upon exposure to UV. Removal of DNA lesions by treatment of these cells with photolyase restores their ability to induce a normal immunoresponse (Vink et al., 1996). Evidence for the role of DNA lesions in immunosuppression was further substantiated by the observation that application of liposomes containing T4 endonuclease V (T4N5) to the mouse skin, enhancing repair of pyrimidine dimers, resulted in similar prevention of suppression of both CHS and DTH (Kripke, 1992). Also, T4N5 treatment prevented the loss of Langerhans cells from the epidermis and secretion of IL-10 and TNF-α from keratinocytes. Finally, it has been suggested that thymine dinucleotides in liposomes, when applied to the skin, cause a suppression of CHS (Cruz et al., 2000). Taken together, ample evidence exists putting forward an important role for DNA lesions in UV-induced immunosuppression. How this relates to the role of UCA, however, is still to be elucidated.

Conclusions

It is evident from the previously discussed topics that exposure to ultraviolet light affects a great variety of processes. The danger of exposure to UV light for human health is highlighted by the substantial incidence of skin cancer in the Caucasian population. Many studies have suggested an important role for DNA damage in this process. To what extent DNA damage exerts this role, and which specific lesions are responsible, however, is not known. In this thesis, we therefore set out to determine the relative contribution of the two UV-induced lesions, CPDs and 6-4PPs, to acute and chronic effects of UV exposure. To this end, we have generated a set of mouse models transgenically expressing photolyase genes. Photoreactivation of CPDs, 6-4PPs or both lesions allows investigation of the effects of a single type of lesion.

Furthermore, to determine the role of basal keratinocytes in the skin's responses to UV, we have generated mice expressing photolyase genes from a basal keratinocyte specific promoter. Exposure of these animals to photoreactivating light results in damage removal from basal keratinocytes only, whereas DNA lesions in other cell types in the skin will be repaired, if at all, by the slower NER. Experiments with these mice will provide a unique insight into the role of keratinocytes in the DNA damage responses upon UV.

Finally, investigation of the mechanisms behind the detrimental effects of UV will provide a basis for further understanding and, possibly, treatment or prevention of cancer.

References

Aboussekhra, A., Biggerstaff, M., Shivji, M.K., Vilpo, J.A., Moncollin, V., Podust, V.N., Protic, M., Hubscher, U., Egly, J.M. and Wood, R.D. (1995) Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell, 80, 859-868.

Aboussekhra, A. and Thoma, F. (1999) TATA-binding protein promotes the selective formation of UV-induced (6- 4)-photoproducts and modulates DNA repair in the TATA box. Embo J, 18, 433-443.

Applegate, L.A., Ley, R.D., Alcalay, J. and Kripke, M.L. (1989) Identification of the molecular target for the suppression of contact hypersensitivity by ultraviolet radiation. J Exp Med, 170, 1117-1131.

Armstrong, B.K. and Kricker, A. (2001) The epidemiology of UV induced skin cancer. J Photochem Photobiol B, 63, 8-18.

Armstrong, B.K. and McMichael, A.J. (1984) Cancer in migrants. Med J Aust, 140, 3-4.

Asahina, H., Han, Z., Kawanishi, M., Kato, T., Jr., Ayaki, H., Todo, T., Yagi, T., Takebe, H., Ikenaga, M. and Kimura, S.H. (1999) Expression of a mammalian DNA photolyase confers light-dependent repair activity and reduces mutations of UV-irradiated shuttle vectors in xeroderma pigmentosum cells. Mutat Res, 435, 255-262.

Baynton, K. and Fuchs, R.P. (2000) Lesions in DNA: hurdles for polymerases. Trends Biochem Sci, 25, 74-79.

Becker, M.M. and Wang, J.C. (1984) Use of light for footprinting DNA in vivo. Nature, 309, 682-687.

Becker, M.M. and Wang, Z. (1989) Origin of ultraviolet damage in DNA. J Mol Biol, 210, 429-438.

Beissert, S., Bluestone, J.A., Mindt, I., Voskort, M., Metze, D., Mehling, A., Luger, T.A., Schwarz, T. and Grabbe, S. (1999) Reduced ultraviolet-induced carcinogenesis in mice with a functional disruption in B7-mediated costimulation. J Immunol, 163, 6725-6731.

Beissert, S., Bluestone, J.A., Mindt, I., Voskort, M., Metze, D., Mehling, A., Luger, T.A., Schwarz, T., Grabbe, S. (2001) Reduced Ultraviolet-Induced Carcinogenesis in Mice with a functional disruption in B7-Mediated Costimulation. J Immunol, 163, 6725-6731.

Beissert, S., Ullrich, S.E., Hosoi, J., Granstein, R.D. (1995) Supernatants from UVB radiation exposed keratinocytes inhibit Langerhans cell presentation of tumor-associated antigens via IL-10 content. J Leukoc Biol, 58, 234-240.

Berg, R.J., de Vries, A., van Steeg, H. and de Gruijl, F.R. (1997) Relative susceptibilities of XPA knockout mice and their heterozygous and wild-type littermates to UVB-induced skin cancer. Cancer Res, 57, 581-584.

Berg, R.J., Ruven, H.J., Sands, A.T., de Gruijl, F.R. and Mullenders, L.H. (1998) Defective global genome repair in XPC mice is associated with skin cancer susceptibility but not with sensitivity to UVB induced erythema and edema. J Invest Dermatol, 110, 405-409.

Bodak, N., Queille, S., Avril, M. F., Bouadjar, B., Drougard C., Sarasin A., Daya-Grosjean, L. (1999) High levels of patched gene mutations in basal cell carcinoma from patients with xeroderma pigmentosum. Proc Natl Acad Sci U S A, 96, 5117-5122.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell, 40, 359-369.

Bootsma, D. and Hoeijmakers, J.H. (1991) The genetic basis of xeroderma pigmentosum. Ann Genet, 34, 143-150.

Bootsma, D., Kraemer,K.H., Cleaver,J.E., Hoeijmakers,J.H.J. (1998) Nucleotide excision repair syndromes: xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. In Vogelstein, B., Kinzler, K.W. (ed.), The genetic basis of human cancer. McGraw-Hill New-York, pp. 245-274.

Bootsma, D., Kraemer,K.H., Cleaver,J.E., Hoeijmakers,J.H.J. (2001) Nucleotide excision repair syndromes: xeroderma pigmentosum, Cockayne syndrome amd trichothiodystrophy. In Scriver, C.R., Beaudet,A.L., Sly,W.S., Valle,D. (ed.), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 677-703.

Boussiotis, V.A., Gribben, J.G., Freeman, G.J. and Nadler, L.M. (1994) Blockade of the CD28 co-stimulatory pathway: a means to induce tolerance. Curr Opin Immunol, 6, 797-807.

Brash, D.E., Rudolph, J.A., Simon, J.A., Lin, A., McKenna, G.J., Baden, H.P., Halperin, A.J. and Ponten, J. (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci U S A, 88, 10124-10128.

Brown, D.W., Libertini, L.J., Suquet, C., Small, E.W. and Smerdon, M.J. (1993) Unfolding of nucleosome cores dramatically changes the distribution of ultraviolet photoproducts in DNA. Biochemistry, 32, 10527-10531.

Budd, M.E. and Campbell, J.L. (1995) DNA polymerases required for repair of UV-induced damage in Saccharomyces cerevisiae. Mol Cell Biol, 15, 2173-2179.

Buschta-Hedayat, N., Buterin, T., Hess, M.T., Missura, M. and Naegeli, H. (1999) Recognition of nonhybridizing base pairs during nucleotide excision repair of DNA. Proc Natl Acad Sci U S A, 96, 6090-6095.

Cadet, J., Berger, M., Douki, T. and Ravanat, J.L. (1997) Oxidative damage to DNA: formation, measurement, and biological significance. Rev Physiol Biochem Pharmacol, 131, 1-87.

Carell, T., Burgdorf, L.T., Kundu, L.M. and Cichon, M. (2001) The mechanism of action of DNA photolyases. Curr Opin Chem Biol, 5, 491-498.

Ciarrocchi, G. and Pedrini, A.M. (1982) Determination of pyrimidine dimer unwinding angle by measurement of DNA electrophoretic mobility. J Mol Biol, 155, 177-183.

Conforti, G., Nardo, T., D'Incalci, M. and Stefanini, M. (2000) Proneness to UV-induced apoptosis in human fibroblasts defective in transcription coupled repair is associated with the lack of Mdm2 transactivation. Oncogene, 19, 2714-2720.

Cruz, P.D., Jr., Leverkus, M., Dougherty, I., Gleason, M.J., Eller, M., Yaar, M. and Gilchrest, B.A. (2000) Thymidine dinucleotides inhibit contact hypersensitivity and activate the gene for tumor necrosis factor alpha1. J Invest Dermatol, 114, 253-258.

De Fabo, E.C. and Noonan, F.P. (1983) Mechanism of immune suppression by ultraviolet irradiation in vivo. I. Evidence for the existence of a unique photoreceptor in skin and its role in photoimmunology. J Exp Med, 158, 84-98.

De Gruijl, F.R., Longstreth, J., Norval, M., Cullen, A.P., Slaper, H., Kripke, M.L., Takizawa, Y. and van der Leun, J.C. (2003) Health effects from stratospheric ozone depletion and interactions with climate change. Photochem Photobiol Sci, 2, 16-28.

De Gruijl, F.R., Sterenborg, H.J., Forbes, P.D., Davies, R.E., Cole, C., Kelfkens, G., van Weelden, H., Slaper, H. and van der Leun, J.C. (1993) Wavelength dependence of skin cancer induction by ultraviolet irradiation of albino hairless mice. Cancer Res, 53, 53-60.

De Gruijl, F.R. and van der Leun, J.C. (1982) Systemic influence of pre-irradiation of a limited skin area on UV-tumorigenesis. Photochem Photobiol, 35, 379-383.

De Gruijl, F.R. and Van Der Leun, J.C. (1983) Follow up on systemic influence of partial pre-irradiation on UV-tumorigenesis. Photochem Photobiol, 38, 381-383.

De Laat, W.L., Jaspers, N.G. and Hoeijmakers, J.H. (1999) Molecular mechanism of nucleotide excision repair. Genes Dev, 13, 768-785.

Diffey, B.L., Oakley, A.M. (1987) The onset of ultraviolet erythema. Br. J. Dermatol., 116, 184-187.

Douki, T. and Cadet, J. (2001) Individual determination of the yield of the main UV-induced dimeric pyrimidine photoproducts in DNA suggests a high mutagenicity of CC photolesions. Biochemistry, 40, 2495-2501.

Douki, T., Court, M. and Cadet, J. (2000a) Electrospray-mass spectrometry characterization and measurement of far- UV-induced thymine photoproducts. J Photochem Photobiol B, 54, 145-154.

Douki, T., Court, M., Sauvaigo, S., Odin, F. and Cadet, J. (2000b) Formation of the main UV-induced thymine dimeric lesions within isolated and cellular DNA as measured by high performance liquid chromatography-tandem mass spectrometry. J Biol Chem, 275, 11678-11685.

Drapkin, R., Reardon, J.T., Ansari, A., Huang, J.C., Zawel, L., Ahn, K., Sancar, A. and Reinberg, D. (1994) Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. Nature, 368, 769-772.

Drobetsky, E.A., Grosovsky, A.J. and Glickman, B.W. (1987) The specificity of UV-induced mutations at an endogenous locus in mammalian cells. Proc Natl Acad Sci U S A, 84, 9103-9107.

Drobetsky, E.A. and Sage, E. (1993) UV-induced G:C-->A:T transitions at the APRT locus of Chinese hamster ovary cells cluster at frequently damaged 5'-TCC-3' sequences. Mutat Res, 289, 131-138.

Eker, A.P., Dekker, R.H. and Berends, W. (1981) Photoreactivating enzyme from Streptomyces griseus-IV. On the nature of the chromophoric cofactor in Streptomyces griseus photoreactivating enzyme. Photochem Photobiol, 33, 65-72.

Eker, A.P., Hessels, J. K. C., van de Velde J. (1988) Photoreactivating enzyme from the green alga Scenedesmus acutus. Evidence for the presence of two different flavin chromophores. Biochemistry, 27, 1758-1765.

el Ghorr AA, N.M. (1995) A Monoclonal antibody to cis-uracanic acid prevents the ultraviolet-induced changes in Langerhans cells and delayed hypersensitivity responses in mice, although not preventing dendritic cell accumulation in lymph nodes draining the site of irradiation and contact hypersensitivity responses. J. Invest. Dermatol., 105, 264-268.

English, D.R., Armstrong, B.K., Kricker, A., Winter, M.G., Heenan, P.J. and Randell, P.L. (1998) Demographic characteristics, pigmentary and cutaneous risk factors for squamous cell carcinoma of the skin: a case-control study. Int J Cancer, 76, 628-634.

Enk, A.H., Angeloni, V.L., Udey, M.C. and Katz, S.I. (1993) Inhibition of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. J Immunol, 151, 2390-2398.

Enk, A.H. and Katz, S.I. (1992) Early molecular events in the induction phase of contact sensitivity. Proc Natl Acad Sci U S A, 89, 1398-1402.

Fisher M. S., K.M.L. (1977) Systemic alteration induced in mice by ultraviolet light irradiation and its relationship to ultraviolet carcinogenesis. Proc Natl Acad Sci U S A, 74, 1688-1692.

Fisher, M.S. and Kripke, M.L. (1978) Further studies on the tumor-specific suppressor cells induced by ultraviolet radiation. J Immunol, 121, 1139-1144.

Fisher, M.S. and Kripke, M.L. (1982) Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice. Science, 216, 1133-1134.

Flegg, P.J. (1990) Potential risks of ultraviolet radiation in HIV infection. Int J STD AIDS, 1, 46-48.

Friedberg, E., Walker, G.C., Siede, W. (ed.). (1995) DNA repair and mutagenesis. ASM press, Washington DC.

Friedberg, E.C. (2003) DNA damage and repair. Nature, 421, 436-440.

Friedberg, E.C. and Gerlach, V.L. (1999) Novel DNA polymerases offer clues to the molecular basis of mutagenesis. Cell, 98, 413-416.

Friedberg, E.C. and Meira, L.B. (2003) Database of mouse strains carrying targeted mutations in genes affecting biological responses to DNA damage. Version 5. DNA Repair (Amst), 2, 501-530.

Gailani, M.R., Leffell, D.J., Ziegler, A., Gross, E.G., Brash, D.E. and Bale, A.E. (1996) Relationship between sunlight exposure and a key genetic alteration in basal cell carcinoma. J Natl Cancer Inst, 88, 349-354.

Gaillard, H., Fitzgerald, D. J., Smith, C. L., Peterson, C. L., Richmond, T. J., Thoma, F. (2003) Chromatin remodeling activities act on UV-damaged nucleosomes and modulate DNA-damage accessibility to photolyase. J Biol Chem

Gale, J.M., Nissen, K.A. and Smerdon, M.J. (1987) UV-induced formation of pyrimidine dimers in nucleosome core DNA is strongly modulated with a period of 10.3 bases. Proc Natl Acad Sci U S A, 84, 6644-6648.

Gale, J.M. and Smerdon, M.J. (1990) UV induced (6-4) photoproducts are distributed differently than cyclobutane dimers in nucleosomes. Photochem Photobiol, 51, 411-417.

Garssen, J., van Steeg, H., de Gruijl, F., de Boer, J., van der Horst, G.T., van Kranen, H., van Loveren, H., van Dijk, M., Fluitman, A., Weeda, G. and Hoeijmakers, J.H. (2000) Transcription-coupled and global genome repair differentially influence UV-B-induced acute skin effects and systemic immunosuppression. J Immunol, 164, 6199-6205.

Gilchrest, B.A. and Eller, M.S. (1999) DNA photodamage stimulates melanogenesis and other photoprotective responses. J Investig Dermatol Symp Proc, 4, 35-40.

Gilchrest, B.A., Park, H.Y., Eller, M.S. and Yaar, M. (1996) Mechanisms of ultraviolet light-induced pigmentation. Photochem Photobiol, 63, 1-10.

Gilchrest, B.A., Zhai, A., Eller, S.E., Yarosh, D.B., Yaar, M. (1993) Treatment of human Melanocyte and S91 Melanoma cells with the DNA repair enzyme T4 Endonuclease V Enhances Melanogenesis After Ultraviolet Irradiation. J Invest Dermatol, 101, 666-672.

Hart, P.H., Jaksic, A., Swift, G., Norval, M., el-Gohrr, A.A., Finlay-Jones, J.J. (1997) Histamine involvement in UVB- and cis-urocanic acid-induced systemic suppression of contact hypersensitivity responses. Immunology, 91, 601-608.

Harteveld, M.M., Bouwes Bavinck, J.N., Kootte, A.M.M., Vermeer, B.J., Vandenbroucke J.P. (1990) Incidence of skin cancer after renal transplantation in the Netherlands. Transplantation, 49, 506-509.

Hitomi, K., Kim, S.T., Iwai, S., Harima, N., Otoshi, E., Ikenaga, M. and Todo, T. (1997) Binding and catalytic properties of Xenopus (6-4) photolyase. J Biol Chem, 272, 32591-32598.

Hoeijmakers, J.H. (2001) Genome maintenance mechanisms for preventing cancer. Nature, 411, 366-374.

Holman, C.D. and Armstrong, B.K. (1984) Pigmentary traits, ethnic origin, benign nevi, and family history as risk factors for cutaneous malignant melanoma. J Natl Cancer Inst, 72, 257-266.

Hubscher, U., Nasheuer, H.P. and Syvaoja, J.E. (2000) Eukaryotic DNA polymerases, a growing family. Trends Biochem Sci, 25, 143-147.

Hwang, B.J., Ford, J.M., Hanawalt, P.C. and Chu, G. (1999) Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. Proc Natl Acad Sci U S A, 96, 424-428.

Iwatsuki, N., Joe, C.O. and Werbin, H. (1980) Evidence that deoxyribonucleic acid photolyase from baker's yeast is a flavoprotein. Biochemistry, 19, 1172-1176.

Jakob, T. and Udey, M.C. (1998) Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells in vivo. J Immunol, 160, 4067-4073.

Johnson, J.L., Hamm-Alvarez, S., Payne, G., Sancar, G.B., Rajagopalan, K.V. and Sancar, A. (1988) Identification of the second chromophore of Escherichia coli and yeast DNA photolyases as 5,10-methenyltetrahydrofolate. Proc Natl Acad Sci U S A, 85, 2046-2050.

Johnson, R.E.K., C.M. Prakash, S. Prakash, L. (1999) hRad30 Mutations in the variant form of xeroderma pigentosum. Science, 285, 263-265.

Jorns, M.S., Sancar, G.B. and Sancar, A. (1984) Identification of a neutral flavin radical and characterization of a second chromophore in Escherichia coli DNA photolyase. Biochemistry, 23, 2673-2679.

Kelner, A. (1949a) Effects of visible light on the recovery of Streptomyces griseus conidia from ultraviolet radiation injury. Proc Natl Acad Sci U S A, 35, 73-79.

Kelner, A. (1949b) Photoreactivation of ultraviolet-irradiated Escherichia coli with special reference to the dose-reduction principle and to ultraviolet-induced mutation. J Bacteriol, 58, 511-522.

Kim, J.K. and Choi, B.S. (1995) The solution structure of DNA duplex-decamer containing the (6-4) photoproduct of thymidylyl(3'-->5')thymidine by NMR and relaxation matrix refinement. Eur J Biochem, 228, 849-854.

Kim, S.T., Heelis, P.F., Okamura, T., Hirata, Y., Mataga, N. and Sancar, A. (1991) Determination of rates and yields of interchromophore (folate----flavin) energy transfer and intermolecular (flavin----DNA) electron transfer in Escherichia coli photolyase by time-resolved fluorescence and absorption spectroscopy. Biochemistry, 30, 11262-11270.

Kim, S.T., Heelis, P.F. and Sancar, A. (1992) Energy transfer (deazaflavin-->FADH2) and electron transfer (FADH2-->T <> T) kinetics in Anacystis nidulans photolyase. Biochemistry, 31, 11244-11248.

Kim, S.T., Malhotra, K., Smith, C.A., Taylor, J.S. and Sancar, A. (1994) Characterization of (6-4) photoproduct DNA photolyase. J Biol Chem, 269, 8535-8540.

Kobayashi, T., Takao, M., Oikawa, A. and Yasui, A. (1990) Increased UV sensitivity of Escherichia coli cells after introduction of foreign photolyase genes. Mutat Res, 236, 27-34.

Kobayashi, Y. (1997) Langerhans' cells produce type IV collagenase (MMP-9) following epicutaneous stimulation with haptens. Immunology, 90, 496-501.

Kolgen, W., Both, H., van Weelden, H., Guikers, K.L., Bruijnzeel-Koomen, C.A., Knol, E.F., van Vloten, W.A. and De Gruijl, F.R. (2002) Epidermal langerhans cell depletion after artificial ultraviolet B irradiation of human skin in vivo: apoptosis versus migration. J Invest Dermatol, 118, 812-817.

Kraemer, K.H. (1997) Sunlight and skin cancer: another link revealed. Proc Natl Acad Sci U S A, 94, 11-14.

Kraemer, K.H., Lee, M.M. and Scotto, J. (1984) DNA repair protects against cutaneous and internal neoplasia: evidence from xeroderma pigmentosum. Carcinogenesis, 5, 511-514.

Kricker, A., Armstrong, B.K., English, D.R. and Heenan, P.J. (1991) Pigmentary and cutaneous risk factors for non-melanocytic skin cancer-- a case-control study. Int J Cancer, 48, 650-662.

Kripke, M.L., Cox P.A., Alas L.G., Yarosh D.B. (1992) Pyrimidine dimers in DNA initiate systemic immunosuppression in UV-irradiated mice. Proc Natl Acad Sci U S A, 89, 7516-7520.

Kripke, M.L., Morison, W.L. and Parrish, J.A. (1982) Induction and transplantation of murine skin cancers induced by methoxsalen plus ultraviolet (320-400 nm) radiation. J Natl Cancer Inst, 68, 685-690.

Kripke, M. L., (1974) Antigenicity of murine skin tumors induced by ultraviolet light. J Natl Cancer Inst, 5, 1333-1336

Krueger, G.G. (1989) A perspective of psoriasis as an aberration in skin modified to expression by the inflammatory/repair system. Immunol Ser, 46, 425-445.

Kuraoka, I., Bender, C., Romieu, A., Cadet, J., Wood, R.D. and Lindahl, T. (2000) Removal of oxygen free-radical-induced 5',8-purine cyclodeoxynucleosides from DNA by the nucleotide excision-repair pathway in human cells. Proc Natl Acad Sci U S A, 97, 3832-3837.

Kurimoto, I., Streilein, J.W. (1992) Deleterious effects of cis-urocanic acid and UVB irradiation on Langerhans cells and on induction of contact hypersensitivity are mediated by tumor necrosis factor-alpha. J Invest Dermatol, 99, 69S-70S.

Le Page, F., Kwoh, E.E., Avrutskaya, A., Gentil, A., Leadon, S.A., Sarasin, A. and Cooper, P.K. (2000) Transcription-coupled repair of 8-oxoguanine: requirement for XPG, TFIIH, and CSB and implications for Cockayne syndrome. Cell, 101, 159-171.

Lenschow, D.J., Zeng, Y., Thistlethwaite, J.R., Montag, A., Brady, W., Gibson, M.G., Linsley, P.S. and Bluestone, J.A. (1992) Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. Science, 257, 789-792.

Li, L., Lu, X., Peterson, C.A. and Legerski, R.J. (1995) An interaction between the DNA repair factor XPA and replication protein A appears essential for nucleotide excision repair. Mol Cell Biol, 15, 5396-5402.

Lichtenauer-Kaligis, E.G.R., Thijssen, J., den Dulk, H., van de Putte, P., Giphart-Gassler, M., Tasseron-de Jong, J. G. (1995) Mutat Res, 326, 131-146.

Ljungman, M. and Zhang, F. (1996) Blockage of RNA polymerase as a possible trigger for u.v. light-induced apoptosis. Oncogene, 13, 823-831.

Logan, G., WIlhelm, D.L. (1963) Ultraviolet injury as an experimental model of the imflammatory response. Nature, 198, 968-969.

Longstreth, J. (1988) Cutaneous malignant melanoma and ultraviolet radiation: a review. Cancer Metastasis Rev, 7, 321-333.

Lukas, M., Stossel, H., Hefel, L., Imamura, S., Fritsch, P., Sepp, N.T., Schuler, G. and Romani, N. (1996) Human cutaneous dendritic cells migrate through dermal lymphatic vessels in a skin organ culture model. J Invest Dermatol, 106, 1293-1299.

Lyamichev, V. (1991) Unusual conformation of (dA)n.(dT)n-tracts as revealed by cyclobutane thymine-thymine dimer formation. Nucleic Acids Res, 19, 4491-4496.

Masutani, C., Araki, M., Yamada, A., Kusumoto, R., Nogimori, T., Maekawa, T., Iwai, S. and Hanaoka, F. (1999a) Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity. Embo J, 18, 3491-3501.

Masutani, C., Kusumoto, R., Iwai, S. and Hanaoka, F. (2000) Mechanisms of accurate translesion synthesis by human DNA polymerase eta. Embo J, 19, 3100-3109.

Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K. and Hanaoka, F. (1999b) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. Nature, 399, 700-704.

Miller, J.H. (1985) Mutagenic specificity of ultraviolet light. J Mol Biol, 182, 45-65.

Mitchell, D.L. (1988) The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem Photobiol, 48, 51-57.

Moodycliffe, A.M., Bucana C.D., Kripke M.L., Norval M., Ullrich S.E. (1996) Differential effects of a monoclonal antibody to cis-urocanic acid on the suppression of delayed and contact hypersensitivity following ultraviolet radiation. J.Immunol., 157, 2891-2899.

Moore, R.J., Owens, D.M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, B., Pasparakis, M., Kollias, G. and Balkwill, F. (1999) Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. Nat Med, 5, 828-831.

Muir C., W.J., Mack T., Powell J., Whelan S. (1987) Cancer incidence in five continents. In. International Agency for research on cancer, Lyon, Vol. 5.

Nance, M.A. and Berry, S.A. (1992) Cockayne syndrome: review of 140 cases. Am J Med Genet, 42, 68-84.

Niggli, H.J. and Cerutti, P.A. (1982) Nucleosomal distribution of thymine photodimers following far- and near-ultraviolet irradiation. Biochem Biophys Res Commun, 105, 1215-1223.

Noonan, F.P. and De Fabo, E.C. (1992) Immunosuppression by ultraviolet B radiation: initiation by urocanic acid. Immunol Today, 13, 250-254.

Noonan, F.P., De Fabo, E.C. and Kripke, M.L. (1981) Suppression of contact hypersensitivity by UV radiation and its relationship to UV-induced suppression of tumor immunity. Photochem Photobiol, 34, 683-689.

Olson, R.J., Gaylor, J., Everett, M.A. (1973) Skin color, melanin and erythema. Arch Dermatol, 108, 541-544.

Otoshi, E., Yagi, T., Mori, T., Matsunaga, T., Nikaido, O., Kim, S.T., Hitomi, K., Ikenaga, M. and Todo, T. (2000) Respective roles of cyclobutane pyrimidine dimers, (6-4)photoproducts, and minor photoproducts in ultraviolet mutagenesis of repair-deficient xeroderma pigmentosum A cells. Cancer Res, 60, 1729-1735.

Ouhtit, A., Muller H.K., Davis, D.W., Ullrich, S.E., McConkey, D., Ananthaswamy, H.N. (2000) Temporal events in skin injury and the early adaptive responses in ultraviolet-irradiated mouse skin. Am J Pathol, 156, 201-207.

Park, H.W., Kim, S.T., Sancar, A. and Deisenhofer, J. (1995) Crystal structure of DNA photolyase from Escherichia coli. Science, 268, 1866-1872.

Parkin, D.M. and Iscovich, J. (1997) Risk of cancer in migrants and their descendants in Israel: II. Carcinomas and germ-cell tumours. Int J Cancer, 70, 654-660.

Parrish, J.A., Jaenicke, K.F. and Anderson, R.R. (1982) Erythema and melanogenesis action spectra of normal human skin. Photochem Photobiol, 36, 187-191.

Pearse, A.D., Gaskell, S.A., Marks, R. (1987) Epidermal changes in human skin following irradiation with either UVB or UVA. J Invest Dermatol, 1987.

Pehrson, J.R. (1989) Thymine dimer formation as a probe of the path of DNA in and between nucleosomes in intact chromatin. Proc Natl Acad Sci U S A, 86, 9149-9153.

Pehrson, J.R. (1995) Probing the conformation of nucleosome linker DNA in situ with pyrimidine dimer formation. J Biol Chem, 270, 22440-22444.

Pendrys, J. P. (1983) A model of the kinetics of photorepair in chick embryo fibroblasts. Mutat Res, 122, 129-133

Pfeifer, G.P., Drouin, R., Riggs, A.D. and Holmquist, G.P. (1992) Binding of transcription factors creates hot spots for UV photoproducts in vivo. Mol Cell Biol, 12, 1798-1804.

Ponten, F., Berg, C., Ahmadian, A., Ren, Z.P., Nister, M., Lundeberg, J., Uhlen, M. and Ponten, J. (1997) Molecular pathology in basal cell cancer with p53 as a genetic marker. Oncogene, 15, 1059-1067.

Price, V.H., Odom, R.B., Ward, W.H. and Jones, F.T. (1980) Trichothiodystrophy: sulfur-deficient brittle hair as a marker for a neuroectodermal symptom complex. Arch Dermatol, 116, 1375-1384.

Reeve, V.E., Greenoak, G.E., Canfield, P.J., Boehm-Wilcox, C. and Gallagher, C.H. (1989) Topical urocanic acid enhances UV-induced tumour yield and malignancy in the hairless mouse. Photochem Photobiol, 49, 459-464.

Ross, J.A., Howie, S.E., Norval, M., Maingay, J. and Simpson, T.J. (1986) Ultraviolet-irradiated urocanic acid suppresses delayed-type hypersensitivity to herpes simplex virus in mice. J Invest Dermatol, 87, 630-633.

Rowden, G., Lewis, M.G. and Sullivan, A.K. (1977) Ia antigen expression on human epidermal Langerhans cells. Nature, 268, 247-248.

Sancar, A. (1996) No "End of History" for photolyases. Science, 272, 48-49.

Sancar, A., Franklin, K.A. and Sancar, G.B. (1984a) Escherichia coli DNA photolyase stimulates uvrABC excision nuclease in vitro. Proc Natl Acad Sci U S A, 81, 7397-7401.

Sancar, A. and Sancar, G.B. (1984) Escherichia coli DNA photolyase is a flavoprotein. J Mol Biol, 172, 223-227.

Sancar, G.B. and Smith, F.W. (1989) Interactions between yeast photolyase and nucleotide excision repair proteins in Saccharomyces cerevisiae and Escherichia coli. Mol Cell Biol, 9, 4767-4776.

Sancar, G.B., Smith, F.W., Lorence, M.C., Rupert, C.S. and Sancar, A. (1984b) Sequences of the Escherichia coli photolyase gene and protein. J Biol Chem, 259, 6033-6038.

Schaeffer, L., Moncollin, V., Roy, R., Staub, A., Mezzina, M., Sarasin, A., Weeda, G., Hoeijmakers, J.H. and Egly, J.M. (1994) The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. Embo J, 13, 2388-2392.

Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J.H., Chambon, P. and Egly, J.M. (1993) DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science, 260, 58-63.

Scotto, J., Fears, T.R., Fraumeni, J.F. (1983) Incidence of Nonmelanoma Skin Cancer in the United States. Department of Health and Human Services, Washington.

Selleck, S.B. and Majors, J. (1987a) Photofootprinting in vivo detects transcription-dependent changes in yeast TATA boxes. Nature, 325, 173-177.

Selleck, S.B. and Majors, J.E. (1987b) In vivo DNA-binding properties of a yeast transcription activator protein. Mol Cell Biol, 7, 3260-3267.

Shelley, W.B. and Juhlin, L. (1976) Langerhans cells form a reticuloepithelial trap for external contact antigens. Nature, 261, 46-47.

Shivji, M.K., Podust, V.N., Hubscher, U. and Wood, R.D. (1995) Nucleotide excision repair DNA synthesis by DNA polymerase epsilon in the presence of PCNA, RFC, and RPA. Biochemistry, 34, 5011-5017.

Sijbers, A.M., de Laat, W.L., Ariza, R.R., Biggerstaff, M., Wei, Y.F., Moggs, J.G., Carter, K.C., Shell, B.K., Evans, E., de Jong, M.C., Rademakers, S., de Rooij, J., Jaspers, N.G., Hoeijmakers, J.H. and Wood, R.D. (1996) Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell, 86, 811-822.

Slaper, H., Velders, G.J., Daniel, J.S., de Gruijl, F.R. and van der Leun, J.C. (1996) Estimates of ozone depletion and skin cancer incidence to examine the Vienna Convention achievements. Nature, 384, 256-258.

Smith, M.L. and Fornace, A.J., Jr. (1997) p53-mediated protective responses to UV irradiation. Proc Natl Acad Sci U S A, 94, 12255-12257.

Spellman, C.W. and Daynes, R.A. (1978) Properties of ultraviolet light-induced suppressor lymphocytes within a syngeneic tumor system. Cell Immunol, 36, 383-387.

Stigger, E., Drissi, R. and Lee, S.H. (1998) Functional analysis of human replication protein A in nucleotide excision repair. J Biol Chem, 273, 9337-9343.

Sugasawa, K., Okamoto, T., Shimizu, Y., Masutani, C., Iwai, S. and Hanaoka, F. (2001) A multistep damage recognition mechanism for global genomic nucleotide excision repair. Genes Dev, 15, 507-521.

Suter, B., Livingstone-Zatchej, M. and Thoma, F. (1997) Chromatin structure modulates DNA repair by photolyase in vivo. Embo J, 16, 2150-2160.

Tamada, T., Kitadokoro, K., Higuchi, Y., Inaka, K., Yasui, A., de Ruiter, P.E., Eker, A.P. and Miki, K. (1997a) Crystal structure of DNA photolyase from Anacystis nidulans. Nat Struct Biol, 4, 887-891.

Tamada, T., Kitadokoro, K., Higuchi, Y., Inaka, K., Yasui, A., de Ruiter, P.E., Eker, A.P. and Miki, K. (1997b) Crystal structure of DNA photolyase from Anacystis nidulans. Nat Struct Biol, 4, 887-891.

Tang, A., Judge, T.A., Nickoloff, B.J. and Turka, L.A. (1996) Suppression of murine allergic contact dermatitis by CTLA4Ig. Tolerance induction of Th2 responses requires additional blockade of CD40-ligand. J Immunol, 157, 117-125.

Teunissen, M.B., De Jager, M.H., Kapsenberg, M.L. and Bos, J.D. (1991) Inhibitory effect of cyclosporin A on antigen and alloantigen presenting capacity of human epidermal Langerhans cells. Br J Dermatol, 125, 309-316.

Thoma, F. (1999) Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair. Embo J, 18, 6585-6598.

Thompson, C.B. (1995) Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? Cell, 81, 979-982.

Tissier, A., Frank, E.G., McDonald, J.P., Iwai, S., Hanaoka, F. and Woodgate, R. (2000) Misinsertion and bypass of thymine-thymine dimers by human DNA polymerase iota. Embo J, 19, 5259-5266.

Todo, T. (1999) Functional diversity of the DNA photolyase/blue light receptor family. Mutat Res, 434, 89-97.

Todo, T., Kim, S.T., Hitomi, K., Otoshi, E., Inui, T., Morioka, H., Kobayashi, H., Ohtsuka, E., Toh, H. and Ikenaga, M. (1997) Flavin adenine dinucleotide as a chromophore of the Xenopus (6-4)photolyase. Nucleic Acids Res, 25, 764-768.

Toews, G.B., Bergstresser, P.R. and Streilein, J.W. (1980) Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. J Immunol, 124, 445-453.

Tornaletti, S. and Hanawalt, P.C. (1999) Effect of DNA lesions on transcription elongation. Biochimie, 81, 139-146.

Tornaletti, S. and Pfeifer, G.P. (1995) UV light as a footprinting agent: modulation of UV-induced DNA damage by transcription factors bound at the promoters of three human genes. J Mol Biol, 249, 714-728.

Tron, V.A., Trotter, M.J., Tang, L., Krajewska, M., Reed, J.C., Ho, V.C. and Li, G. (1998) p53-regulated apoptosis is differentiation dependent in ultraviolet B-irradiated mouse keratinocytes. Am J Pathol, 153, 579-585.

van de Merwe, W.P. and Bronk, B.V. (1981) Increased efficiency of photoreversal of UV-induced dimers in the DNA of chick embryo fibroblasts with post-UV dark time. Mutat Res, 84, 429-441.

van der Horst, G.T., van Steeg, H., Berg, R.J., van Gool, A.J., de Wit, J., Weeda, G., Morreau, H., Beems, R.B., van Kreijl, C.F., de Gruijl, F.R., Bootsma, D. and Hoeijmakers, J.H. (1997) Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. Cell, 89, 425-435.

van der Leun, J.C. and de Gruijl, F.R. (2002) Climate change and skin cancer. Photochem Photobiol Sci, 1, 324-326.

van der Leun, J.C., de Gruijl, F.R., Tevini, M. and Worrest, R.C. (1993) Ultraviolet carcinogenesis: environmental effects of ozone depletion. Recent Results Cancer Res, 128, 263-267.

van Kranen, H.J. and de Gruijl, F.R. (1999) Mutations in cancer genes of UV-induced skin tumors of hairless mice. J Epidemiol, 9, S58-65.

van Noort, J., Orsini, F., Eker, A., Wyman, C., de Grooth, B. and Greve, J. (1999) DNA bending by photolyase in specific and non-specific complexes studied by atomic force microscopy. Nucleic Acids Res, 27, 3875-3880.

van Noort, S.J., van der Werf, K.O., Eker, A.P., Wyman, C., de Grooth, B.G., van Hulst, N.F. and Greve, J. (1998) Direct visualization of dynamic protein-DNA interactions with a dedicated atomic force microscope. Biophys J, 74, 2840-2849.

Vassylyev, D.G., Kashiwagi, T., Mikami, Y., Ariyoshi, M., Iwai, S., Ohtsuka, E. and Morikawa, K. (1995) Atomic model of a pyrimidine dimer excision repair enzyme complexed with a DNA substrate: structural basis for damaged DNA recognition. Cell, 83, 773-782.

Vink, A.A., Yarosh, D.B. and Kripke, M.L. (1996) Chromophore for UV-induced immunosuppression: DNA. Photochem Photobiol, 63, 383-386.

Wang, C.I. and Taylor, J.S. (1991) Site-specific effect of thymine dimer formation on dAn.dTn tract bending and its biological implications. Proc Natl Acad Sci U S A, 88, 9072-9076.

Weinlich, G., Heine, M., Stossel, H., Zanella, M., Stoitzner, P., Ortner, U., Smolle, J., Koch, F., Sepp, N.T., Schuler, G. and Romani, N. (1998) Entry into afferent lymphatics and maturation in situ of migrating murine cutaneous dendritic cells. J Invest Dermatol, 110, 441-448.

Wikonkal, N.M. and Brash, D.E. (1999) Ultraviolet radiation induced signature mutations in photocarcinogenesis. J Investig Dermatol Symp Proc, 4, 6-10.

Winkler, G.S., Araujo, S.J., Fiedler, U., Vermeulen, W., Coin, F., Egly, J.M., Hoeijmakers, J.H., Wood, R.D., Timmers, H.T. and Weeda, G. (2000) TFIIH with inactive XPD helicase functions in transcription initiation but is defective in DNA repair. J Biol Chem, 275, 4258-4266.

Wolf, P., cox P., Yarosh D.B., Kripke M.L. (1995) Sunscreens and T4N5 liposomes differ in their ability to protect against ultraviolet-induced sunburn cell formation, alterations of dendritic epidermal cells, and local suppression of contact hypersensitivity. J Invest Dermatol, 104, 287-292.

Wood, R.D. (1999) DNA repair. Variants on a theme. Nature, 399, 639-640.

Woodgate, R. (1999) A plethora of lesion-replicating DNA polymerases. Genes Dev, 13, 2191-2195.

Yamaizumi, M. and Sugano, T. (1994) U.v.-induced nuclear accumulation of p53 is evoked through DNA damage of actively transcribed genes independent of the cell cycle. Oncogene, 9, 2775-2784.

Yamamoto, K., Satake, M., Shinigawa, H., Fujiwara, Y. (1983) Amelioration of ultraviolet sensitivity of an Escherichia coli recA mutant in the dark by photoreactivating enzyme. Mol Gen Genet, 190, 511-515.

Yarosh, D., Bucana, C., Cox, P., Alas, L., Kibitel, J. and Kripke, M. (1994) Localization of liposomes containing a DNA repair enzyme in murine skin. J Invest Dermatol, 103, 461-468.

Yasuhira, S. and Yasui, A. (1992) Visible light-inducible photolyase gene from the goldfish Carassius auratus. J Biol Chem, 267, 25644-25647.

Yasui, A., Eker, A.P., Yasuhira, S., Yajima, H., Kobayashi, T., Takao, M. and Oikawa, A. (1994a) A new class of DNA photolyases present in various organisms including aplacental mammals. Embo J, 13, 6143-6151.

Yasui, A., Eker, A.P., Yasuhira, S., Yajima, H., Kobayashi, T., Takao, M. and Oikawa, A. (1994b) A new class of DNA photolyases present in various organisms including aplacental mammals. Embo J, 13, 6143-6151.

Yasui, A., Eker,A.P.M. (1997) DNA photolyases. In Nickoloff, J.A., Hoekstra,M.F. (ed.), DNA Damage and Repair: Biochemistry, Genetics and Cell Biology. Humana Press, Totowa, Vol. 2, pp. 9-32.

You, Y.H., Lee, D.H., Yoon, J.H., Nakajima, S., Yasui, A. and Pfeifer, G.P. (2001a) Cyclobutane pyrimidine dimers are responsible for the vast majority of mutations induced by UVB irradiation in mammalian cells. J Biol Chem, 276, 44688-44694.

You, Y.H., Lee, D.H., Yoon, J.H., Nakajima, S., Yasui, A. and Pfeifer, G.P. (2001b) Cyclobutane pyrimidine dimers are responsible for the vast majority of mutations induced by UVB irradiation in mammalian cells. J Biol Chem, 276, 44688-44694.

Ziegler, A., Jonason, A.S., Leffell, D.J., Simon, J.A., Sharma, H.W., Kimmelman, J., Remington, L., Jacks, T. and Brash, D.E. (1994) Sunburn and p53 in the onset of skin cancer. Nature, 372, 773-776.

Ziegler, A., Leffell, D.J., Kunala, S., Sharma, H.W., Gailani, M., Simon, J.A., Halperin, A.J., Baden, H.P., Shapiro, P.E., Bale, A.E. and et al. (1993) Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. Proc Natl Acad Sci U S A, 90, 4216-4220.

Zmudzka, B.Z. and Beer, J.Z. (1990) Activation of human immunodeficiency virus by ultraviolet radiation. Photochem Photobiol, 52, 1153-1162.

Zwetsloot, J.C., Vermeulen, W., Hoeijmakers, J.H., Yasui, A., Eker, A.P. and Bootsma, D. (1985) Microinjected photoreactivating enzymes from Anacystis and Saccharomyces monomerize dimers in chromatin of human cells. Mutat Res, 146, 71-77.

Chapter Two

Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice

Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice

During evolution, placental mammals appear to have lost CPD photolyase, an enzyme that efficiently removes UV-induced cyclobutane pyrimidine dimers (CPDs) from the DNA in a light-dependent manner and that is found in all kingdoms. As a consequence they have to rely solely on the more complex and for this lesion less efficient nucleotide excision repair pathway. To assess the contribution of poor repair of this major UV damage to the various biological effects of UV, we generated mice expressing a marsupial CPD photolyase transgene. Expression from the ubiquitous β-actin promoter allowed rapid repair of CPDs in epidermis and dermis. UV-exposed cultured dermal fibroblasts from these mice display a superior survival when treated with photoreactivating light. Moreover, photoreactivation of CPDs in the intact skin dramatically reduces acute UV effects like erythema (sunburn), hyperplasia and apoptosis. Thus, providing mice with CPD photolyase significantly improves repair and uncovers the biological effects of CPD lesions.

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Introduction

Absorption of ultraviolet light (UV) energy by DNA induces various types of lesions. Although single- and double-strand breaks as well as DNA-protein cross-links can occur, more than 99% of the UV-induced damage consists of chemical base modifications, with cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs) as the most frequent type of photolesions (Mitchell, 1988). CPDs and 6-4PPs occur at two adjacent pyrimidines in the DNA and affect proper basepairing, which results in interference with key cellular processes like transcription and replication. Lesions in the DNA can lead to a reduction of RNA synthesis, arrest of cell cycle progression, and induction of apoptosis. Moreover, persisting DNA damage can give rise to gene mutations that may allow cells to escape from controlled growth, which ultimately may lead to cancer (Friedberg, 1995).

To counteract the deleterious effects of DNA damage produced by endogenous and environmental genotoxic agents (including UV-induced lesions), all organisms have developed a complex network of repair systems with complementary substrate specificity that keeps the DNA under continuous surveillance (Friedberg, 1995; Hoeijmakers, 2001). Removal of photolesions from the DNA is performed by the versatile and evolutionary highly conserved nucleotide excision repair (NER) pathway. NER is a complex multi-step process and involves the concerted action of 30 or so proteins to sequentially execute damage recognition, chromatin remodeling, excision of a small oligonucleotide containing the damage, and gap-filling DNA synthesis followed by strand ligation (de Laat et al., 1999; Friedberg, 1995; Hoeijmakers, 2001; Wood, 1999). The NER system is comprised of two subpathways. Global genome NER (GG-NER) operates genome wide but has the disadvantage that certain types of damage (like UV-induced CPDs) are less well recognized and accordingly less efficiently repaired. To avoid that such lesions hamper transcription too long by stalling RNA polymerase II, a distinct NER subpathway has evolved, called Transcription-Coupled Repair (TC-NER). This process directs the repair machinery preferentially to the blocked polymerase on the template strand of actively transcribed DNA and operates as a selective backup system for lesions that are slowly or not at all repaired by GG-NER (Tornaletti and Hanawalt, 1999).

Inherited defects in NER genes cause photosensitive disorders such as xeroderma pigmentosum (XP, subdivided over 7 complementation groups: XP-A through G) and Cockayne syndrome (CS, complementation groups A and B) (for a review see Bootsma (2001)). All XP patients are deficient in GG-NER and, with the exception of XP group C and E, also in TC-NER. A specific defect in TC-NER is encountered in CS (van Hoffen et al., 1993; Venema et al., 1990). XP and CS patients display an increased sensitivity of the skin to UV light. In addition, XP patients have a highly elevated risk of developing UV-induced skin cancer. Obviously, the repair of UV-induced DNA damage strongly reduces the many undesirable consequences of UV exposure.

It has proven difficult to disentangle the individual contribution of different types of UV lesions (*i.e.* CPDs and 6-4PPs) to the range of deleterious effects exerted by UV irradiation. CPDs are known to be repaired much slower by NER than 6-4PPs (Mitchell, 1988). In NER-proficient human fibroblasts, repair of most of the CPD lesions introduced by a modest dose of UV light takes >24 hrs, while 6-4PPs are removed within a few hours (Bohr et al., 1985). The GG-NER system is responsible for CPD removal anywhere in the genome, whereas CPDs that stall elongating RNA polymerase are selectively eliminated by TC-NER (Bohr et al., 1985; Mellon et al., 1987; van Hoffen et al., 1993). Recognition and

repair of CPDs by GG-NER requires p53 dependent upregulation of the p48 subunit of the XP-E p48-p125 UV-DDB heterodimer (Hwang et al., 1999). Importantly, the *p48* gene is not induced in rodent cells (Hwang et al., 1999); therefore repair of CPDs is limited to the transcribed strand (TS) of active genes by TC-NER (Bohr et al., 1985; van der Horst et al., 1997). In contrast to CPD repair, both human and rodent cells efficiently remove 6-4PPs from their genome (Mitchell, 1988; van Hoffen et al., 1995) mainly by GG-NER. In the epidermis of man and mice, keratinocytes remove CPDs and 6-4PPs in the same fashion as cultured fibroblasts (Hanawalt et al., 1981; Mitchell et al., 1990; Qin et al., 1995; Ruven et al., 1994). Some studies have pointed to the CPDs as the most cytotoxic and carcinogenic lesions (Broughton et al., 1990; Chigancas et al., 2000; Nishigaki et al., 1998) but to date the actual magnitude of the biological effects provoked by the different types of UV-induced DNA lesions has remained unclear.

Many organisms of all kingdoms mount an additional repair system to remove UV lesions, called photoreactivation. In contrast to the complex NER pathway, photoreactivation is performed by photolyases that rapidly convert UV lesions back to the original undamaged bases in a simple enzymatic reaction using visible light as an energy source. To perform this reaction, photolyases are equipped with two different chromophoric cofactors. Depending on the photolyase, either 5,10,-methyl tetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF) serve as light-harvesting antennas, that pass on energy to reduced FAD, the chromophore that acts as the reaction center in dimer splitting (for a review, see Yasui and Eker (1997)). Notably, photolyases show substrate specificity for either CPDs (CPD photolyase) or 6-4PPs (6-4PP photolyase). Photolyases occur in bacteria, lower eukaryotes, plants and many animals including marsupials. Remarkably, despite the strong evolutionary conservation, early development in evolution and presence of this efficient repair system in non-placental mammals, photoreactivation is absent in placental mammals like mice and humans, implying that photolyase genes have been lost during early development of placental mammals. The inability to photoreactivate CPDs and 6-4PPs leaves placental mammals with only the NER system for removal of these photolesions. Here we report the generation and characterization of mice that express the CPD photolyase transgene from the marsupial *Potorous tridactylus*.

Materials and methods

Generation of CPD photolyase transgenic mice

Constructs for the generation of CPD photolyase transgenic mice were cloned in the vector pSP72 (Promega) and contained the chicken β-actin promoter with the cytomegalovirus (CMV) enhancer (from pCY4B, kindly provided by Dr. J. Miyazaki, Osaka, Japan) followed by the *Potorous tridactylus* CPD photolyase cDNA. At the 3' end, we inserted exon 2 (last 22 basepairs), intron 2, exon 3 and the 3' UTR (including the polyadenylation signal) of the human *β-globin* gene. The expression constructs were excised from the plasmid using appropriate restriction enzymes, separated from the vector DNA by agarose gel electrophoresis, isolated from gel with the GeneClean II kit (Bio101), and further purified using elutip-D-mini columns (Schleicher and Schuell). The fragment was dissolved in injection buffer (10 mM Tris-HCl pH 7.5, 0.08 mM EDTA) and injected in the pronucleus of fertilized eggs derived from FVB/N intercrosses as described (Hogan, 1994).

Transgenic animals were identified by Southern blot analysis of genomic tail DNA, using the CPD photolyase cDNA as a probe. To estimate the number of integrated copies, equal amounts of genomic DNA from transgenic animals were subjected to Southern blot analysis. As a standard, we used equal amounts of wildtype genomic DNA supplemented with 0, 10, 30, or 100 pg of the corresponding CPD photolyase expression construct. The hybridization signal obtained with the CPD photolyase cDNA probe was quantified using a Molecular Dynamics phospho-imager and ImageQuant software. After comparison of signal intensities, the transgene copy number could be estimated using the supplemented reference samples. The site of chromosomal integration was determined by FISH analysis of metaphase chromosome spreads of transgenic mouse cell lines as described by Arnoldus et al. (1990), using the CPD photolyase cDNA probe.
Routine genotyping of mice was performed by PCR analysis. Primer set 5'-TGA GAC TCA TCT CCC AGG AC-3' (forward primer) and 5'-CAC CAA TGC CAT GTG TTT GC-3' (reverse primer) was used to amplify a 321 bp fragment of the CPD photolyase coding region.

RNA isolation and RT-PCR
Mouse skin RNA was isolated from a 0.5-1.0 cm^2 skin section, after hairs had been plucked from the back of the sacrificed mouse. After addition of 2-3 ml of an ice-cold 3 M LiCl, 6 M urea solution, the skin sample was immediately homogenized on ice using a Heidolph Diax 600 homogenizer set at 22000 rpm. The homogenate was left at 4°C overnight, and after centrifugation at 113000 g for 20 minutes the pellet was resuspended in 0.5 ml 10 mM Tris/HCl, 0.1 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K and incubated at 37°C for 30 minutes. Total RNA was obtained by subsequent phenol extraction, centrifugation and ethanol precipitation of the supernatant. The pellet was dissolved in 10 mM Tris/HCl, 0.1 mM EDTA. cDNA synthesis was performed using Superscript II RNase H reverse transcriptase (RT) (Life Technologies) according to the protocol of the supplier. A standard PCR reaction was performed on the cDNA using a forward primer in the photolyase transgene (5'-GAT CTT CGG AAA GAT CCG C-3') and a reverse primer in exon 3 of the *β-globin* gene (5'-TGG ACA GCA AGA AAG CGA G-3'). The presence of introns in the photolyase transgene allowed discrimination of the cDNA derived PCR product from possible genomic DNA contaminations in the isolated RNA.

Isolation and culturing of dermal fibroblasts
Primary mouse dermal fibroblasts (MDFs) were isolated from the skin according to the protocol of Mémet et al. (1997). In short, after removal of hairs from a small area on the back of an anaesthetized mouse, a small piece of skin (about 0.3 cm^2) was removed. The tissue was minced, transferred to a PBS solution containing 0.125% trypsin, 1 mg/ml collagenase type V and 0.3 mg/ml hyaluronidase, and incubated at 37°C for 15 minutes after which the cell suspension was collected. A fresh enzyme solution was added to the remaining pieces of skin and the procedure was repeated two more times. Cell suspensions from one mouse line were pooled and centrifuged, after which the pellet was resuspended in medium (40% Dulbecco's Modified Eagle's Medium (DMEM), 40% HAM-F10, 20% Fetal Calf Serum (FCS), 1% penicillin + streptomycin). Cells were transferred to 10 cm dishes and cultured at 37°C, 5% CO_2.

Immunological procedures

A fusion protein of *P. tridactylus* CPD photolyase and glutathion S-transferase (GST) was overexpressed in *E. coli*, purified (Yasui et al., 1994), and used for immunization of two rabbits. The serum was affinity purified against immobilized CPD photolyase.

Western blot analysis. Protein extracts were prepared by harvesting MDFs in sample buffer (2% SDS, 0.1 M Tris pH 6.8, 4% β-mercaptoethanol, 15% glycerol) containing 1 mM PMSF. The protein extract was heat denatured, subjected to SDS-PAGE on a 10% gel and transferred to nitrocellulose. The nitrocellulose blot was incubated for 1 h in non-fat milk, washed with PBGTNa (PBS containing 0.5% BSA (Sigma), 0.05% gelatin, 0.05% Tween-20, and 300 mM NaCl), incubated overnight with the anti-CPD photolyase polyclonal antibody diluted in PBGTNa, washed 4 x 5 min in PBGTNa, incubated 2 h with the secondary antibody Goat anti-Rabbit coupled to alkaline phosphatase, washed 2 x 5 min with PBGTNa and 2 x 5 min with PBS. Blots were stained using the BCIP/NBT method (BioRad).

Immunofluorescent detection of CPD photolyase. Coverslips with attached MDFs were rinsed once in PBS and fixed with 2% paraformaldehyde in PBS for 15 min. After fixation, cells were washed twice with PBS and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min. Cells were subsequently washed twice in PBS, incubated in PBS containing 100 mM glycine (Sigma) for 10 min, incubated in PBG (PBS containing 0.5% BSA (Sigma) and 0.05% gelatin) for 10 min, and incubated overnight at 4°C with the anti-CPD photolyase polyclonal antibody diluted in PBG. The coverslips were washed 4 x 5 min with PBG and incubated with goat anti-Rabbit coupled to FITC for 2 h, washed 2 x 5 min with PBG and 2 x 5 min with PBS. Finally, coverslips were incubated for 5 min with DAPI (0.2 µg/ml) in PBS, washed with PBS and mounted in Vectashield (Vector Laboratories, USA). All steps were performed at room temperature unless stated otherwise.

DNA repair assays

Photoreactivation in MDFs. Cells grown on coverslips were washed with PBS, exposed to 20 J/m^2 UV-C (Philips TUV germicidal lamp), and subsequently kept in Hank's buffer (137 mM NaCl, 5.4 mM KCl, 4.4 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 0.81 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 1 g/l glucose, pH 7.4). Photoreactivation was performed by exposing cells for 1 h to light from 4 white fluorescent tubes (Philips TLD 18W/54) at a distance of 15 cm and shielded by a 5 mm glass filter. Control cells were given the same treatment, except that dishes were covered with two layers of aluminum foil and also put under the same fluorescent lamps. Next, cells were fixed in a mixture of 75% methanol and 25% acetic acid for 1 h, subsequently incubated in 70% ethanol (1 h) and 70% ethanol containing 0.07 N NaOH (4 min), and washed 3 x 5 min with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4). Next, cells were treated with 1 µg/ml proteinase K in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4 (10 min), incubated for 30 min with TBSF (TBS containing 1% fetal calf serum (FCS)), and incubated overnight at 4°C with an antibody raised against CPD or 6-4PP photolesions (antibodies TDM2 and 64M2 respectively (Mori et al., 1991)) diluted in TBSF. Coverslips were washed 4 x 5 min with TBSF, incubated for 2 h with goat anti-Mouse conjugated to FITC, washed 2 x 5 min with TBSF, and washed 2 x 5 min with PBS. Finally, coverslips were incubated for 5 min with DAPI (0.2 µg/ml) in PBS, washed with PBS, and mounted in Vectashield (Vector Laboratories, USA). Cells were analyzed using a Leica DMRBE epifluorescence microscope.

UV survival. UV radiation sensitivity was determined as described (Sijbers et al., 1996), except that a photoreactivation step was incorporated in the assay. MDFs were grown in 6-well culture plates (Costar), washed with PBS, exposed to different doses of UV-C light (Philips TUV germicidal lamp), and subsequently kept in Hank's buffer. Next cells were either exposed to photoreactivating light or kept in the dark for 1 h as described above, after which cell culturing was continued in the dark. After two days, the number of proliferating cells was determined from the amount of radioactivity incorporated during a 3 hour pulse with [^{3}H]-thymidine. Cell survival is expressed as the percentage of radioactivity in exposed cells relative to that in untreated cells.

Unscheduled DNA Synthesis. UV-induced global genome repair was assayed using the unscheduled DNA synthesis (UDS) as described (Vermeulen et al., 1994). Cells were exposed to 16 J/m^2 of UV-C light and labeled for 3 h with [methyl-^{3}H]thymidine (10 μM, specific activity 40-60 Ci/mmole), followed by processing for autoradiography. Repair capacity was quantified by grain counting after autoradiographic exposure.

Photoreactivation in the mouse skin. Mice were anaesthetized and hairs were removed from a small area on the back of the animal. One third of the hairless area was covered with black non-adhesive tape and the remaining area was exposed to the light of two Philips TL-12 (40W) tubes emitting UV-B light. Typically, one MED was obtained with an exposure of 2 min, at a distance of 21 cm. Subsequently, half of the UV-exposed area was covered with tape and the mice were exposed for 3 h to the light of 4 Cool White fluorescent tubes (GE Lightning Polylux XL F36W/840 lamps) filtered through 5 mm of glass. Skin samples were taken from the unexposed area, the UV-irradiated area that was covered, and the UV-irradiated area that was exposed to photoreactivating light. The skin samples were fixed for 1 hour in a mixture of 75% methanol and 25% acetic acid, incubated in 70% ethanol for 1 hour, followed by 5% sucrose (1 h), and snapfrozen in TissueTek (Sakura) by immersion in liquid nitrogen. Cryosections of 8 μm were made with a Leica microtome (Jung CM3000) and deposited on SuperFrost (Menzel-Glazer) glass slides. Sections on slides were incubated in 70% ethanol containing 0.07 N NaOH for 4 min and subsequently washed, incubated and labeled like MDFs as described above.

Apoptosis

For detection of apoptotic cells in the skin, we used a TdT-mediated dUTP Nick-End Labeling (TUNEL) assay (Fluorescein Apoptosis Detection System, Promega). Mice were exposed to UV and photoreactivating light as described above, and subsequently kept in the dark. Skin samples, taken 40 h after UV exposure, were fixed overnight in 4% paraformaldehyde, washed in PBS, and embedded in paraffin. Skin sections (5 μm) were deparafinized and incubated as described by the manufacturer.

Erythema and hyperplasia

Mice were anaesthetized and an area on the back was depilated by plucking. After recovery, animals were either kept in the dark or exposed to UV-B (1.5 MED per day), immediately followed by photoreactivating light (3 h) for four consecutive days. Except for the above treatment, animals were continuously kept in the dark. Seven days after the start of the experiment, mice were physically examined for the formation of erythema. For analysis of epidermal hyperplasia, skin biopsies were taken and processed for routine histology.

Results

Generation of β-actin promoter-driven CPD photolyase transgenic mouse and cell lines

To generate mice ubiquitously expressing a *CPD photolyase* transgene, we assembled a construct containing the *Potorous tridactylus* CPD photolyase cDNA (Yasui et al., 1994), preceded by a CMV enhancer containing chicken β-actin promoter and hooked up to human genomic *β-globin* sequences, including exons 2 and 3, intron 2, the 3' untranslated region and the polyadenylation signal (Fig. 1A). After oocyte injections, we obtained two independent β-actin promoter-driven photolyase mouse lines, designated β-act-CPD-1 and β-act-CPD-2 (carrying 2 and 3 copies of the transgene respectively, data not shown). FISH analysis of metaphase chromosome spreads of Mouse Dermal Fibroblasts (MDFs) isolated from β-act-CPD-1 and β-act-CPD-2 animals revealed integration of the photolyase transgene on chromosome 3C and 15A2 respectively (data not shown).

RT-PCR analysis of total skin RNA showed expression and correct splicing of the CPD photolyase mRNA (Fig. 1B). In addition, immunoblot analysis of protein extracts from wildtype, β-act-CPD-1 and β-act-CPD-2 MDFs, using a polyclonal antibody raised against the *Potorous* CPD photolyase, showed a band of the expected size (61 kD), indicating expression of the full-length protein (Fig. 1C). Immunocytochemical analysis of these cultured MDFs revealed a fluorescent signal in the nuclei of transgenic cell lines only, indicating correct nuclear localization of the CPD photolyase protein, probably due to a putative nuclear localization signal sequence (24-ARKKRK-29; see Yasui et al. (1994)) in the N-terminus of the protein (Fig. 1D). Taken together, these data demonstrate that the two independent CPD photolyase mouse lines properly express the transgene.

To facilitate genotyping, and to avoid the potential risk of adverse side effects originating from inactivation of loci at the transgene integration site mouse strains were kept in heterozygous state. Importantly, β-act-CPD photolyase transgenic mice are born at a Mendelian ratio, are fertile, and up till the age of 1 year do not show any overt phenotype. This indicates that insertion of the CPD photolyase transgene does not interfere with development, viability and fertility. Moreover, β-act-CPD-1 and β-act-CPD-2 cells and animals are indistinguishable for all parameters tested.

Photoreactivation of CPDs in cultured CPD photolyase fibroblasts

The transgenically expressed CPD photolyase is expected to allow lesion-specific repair of CPDs upon exposure to photoreactivating light. To investigate light-dependent removal of CPDs in MDFs, we have applied an immunocytochemical assay using antibodies specific for either CPDs or 6-4PPs (Mori et al., 1991). Cells were irradiated with 20 J/m^2 UV-C light and, as expected, for both antibodies a bright immunofluorescent signal could be detected in the nucleus of UV-treated cells, while non-irradiated cells remained unlabeled. This confirms the presence of both CPDs and 6-4PPs immediately after UV exposure (Fig. 2). When cells were subsequently kept in the dark for 1 hour (thus withholding the photolyase from the energy source required for enzymatic activity), CPD and 6-4PP staining remained present. In line with the known repair rates for both photolesions in rodents (Bohr et al., 1985; Mitchell, 1988), this indicates that in the first hour after UV exposure (TC-)NER can only remove a small fraction of the induced photolesions. In marked contrast, however, after exposure of UV-irradiated cells to one hour of photoreactivating light CPD-labeling was hardly detectable, while the signal for 6-4PPs was comparable to that observed in cells kept in the dark (Fig. 2). The lesion-specific and light-dependent

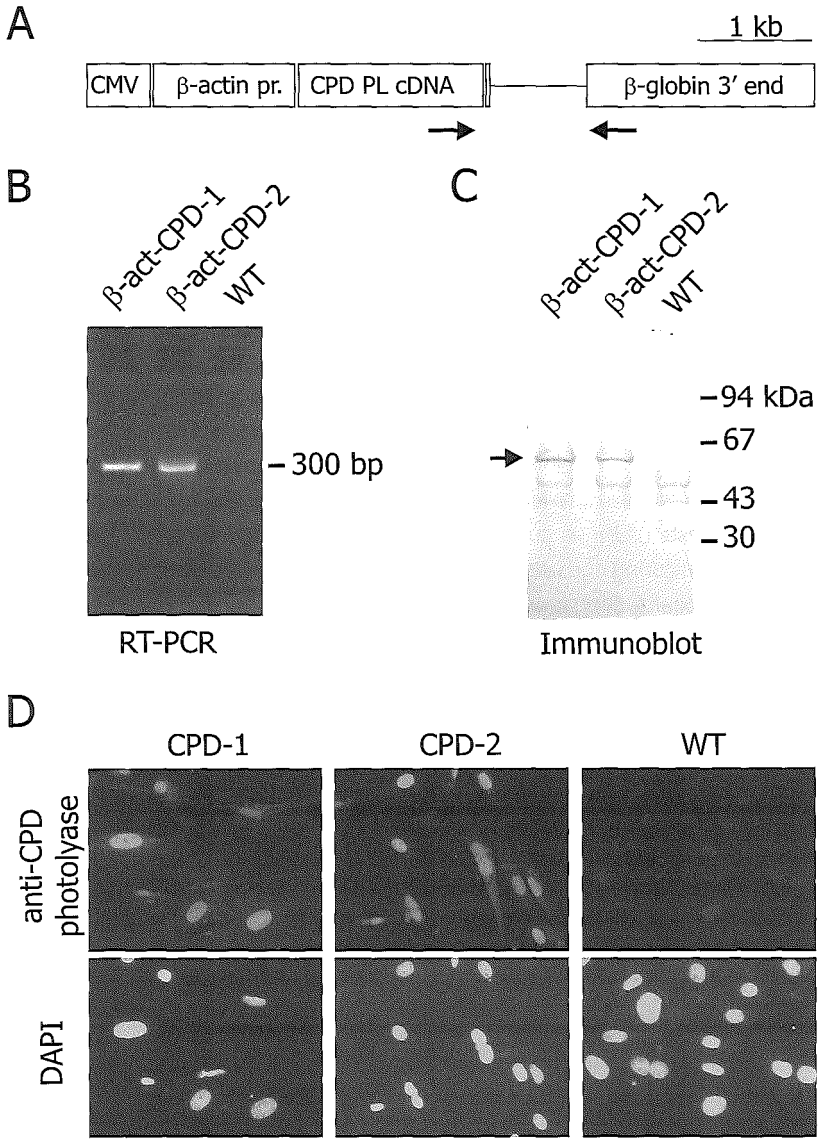

Figure 1
Expression of the β-actin CPD photolyase gene in transgenic mice.
A. Expression construct for the generation of β-act-CPD photolyase transgenic mice, containing the CMV enhancer and chicken β-actin promoter, the *Potorous tridactylus* CPD photolyase cDNA, and human genomic β-globin sequences, including exons 2 and 3, intron 2, the 3' untranslated region and the polyadenylation signal. Arrows indicate the position of the primers used for the RT-PCR experiment. B. RT-PCR on skin extracts of β-act-CPD photolyase transgenic mice results in a 300 bp band. C. Immunoblot analysis of protein extracts (30 mg per lane) from cultured fibroblasts obtained from β-act-CPD photolyase transgenic mice. The arrow indicates the position of the 62 kDa photolyase protein. D. Immunocytochemical detection of CPD photolyase in cultured mouse dermal fibroblasts, using an affinity-purified anti-CPD photolyase. Nuclei are visualized by DAPI staining.

removal of CPDs clearly demonstrates that the marsupial CPD photolyase is enzymatically active in mouse cells.

In repair-proficient mammalian cells, UV-induced DNA lesions are exclusively repaired by NER. From the previous experiment it is evident that CPDs in UV-C exposed transgenic MDFs are rapidly removed by the CPD photolyase. Accordingly, it is expected that cells show reduced UV-induced NER activity after photoreactivation of CPD lesions. NER activity can be measured in cultured cells by the unscheduled DNA synthesis (UDS) assay, which quantifies the incorporation of radiolabeled thymidine in newly synthesized DNA in repair patches. As shown in Fig. 3A, the relative UDS level in β-act-CPD-1

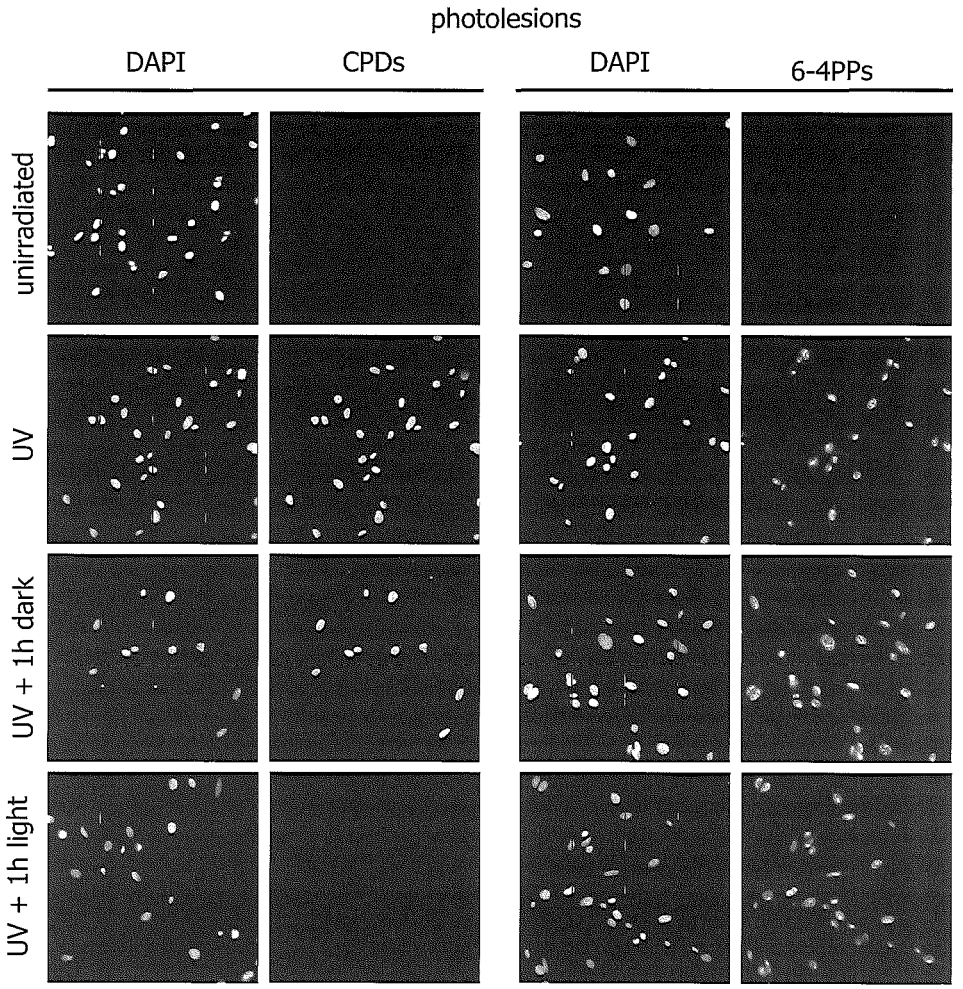

Figure 2
Photoreactivation of CPDs in cultured CPD photolyase transgenic fibroblasts.
Induction of CPD and 6-4PP lesions in cultured mouse dermal fibroblasts (MDFs) from β-act-CPD-1 photolyase mice by 20 J/m² of UV-C light and the effect of subsequent exposure of cells to photoreactivating light for 1 h. Photolesions were detected by immunofluorescent labeling, using CPD or 6-4PP specific antibodies and FITC-conjugated goat anti-mouse antibodies. Nuclei were visualized by DAPI staining.

MDFs that were exposed to UV light and subsequently kept in the dark for 1 hour drops to 75 % of the UDS activity measured immediately after exposure to UV light. This difference reflects the NER activity during the first hour following UV exposure. As predicted, relative UDS even decreased further when UV-exposed cells were given 1 hour of photoreactivating light. The reduction in NER activity confirms the effectiveness of the CPD photolyase and reveals the fraction of UDS derived from CPD repair.
Next, we studied whether the fast removal of CPDs renders MDFs supplemented with a CPD photoreactivating system less sensitive to UV-C light. As shown in Fig. 3B, treating the photolyase MDFs with photoreactivating light gave rise to a higher percentage of surviving cells over the whole range of UV doses tested, when compared to transgenic and wildtype cells that were not treated with photoreactivating light. The dose-reducing effect of the CPD photolyase is about two-fold, meaning that photoreactivation increases the UV survival to the level normally observed at half the UV dose. Thus, NER-proficient mouse cells significantly benefit from CPD photoreactivation.

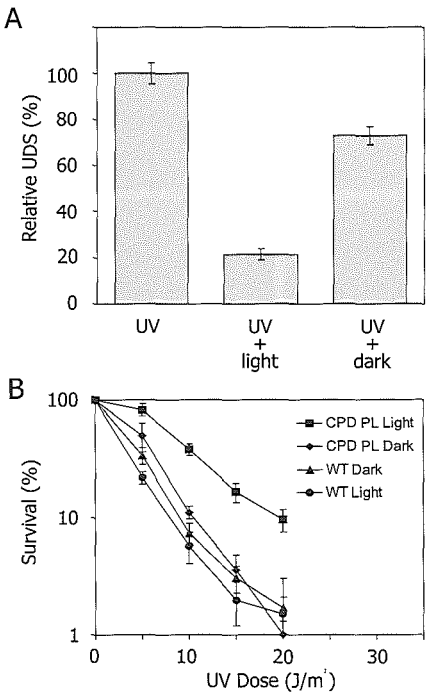

Figure 3
Effect of photoreactivation of CPDs in MDFs on NER activity and cellular survival.
A. NER activity in cultured mouse dermal fibroblasts (MDFs) from β-act-CPD-1 photolyase mice exposed to 16 J/m² of UV-C light and the effect of subsequent exposure of cells to 1hr of photoreactivating light. Unscheduled DNA Synthesis (UDS) was measured by the incorporation of [³H] thymidine, followed by counting of grains above nuclei of non-dividing cells. UDS levels are expressed as the percentage of activity observed immediately after UV exposure. B. UV survival of primary β-act-CPD-1 MDFs after exposure to increasing doses of UV-C light and with or without 1 h exposure to photoreactivating light. Error bars indicate the standard error of the mean. Similar results were obtained with β-act-CPD-2 MDFs.

Photoreactivation of CPDs in the mouse skin

To investigate whether the skin, the prime target of UV light in the intact animal, also profits from the addition of the expressed CPD photolyase transgene, we analyzed the repair of CPDs in the dermis and epidermis of β-actin promoter-driven CPD photolyase transgenic mice. To this end, the immunofluorescent assay used to visualize photolesions in cultured MDFs, was adapted for use on skin sections (see Material and Methods section). A depilated area on the back of photolyase mice was covered for one third while the remaining part was exposed to 1 MED (minimal erythemal dose; the UV dose at which erythema starts to appear) of UV-B light. Next, half of the UV-exposed area as well as the non UV-exposed area was covered, while the remaining part of the skin was exposed to

photoreactivating light for 3 hours. As expected, the non UV-exposed part of the skin did not show any CPD-labeling (data not shown), whereas the UV-irradiated part of the skin contained a clear nuclear labeling of CPDs in the epidermis and the upper part of the dermis (Fig. 4, top panels). Importantly, exposure of the UV-irradiated skin to photoreactivating light resulted in a strong decrease in CPD labeling in epidermis and dermis.
In contrast, labeling of 6-4PPs was not detectably reduced upon photoreactivation (data not shown). These data clearly demonstrate that the ubiquitously expressed CPD photolyase is active in all epidermal and dermal cells and that the enzyme can specifically photoreactivate the majority of CPDs in these cells within 3 hours following UV exposure, leaving the 6-4PPs unaffected. In addition, these data show that both UV-B light and photoreactivating light do penetrate the epidermis and reach into the dermis.

Reduction of acute skin effects by photoreactivation of CPDs
Exposure of the skin to (repeated) UV light induces apoptosis in the epidermis, accompanied by redness (erythema, commonly known as sunburn), swelling (edema), and followed several days later by thickening of the epidermis (hyperplasia). To investigate the effect of CPD photoreactivation on induction of apoptosis, the depilated dorsal skin of CPD photolyase mice was exposed to a single dose of 1 MED UV-B light and partly exposed to photoreactivating light for 3 hours as described in the previous section. Next, animals were kept in the dark and after 40 hours skin samples were processed for analysis of apoptosis by a TUNEL assay. In the absence of photoreactivating light, we observed a strong induction of apoptosis in the epidermis of UV-exposed skin with few apoptotic dermal cells (Fig. 5, middle panels), a staining pattern very similar to that observed in UV-exposed wildtype animals

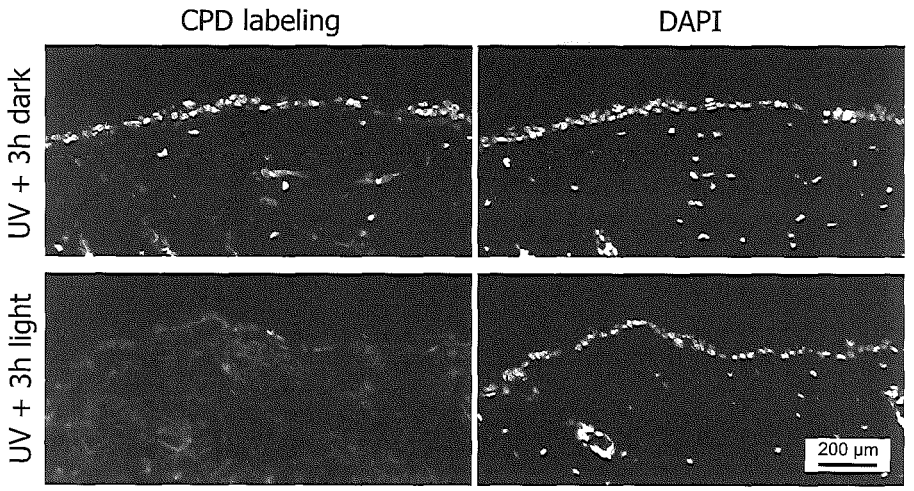

Figure 4
Photoreactivation of CPDs in the skin of β-act-CPD photolyase transgenic mice.
Induction of CPD lesions in the depilated dorsal skin of β-act-CPD-1 photolyase mice by 1 MED UV-B light and the effect of subsequent exposure to photoreactivating light for 3 h. Photolesions were detected by immunofluorescent labeling, using CPD specific antibodies and FITC-conjugated goat anti-mouse antibodies. Nuclei are visualized by DAPI staining.

(data not shown). The non UV-exposed area of the skin remained unstained (Fig. 5, top panels), indicating that apoptosis was specifically induced by exposure to UV light. In marked contrast, the UV-exposed skin showed little or no apoptotic signal when treated with photoreactivating light (Fig. 5, bottom panels). Similar results were obtained when animals were exposed to a UV-B dose of 1.5 MED or when the TUNEL assay was performed 24 hour after UV irradiation (data not shown). Thus, photoreactivation of CPDs in CPD photolyase transgenic mice clearly reduces the apoptotic response.

To study induction of erythema and hyperplasia, we exposed the depilated dorsal skin of CPD photolyase mice to 1.5 MED UV-B light per day for four consecutive days. Animals were continuously kept in the dark, except for animals that received 3 hours of photoreactivating light immediately after each UV exposure. Three days after the last UV exposure, a clear redness and swelling was observed on the back of UV-treated animals that had not been exposed to photoreactivating light (Fig. 6A). In

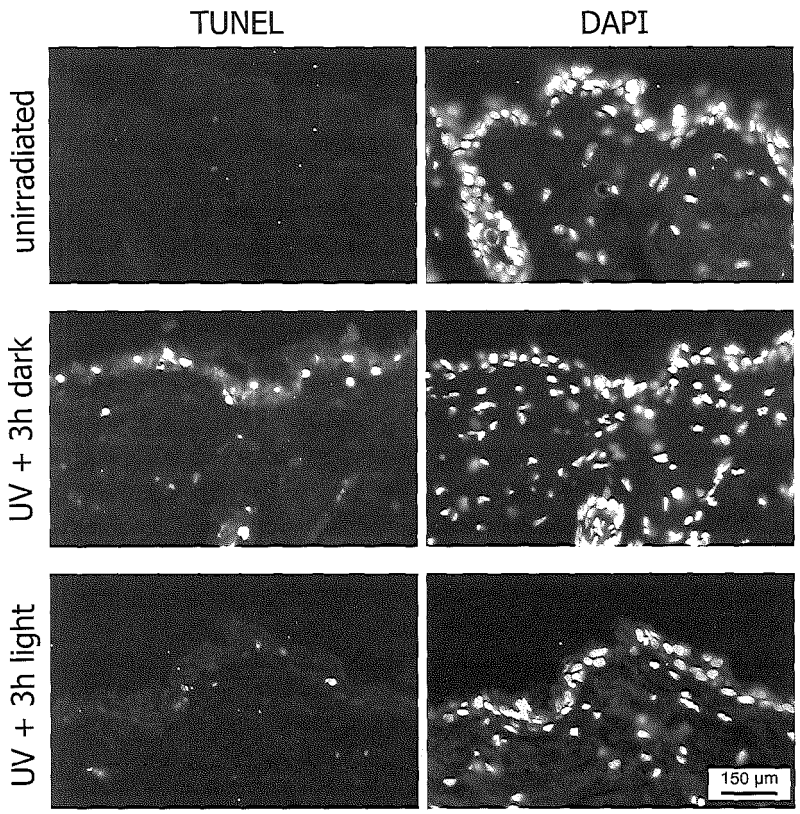

Figure 5
Effect of CPD photoreactivation on UV-induced apoptosis in the skin of β-act-CPD photolyase transgenic mice.
Induction of apoptosis in the depilated dorsal skin of β-act-CPD-1 mice exposed to 1 MED dose of UV-B light, without (middle panels) or with (bottom panels) subsequent treatment of animals with photoreactivating light for 3 h. Non-UV-exposed animals were used as a control (upper panels). Except for the photoreactivation step, animals were kept in the dark immediately after UV treatment. Apoptosis was measured 40 h after UV exposure by a TUNEL assay and nuclei are visualized by DAPI staining.

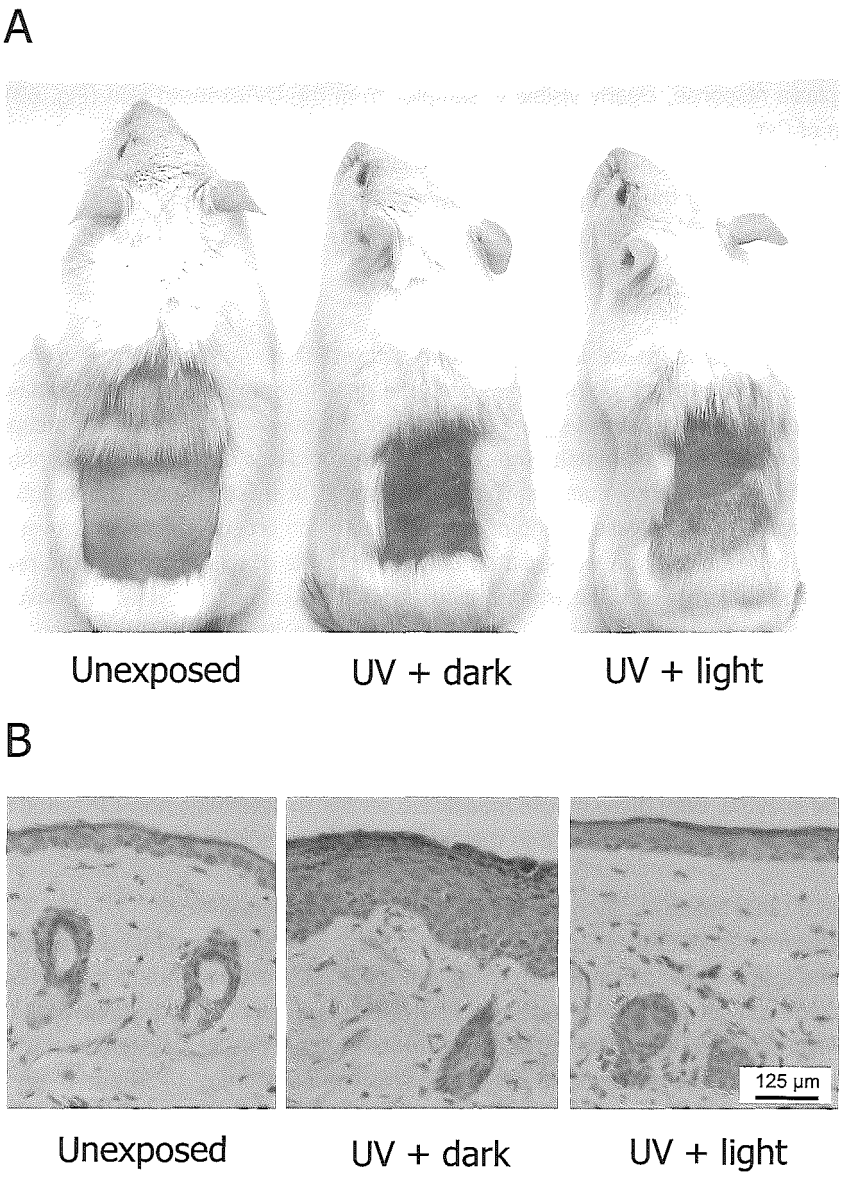

Figure 6
Effect of CPD photoreactivation on UV-induced erythema and hyperplasia in the skin of β-act-CPD photolyase transgenic mice.
The depilated back of β-act-CPD-1 transgenic mice was exposed to UV-B light for four consecutive days (1.5 MED/day) and were either given 3 h of photoreactivating light or kept in the dark. As a control non-UV-exposed animals were used. Animals were sacrificed 3 days after the last exposure and, except for the photoreactivation step, had been kept in the dark throughout the experiment. A. Appearance of the dorsal skin of non-UV-exposed (left), UV-exposed (middle) and UV- and photoreactivating light exposed animals (right), showing clear erythema when photoreactivation of CPDs is omitted. B. shows representative examples of hematoxilin/eosin stained sections of the dorsal skin of the mice shown in panel A. Note the thick epidermal layer in UV-exposed skin that had not received photoreactivating light, indicative for the induction of hyperplasia.

marked contrast, animals exposed to photoreactivating light did not display detectable swelling and only showed slight discoloration of the skin when compared to the unirradiated transgenic mice, used as a control (Fig. 6A). Histological examination of skin sections from these mice revealed a strong epidermal hyperplasia response, clearly visible in samples from the UV-exposed skin (Fig. 6B). Upon photoreactivation of CPDs, however, epidermal hyperplasia was greatly reduced.
Taken together, these data demonstrate that fast removal of CPDs by photoreactivation has a clear protective effect on the skin of the CPD photolyase transgenic mice.

Discussion

Photolyase transgenic mice
We have generated transgenic mouse lines that express the CPD photolyase from *P. tridactylus* in all tissues using the β-actin promoter. Whereas photolyase genes from numerous species have been identified, we have opted for the *Potorous* gene as from an evolutionary point of view marsupials are most closely related to placental mammals. Interestingly, we could not obtain β-act-CPD photolyase transgenic mouse lines at high copy number. This suggests that at least in some tissues ubiquitous overexpression of the CPD photolyase might not be favorable for proper embryonic development. As photolyases have some affinity for unirradiated DNA, high levels of this enzyme in the cell might interfere with key cellular processes like transcription and replication. Importantly, we have shown that the CPD photolyase transgenic mice can specifically and rapidly remove UV-induced CPDs by photoreactivation. This allowed the light-dependent and lesion-specific removal of CPDs from all parts of the skin. The proper functioning of the heterologous photolyase in the mouse implies that the chromophoric co-factors necessary for light-dependent catalysis of dimer splitting (FAD and possibly 5,10-methenyltetrahydrofolate; see Yasui et al. (1994)) are available in mouse cells and get properly incorporated in the enzyme. With these mouse models we set out to determine how CPDs and 6-4PPs, the two main types of UV lesions, contribute to the acute deleterious effects of UV exposure.

Photoreactivation of CPDs increases UV resistance of cultured mouse cells
Using cultured MDFs from β-act-CPD photolyase transgenic mice, we have shown that photoreactivation occurs fast and in a lesion-specific manner. Up to 20 J/m^2 of UV-C, a dose sufficient to kill more than 95% of the cells, the majority of CPD lesions could be removed by photoreactivation within 1 hour, leaving the 6-4PPs unaffected. Within this period, the slower endogenous NER system is only capable of dealing with a fraction of the photolesions, as evident from the amount of CPDs and 6-4PPs left in the cells when photoreactivating light is omitted. Moreover, in rodents NER of CPD lesions is restricted to the transcribed strand of active genes. Yet, we have shown that rapid photoreactivation of CPDs in β-actin CPD photolyase transgenic mouse cells has a pronounced effect on NER, as evident from the approximately 3-fold (light-dependent) reduction in UDS. A comparable reduction in UDS has been observed after microinjection of *Anacystis nidulans* CPD photolyase into cultured human fibroblasts (Zwetsloot et al., 1986; Zwetsloot et al., 1985), which is somewhat surprising as rodent cells, in contrast to human cells, hardly remove CPD lesions by GG-NER. Apparently, TC-NER of CPD lesions constitutes a prominent portion of NER activity in the first hour after UV exposure.
Importantly, we have shown that photoreactivation of CPDs makes the mouse cells more resistant to the killing effects of UV exposure. This observation is in full agreement with previous studies on cultured

cells from organisms with a natural photolyase gene (Rosenstein and Setlow, 1980; van Zeeland et al., 1980; Wade and Trosko, 1983) or photolyase-transfected HeLa cells (Chigancas et al., 2000). The photoreactivating light-mediated reduction in UV sensitivity of transgenic CPD photolyase mouse cells might be explained by a genome-wide reduction in CPD damage load by photoreactivation, and/or an increased clearance of CPDs from the transcribed strand of active genes. Several observations support the latter option. First, overexpression of the *p48* gene in rodent cells markedly stimulates GG-NER-mediated removal of CPDs from the genome, but hardly improves UV survival (Tang et al., 2000). Furthermore, TC-NER deficient cells from patients and rodents with the repair disorder Cockayne syndrome are very sensitive to UV. Moreover, UV-induced apoptosis is primarily triggered by RNA polymerase II stalled at lesions in the template strand of transcribed genes (Conforti et al., 2000; Ljungman and Zhang, 1996; Yamaizumi and Sugano, 1994). Thus, enhanced removal of CPDs from the transcribed strand of active genes appears the major determinant in the improved UV resistance of CPD photolyase mouse cells. Yet, we cannot exclude that also the genome wide photoreactivation of CPDs to some extent contributes to enhanced UV resistance. After removal of CPDs by photoreactivation, the GG-NER and TC-NER pathways can fully concentrate on the repair of 6-4PPs, which therefore may be more efficiently removed from transcribed DNA.

CPD photoreactivation reduces acute skin effects

In this study we have focused on the role of CPDs in the induction of acute effects in the UV-exposed skin: apoptosis, erythema, and hyperplasia. Although some studies point to 6-4PPs as the most cytotoxic lesions (Mitchell, 1988), CPDs are the most abundant damage in the DNA after UV exposure and have been implicated as the cause of UV-induced cell death (Broughton et al., 1990; Chigancas et al., 2000; Nishigaki et al., 1998). Analogous to our results obtained with MDFs, the intact skin of β-actin promoter-driven CPD photolyase transgenic mice shows an efficient light-dependent removal of CPDs by the photolyase, resulting in a reduction of cell death via apoptosis. It must therefore be concluded that the presence of CPDs prominently contributes to the triggering of apoptosis in the skin. Photoreactivation of CPDs in epidermis and dermis also reduces other acute skin effects of (repeated) exposure to UV light, such as erythema, edema and hyperplasia. UV mediated induction of the apoptotic pathway in the intact mouse skin, similar to cultured cells, appears to originate from RNA polymerase II blocking lesions in the transcribed strand. GG-NER deficient XPC mice can accumulate photolesions in their genome without inducing apoptosis and hyperplasia, whereas TC-NER deficient CSB mice (as well as XPA mice with a complete NER defect) already display these acute effects at low UV doses (Brash et al., 2001; van Oosten et al., 2000).

The effect of photoreactivation of CPDs in β-act-CPD photolyase-transgenic mice matches well with previous studies involving animals with an endogenous CPD photolyase. In the South-American opossum *Monodelphis domestica,* photoreactivation has been shown to clearly protect the skin and eye from aforementioned acute effects (Applegate et al., 1985; Ley and Applegate, 1985; Ley et al., 1991). Recently, topical application of CPD photolyase-containing liposomes has been shown to add a higher level of protection to the UV-exposed human skin (Stege et al., 2000). Although on average less than half of the CPDs could be repaired by this approach, several immunological and cytotoxic effects of UV light, such as suppression of ICAM-I, immunosuppression as well as erythema and sunburn-cell formation, could be ablated.

The potential of photolyase mice

In the present study we have focussed on the consequences of photoreactivation of CPDs, and thus the contribution of 6-4PPs and other (non-CPD) types of lesions to acute effects in the mouse skin. It is obvious, however, that an important application of the CPD photolyase transgenic mouse models involves a study of the specific contribution of CPDs and 6-4PPs, as well as their position in the genome (*i.e.* transcribed *vs.* non-transcribed DNA), to other short- and long-term effects of UV exposure like immune suppression, interference with cell cycle progression, mutagenesis, and importantly, carcinogenesis. A recent study by You and coworkers (2001) has shown that photoreactivation of CPDs rather than 6-4PPs reduces the mutation frequency in NER-proficient cultured mouse embryonic fibroblasts carrying the BigBlue© mutation reporter system. Currently, we are in the process of crossing the CPD photolyase mice with totally NER-deficient XPA, GG-NER-deficient XPC, and TC-NER-deficient CSB animals (all in a hairless genetic background), proven to develop skin cancer upon chronic exposure to UV light (Cheo et al., 1997; de Vries et al., 1995; Nakane et al., 1995; van der Horst et al., 1997). The possibility to investigate the effects of different UV lesions by photoreactivation of a specific type of damage is also becoming increasingly valuable now the mechanisms of damage tolerance, including translesion synthesis and recombination repair, are being unraveled (for a review see Lehmann (2000)). Many DNA polymerases have been discovered, each with their own accuracy (and presumably specificity) and ability to perform translesion DNA synthesis on different DNA lesions. Once mutant mouse models for these polymerases become available, they can be crossed with photolyase mice. Detailed analysis of such mice may shed new light on the mutagenic and carcinogenic properties of different types of UV-induced damage in the skin and the role of translesion synthesis therein.

In conclusion, we have complemented a placental mammal with a photoreactivation repair system for light-mediated damage reversal of UV-induced CPD lesions. We have shown that mice expressing a CPD photolyase tolerate higher doses of UV irradiation before developing erythema, sunburn and other short-term effects of UV light. Obviously, it is important to gain complete insight in the fate of the various photolesions, not only the efficiency of repair by TC- and GG-NER, but also the way they are dealt with by the various error-free and error-prone DNA translesion synthesizing polymerases.

Finally, the above objectives clearly highlight the need for the counterpart of our CPD photolyase mice, i.e. animals that express a 6-4PP photolyase and can specifically remove this lesion in a light-dependent manner. Recently we have generated transgenic mice that carry the *Arabidopsis thaliana* 6-4PP photolyase and specifically remove 6-4PPs from their genome in a light-dependent manner. We are currently comparing the properties of CPD photolyase animals with those of 6-4PP photolyase and CPD photolyase/6-4PP photolyase (double) transgenic mice.

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References

Applegate, L.A., Stuart, T.D. and Ley, R.D. (1985) Ultraviolet radiation-induced histopathological changes in the skin of the marsupial Monodelphis domestica. I. The effects of acute and chronic exposures and of photoreactivation treatment. Br J Dermatol, 113, 219-227.

Arnoldus, E.P., Wiegant, J., Noordermeer, I.A., Wessels, J.W., Beverstock, G.C., Grosveld, G.C., van der Ploeg, M. and Raap, A.K. (1990) Detection of the Philadelphia chromosome in interphase nuclei. Cytogenet Cell Genet, 54, 108-111.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell, 40, 359-369.

Bootsma, D., Kraemer,K.H., Cleaver,J.E., Hoeijmakers,J.H.J. (2001) Nucleotide excision repair syndromes: xeroderma pigmentosum, Cockayne syndrome amd trichothiodystrophy. In Scriver, C.R., Beaudet,A.L., Sly,W.S., Valle,D. (ed.), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 677-703.

Brash, D.E., Wikonkal, N.M., Remenyik, E., van der Horst, G.T., Friedberg, E.C., Cheo, D.L., van Steeg, H., Westerman, A. and van Kranen, H.J. (2001) The DNA damage signal for Mdm2 regulation, Trp53 induction, and sunburn cell formation in vivo originates from actively transcribed genes. J Invest Dermatol, 117, 1234-1240.

Broughton, B.C., Lehmann, A.R., Harcourt, S.A., Arlett, C.F., Sarasin, A., Kleijer, W.J., Beemer, F.A., Nairn, R. and Mitchell, D.L. (1990) Relationship between pyrimidine dimers, 6-4 photoproducts, repair synthesis and cell survival: studies using cells from patients with trichothiodystrophy. Mutat Res, 235, 33-40.

Cheo, D.L., Ruven, H.J., Meira, L.B., Hammer, R.E., Burns, D.K., Tappe, N.J., van Zeeland, A.A., Mullenders, L.H. and Friedberg, E.C. (1997) Characterization of defective nucleotide excision repair in XPC mutant mice. Mutat Res, 374, 1-9.

Chigancas, V., Miyaji, E.N., Muotri, A.R., de Fatima Jacysyn, J., Amarante-Mendes, G.P., Yasui, A. and Menck, C.F. (2000) Photorepair prevents ultraviolet-induced apoptosis in human cells expressing the marsupial photolyase gene. Cancer Res, 60, 2458-2463.

Conforti, G., Nardo, T., D'Incalci, M. and Stefanini, M. (2000) Proneness to UV-induced apoptosis in human fibroblasts defective in transcription coupled repair is associated with the lack of Mdm2 transactivation. Oncogene, 19, 2714-2720.

de Laat, W.L., Jaspers, N.G. and Hoeijmakers, J.H. (1999) Molecular mechanism of nucleotide excision repair. Genes Dev, 13, 768-785.

de Vries, A., van Oostrom, C.T., Hofhuis, F.M., Dortant, P.M., Berg, R.J., de Gruijl, F.R., Wester, P.W., van Kreijl, C.F., Capel, P.J., van Steeg, H. and et al. (1995) Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. Nature, 377, 169-173.

Friedberg, E., Walker, G.C., Siede, W. (ed.). (1995) DNA repair and mutagenesis. ASM press, Washington DC.

Hanawalt, P.C., Liu, S.C. and Parsons, C.S. (1981) DNA repair responses in human skin cells. J Invest Dermatol, 77, 86-90.

Hoeijmakers, J.H. (2001) Genome maintenance mechanisms for preventing cancer. Nature, 411, 366-374.

Hogan, B., Beddington,R., Costantini,F. and Lacy,E. (1994) Manipulating the mouse embryo Section E: Production of transgenic mice. In. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 212-252.

Hwang, B.J., Ford, J.M., Hanawalt, P.C. and Chu, G. (1999) Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. Proc Natl Acad Sci U S A, 96, 424-428.

Lehmann, A.R. (2000) Replication of UV-damaged DNA: new insights into links between DNA polymerases, mutagenesis and human disease. Gene, 253, 1-12.

Ley, R.D. and Applegate, L.A. (1985) Ultraviolet radiation-induced histopathologic changes in the skin of the marsupial Monodelphis domestica. II. Quantitative studies of the photoreactivation of induced hyperplasia and sunburn cell formation. J Invest Dermatol, 85, 365-367.

Ley, R.D., Applegate, L.A., Fry, R.J. and Sanchez, A.B. (1991) Photoreactivation of ultraviolet radiation-induced skin and eye tumors of Monodelphis domestica. Cancer Res, 51, 6539-6542.

Ljungman, M. and Zhang, F. (1996) Blockage of RNA polymerase as a possible trigger for u.v. light-induced apoptosis. Oncogene, 13, 823-831.

Mellon, I., Spivak, G. and Hanawalt, P.C. (1987) Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell, 51, 241-249.

Memet, S., Lilienbaum,A. and Israel,A. (1997) Rapid isolation of mouse primary fibroblasts: a tool for the analysis of transgenic mice. Technical Tips Online.

Mitchell, D.L. (1988) The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem Photobiol, 48, 51-57.

Mitchell, D.L., Cleaver, J.E. and Epstein, J.H. (1990) Repair of pyrimidine(6-4)pyrimidone photoproducts in mouse skin. J Invest Dermatol, 95, 55-59.

Mori, T., Nakane, M., Hattori, T., Matsunaga, T., Ihara, M. and Nikaido, O. (1991) Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6-4)photoproduct from the same mouse immunized with ultraviolet-irradiated DNA. Photochem Photobiol, 54, 225-232.

Nakane, H., Takeuchi, S., Yuba, S., Saijo, M., Nakatsu, Y., Murai, H., Nakatsuru, Y., Ishikawa, T., Hirota, S., Kitamura, Y. and et al. (1995) High incidence of ultraviolet-B-or chemical-carcinogen-induced skin tumours in mice lacking the xeroderma pigmentosum group A gene. Nature, 377, 165-168.

Nishigaki, R., Mitani, H. and Shima, A. (1998) Evasion of UVC-induced apoptosis by photorepair of cyclobutane pyrimidine dimers. Exp Cell Res, 244, 43-53.

Qin, X., Zhang, S., Oda, H., Nakatsuru, Y., Shimizu, S., Yamazaki, Y., Nikaido, O. and Ishikawa, T. (1995) Quantitative detection of ultraviolet light-induced photoproducts in mouse skin by immunohistochemistry. Jpn J Cancer Res, 86, 1041-1048.

Rosenstein, B.S. and Setlow, R.B. (1980) DNA repair after ultraviolet irradiation of ICR 2A frog cells. Pyrimidine dimers are long acting blocks to nascent DNA synthesis. Biophys J, 31, 195-205.

Ruven, H.J., Seelen, C.M., Lohman, P.H., van Kranen, H., van Zeeland, A.A. and Mullenders, L.H. (1994) Strand-specific removal of cyclobutane pyrimidine dimers from the p53 gene in the epidermis of UVB-irradiated hairless mice. Oncogene, 9, 3427-3432.

Sijbers, A.M., de Laat, W.L., Ariza, R.R., Biggerstaff, M., Wei, Y.F., Moggs, J.G., Carter, K.C., Shell, B.K., Evans, E., de Jong, M.C., Rademakers, S., de Rooij, J., Jaspers, N.G., Hoeijmakers, J.H. and Wood, R.D. (1996) Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell, 86, 811-822.

Stege, H., Roza, L., Vink, A.A., Grewe, M., Ruzicka, T., Grether-Beck, S. and Krutmann, J. (2000) Enzyme plus light therapy to repair DNA damage in ultraviolet-B-irradiated human skin. Proc Natl Acad Sci U S A, 97, 1790-1795.

Tang, J.Y., Hwang, B.J., Ford, J.M., Hanawalt, P.C. and Chu, G. (2000) Xeroderma pigmentosum p48 gene enhances global genomic repair and suppresses UV-induced mutagenesis. Mol Cell, 5, 737-744.

Tornaletti, S. and Hanawalt, P.C. (1999) Effect of DNA lesions on transcription elongation. Biochimie, 81, 139-146.

van der Horst, G.T., van Steeg, H., Berg, R.J., van Gool, A.J., de Wit, J., Weeda, G., Morreau, H., Beems, R.B., van Kreijl, C.F., de Gruijl, F.R., Bootsma, D. and Hoeijmakers, J.H. (1997) Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. Cell, 89, 425-435.

van Hoffen, A., Natarajan, A.T., Mayne, L.V., van Zeeland, A.A., Mullenders, L.H. and Venema, J. (1993) Deficient repair of the transcribed strand of active genes in Cockayne's syndrome cells. Nucleic Acids Res, 21, 5890-5895.

van Hoffen, A., Venema, J., Meschini, R., van Zeeland, A.A. and Mullenders, L.H. (1995) Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6-4 photoproducts with equal efficiency and in a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts. Embo J, 14, 360-367.

van Oosten, M., Rebel, H., Friedberg, E.C., van Steeg, H., van der Horst, G.T., van Kranen, H.J., Westerman, A., van Zeeland, A.A., Mullenders, L.H. and de Gruijl, F.R. (2000) Differential role of transcription-coupled repair in UVB-induced G2 arrest and apoptosis in mouse epidermis. Proc Natl Acad Sci U S A, 97, 11268-11273.

van Zeeland, A.A., Natarajan, A.T., Verdegaal-Immerzeel, E.A. and Filon, A.R. (1980) Photoreactivation of UV induced cell killing, chromosome aberrations, sister chromatid exchanges, mutations and pyrimidine dimers in Xenopus laevis fibroblasts. Mol Gen Genet, 180, 495-500.

Venema, J., Mullenders, L.H., Natarajan, A.T., van Zeeland, A.A. and Mayne, L.V. (1990) The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. Proc Natl Acad Sci U S A, 87, 4707-4711.

Vermeulen, W., Scott, R. J., Rodgers, S., Muller, H. J., Cole, J., Arlett, C. F., Kleijer, W. J., Bootsma, D., Hoeijmakers, J. H., Weeda, G. (1994) Clinical heterogeneity within xeroderma pigmentosum associated with mutations in the DNA repair and transcription gene ERCC3. Am J Hum Genet, 54, 191-200

Wade, M.H. and Trosko, J.E. (1983) Enhanced survival and decreased mutation frequency after photoreactivation of UV damage in rat kangaroo cells. Mutat Res, 112, 231-243.

Wood, R.D. (1999) DNA repair. Variants on a theme. Nature, 399, 639-640.

Yamaizumi, M. and Sugano, T. (1994) U.v.-induced nuclear accumulation of p53 is evoked through DNA damage of actively transcribed genes independent of the cell cycle. Oncogene, 9, 2775-2784.

Yasui, A., Eker, A.P., Yasuhira, S., Yajima, H., Kobayashi, T., Takao, M. and Oikawa, A. (1994) A new class of DNA photolyases present in various organisms including aplacental mammals. Embo J, 13, 6143-6151.

Yasui, A., Eker,A.P.M. (1997) DNA photolyases. In Nickoloff, J.A., Hoekstra,M.F. (ed.), DNA Damage and Repair: Biochemistry, Genetics and Cell Biology. Humana Press, Totowa, Vol. 2, pp. 9-32.

You, Y.H., Lee, D.H., Yoon, J.H., Nakajima, S., Yasui, A. and Pfeifer, G.P. (2001) Cyclobutane pyrimidine dimers are responsible for the vast majority of mutations induced by UVB irradiation in mammalian cells. J Biol Chem, 276, 44688-44694.

Zwetsloot, J.C., Hoeymakers, J.H., Vermeulen, W., Eker, A.P. and Bootsma, D. (1986) Unscheduled DNA synthesis in xeroderma pigmentosum cells after microinjection of yeast photoreactivating enzyme. Mutat Res, 165, 109-115.

Zwetsloot, J.C., Vermeulen, W., Hoeijmakers, J.H., Yasui, A., Eker, A.P. and Bootsma, D. (1985) Microinjected photoreactivating enzymes from Anacystis and Saccharomyces monomerize dimers in chromatin of human cells. Mutat Res, 146, 71-77.

Chapter Three

Photolyase transgenic mice reveal a prominent role of cyclobutane pyrimidine dimer-type of photolesions in UV-induced mutagenesis

Photolyase transgenic mice reveal a prominent role of cyclobutane pyrimidine dimer-type of photolesions in UV-induced mutagenesis

To date, the hazardous effects of exposure to sunlight received much attention due to the increasing incidence of skin carcinomas. In particular, UV-induced DNA lesions are held responsible for this. To assess the contribution of UV-induced DNA lesions (cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs)) to the detrimental effects of UV light, we generated mice transgenically expressing 6-4PP-photolyase. Photolyase enzymes specifically repair UV-induced lesions using visible light as a source of energy. Placental mammals, however, lack these enzymes. We show expression of 6-4PP-photolyase and, importantly, light-dependent repair of 6-4PPs in cultured cells and mouse skin. With the previously generated CPD-photolyase mice we now have the unique opportunity to investigate the relative contribution of CPDs versus 6-4PPs in UV-responses. Here, we provide evidence that CPD lesions are the main cause of UV-induced acute skin effects including erythema and hyperplasia. In addition, we demonstrate that enhanced removal of CPDs by photoreactivation moderately reduces the mutation load in the skin while resulting in an almost complete protection from the induction of pre-carcinogenic lesions. Our data shed light on the mechanisms behind the detrimental effects of UV, highlighting that the photolyase enzymes, possibly lost in mammalian evolution, could offer protection from sunlight-induced skin cancer.

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Introduction

The integrity of the genome is continuously threatened by a variety of endogenous (e.g. oxidative stress) and environmental agents that damage the DNA. Exposure of the skin to ultraviolet (UV) light induces cross-links between the base moieties of adjacent pyrimidines, resulting in the formation of two main types of dimers: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) (Mitchell, 1988b). These photolesions, like other categories of DNA damage, can interfere with the vital cellular processes of transcription and replication, resulting in reduction of RNA synthesis, arrest of cell cycle progression, and induction of apoptosis. Acute skin effects of UV light involve induction of erythema (commonly known as sunburn), edema and hyperplasia. Importantly, replication of UV-damaged DNA can induce mutations that ultimately may lead to the formation of skin cancer (Friedberg et al., 1995).

In mammals, the deleterious effects of the main photolesions (and a wide range of other helix-distorting DNA lesions) are kept within bounds by Nucleotide Excision Repair (NER), one of the repair systems that continuously guard the genome and remove lesions from the DNA (Friedberg, 1995; Hoeijmakers, 2001). The relevance of this repair mechanism for human health is highlighted by the inherited disorders xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy (TTD), in which mutations in NER genes cause increased sensitivity of the skin to UV and, in the case of xeroderma pigmentosum, an over 1000-fold increased susceptibility to sunlight induced skin cancer (Bootsma et al., 2001).

NER proceeds via a complex multi-step reaction, involving the concerted action of at least 30 proteins and encompassing the following stages: (I) damage recognition, (II) chromatin remodeling, (III) DNA unwinding, (IV) excision of a small oligonucleotide containing the damage, (V) gap-filling DNA synthesis, and (VI) strand ligation (de Laat et al., 1999; Friedberg, 1995; Hoeijmakers, 2001; Wood, 1996). NER consists of two sub-pathways: global genome NER (GG-NER) and transcription coupled NER (TC-NER). GG-NER can repair helix-distorting lesions throughout the entire genome, but is hampered by the fact that certain types of damage are less well recognized by the main (GG-NER-specific) damage sensing XPC-HR23B complex, and accordingly less efficiently repaired. To prevent that such lesions, when present in the transcribed strand of active genes, can block transcription elongation for too long, TC-NER can preferentially remove transcription-blocking DNA injury using stalled RNA polymerase II as the damage sensor (Bohr et al., 1985; Mellon et al., 1987; van Hoffen et al., 1993). Thus, TC-NER acts as an efficient back-up system for lesions that are slowly or not at all repaired by GG-NER.

Due to the distinct helix-deforming characteristics of UV-induced CPDs and 6-4PPs, considerable differences exist in the efficiency at which these photoproducts are processed by the mammalian NER system. Human and rodent cells, when exposed to a moderate dose of UV-light, efficiently remove helix-distorting 6-4PPs from their genome (>75% within 4 hr) mainly by GG-NER (Mitchell, 1988b; van Hoffen et al., 1993). On the other hand, GG-NER-mediated repair of the only mildly helix-distorting CPD lesions proceeds considerably slower in human fibroblasts (>60% repair in 24 h) involving the XPE-UV-DDB2 auxiliary complex, and is virtually absent in rodent cells (Bohr et al., 1985; Mitchell, 1988b). Apart from the evolutionary highly conserved NER pathway, nature has evolved an additional repair mechanism specifically for repair of UV-induced DNA lesions, called photoreactivation. The latter reaction involves photolyases, enzymes that bind to CPDs or 6-4PPs in a lesion-specific manner and

rapidly revert the dimer back to undamaged bases, using the energy of visible light (Yasui and Eker, 1997). Photolyases are highly conserved and are present in various kingdoms including bacteria, lower eukaryotes, plants and many animals (including marsupials), rendering photoreactivation likely to be a very ancient repair system that probably has existed from very early on in evolution. Intriguingly, placental mammals do not possess photolyases (Todo et al., 1996; van der Spek et al., 1996). Evolution appears to have recruited the 6-4PP-photolyase gene for the purpose of driving the biological clock (Okamura et al., 1999; van der Horst et al., 1999). Possibly, placental mammals have gone through a nocturnal phase during evolution and consequently have lost this important genome protection mechanism against sunlight. As a consequence, man and mice rely solely on the complex NER system for removal of UV-induced damage.

Since the number of CPDs induced by UV is approximately three fold higher than the number of 6-4PPs, much of the mutagenic and carcinogenic potential of the UV component of sunlight is expected to arise from CPDs (Broughton et al., 1990; Chigancas et al., 2002; Chigancas et al., 2000; Nishigaki et al., 1998). However, studies with site-specific lesions have shown the 6-4PP to be potentially much more mutagenic (Mitchell, 1988b). As 6-4PPs and CPDs are induced simultaneously at similar locations and the NER system eliminates both (albeit with different kinetics), it has been impossible to determine in an unequivocal fashion the relative contribution of CPDs and 6-4PPs to cytotoxicity, mutagenesis, and carcinogenesis at the level of the intact organism. Making use of the substrate-specificity and light-dependent action of photolyases and the absence of this repair mechanism in placental mammals, we have recently started to explore the biological effects of CPDs and 6-4PPs in the skin of the intact animal. Using transgenic mice that ubiquitously express a marsupial CPD-photolyase, we have shown that accelerated repair of CPD lesions by photoreactivation dramatically reduces acute UV-mediated skin effects like erythema (sunburn), hyperplasia and apoptosis (Schul et al., 2002). This increased tolerance of animals to higher doses of UV indicates a crucial role for CPDs in these processes.

Evidently, our approach also requires the counterpart of the CPD-photolyase transgenic mouse; a mouse expressing a pyrimidine (6-4) pyrimidone-specific photolyase (6-4PP-PL), enabling light-dependent investigation of the consequences of this specific lesion. Here, we report the generation of mice expressing the *Arabidopsis thaliana* 6-4PP-PL from the ubiquitous β-actin promoter. This mouse, in combination with the CPD-photolyase mice and animals expressing both CPD-PL and 6-4PP-PL, offers an exceptional opportunity to examine the individual effects of CPDs versus 6-4PPs in UV-induced responses such as mutagenesis and carcinogenesis.

Materials and Methods

Generation of 6-4PP photolyase transgenic mice

The construct for the generation of 6-4PP-PL transgenic mice was cloned in the vector pSP72 (Promega) and contained the chicken β-actin promoter with the CMV enhancer (from pCY4B, kindly provided by Dr J. Miyazaki, Osaka, Japan), followed by the *Arabidopsis thaliana* 6-4PP-PL cDNA (Nakajima et al., 1998). At the 3′ end, exon 2 (the last 22 bp), intron 2, exon 3 and the 3′ untranslated region (including the polyadenylation signal) of the human *β-globin* gene were inserted. The expression constructs

were excised from the plasmid using *SalI*, separated from vector DNA by agarose gel electrophoresis, isolated from the gel with the GeneClean II Kit (Bio 101) and further purified using elutip-D-mini columns (Schleicher and Schuell, Germany). The fragment was dissolved in injection buffer (10 mM Tris-HCl pH 7.5, 0.08 mM EDTA) and injected in the pronucleus of fertilized eggs derived from FVB/N intercrosses as described previously (Hogan, 1994).
Transgenic animals were identified by Southern blot analysis of genomic tail DNA, using the 6-4PP-PL cDNA as a probe. To estimate the number of integrated copies, equal amounts of genomic DNA from transgenic mice were subjected to Southern blot analysis. As a standard, we used equal amounts of genomic tail DNA supplemented with 0, 10, 30 or 100 pg of the corresponding 6-4PP-PL expression construct. The hybridization signal obtained with the 6-4PP-PL cDNA probe was quantified using a Molecular Dynamics PhosphorImager and ImageQuant software. After comparison of signal intensities, the transgene copy number was estimated using the supplemented reference samples. The site of chromosomal integration was determined by FISH analysis of metaphase chromosome spreads of transgenic mouse lines as described by Arnoldus et al. (1990) using the 6-4PP-PL cDNA probe.
Routine genotyping of mice was performed by PCR analysis. Primer set 5'-GCACGATTCAGCAAGCAAGG-3' (forward primer) and 5'-CGGTACCTCTACCTATTTGAGTTTTG-3' (reverse primer) were used to amplify a 200 basepair fragment of the 6-4PP-PL coding region.

RNA isolation and RT-PCR
Mouse skin RNA was isolated and cDNA synthesis was performed using Superscript II RNAseH-reverse transcriptase (RT; Life Technologies) according to the protocol of the supplier. A PCR reaction was performed on the cDNA using a forward primer in the photolyase transgene (5'-GCACGATTCAGCAAGCAAGG-3') and a reverse primer in exon 3 of the *β-globin* gene (5'-TGGACAGCAAGAAAGCGAG-3'). The presence of introns in the β-globin moiety of the photolyase transgene allows discrimination between the cDNA-derived PCR product and possible genomic DNA contamination.

Isolation and culturing of dermal fibroblasts
Primary MDFs were isolated according to the protocol of Mémet et al. (Mémet, 1997). In short, after removal of hairs from the back of a mouse, a small piece of skin (0.3 cm^2) was isolated. The tissue was minced, transferred to a phosphate-buffered saline (PBS) solution containing 0.125% trypsin, 1 mg/ml collagenase type V and 0.3 mg/ml hyaluronidase, and incubated at 37°C for 15 min, after which the cell suspension was collected. A fresh enzyme solution was added to the remaining pieces of skin and the procedure was repeated twice. Cell suspensions were centrifuged after which the pellets were resuspended in medium (40% Dulbecco's modified Eagle's medium (DMEM), 40% HAM-F10, 20% fetal calf serum (FCS), 1% penicillin and streptomycin). Cells were transferred to 10 cm dishes and cultured at 37°C in 5% CO_2.

Immunological procedures

Generation of antibodies

A fusion protein of *A. thaliana* 6-4PP-PL and maltose binding protein (MBP) was overexpressed in *E. coli*, purified, digested with factor Xa and used for immunization of two rabbits. The serum was affinity purified against membrane-immobilized 6-4PP-PL. The generation of antibodies TDM2 and 64M2 recognizing the CPD and 6-4 photoproducts respectively is described in Mori et al. (1991).

Western blot analysis

Protein extracts were prepared by harvesting MDFs in sample buffer (2% SDS, 0.1 M Tris (pH 6.8), 4% β-mercapthoethanol, 15% glycerol) containing 1 mM PMSF. The protein extract was heat denatured, subjected to SDS-PAGE on a 10% gel and transferred to nitrocellulose. The nitrocellulose blot was incubated for 1 h in non-fat milk, washed with PBGTNa (PBS containing 0.5% BSA (Sigma), 0.05% gelatin, 0.05% Tween-20, and 300 mM NaCl), incubated overnight with the anti-6-4PP-photolyase polyclonal antibody diluted in PBGTNa, washed 4 x 5 min in PBGTNa, incubated 2 h with the secondary antibody goat anti-rabbit coupled to alkaline phosphatase, washed 2 x 5 min with PBGTNa and 2 x 5 min with PBS. Blots were stained using the BCIP/NBT method (BioRad).

Immunofluorescent detection of 6-4PP photolyase

Coverslips with attached MDFs were rinsed once in PBS and fixed with 2% paraformaldehyde in PBS for 15 min. After fixation cells were washed twice with PBS and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min. Cells were subsequently washed twice in PBS, incubated in PBS containing 100 mM glycine (Sigma) for 10 min, incubated in PBG (PBS containing 0.5% BSA (Sigma) and 0.05% gelatin) for 10 min, and incubated overnight at 4°C with the anti-6-4PP-photolyase polyclonal antibody diluted in PBG. The coverslips were washed 4 x 5 min with PBG and incubated with goat anti-rabbit coupled to FITC for 2 h, washed 2 x 5 min with PBG and 2 x 5 min with PBS. Finally, coverslips were incubated for 5 min with DAPI (0.2 µg/ml) in PBS, washed with PBS, and mounted in Vectashield (Vector Laboratories, USA).

Immunofluorescent detection of CPDs and 6-4PPs in MDFs

Cells were grown on coverslips and washed with PBS, exposed to 20 J/m^2 UV-C (Philips TUV germicidal lamp) and subsequently kept in Hank's buffer (137 mM NaCl, 5.4 mM KCl, 4.4 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 0.81 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 1 g/l glucose, pH 7.4). Photoreactivation was performed by exposing cells for 1 h to light from 4 white fluorescent tubes (Philips TLD 18W/54) at a distance of 15 cm and shielded by a 5 mm glass filter. Non-photoreactivated cells were given the same treatment except that dishes were covered with two layers of aluminum foil and put under the same fluorescent lamps. Immunocytochemical staining of CPDs and 6-4PPs using the antibodies TDM2 and 64M2 respectively was performed as described previously (Schul et al., 2002).

DNA repair assays

UV survival

UV radiation sensitivity was determined as described previously (Sijbers et al., 1996), except that a photoreactivation step was incorporated in the assay. MDFs were grown in 6-well culture plates (Costar), washed with PBS, exposed to different doses of UV-C light (Philips TUV germicidal lamp) and subsequently kept in Hank's buffer. Next, the cells were either exposed to photoreactivating light or kept in the dark for 1 h as described above, after which cell culturing was continued in the dark. After

Results

Generation of 6-4PP-specific photolyase transgenic mouse and cell lines

To obtain mice ubiquitously expressing a 6-4PP-specific photolyase transgene (β-act-6-4PP-PL mice), we have generated a construct containing the *Arabidopsis thaliana* 6-4PP photolyase cDNA (Nakajima et al., 1998), preceded by the chicken β-actin promoter and CMV enhancer, and completed with the 3' part of the human β-globin gene (i.e. exons 2 and 3, intron 2, the 3' untranslated region and the polyadenylation signal) behind the photolyase cDNA (Figure 1A). After pronuclear injection of the construct in fertilized oocytes and transfer of the injected eggs to pseudopregnant foster mothers, we obtained several independent β-act-6-4PP-PL transgenic founder lines for further breeding. In all cases the transgene was transmitted in a Mendelian fashion and heterozygous offspring was fertile and did not show an overt phenotype (up to an age of 6 months). We further investigated a 6-4PP-PL mouse line containing three copies of the transgene (determined by Southern blot analysis; data not shown). FISH analysis of metaphase chromosome spreads of mouse dermal fibroblasts (MDFs) from this mouse line revealed integration of the transgene at chromosome 7F3-4 (data not shown). Unless stated otherwise, in this article 6-4PP-PL refers to the ubiquitously expressing β-act-6-4PP-PL.

To investigate whether the 6-4PP-PL transgene was expressed, we first performed RT-PCR analysis on total skin RNA (for details, see experimental procedures). The presence of a 300 bp PCR fragment revealed proper transcription and splicing of the transgene (Figure 1B). Immunoblot analysis of proteins extracted from wildtype and 6-4PP-PL transgenic MDFs using a polyclonal antibody raised against the 6-4PP-PL, showed a band of the expected size (60 kD) in transgenic cells only (Figure 1C), whereas immunocytochemical analysis of these cells revealed a fluorescent signal in the nucleus (Figure 1D). Taken together, these data demonstrate we have successfully generated a transgenic mouse line that expresses the full length 6-4PP-PL protein in the nucleus.

Specific photoreactivation of 6-4 photoproducts in cultured 6-4PP-PL dermal fibroblasts

To investigate whether expression of the 6-4PP-PL transgene results in light-dependent and specific repair of 6-4PPs, 6-4PP-PL MDFs were subjected to an immunocytochemical repair assay using antibodies specific for CPDs or 6-4PPs (Mori et al., 1991). As shown in Figure 2, immediately after exposure of cells to 20 J/m^2 UV-C bright immunofluorescent signals were detected in the nucleus, indicating induction of both CPDs and 6-4PPs. When cells were subsequently kept in the dark, thus withholding the photolyase from its source of energy, both photoproducts remained present. This indicates that, in line with previous reports (Bohr et al., 1985; Mitchell, 1988a), NER-mediated removal of CPD and 6-4PP lesions is relatively slow compared to photoreactivation, leaving most of the lesions unrepaired after one hour. In marked contrast, however, exposure of UV-irradiated 6-4PP-PL MDFs to 1 hour of photoreactivating light resulted in a strong reduction of 6-4PP levels, whereas levels of CPDs remained unchanged. These data demonstrate that the *A. thaliana* 6-4PP-PL efficiently and specifically removes 6-4PPs in this transgenic mouse system.

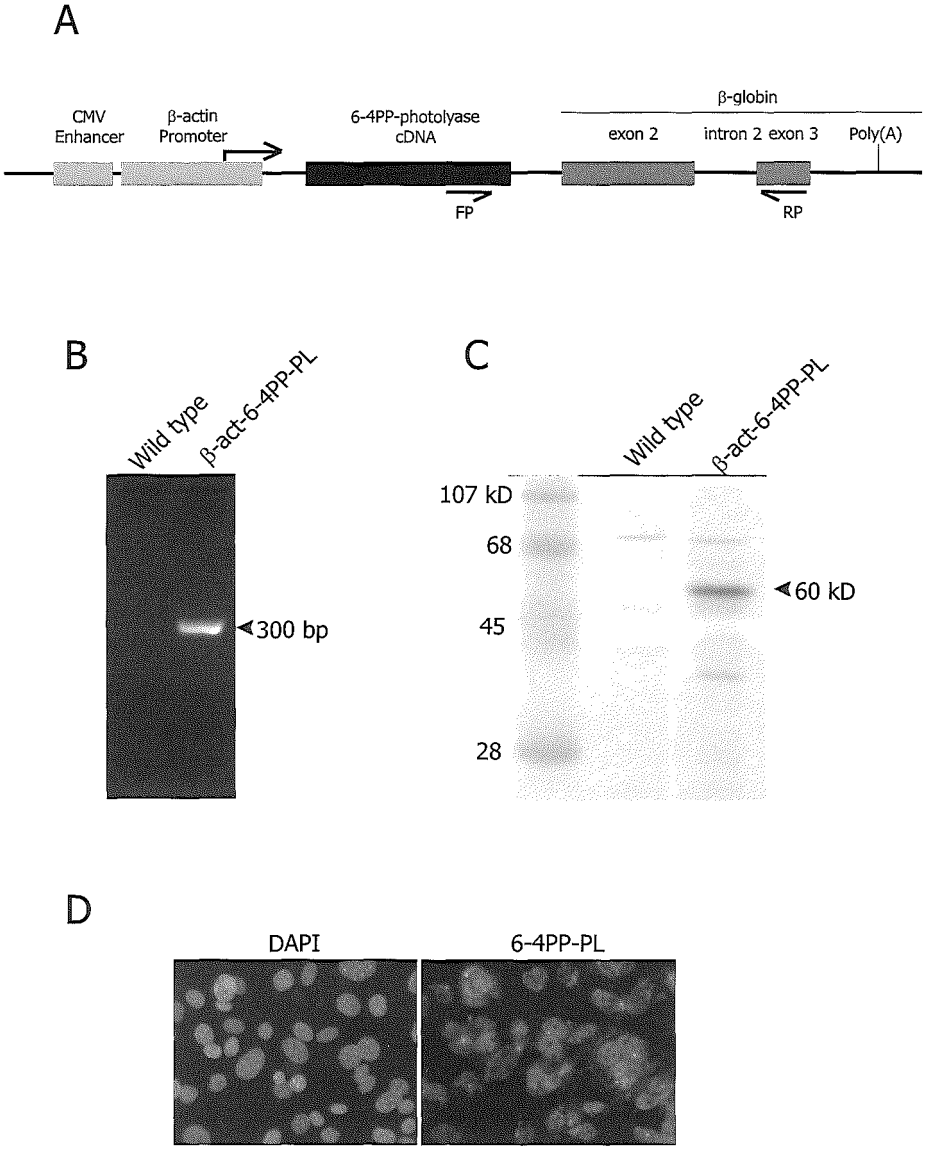


Figure 1
Expression of *Arabidopsis thaliana* 6-4PP photolyase in transgenic mice.
A. Expression construct for the generation of transgenic mice, containing the *Arabidopsis thaliana* 6-4PP-photolyase cDNA, the CMV enhancer and chicken β-actin promoter and the human β-globin exon 2, intron 2 and exon 3 including the poly(A) signal. Arrows indicate primers used for the RT-PCR: FP: forward primer, RP: reverse primer. B. RT-PCR on skin extracts shows the presence of photolyase mRNA in a transgenic photolyase line. C. Immunoblot on protein extracts from cultured fibroblasts shows the presence of the 60 kD 6-4PP photolyase. D. Immunocytochemical detection of 6-4PP photolyase in cultured fibroblasts. Nuclei are visualized by DAPI staining.

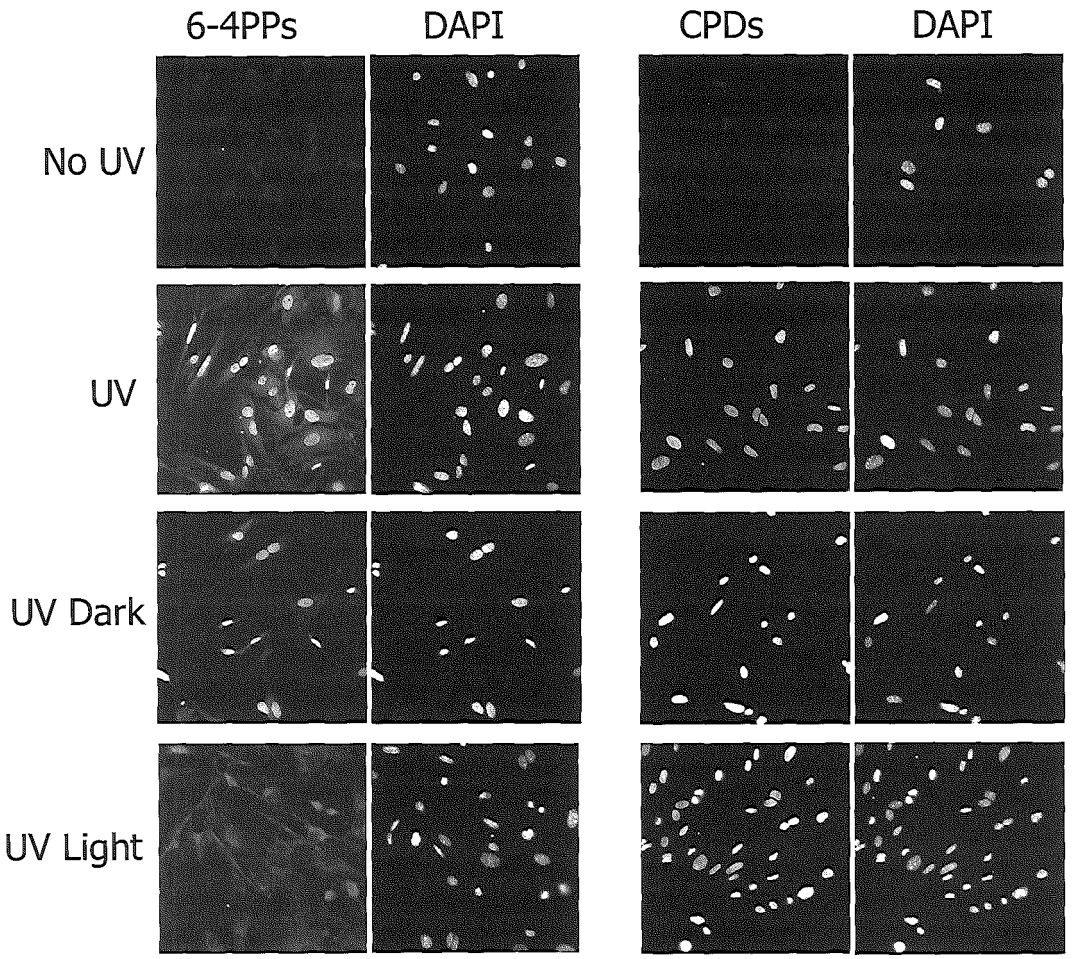


Figure 2
Photoreactivation of 6-4PPs in cultured transgenic fibroblasts.
Induction of CPD and 6-4PP lesions in cultured MDFs from 6-4PP-PL transgenic mice by 20 J/m^2 of UV-C light and subsequent exposure to photoreactivating light for 1 hour. Photolesions were detected by immunofluorescent labeling using CPD- or 6-4PP-specific antibodies and FITC-conjugated secondary antibodies. Nuclei are visualized by DAPI staining.

Effects of CPD and/or 6-4PP photoreactivation on UV-exposed dermal fibroblasts

Prior to investigating the contribution of 6-4PPs to UV-induced cell death, we have completed our panel of CPD-PL and 6-4PP-PL transgenic MDF lines with the isolation of cell lines expressing both photolyases. To this end, 6-4PP-PL mice were bred with CPD-PL mice to obtain double transgenic animals. Like the parent transgenic mouse lines, CPD-PL/6-4PP-PL mice were born in a Mendelian ratio and lack an apparent spontaneous phenotype. As expected on the basis of results obtained with single transgenic cell-lines, UV-irradiated MDFs isolated from these mice show light-dependent removal of both CPDs and 6-4PPs (data not shown).

The UV sensitivity of the MDF lines was determined using the ^{3}H-thymidine incorporation-based UV survival assay (Sijbers et al., 1996). Figure 3A shows the fraction of surviving CPD-PL, 6-4PP-

PL and CPD-PL/6-4PP-PL cells upon exposure to increasing doses of UV-C. In line with our previous observations, CPD-PL MDFs display wildtype UV-sensitivity when kept in the dark, while fast repair of CPDs by exposure of cells to photoreactivating light dramatically reduces the level of cell death, as evident from the enhanced cellular survival (Schul et al., 2002). Similarly, the newly generated 6-4PP-PL and CPD-PL/6-4PP-PL MDFs demonstrate a UV-sensitivity in the wildtype range when kept in the dark. Importantly, photoreactivation of 6-4PPs in UV-exposed 6-4PP-PL MDFs did not result in an enhanced cellular survival.

Similarly, photoreactivation of both CPDs and 6-4PPs in UV-exposed CPD-PL/6-4PP-PL MDFs reveals a UV-sensitivity comparable to that observed in CPD-PL cells. Thus, in the UV-dose range tested no additive effect of photoreactivation of 6-4PPs could be observed, pointing towards CPDs as the major trigger for UV-induced cell death.

Depending on the dose, UV-exposed fibroblasts undergo a transient reduction in RNA synthesis. To investigate which class of UV-induced lesions is responsible for this reduction in the overall rate of transcription, we have measured the relative incorporation of ^{3}H-uridine (indicative for the level of RNA synthesis) in UV-treated photolyase transgenic cells that were either kept in the dark, or exposed to photoreactivating light. As expected, when kept in the dark, CPD-PL, 6-4PP-PL and CPD-PL/6-4PP-PL MDFs showed a reduced level of RNA synthesis 15 hours after exposure to 10 J/m^2 of UV-C (Figure 3B). However, photoreactivation of CPDs in UV-exposed CPD-PL cells largely reduced the drop in RNA synthesis. In marked contrast, exposure of 6-4PP-PL cells to photoreactivating light did not affect the UV-mediated inhibition of RNA synthesis. When UV-exposed CPD-PL/6-4PP-PL MDFs were treated with photoreactivating light, we noticed an attenuation of the inhibiting effect of UV on RNA synthesis, comparable to that observed when only CPDs were photoreactivated (Figure 3B). Taken together, these findings suggest that UV-induced transcription inhibition

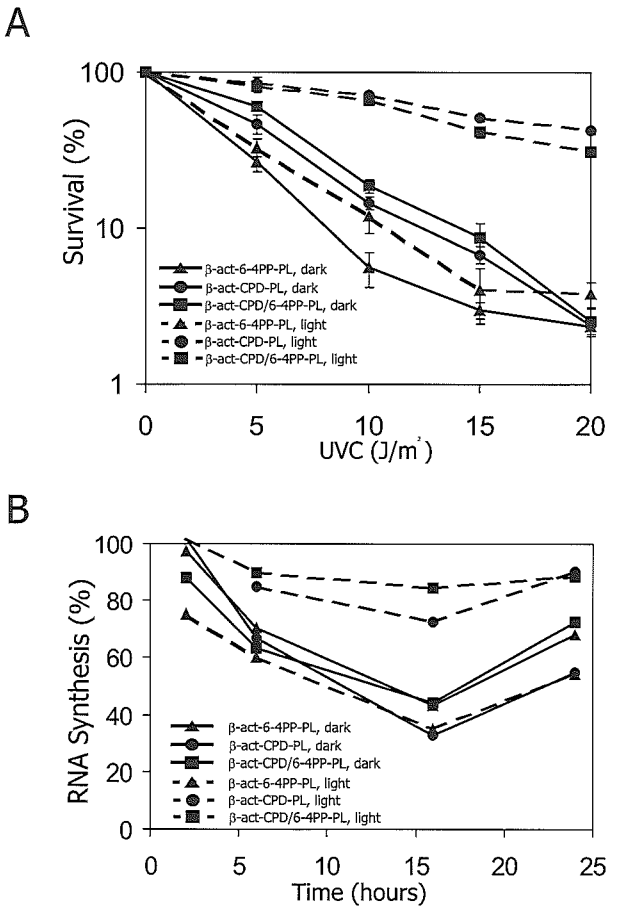


Figure 3
Effect of photoreactivation of photoproducts on cellular survival and RNA synthesis.
A. UV survival of CPD-PL, 6-4PP-PL and double transgenic (CPD-PL/6-4PP-PL) MDFs upon increasing doses of UV-C light, with or without subsequent exposure to photoreactivating light for one hour. B. RNA synthesis of CPD-PL, 6-4PP-PL and double transgenic (CPD-PL/6-4PP-PL) MDFs followed in time after a single dose of 10 J/m^2 of UV-C light with or without subsequent exposure to photoreactivating light.

and cell death in NER-proficient rodent fibroblasts can be mainly ascribed to CPD lesions in the DNA, whereas 6-4PPs appear of minor importance in these processes.

Contribution of 6-4 photoproducts to induction of acute skin effects

Previously, we have shown that photoreactivation of CPD lesions in UV-exposed CPD-PL mice markedly suppresses acute skin effects (Schul et al., 2002). Prior to studying the consequence of rapid removal of 6-4PPs on acute UV-mediated skin effects, we tested whether transgenic 6-4PP-PL mice are able to remove 6-4PPs from the dermis and epidermis. To this end, the immunocytochemical assay for detection of 6-4PPs was optimized for use in tissue (skin) sections (for details, see experimental procedures). When the depilated back of 6-4PP-PL mice was exposed to 1 MED (minimal erythemal dose) of UV-B and animals were subsequently kept in the dark for 3 hours, we observed bright fluorescent nuclear signals in epidermal and (upper) dermal cells, showing the presence of 6-4PPs (Figure 4, top). Despite a functional NER mechanism in these animals, 6-4PP levels were not detectably reduced within these three hours. Importantly, exposure of UV-treated animals to photoreactivating light for 3 hours appeared sufficient to cause a strong reduction in 6-4PP levels in dermis and epidermis (Figure 4, bottom). Immunohistochemical staining with CPD antibodies revealed that photoreactivating light did not affect the level of CPD lesions in UV-exposed 6-4PP-PL mice (data not shown). In conclusion, the *A. thaliana* 6-4PP-PL is active in epidermal as well as dermal cells and can specifically photoreactivate the majority of 6-4PPs within 3 hours, leaving the CPDs unaffected.

Exposure of skin to UV light results in the formation of so called sunburn cells, due to an apoptotic response of keratinocytes. Apoptosis has been shown to result from damage in transcribed genes that block RNA polymerase during transcription (Conforti et al., 2000; Ljungman and Zhang, 1996; Yamaizumi and Sugano, 1994). Previously, we have shown that photoreactivation of CPD lesions largely ablates the UV-induced apoptosis in the mouse epidermis (Schul et al., 2002). To investigate whether rapid removal of 6-4PPs also suppresses induction of apoptosis, we have exposed 6-4PP-PL mice to

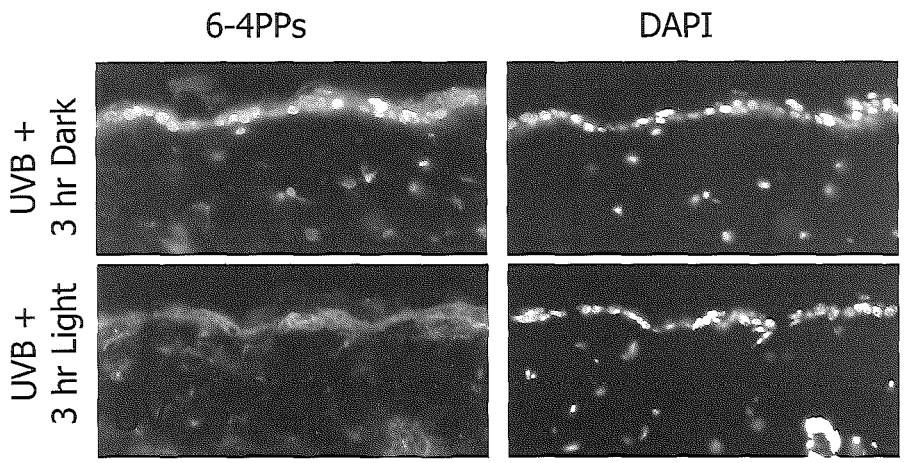


Figure 4
Photoreactivation of 6-4PPs in the skin of 6-4PP photolyase transgenic mice.
Induction of 6-4PPs in the depilated dorsal skin of 6-4PP-PL mice by 1 MED UV-B light and the effect of subsequent exposure to 3 hours of photoreactivating light. Photolesions were detected by immunofluorescent labeling using 6-4PP-specific antibodies and FITC-conjugated secondary antibodies. Nuclei are visualized by DAPI staining.

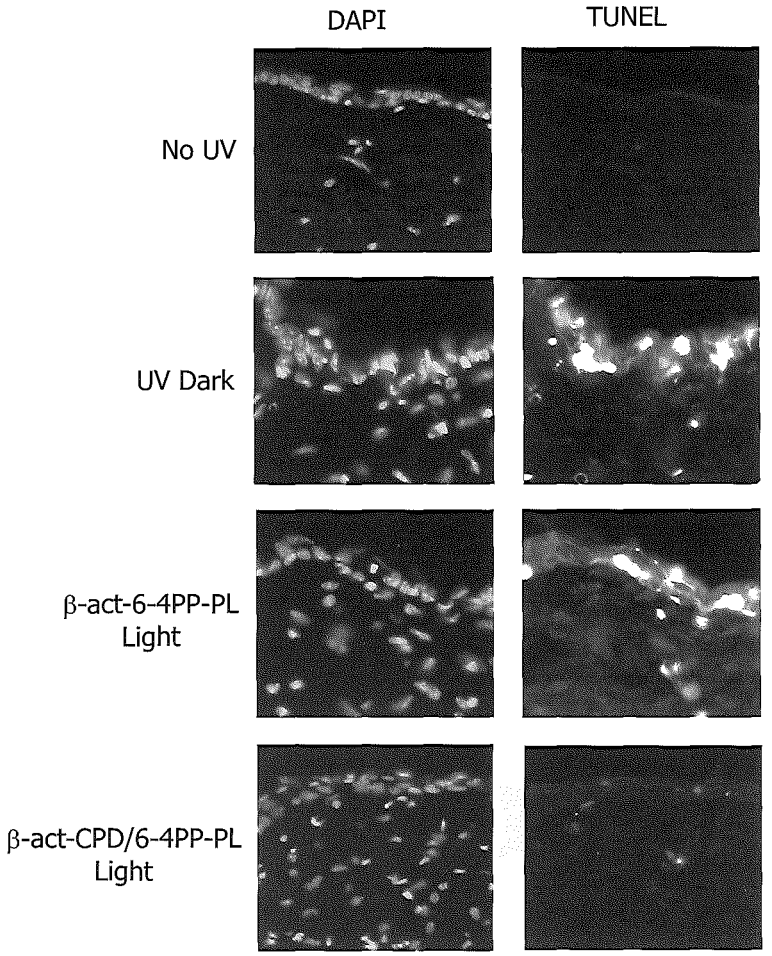
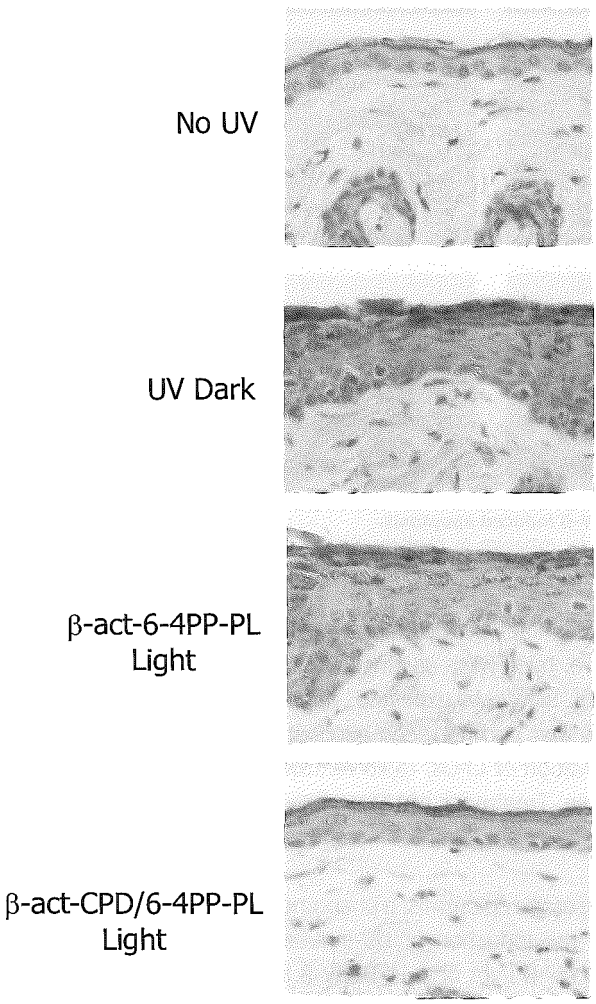


Figure 5
Effect of photoreactivation of 6-4PPs and CPDs on UV-B induced apoptosis.
Photolyase mice were exposed to 1 MED UV-B, followed by exposure to photoreactivating light for three hours or darkness. Apoptosis was measured 40 hours after UV exposure by a TUNEL assay.

1 MED UV-B. Subsequently, animals were either kept in the dark or exposed to photoreactivating light for 3 hours. Forty hours after UV exposure, mice were sacrificed and skin biopsies were further processed for analysis of apoptosis by a TUNEL assay. As expected, apoptotic nuclei were visible in the UV-exposed epidermis of 6-4PP-PL animals that had not been exposed to photoreactivating light (Figure 5). However, in contrast to photoreactivation of CPDs, rapid removal of 6-4PPs by photolyase did not result in a decreased apoptotic response. Treatment of UV-exposed CPD-PL/6-4PP-PL double transgenic mice with photoreactivating light reduced the apoptotic response to a level comparable to that observed when only CPDs were removed.

Repeated exposure to UV can result in thickening of the epidermis, a phenomenon known as hyperplasia. To investigate whether 6-4PPs play a role in the induction of UV-induced hyperplasia, photolyase transgenic mice were either exposed to 1 MED UV-B light or 1 MED UV-B light and photoreactivating

light for four consecutive days. One week after the start of the experiment, animals were sacrificed. As shown in Figure 6, haematoxylin/eosin-stained skin sections revealed a clear induction of epidermal hyperplasia upon UV irradiation in the absence of photoreactivation of CPDs and/or 6-4PPs. As reported previously, light-mediated CPD removal in UV-exposed CPD-PL mice prevents the induction of hyperplasia (Schul et al., 2002). The skin of UV-exposed 6-4PP-PL mice that had received photoreactivating light, however, showed no reduction in epidermal hyperplasia, comparable to what was observed in mice that had been kept in the dark. Thus, removal of 6-4PPs does not significantly reduce onset of hyperplasia. On the other hand, photoreactivation of both CPDs and 6-4PPs in double transgenic animals again resulted in a strong inhibition of UV-induced hyperplasia, as observed when only CPDs have been removed by photolyase. These findings point towards CPDs as the major trigger for UV-induced apoptosis and hyperplasia in NER-proficient mice.



Contribution of CPDs and 6-4PPs to mutagenic events

6-4PPs and, to a lesser extent, CPDs are potentially mutagenic lesions (Mitchell, 1988a). To investigate whether rapid removal of CPDs and/or 6-4PPs by photoreactivation affects the UV-induced mutation frequency, photolyase mice were bred with *LacZ* reporter mice (Dolle et al., 1996) in an SKH1 hairless background. These animals contain a repeat of 20 copies of a *LacZ* reporter plasmid in their genome. In a typical experiment, animals are exposed to 1 MED UVB-light and after two weeks (the time window required to allow fixation of mutations) genomic DNA is extracted from the epidermis. Next, *LacZ* plasmids are isolated by restriction-enzyme digestion followed by sealing by ligation. After ligation, plasmids are transformed into a *LacZ-/GalE- E. coli* strain, allowing determination of mutation frequencies by growing the cells

Figure 6
Effect of photoreactivation of 6-4PPs and CPDs on UV-B induced epidermal hyperplasia.
Mice were exposed to 1 MED UV-B for four subsequent days, followed by exposure to photoreactivating light (3 hours) or darkness. Four days after the last exposure mice were sacrificed and skin sections were stained with haematoxylin and eosin.

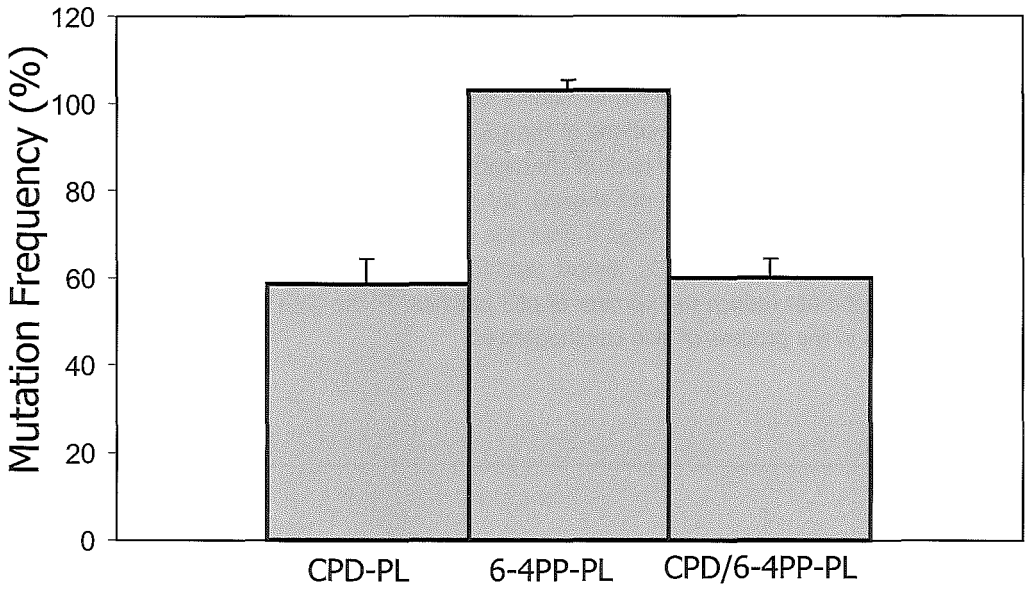


Figure 7
Effect of photoreactivation of 6-4PPs and CPDs on UV-B induced mutation frequency.
Mice received a single dose of 1 MED UV-B followed by exposure to photoreactivating light (3 hours) or darkness, after which 2 weeks were allowed for mutation fixation. The epidermal mutation frequency in the *LacZ* reporter gene of mice exposed to photoreactivating light was determined and expressed as a percentage of the frequency in mice that were kept in the dark.

on selective plates. Typically, mutation frequencies of 150.10^{-5} were observed for photolyase mice exposed to 1 MED UV and subsequently kept in the dark. Analysis of epidermal DNA of non-irradiated mice yielded a background mutation frequency of 15.10^{-5}. Figure 7 shows the mutation frequencies in UV-exposed CPD-PL, 6-4PP-PL and CPD-PL/6-4PP-PL mice, expressed as a percentage of the mutation frequency observed in animals that have not been exposed to photoreactivating light. As expected, the UV-induced mutation frequency in wildtype mice (lacking photolyase) is not affected by exposure to photoreactivating light (data not shown). Remarkably, near complete photoreactivation of CPDs (as determined by immunohistochemistry) in CPD-PL mice only reduced the UV-induced mutation frequency in the epidermis by some 40%. In contrast, removal of 6-4PPs does not result in a decreased mutation frequency. When both 6-4PPs and CPDs are photoreactivated in UV-exposed CPD-PL/6-4PP-PL mice, we observed a mutation frequency similar to that found in CPD-PL mice. Thus, in a NER-proficient background rapid removal of CPDs rather than 6-4PPs by photoreactivation reduces the UV-induced mutation frequency.

Contribution of CPDs and 6-4PPs to p53 patch formation

Mutations in the *p53* tumor suppressor gene have been reported to play a crucial role in the early development of squamous cell carcinomas. Notably, mutant p53 protein can be immunohistochemically detected in clusters of preneoplastic epidermal cells induced upon chronic treatment with UV, and the

number of these "p53 patches" appears a direct measure for UV-induced tumor risk (Berg et al., 1996; Rebel et al., 2001). To investigate the protective effect of rapid photoreactivation of CPDs and 6-4PPs on the process of carcinogenesis, hairless photolyase mice were given a daily dose of UV-B (1 MED) with or without a subsequent treatment with photoreactivating light. Twenty-one days after the first exposure, mice were sacrificed and epidermal sheets were isolated and stained for the presence of cell clusters expressing mutant conformations of the p53 protein. Figure 8 shows the number of p53 patches observed in a fixed area on the back of UV-treated photolyase mice. Whereas in non-exposed animals p53 patches were absent (data not shown), UV irradiation resulted in a clear induction of p53 patches in both CPD-PL and 6-4PP-PL mice. A dramatic reduction in the number of p53 patches was observed upon photoreactivation of CPDs, almost reaching the level of unirradiated controls. Removal of 6-4PPs, however, did not reduce the number of p53 patches. These data suggest a substantial role for unrepaired CPDs in the process of skin carcinogenesis.

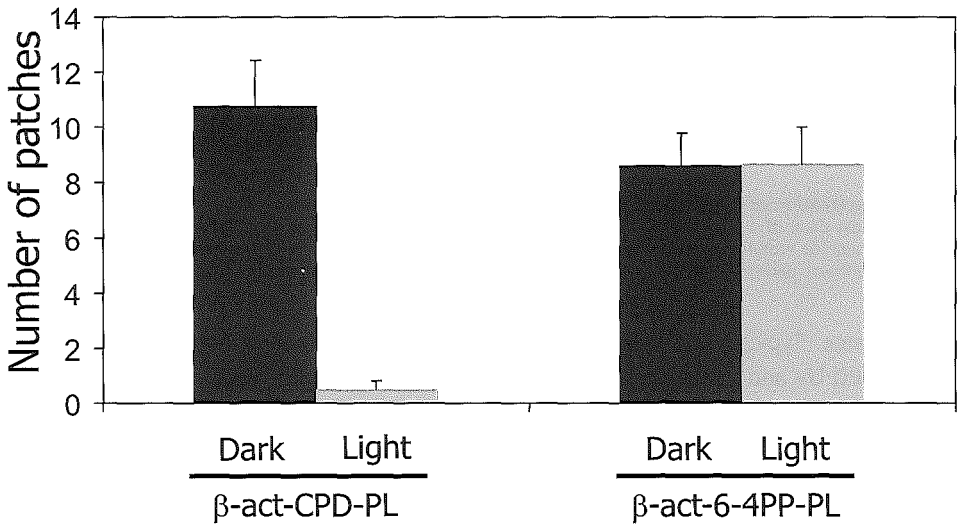


Figure 8
Effect of photoreactivation of 6-4PPs or CPDs on induction of p53 patches.
Photolyase mice were exposed to 1 MED UV-B daily, followed by exposure to photoreactivating light (3 hours) or darkness. After 21 days, mice were sacrificed and epidermal sheets were isolated. The epidermis was further processed for staining of mutant p53 and mounted on glass slides. Clusters of more than 10 cells that show a positive staining were marked as p53 patches. The number of p53 patches per 5.4 cm^2 was counted.

Discussion

Photolyase transgenic mice

Unrepaired CPD and 6-4PP DNA lesions have been implicated as the major trigger of acute and long-term effects resulting from exposure of the skin to ultraviolet light (i.e. sunburn, mutagenesis, immunesuppression, skin carcinogenesis). To improve our understanding of the relative contribution of these two photolesions to the various UV-mediated skin effects, we have set out to generate a panel of transgenic mouse lines, supplemented with photolyases for rapid (compared to the relatively slow acting NER system) and lesion-specific removal of CPDs and 6-4PPs. Previously, using *Potorous tridactylis* CPD photolyase transgenic mouse models, we have shown that rapid photoreactivation of CPDs prevents the onset of apoptosis, epidermal hyperplasia and erythema (sunburn), pointing to CPDs as a major trigger for acute skin effects (Schul et al., 2002). However, these studies neglected the contribution of the even more helix-distorting 6-4PPs on these biological endpoints. To define the consequences of 6-4PPs in DNA, we have generated transgenic mice carrying the *Arabidopsis thaliana* 6-4PP photolyase under control of a ubiquitous β-actin promoter, containing a CMV enhancer sequence. Although other, more related 6-4PP photolyase cDNAs are available (such as the *Drosophila melanogaster* 6-4PP photolyase), we have chosen to use the plant 6-4PP photolyase cDNA for the generation of transgenic mice as it was previously shown to be active in transiently transfected mammalian cells. Several 6-4PP-PL mouse lines were obtained that markedly resembled CPD-PL mice in containing the photolyase transgene only at low (2-3) copy number. The inability to obtain high copy-number CPD-PL mice was explained by assuming that ubiquitous overexpression of the photolyase transgene would negatively affect proper embryonic development through interference of high levels of this DNA binding protein with key cellular processes like transcription and replication (Schul et al., 2002). A similar situation may hold for overexpression of the 6-4PP PL transgene. Importantly, 6-4PP-PL mice did not show any overt phenotype.

We have shown that 6-4PP-PL mice properly express the transgene at the mRNA as well as at the protein level. Moreover, the heterologous 6-4PP photolyase localizes in the nucleus and is biologically active, as shown by the ability of transgenic mice and corresponding dermal fibroblast lines to efficiently and specifically remove 6-4PPs in a light-dependent manner. Thus, the *A. thaliana* enzyme is functional in the mouse, and the 6-4PP-PL mouse model now allows investigation of the biological consequences of unrepaired 6-4PPs in the mouse skin.

CPDs, rather than 6-4PPs, form the main trigger for UV-induced cell death and transcription inhibition

UV-induced cell death has been reported to originate from transcription-blocking DNA lesions in the transcribed strand of active genes that trigger a cell-type specific apoptotic response (Ljungman and Zhang, 1996; Ljungman et al., 1999; Yamaizumi and Sugano, 1994). Moreover, lesions that block elongating RNA polymerase II (RNApolII) have been shown to induce ubiquitination and degradation of the large subunit of RNApolII (Bregman et al., 1996; Lee et al., 2002; Ratner et al., 1998). This causes an overall inhibition of transcription by repression of transcription initiation (Rockx et al., 2000), thereby protecting the cell from transcribing damaged genes. Since both CPDs and 6-4PPs can potentially block RNApolII (Friedberg et al., 1995), rapid photoreactivation of either class of photolesions is expected to ablate UV-induced cell killing and transcription inhibition.

Using cultured MDFs from CPD-PL and 6-4PP-PL transgenic mice, we have shown that photolyase removes the majority of CPDs and 6-4PPs respectively within 1 hour in a lesion-specific manner. Whereas photoreactivation of CPD lesions in UV-exposed cells results in a markedly improved cellular survival, photoreactivation of 6-4PPs apparently does not affect UV-resistance. Similarly, rapid CPD removal by photolyase reduces UV-mediated transcription inhibition, whereas photoreactivation of 6-4PPs had no effect, even in a CPD-PL transgenic background. The different response of UV-exposed cells to photoreactivation of CPDs or 6-4PPs might be explained to some extent by the fact that the number of CPDs induced by UV is approximately three-fold higher than the number of 6-4PPs. More likely, however, as RNApolII blocking lesions form the major trigger of apoptosis and overall suppression of transcription initiation (see above), the difference originates from the efficiency at which NER can remove CPDs and 6-4PPs from the template strand of active genes in these photolyase transgenic cells. Whereas rodent cells relatively rapidly remove 6-4PPs by both the global genome and transcription-coupled repair subpathways of NER, CPDs are only removed by transcription-coupled repair. Providing NER-proficient mouse cells with CPD photolyase may therefore be significantly more effective than introduction of a 6-4PP photolyase. In conclusion, our data provide evidence that at a given dose and in a NER-proficient background, CPDs rather than 6-4PPs are the main trigger of cell death and inhibition of transcription initiation. The data obtained with the survival studies confirm previously performed transfection studies and studies on marsupial systems where CPDs were reported to be the main intermediate in UV-induced apoptosis (Chigancas et al., 2000; Miyaji and Menck, 1998; Nishigaki et al., 1998).

To unequivocally establish the relative intrinsic cytotoxic effects of unrepaired CPDs and 6-4PPs, similar experiments need to be performed in totally NER deficient (XPA) cells supplemented with either CPD-PL, 6-4PP-PL or both photolyases. Since repair of UV lesions in these cells is completely dependent on photoreactivation, the UV dose can be adapted in such a way that equal amounts of unrepaired CPDs or 6-4PPs remain present in the genome, thereby enabling a true comparison of cytotoxic effects of these lesions.

Photoreactivation of CPD and/or 6-4PPs in the UV exposed skin

Overexposure of the skin to UV-light causes basal keratinocytes to undergo apoptosis, as shown by the dose-dependent occurrence of sunburn cells in the epidermis. Upon repeated exposure, however, keratinocytes start to proliferate resulting in epidermal hyperplasia. Previously, using CPD-PL transgenic mice, we have shown a considerable contribution of CPDs to the induction of both UV-induced epidermal apoptosis and hyperplasia (Schul et al., 2002). Here we provide evidence that photoreactivation of 6-4PPs does not detectably decrease the magnitude of these processes, which further points towards CPDs as the major cause for apoptosis and hyperplasia in the epidermis of the murine skin. Yet, we cannot exclude a potential role for 6-4PPs in the induction apoptosis and hyperplasia since CPDs are more abundant than 6-4PPs and the remaining CPDs in these experiments might result in an overruling response. To properly address this issue, these experiments should be performed in a totally NER-deficient (XPA) background. This will permit selective, light-controlled elimination of one class of UV-induced pyrimidine dimer in order to be able to assess the biological effect of near equal doses of the remaining type of UV lesion.

Mutagenic and carcinogenic properties of CPDs and 6-4PPs

CPDs have been suggested to be the main intermediate in UV-induced mutagenesis and carcinogenesis since the number of these lesions exceeds the number of 6-4PPs three-fold (Broughton et al., 1990; Chigancas et al., 2002; Chigancas et al., 2000; Nishigaki et al., 1998). However, studies with site-specific lesions have shown the 6-4PP to be potentially much more mutagenic than CPDs, which may in part be due to the fact that polymerase η (*Rad30*, XPV) can bypass 5'-TT-CPDs and 5'-TC-CPDs lesions in an error-free manner, in contrast to 6-4PPs. The relative contribution of 6-4PPs and CPDs to in vivo mutagenic events in the mouse epidermis was investigated in photolyase mice, supplemented with a *LacZ* mutation reporter plasmid. Following treatment of the animals with UV, plasmids were efficiently recovered from genomic DNA and transformed into bacteria, allowing simple detection of the UV-induced mutation frequency. We have shown that photoreactivation of CPDs reduces the mutation frequency in the epidermis of UV-exposed animals. Despite the fact that most of the CPDs are removed by treatment of UV-exposed CPD-PL animals with photoreactivating light (Schul et al., 2002), the reduction in mutation frequency is limited to some 40%. This relatively small reduction of UV-induced mutations upon photoreactivation of CPDs may be well explained by the finding that photoreactivation of CPDs eliminates the apoptotic trigger, thereby suppressing the elimination of pre-mutagenic cells in the epidermis. Also, the LacZ reporter might reside in an inactive location in the genome that is not easily accessible for the CPD-photolyase. In marked contrast, rapid removal of 6-4PPs by photoreactivation did not affect the UV-induced mutation frequency, whereas photoreactivation of both CPDs and 6-4PPs did not have a more prominent effect than photoreactivation of CPDs alone. These data fit well with data provided by You and coworkers (You et al., 2001), showing that photoreactivation of CPDs rather than 6-4PPs reduces the mutation frequency in NER-proficient cultured mouse embryonic fibroblasts carrying the BigBlue© mutation reporter system.

The lack of a reduced mutation rate upon the photoreactivation of 6-4PPs is presumably caused by the ability of NER to efficiently remove these lesions, prohibiting fixation of mutations. In order to study the *in vivo* mutagenicity of these lesions, similar experiments are currently being performed in mice deficient in NER. It should be noted, however, that in the reporter mice used in our experiments, the repeat of *LacZ* genes is not transcribed. As mentioned previously, repair of CPDs in non-transcribed strands by NER is virtually absent. Therefore, whereas we show that enhanced CPD removal significantly reduces the mutation frequency in a non-transcribed gene, the effect of photoreactivation of CPDs on the mutation load in transcribed genes is potentially milder since NER may deal with these lesions.

Induction of mutations is of crucial importance in the multi-step process of carcinogenesis. The frequency of clusters of cells containing mutated p53 protein has been shown to correlate well with rates of carcinoma induction in murine skin (Berg et al., 1996; Rebel et al., 2001). Since photoreactivation of CPDs reduces the epidermal mutation frequency, less p53 patches are expected to arise in CPD-PL mice. Indeed, a dramatic reduction in the formation of p53 patches is observed upon photoreactivation of CPDs. The reduction in this number of pre-carcinogenic patches is substantially larger than the reduction in mutation frequency, consistent with a multi-hit hypothesis where multiple genes need to be mutated before tumors arise. Assuming e.g. that mutations in 4 genes are required for skin carcinomas to occur, lowering the chance a single gene is mutated to 60% would result in $(0.60)^4$= 13% chance that all four genes are hit and carcinoma would develop. This is consistent with the observed dramatic reduction in p53 patch formation upon photoreactivation of CPDs. Additionally,

the discrepancy of the reduction in mutation frequency with the reduction in p53 patch formation might arise from the different transcriptional states of the *LacZ* gene versus the *p53* gene. The *p53* gene is actively transcribed upon exposure to UV and may therefore be more accessible to DNA repair mechanisms like NER and photolyase.

Concluding remarks

Previous work to disentangle the effects of CPDs and 6-4PPs has focused on transfection studies of cultured cells with photolyases, revealing important information on the cytotoxic and genotoxic effects of these lesions at the level of the cell. We now have generated a comprehensive set of photolyase transgenic mouse models that enable thorough analysis of the contribution of individual classes of photolesions to UV-induced responses in the prime target of UV light, the skin. We show that, despite enhanced repair of 6-4PPs, no improved resistance to UV is observed in 6-4PP-PL mice. Furthermore, CPDs are the major intermediate in all UV-induced processes studied, including mutagenesis, and, more importantly, carcinogenesis. To reveal the true relative potential of CPDs and 6-4PPs to mediate these processes, as well as their position in the genome (i.e. non-transcribed versus transcribed DNA), we are currently breeding photolyase mice with totally NER-deficient XPA, GG-NER-deficient XPC, and TC-NER-deficient CSB animals (all in a hairless genetic background), proven to develop skin cancer upon chronic exposure to UV light (Cheo et al., 1997; de Vries et al., 1995; Nakane et al., 1995; van der Horst et al., 1997).

The differences we observe in the contribution of CPDs versus 6-4PPs in the induction of UV-induced skin effects in mice may differ from the situation in man. In humans, CPDs can be repaired to some extent from the global genome by GG-NER. Therefore, the differences between enhanced removal of CPDs and 6-4PPs may be less pronounced in man.

We have shown that the CPD photolyase enzyme protects mice from many adverse effects of UV light, most importantly the formation of pre-carcinogenic lesions in the skin. We should be cautious, however, in translating this to actual carcinoma formation. Although p53 patch formation has been shown to correlate well with the formation of squamous cell carcinomas, we do not know whether this holds true in photolyase mice as well, since removal of CPDs may affect more factors in the process of carcinogenesis than merely reducing the occurrence of premutagenic lesions. For example, DNA damage has been implicated to play an important role in suppression of the immune system, a prerequisite for outgrowth of skin carcinomas. By photoreactivation of CPDs, we may have abolished the immune suppression and, as a consequence, allowed the immune surveillance to efficiently remove malignancies. This would lead to an even more pronounced effect of CPD photolyase on prevention of carcinomas than the effect observed on pre-carcinogenic lesions. Finally, to establish the true protective effect of photolyase enzymes, photolyase mice will be exposed to chronic UV treatments followed by exposure to photoreactivating light. This will shed light on potential benefits of the enzyme in skin cancer prevention.

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References

Arnoldus, E.P., Wiegant, J., Noordermeer, I.A., Wessels, J.W., Beverstock, G.C., Grosveld, G.C., van der Ploeg, M. and Raap, A.K. (1990) Detection of the Philadelphia chromosome in interphase nuclei. Cytogenet Cell Genet, 54, 108-111.

Berg, R.J., van Kranen, H.J., Rebel, H.G., de Vries, A., van Vloten, W.A., Van Kreijl, C.F., van der Leun, J.C. and de Gruijl, F.R. (1996) Early p53 alterations in mouse skin carcinogenesis by UVB radiation: immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells. Proc Natl Acad Sci U S A, 93, 274-278.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell, 40, 359-369.

Bootsma,D., Kraemer,K.H., Cleaver,J.E., Hoeijmakers,J.H.J. (2001) Nucleotide excision repair syndromes: xeroderma pigmentosum, Cockayne syndrome amd trichothiodystrophy. The Metabolic and Molecular Bases of Inherited Disease. Scriver,C.R., Beaudet,A.L., Sly,W.S., Valle, D.(eds)

Bregman, D.B., Halaban, R., van Gool, A.J., Henning, K.A., Friedberg, E.C. and Warren, S.L. (1996) UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells. Proc Natl Acad Sci U S A, 93, 11586-11590.

Broughton, B.C., Lehmann, A.R., Harcourt, S.A., Arlett, C.F., Sarasin, A., Kleijer, W.J., Beemer, F.A., Nairn, R. and Mitchell, D.L. (1990) Relationship between pyrimidine dimers, 6-4 photoproducts, repair synthesis and cell survival: studies using cells from patients with trichothiodystrophy. Mutat Res, 235, 33-40.

Cheo, D.L., Ruven, H.J., Meira, L.B., Hammer, R.E., Burns, D.K., Tappe, N.J., van Zeeland, A.A., Mullenders, L.H. and Friedberg, E.C. (1997) Characterization of defective nucleotide excision repair in XPC mutant mice. Mutat Res, 374, 1-9.

Chigancas, V., Batista, L.F., Brumatti, G., Amarante-Mendes, G.P., Yasui, A. and Menck, C.F. (2002) Photorepair of RNA polymerase arrest and apoptosis after ultraviolet irradiation in normal and XPB deficient rodent cells. Cell Death Differ, 9, 1099-1107.

Chigancas, V., Miyaji, E.N., Muotri, A.R., de Fatima Jacysyn, J., Amarante-Mendes, G.P., Yasui, A. and Menck, C.F. (2000) Photorepair prevents ultraviolet-induced apoptosis in human cells expressing the marsupial photolyase gene. Cancer Res, 60, 2458-2463.

Conforti, G., Nardo, T., D'Incalci, M. and Stefanini, M. (2000) Proneness to UV-induced apoptosis in human fibroblasts defective in transcription coupled repair is associated with the lack of Mdm2 transactivation. Oncogene, 19, 2714-2720.

de Laat, W.L., Jaspers, N.G. and Hoeijmakers, J.H. (1999) Molecular mechanism of nucleotide excision repair. Genes Dev, 13, 768-785.

de Vries, A., van Oostrom, C.T., Hofhuis, F.M., Dortant, P.M., Berg, R.J., de Gruijl, F.R., Wester, P.W., van Kreijl, C.F., Capel, P.J., van Steeg, H. and et al. (1995) Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. Nature, 377, 169-173.

Dolle, M.E., Martus, H.J., Gossen, J.A., Boerrigter, M.E. and Vijg, J. (1996) Evaluation of a plasmid-based transgenic mouse model for detecting in vivo mutations. Mutagenesis, 11, 111-118.

Friedberg, E., Walker, G.C., Siede, W. (ed.). (1995) DNA repair and mutagenesis. ASM press, Washington DC.

Hoeijmakers, J.H. (2001) Genome maintenance mechanisms for preventing cancer. Nature, 411, 366-374.

Hogan, B., Beddington,R., Costantini,F. and Lacy,E. (1994) Manipulating the mouse embryo Section E: Production of transgenic mice. In. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 212-252.

Lee, K.B., Wang, D., Lippard, S.J. and Sharp, P.A. (2002) Transcription-coupled and DNA damage-dependent ubiquitination of RNA polymerase II in vitro. Proc Natl Acad Sci U S A, 99, 4239-4244.

Ljungman, M. and Zhang, F. (1996) Blockage of RNA polymerase as a possible trigger for u.v. light-induced apoptosis. Oncogene, 13, 823-831.

Ljungman, M., Zhang, F., Chen, F., Rainbow, A.J. and McKay, B.C. (1999) Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene, 18, 583-592.

Mellon, I., Spivak, G. and Hanawalt, P.C. (1987) Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell, 51, 241-249.

Mémet, S., Lilienbaum,A. and Israel,A. (1997) Rapid isolation of mouse primary fibroblasts: a tool for the analysis of transgenic mice. Technical Tips Online.

Mitchell, D.L. (1988a) The induction and repair of lesions produced by the photolysis of (6-4) photoproducts in normal and UV-hypersensitive human cells. Mutat Res, 194, 227-237.

Mitchell, D.L. (1988b) The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem Photobiol, 48, 51-57.

Miyaji, E.N. and Menck, C.F. (1998) Human Bcl-2 expression delays ultraviolet-induced apoptosis in marsupial cells. Photochem Photobiol, 68, 719-724.

Mori, T., Nakane, M., Hattori, T., Matsunaga, T., Ihara, M. and Nikaido, O. (1991) Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6-4)photoproduct from the same mouse immunized with ultraviolet-irradiated DNA. Photochem Photobiol, 54, 225-232.

Nakajima, S., Sugiyama, M., Iwai, S., Hitomi, K., Otoshi, E., Kim, S.T., Jiang, C.Z., Todo, T., Britt, A.B. and Yamamoto, K. (1998) Cloning and characterization of a gene (UVR3) required for photorepair of 6-4 photoproducts in Arabidopsis thaliana. Nucleic Acids Res, 26, 638-644.

Nakane, H., Takeuchi, S., Yuba, S., Saijo, M., Nakatsu, Y., Murai, H., Nakatsuru, Y., Ishikawa, T., Hirota, S., Kitamura, Y. and et al. (1995) High incidence of ultraviolet-B-or chemical-carcinogen-induced skin tumours in mice lacking the xeroderma pigmentosum group A gene. Nature, 377, 165-168.

Nishigaki, R., Mitani, H. and Shima, A. (1998) Evasion of UVC-induced apoptosis by photorepair of cyclobutane pyrimidine dimers. Exp Cell Res, 244, 43-53.

Okamura, H., Miyake, S., Sumi, Y., Yamaguchi, S., Yasui, A., Muijtjens, M., Hoeijmakers, J.H. and van der Horst, G.T. (1999) Photic induction of mPer1 and mPer2 in cry-deficient mice lacking a biological clock. Science, 286, 2531-2534.

Ratner, J.N., Balasubramanian, B., Corden, J., Warren, S.L. and Bregman, D.B. (1998) Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair. J Biol Chem, 273, 5184-5189.

Rebel, H., Mosnier, L.O., Berg, R.J., Westerman-de Vries, A., van Steeg, H., van Kranen, H.J. and de Gruijl, F.R. (2001) Early p53-positive foci as indicators of tumor risk in ultraviolet- exposed hairless mice: kinetics of induction, effects of DNA repair deficiency, and p53 heterozygosity. Cancer Res, 61, 977-983.

Rockx, D.A., Mason, R., van Hoffen, A., Barton, M.C., Citterio, E., Bregman, D.B., van Zeeland, A.A., Vrieling, H. and Mullenders, L.H. (2000) UV-induced inhibition of transcription involves repression of transcription initiation and phosphorylation of RNA polymerase II. Proc Natl Acad Sci U S A, 97, 10503-10508.

Schul, W., Jans, J., Rijksen, Y.M., Klemann, K.H., Eker, A.P., de Wit, J., Nikaido, O., Nakajima, S., Yasui, A., Hoeijmakers, J.H. and van der Horst, G.T. (2002) Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice. Embo J, 21, 4719-4729.

Sijbers, A.M., de Laat, W.L., Ariza, R.R., Biggerstaff, M., Wei, Y.F., Moggs, J.G., Carter, K.C., Shell, B.K., Evans, E., de Jong, M.C., Rademakers, S., de Rooij, J., Jaspers, N.G., Hoeijmakers, J.H. and Wood, R.D. (1996) Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell, 86, 811-822.

Todo, T., Ryo, H., Yamamoto, K., Toh, H., Inui, T., Ayaki, H., Nomura, T. and Ikenaga, M. (1996) Similarity among the Drosophila (6-4)photolyase, a human photolyase homolog, and the DNA photolyase-blue-light photoreceptor family. Science, 272, 109-112.

van der Horst, G.T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A.P., van Leenen, D., Buijs, R., Bootsma, D., Hoeijmakers, J.H. and Yasui, A. (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature, 398, 627-630.

van der Horst, G.T., van Steeg, H., Berg, R.J., van Gool, A.J., de Wit, J., Weeda, G., Morreau, H., Beems, R.B., van Kreijl, C.F., de Gruijl, F.R., Bootsma, D. and Hoeijmakers, J.H. (1997) Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. Cell, 89, 425-435.

van der Spek, P.J., Kobayashi, K., Bootsma, D., Takao, M., Eker, A.P. and Yasui, A. (1996) Cloning, tissue expression, and mapping of a human photolyase homolog with similarity to plant blue-light receptors. Genomics, 37, 177-182.

van Hoffen, A., Natarajan, A.T., Mayne, L.V., van Zeeland, A.A., Mullenders, L.H. and Venema, J. (1993) Deficient repair of the transcribed strand of active genes in Cockayne's syndrome cells. Nucleic Acids Res, 21, 5890-5895.

Wood, R.D. (1996) DNA repair in eukaryotes. Annu Rev Biochem, 65, 135-167.

Yamaizumi, M. and Sugano, T. (1994) U.v.-induced nuclear accumulation of p53 is evoked through DNA damage of actively transcribed genes independent of the cell cycle. Oncogene, 9, 2775-2784.

Yasui, A., Eker,A.P.M. (1997) DNA photolyases. In DNA Damage and Repair: Biochemistry, Genetics and Cell Biology. Nickoloff,J.A., Hoekstra,M.F. (eds).

You, Y.H., Lee, D.H., Yoon, J.H., Nakajima, S., Yasui, A. and Pfeifer, G.P. (2001) Cyclobutane pyrimidine dimers are responsible for the vast majority of mutations induced by UVB irradiation in mammalian cells. J Biol Chem, 276, 44688-44694.

Chapter Four

Pyrimidine dimers in basal keratinocytes are the primary cause of UV responses in the skin

Pyrimidine dimers in basal keratinocytes are the primary cause of UV responses in the skin

Exposure to ultraviolet (UV) light has a plethora of undesired and harmful consequences, including an increased risk of developing skin cancer. More than 90% of the DNA damage brought about by UV light consists of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). Previously, we generated two transgenic mouse lines ubiquitously expressing photolyases, enzymes that efficiently repair either CPDs or 6-4PPs in a light-controlled manner and that are absent in placental mammals such as mice and man. Utilizing these mice, we can specifically remove either CPDs or 6-4PPs and study the individual contribution of these lesions to UV responses. In addition to these mouse models, we have now generated mice expressing photolyase enzymes from the basal keratinocyte-specific promoter keratin-14 (K14). Here, we show that such mice can rapidly remove specific DNA lesions from basal keratinocytes only. Damage in all other cell types, including fibroblasts, Langerhans cells and more differentiated keratinocytes, is only repaired by the slower NER system. We show that damage in basal keratinocytes is solely responsible for UV damage-induced apoptosis and epidermal hyperplasia. Strikingly, in K14-CPD animals the anti-apoptotic effect appears not limited to the basal region of the epidermis but extends to other skin compartments, suggesting the presence of intercellular apoptotic signals.

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Introduction

Excessive exposure to ultraviolet (UV) light can have many undesired consequences for man. Apart from acute effects such as sunburn, particularly the formation of skin tumors is considered a major threat, demonstrated by the increasing incidences of skin carcinomas in white populations (Armstrong and Kricker (2001), Wassberg et al., (2001), Center for Epidemiology, Sweden (2000)). When UV radiation reaches the skin, it is absorbed by DNA, leading to two major forms of DNA damage: cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) (Mitchell, 1988). Both of these lesions are formed by covalent bonds between two adjacent pyrimidines.

Several repair mechanism have evolved that protect the genome by removing potentially hazardous DNA lesions (Friedberg, 1995; Friedberg, 2003; Hoeijmakers, 2001). In most organisms, nucleotide excision repair (NER) is responsible for removal of UV-induced DNA damage as well as other helix-distorting lesions. NER is divided in two subpathways: global genome NER (GG-NER) and transcription coupled NER (TC-NER), that primarily differ in the way damage is recognized (Bohr et al., 1985; Mellon et al., 1987; van Hoffen et al., 1993). In GG-NER, the protein complex XPC/hHR23B scans the complete genome for deformation of the DNA double helix (Sugasawa et al., 2001). This complex readily recognizes the highly helix distorting 6-4PPs, allowing fast removal of this type of lesion, whereas mildly distorting CPDs are only poorly recognized (Bohr et al., 1985; Mitchell, 1988). In humans, CPD recognition is greatly enhanced due to p53 dependent upregulation of the p48 subunit of the DNA damage binding protein DDB (Hwang et al., 1999). Rodents, however, lack the p53-responsive element in the *p48* gene and are therefore incapable of CPD repair by GG-NER (Hwang et al., 1999; Hwang et al., 1998).

TC-NER is initiated when elongating RNA-polymerase II (RNApolII) is stalled upon transcription-blocking lesions (such as CPDs and 6-4PPs), and acts on the template strand of active genes. Besides RNApolII, the initial step in TC-NER requires the action of the CSB and CSA proteins. Following lesion recognition, the XPB and XPD helicases of the multi-subunit transcription factor TFIIH unwind the helix surrounding the lesion. XPA verifies the damage after which RPA stabilizes the complex. Next, a single strand DNA fragment of approximately 30 nucleotides containing the damage is excised by the endonucleases XPG and XPF/ERCC1. Finally, DNA synthesis of the excised strand and ligation of the nick takes place (de Laat et al., 1999; Friedberg, 1995; Hoeijmakers, 2001; Wood, 1996).

The relevance of this repair mechanism in humans is highlighted by the occurrence of inherited photosensitive diseases associated with a defective NER: xeroderma pigmentosum (XP), the neuro-developmental disorder Cockayne syndrome (CS) and trichothiodystrophy (TTD). In addition to neuro-developmental symptoms, TTD patients show characteristic brittle hair and nails (Bootsma, 2001). Patients exhibit an increased sensitivity of the skin to UV. Furthermore, XP patients show a >1000 fold increased susceptibility to sunlight-induced skin cancer (Bootsma, 2001).

Many organisms are equipped with yet another mechanism for repair of UV-induced DNA lesions: photoreactivation (for a review, see Yasui and Eker (1997)). In contrast to NER, photoreactivation is performed by single enzymes, photolyases, that specifically recognize either CPDs or 6-4PPs. Upon exposure to visible light, which is required as a source of energy, photolyases split the pyrimidine dimer and revert the lesions to their original states. Despite their strong evolutionary conservation and presence in many organisms (including bacteria, plants and marsupials), photolyase enzymes are

absent in placental mammals such as rodents and man, which therefore solely rely on the complex NER system for elimination of UV-induced lesions (for a review, see Yasui and Eker (1997)).
Previously, we have generated transgenic mice ubiquitously expressing either the *Potorous tridactylus* CPD photolyase (β-act-CPD-PL) or the *Arabidopsis thaliana* 6-4PP photolyase (β-act-6-4PP-PL) from the β-actin promoter (Schul et al., 2002). We have shown that the presence of one or both transgenes in these mice allows light-controlled removal of the corresponding type of lesion in the dermis and epidermis. Moreover, we have provided evidence that in a NER proficient background, CPD rather than 6-4PP lesions are responsible for many of the undesired effects of UV light, including sunburn, mutagenesis and the formation of pre-carcinogenic lesions (Schul et al., 2002).
Although the above mouse models serve as an important tool to unravel the contribution of the individual classes of photolesions (i.e. CPDs vs 6-4PPs) to the deleterious outcome of UV exposure on the skin, they do not address the question whether these undesired effects of UV light are elicited by DNA damage accumulating in basal keratinocytes, or whether other cell types (e.g. more differentiated keratinocytes, Langerhans cells or fibroblasts) are of importance in these processes. To address this question, we have now generated mice expressing the *P. tridactylus* CPD or *A. thaliana* 6-4PP photolyase enzymes from the basal keratinocyte-specific promoter keratin-14 (K14). These K14-photolyase (K14-PL) transgenic mice allow rapid light-dependent removal of specific lesions from basal keratinocytes only, whereas DNA lesions in all other cell types (including fibroblasts and more differentiated keratinocytes) can only be removed by the slower NER system. This offers the unique opportunity to dissect the processes occurring in the skin after exposure to UV light, and to investigate whether DNA damage in basal keratinocytes represent the sole trigger for acute UV effects such as apoptosis and epidermal hyperplasia.

Materials and Methods

Generation of K14-photolyase transgenic mice

The construct for the generation of K14-CPD-PL and K14-6-4PP-PL transgenic mice was cloned in the vector pSP72 (Promega) and contained the human keratin-14 promoter (2.3 kb PCR fragment, generated using forward primer 5'-AAGCTTATATTCCATGCTAG-3' and reverse primer 5'-GGATCCTGAGTGAAGAGAAGG-3') followed by the *Potorous tridactylus* CPD-PL cDNA or the *Arabidopsis thaliana* 6-4PP-PL cDNA. At the 3' end, exon 2 (the last 22 bp), intron 2, exon 3 and the 3' untranslated region (including the polyadenylation signal) of the human *β-globin* gene were inserted. The expression constructs were excised from the plasmid using *SalI*, separated from vector DNA by agarose gel electrophoresis, isolated from the gel with the GeneClean II Kit (Bio 101) and further purified using elutip-D-mini columns (Schleicher and Schuell, Germany). The fragment was dissolved in injection buffer (10 mM Tris-HCl pH 7.5, 0.08 mM EDTA) and injected in the pronucleus of fertilized eggs derived from FVB/N intercrosses as described previously (Hogan, 1994).
Transgenic animals were identified by Southern blot analysis of genomic tail DNA, using the CPD-PL and 6-4PP-PL cDNA as a probe. To estimate the number of integrated copies, equal amounts of genomic DNA from transgenic mice were subjected to Southern blot analysis. As a standard, we used equal amounts of genomic tail DNA supplemented with 0, 10, 30 or 100 pg of the corresponding expression construct. The hybridization signal obtained with the CPD-PL or 6-4PP-PL cDNA probe was quantified using a Molecular Dynamics PhosphorImager and ImageQuant software. After comparison of signal

intensities, the transgene copy number was estimated using the supplemented reference samples. Routine genotyping of mice was performed by PCR analysis. Primer set 5'-TGA GAC TCA TCT CCC AGG AC-3' (forward primer) and 5'-CAC CAA TGC CAT GTG TTT GC-3' (reverse primer) was used to amplify a 321 bp fragment of the CPD photolyase coding region. Primer set 5'-GCACGATTCAGCAAGCAAGG-3' (forward primer) and 5'-CGGTACCTCTACCTATTTGAGTTTTG-3' (reverse primer) was used to amplify a 200 basepair fragment of the 6-4PP-PL coding region.

RNA isolation and RT-PCR

Mouse skin RNA was isolated and cDNA synthesis was performed using Superscript II RNAseH$^-$ reverse transcriptase (RT; Life Technologies) according to the protocol of the supplier. A PCR reaction was performed on the cDNA using a forward primer in the photolyase transgene (CPD-PL: 5'-GATCTTCGGAAAGATCCGC-3', 6-4PP-PL: 5'- GCACGATTCAGCAAGCAAGG -3') and a reverse primer in exon 3 of the *β-globin* gene (CPD-PL and 6-4PP-PL: 5'-TGGACAGCAAGAAAGCGAG-3'). The presence of introns in the β-globin moiety of the photolyase transgenes allows discrimination between the cDNA-derived PCR product and possible genomic DNA contamination.

Photoreactivation in mouse skin

Mice were anesthetized and hairs were removed from a small area on the back of the animal. One third of the hairless area was covered with black non-adhesive tape and the remaining area was exposed to the light of two Philips TL-12 (40W) tubes emitting UV-B light. Typically, one MED was obtained with an exposure of 2 min. Subsequently, half of the UV-exposed area was covered with tape and mice were exposed for 3 h to the light of 4 white fluorescent tubes (GE Lightning Polylux XL F36W/840) filtered through 5 mm of glass. Skin samples were taken from the unexposed area, the UV-irradiated area that was covered, and the UV-irradiated area that was exposed to photoreactivating light. Skin sections were stained for presence of CPDs as described previously with TDM2 antibodies (Mori et al., 1991; Schul et al., 2002). 6-4PPs were detected with 64M2 antibodies (Mori et al., 1991) in combination with horse-radish peroxidase (HRP) conjugated secondary antibodies using DAB as a substrate.

Apoptosis

For detection of apoptotic cells in the skin, we used a TUNEL assay (Fluorescein Apoptosis Detection System, Promega). Mice were exposed to UV and photoreactivating light as described above, and subsequently kept in the dark. Skin samples, taken 40 h after UV exposure were fixed overnight in 4% paraformaldehyde, washed in PBS and embedded in paraffin. Skin sections (5 μm) were deparaffinized and incubated as described by the manufacturer.

Hyperplasia

Mice were anesthetized and an area on the back was depilated by plucking. Mice were exposed to 1 MED of UV-B and photoreactivating light for four consecutive days. One week after the start of the experiment, mice were sacrificed and 8 μm skin sections were obtained. Sections were further processed and stained with haematoxylin and eosin.

Results

Generation of keratin-14-photolyase transgenic mouse lines

To investigate whether acute (e.g. apoptosis) and chronic (e.g. cancer) UV effects are mediated through basal keratinocytes or also require damage in other cell types, we modified the expression construct previously used for the generation of mice ubiquitously expressing photolyase transgenes. To this end, we have exchanged the ubiquitously expressing β-actin promoter and accompanying CMV enhancer with the human keratin-14 (K14) promoter (Fig. 1A). The latter promoter has been reported to be only active in basal keratinocytes of the epidermis (Vassar et al., 1989). After oocyte injections, we obtained transgenic mouse lines for both the K14-CPD photolyase and K14-6-4PP photolyase. All mouse lines carried about 25 copies of the photolyase transgene as determined by Southern blot analysis of mouse genomic DNA (data not shown). RT-PCR analysis of total skin RNA revealed that the transgenes are transcribed and properly spliced (Fig. 1B). A similar result was obtained when RT-PCR analysis was performed on cultured basal keratinocytes isolated from these transgenic mouse lines (data not shown). This shows that the CPD photolyase as well as the 6-4PP photolyase transgene is expressed in basal keratinocytes. Transgenic animals are born at the expected Mendelian frequency, are fertile and do not show an aberrant phenotype up to the age of 1 year, indicating that integration of either the CPD-PL or 6-4PP-PL transgene does not interfere with normal development of the animal.

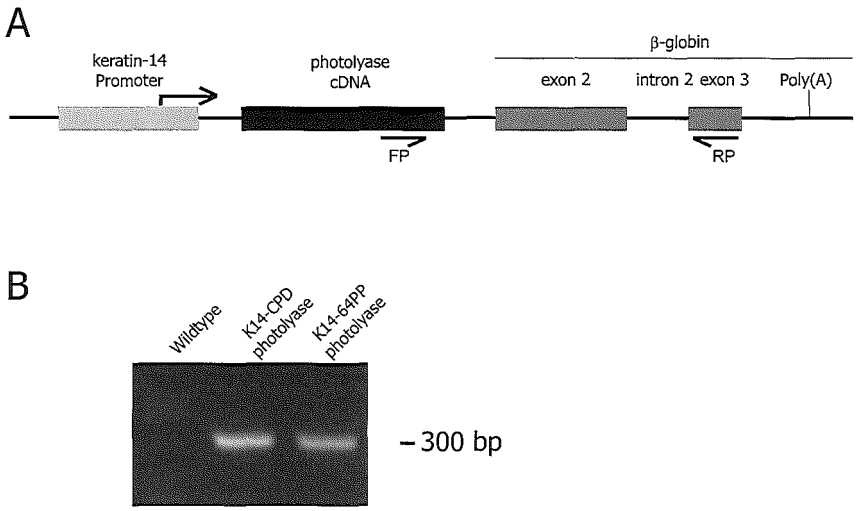

Figure 1
Expression of the K14 photolyase genes in transgenic mice.
A. Expression construct for the generation of K14 photolyase transgenic mice, containing the human K14 promoter, the *Potorous tridactylis* CPD photolyase cDNA OR the *Arabidopsis thaliana* 64PP photolyase cDNA and human genomic *β-globin* sequences, including exons 2 and 3, intron 2, the 3' untranslated region and the polyadenylation signal. Arrows indicate the position of the primers used for the RT-PCR experiment. B. RT-PCR on skin extracts of K14-CPD and K14-64PP photolyase transgenic mice results in a 300 bp band.

Photoreactivation in the mouse skin

To investigate whether the K14 promoter-driven photolyase transgenes are active, we performed photoreactivation experiments on UV-B exposed K14-photolyase mice using an immunocytochemical labeling assay for detection of repair of CPD and 6-4PP lesions in skin sections. One-third of a depilated area on the back of mice was covered, while the remaining part was exposed to 1 MED (minimal erythemal dose) of UV-B light. Next, half of the UV-exposed area was covered while the remaining part of skin was exposed to photoreactivating light for three hours. Skin biopsies were taken and sections were further processed for immunohistochemical staining using antibodies specifically recognizing CPDs (TDM2) or 6-4PPs (64M2).

As expected, the non UV-irradiated skin of photolyase transgenic animals did not show any DNA lesions (data not shown), whereas the UV-exposed part of the skin that had not received photoreactivating light

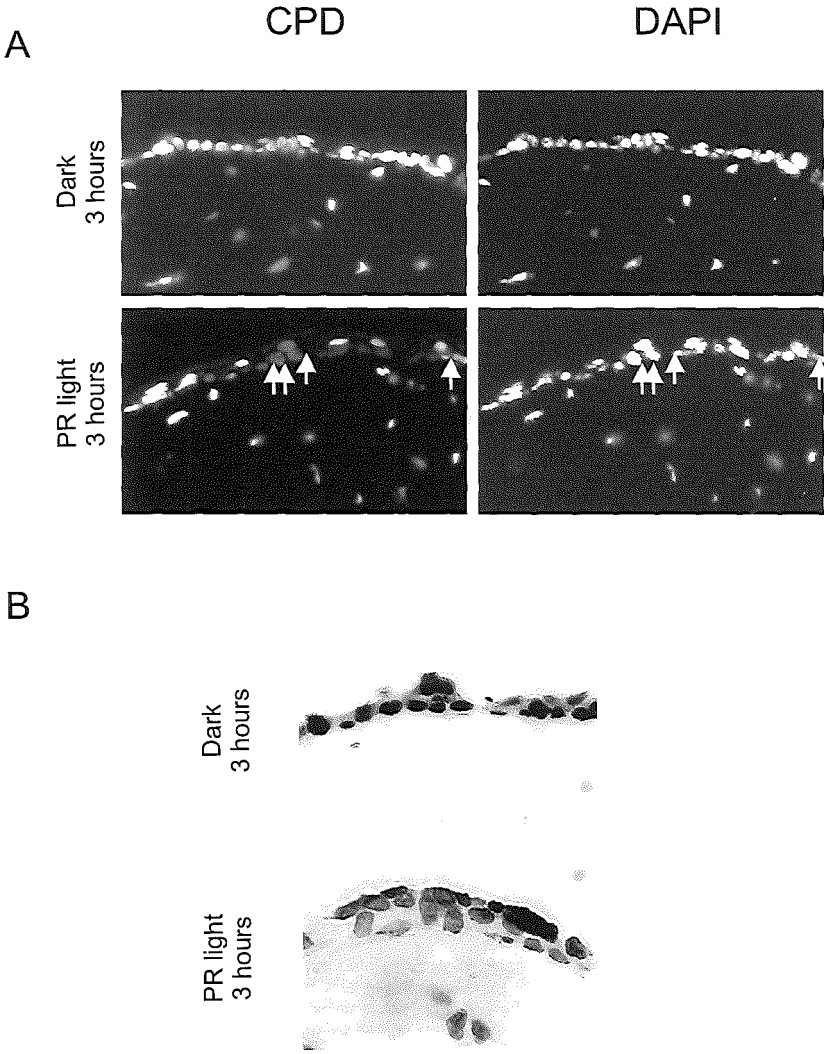

Figure 2
Photoreactivation of CPDs and 6-4PPs in the skin of K14 photolyase mice.
A. CPD lesions in the depilated dorsal skin of K14-CPD photolyase mice following exposure to 1 MED of UV-B light and without (top panels) or with (bottom panels) subsequent exposure to photoreacti-vating light for 3 h. Photolesions were detected by immuno-fluorescent labeling, using CPD specific antibodies and FITC-conjugated goat anti-mouse antibodies. Note the specific absence of labeling in basal keratinocytes and persistence of CPDs in dermal fibroblasts after exposure of mice to photo-reactivating light. Nuclei are visualized by DAPI staining. B. 64PP lesions in the depilated dorsal skin of K14-64PP photolyase mice following exposure to 1 MED of UV-B light and with (top panel) or without (bottom panel) subsequent exposure to photoreactivating light. Photolesions were detected using 64PP specific antibodies and HRP-conjugated secondary antibodies. DAB was used as substrate. Nuclei are visualized by methyl green staining.

displayed a clear labeling of both CPDs and 6-4PPs in the epidermis and the upper part of the dermis (Fig. 2). In photoreactivating light treated areas of the UV-exposed skin of K14-CPD-PL mice, however, CPDs are rapidly removed from the basal keratinocytes whereas 6-4PPs remain present (Fig. 2). The opposite is true for 6-4PP-PL animals. In contrast, lesions in the DNA of dermal cells and keratinocytes in the upper layer of the epidermis are not rapidly removed by exposure to photoreactivating light (Fig. 2). This fits with the notion that the K14 promoter is not active in the dermis, and in the epidermis is switched off once basal keratinocytes start to differentiate (Vassar et al., 1989). With respect to the latter, the turnover of the photolyase protein is apparently sufficiently high to deplete terminally differentiated keratinocytes from photoreactivating capacity. In conclusion, this result shows that it is possible to photoreactivate either CPDs or 6-4PPs in a specific compartment of the skin by using cell type-specific promoter-driven photolyase transgenes. The K14-photolyase transgene now allows removal of UV lesions from proliferating basal and early differentiating keratinocytes in the UV-exposed epidermis, which potentially appears the most dangerous cell type in the development of skin cancer.

Reduction of acute skin effects by photoreactivation

Exposure of the skin to UV light results in the induction of apoptosis in keratinocytes in the epidermis (sunburn cell formation). Previously, using β-actin promoter-driven photolyase transgenic mice, we have shown that UV-induced apoptosis is abolished upon photoreactivation of CPDs, whereas photoreactivation of 6-4PPs did not have any effect. This strongly suggests that CPDs are a crucial trigger for UV-induced apoptosis. To investigate the consequence of rapid removal of CPDs or 6-4PPs by photoreactivation in basal keratinocytes on the induction of apoptosis in the skin, K14-photolyase mice were subjected to a single dose (1 MED) of UV-B light, with or without subsequent photoreactivation. Forty hours after UV exposure, skin biopsies were taken and further processed for a TUNEL assay, enabling detection of apoptotic cells. Light-mediated removal of CPDs from the basal keratinocytes almost completely prevented the apoptotic response in the epidermis (Fig. 3). In contrast, enhanced removal of 6-4PPs by photoreactivation did not affect the level of apoptosis. This is consistent with our previous findings with β-act-6-4PP photolyase mice. Strikingly, not only did we observe a reduction in the apoptotic response throughout the epidermis upon photoreactivation of CPDs, also in the dermis the induction of apoptosis was found to be diminished. This suggests that CPDs in basal keratinocytes are an important trigger for apoptosis in the whole skin, and thus exert a systemic effect.

Repeated exposure of the skin to UV results in the development of epidermal hyperplasia (detected as thickening of the epidermis), which protects the basal layer from future damage. Studies performed on β-act-photolyase mice provided evidence that CPD lesions, and not 6-4PPs, are the cause of epidermal hyperplasia. To study the effect of photolesions in basal keratinocytes on epidermal hyperplasia, K14-photolyase mice were treated with 1 MED UV-B for four consecutive days. Every UV treatment was followed by exposure to photoreactivating light for three hours. As a control, UV treated K14-PL mice were kept in the dark. Three days after the last UV exposure, animals were sacrificed and skin samples were processed for staining with haematoxylin and eosin. As expected, UV exposed K14-PL mice that had not received photoreactivating light show a clear induction of epidermal hyperplasia (Fig. 4). However, upon photoreactivation of CPDs in basal keratinocytes of K14-CPD photolyase mice, only a minor thickening of the epidermis could be observed. This indicates that the basal keratinocyte is the major cell type responsible for UV-induced hyperplasia and confirms previously obtained data

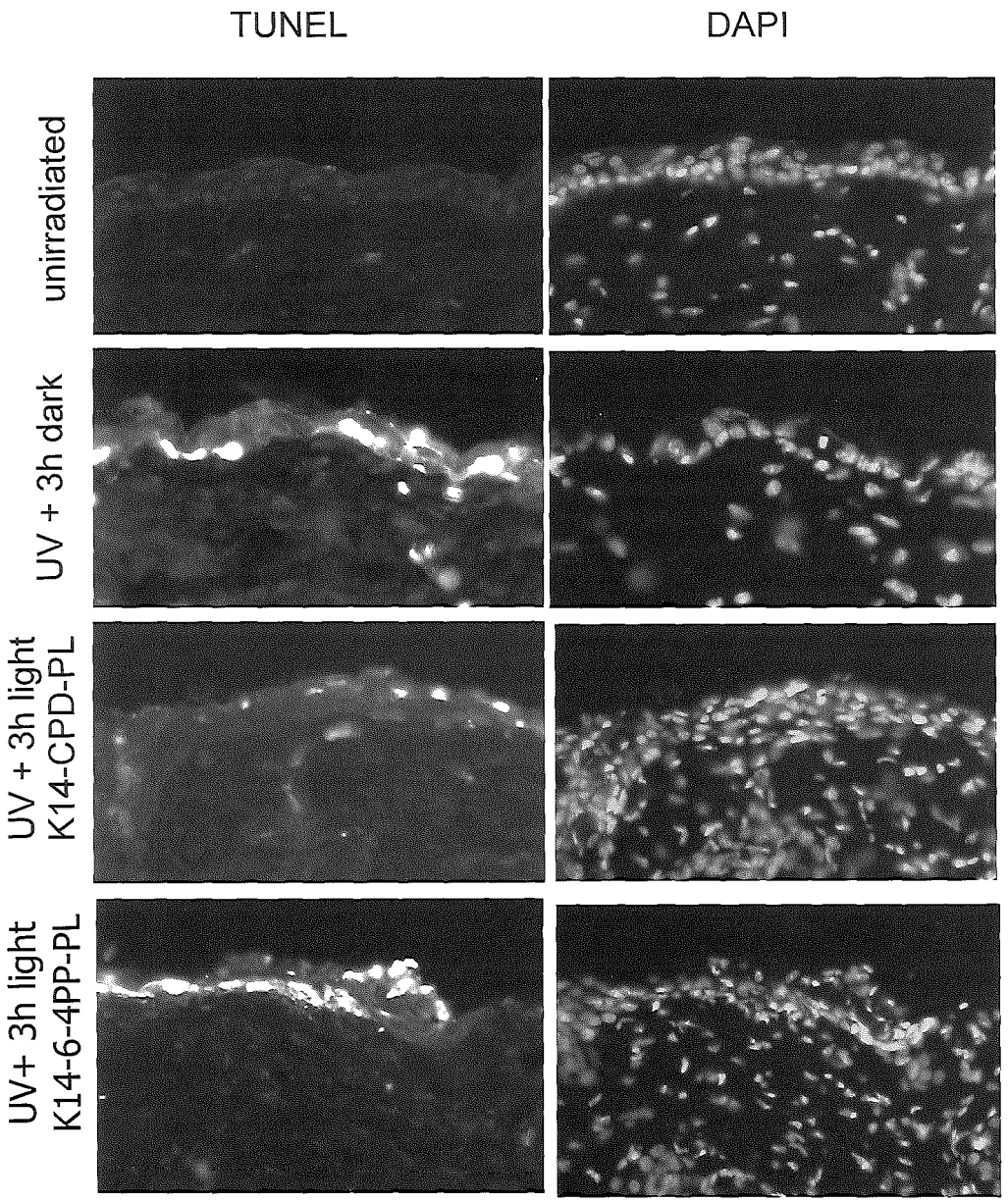

Figure 3
Effect of CPD and 64PP photoreactivation in basal keratinocytes on UV-induced apoptosis in the skin of K14 photolyase transgenic mice.
Induction of apoptosis in the depilated dorsal skin of K14 photolyase mice exposed to 1 MED of UV-B light, without or with subsequent treatment of animals with photoreactivating light for 3 h. Non-UV exposed animals were used as a control (upper panels). Except for the photoreactivation step, animals were kept in the dark immediately after UV treatment. Apoptosis was measured 40 h after UV exposure by a TUNEL assay and nuclei are visualized by DAPI staining.

pointing towards CPDs as the important lesions involved in this process. In contrast to removal of CPDs, photoreactivation of 6-4PPs from basal keratinocytes in K14-6-4PP-PL mice did not alter the hyperplasia response observed in the UV-exposed mouse skin. This finding is in line with the previously reported data obtained with the ubiquitously expressing β-act-6-4PP-PL mouse, where removal of 6-4PPs from all cell types in skin was shown to be insufficient to reduce epidermal hyperplasia.

Discussion

Photolyase transgenic mice

Exposure to ultraviolet light induces many different processes in the skin. Absorption of UV light by DNA in basal keratinocytes has important short and long-term consequences. Upon excessive exposure of the skin, these cells may undergo apoptosis or, if DNA lesions persist in these cells, they become malignant and skin tumors may appear. To investigate to what extent DNA lesions in basal keratinocytes are responsible for UV-induced skin responses, we have generated transgenic mice expressing a CPD or 6-4PP photolyase from the basal keratinocyte-specific keratin-14 (K14) promoter. As expected, exposure of UV-irradiated K14-photolyase animals to photoreactivating light, providing the photolyase enzymes with the energy required for its function, did result in repair of DNA lesions in only a subset of epidermal cells, the basal keratinocytes, whereas lesions persisted in other cell types.

Previously, we have generated mice ubiquitously expressing the CPD or 6-4PP photolyase transgenes from the strong β-actin promoter supplemented with the CMV-enhancer (β-act-photolyase) (Schul et al., 2002). Somewhat surprisingly, we were only able to obtain low copy-number (2-3) transgenic animals with these transgenes. This suggests that high levels of expression of this enzyme may interfere with important processes in the development of mice and lead to lethality. Since photolyases show affinity for (damaged) DNA, these enzymes may affect transcription, replication and DNA repair. Using the weaker and basal keratinocyte specific K14 promoter we now have obtained animals with higher copy-numbers (20-30) of the transgene, indicating that either high expression levels of the transgene or expression in tissues other than keratinocytes may interfere with viability of the animal.

Effects of photoreactivation on apoptosis and hyperplasia

Exposure to UV light results in the formation of apoptotic keratinocytes in the epidermis, also referred to as sunburn cells. This programmed cell death ensures that heavily damaged cells are eliminated from the skin and prevents these cells from becoming malignant. Previously, we have shown that CPDs remaining in the mouse skin are to a large extent responsible for the UV-induced apoptosis (Schul et al., 2002). This may be explained by the fact that 6-4PPs are very efficiently repaired by nucleotide excision repair (NER). Thus, providing mice with a photoreactivation system as a second pathway for repair of 6-4PPs appears not beneficial.

In contrast, rodents do not repair CPDs in non-transcribed DNA via GG-NER. This explains why photoreactivation of CPDs in CPD-PL mice dramatically affects the level of apoptosis, as previously reported (Schul et al., 2002). Apparently, CPDs are the main trigger for UV-induced cell-death.

Here, we show that photoreactivation of CPDs from basal keratinocytes only, is already sufficient to reduce the apoptotic response. Importantly, not only apoptosis in keratinocytes is reduced, also in dermal cells the apoptotic response appears to be diminished. This suggests that UV-induced apoptosis

in the skin is not just a cell-autonomous effect but may act in a systemic manner. In this respect, the Fas-FasLigand (Fas/FasL) system has been implicated to play an important role in intercellular signaling between keratinocytes during UV-induced apoptosis in the skin (Hill et al., 1999; Wehrli et al., 2000 and references therein). Apparently, as evident from this study, signaling from keratinocytes extends to other cell types, such as dermal fibroblasts. The exact nature of this signaling is as of yet unknown. However, analysis of gene expression in skin of K14-PL and β-act-PL mice by a genomics approach may provide clues that help unravel the intercellular signaling pathways involved in UV responses.

Chronic exposure to UV light results in epidermal hyperplasia. Using the ubiquitously expressing β-act-photolyase mice, we previously reported that CPD lesions played a crucial role in this response. Photoreactivation of this lesion almost completely abolished the induction of hyperplasia normally observed after UV exposure, whereas removal of 6-4PPs did not affect this response. It was not known, however, whether epidermal hyperplasia is initiated due to lesions in the basal keratinocytes that stimulate these cells to proliferate, or whether more differentiated keratinocytes or signals from other cell types such as fibroblasts are involved. To address this question, we studied the induction of hyperplasia in exposure of K14-photolyase mice photoreactivating light to rapidly remove CPDs or 6-4PPs in basal keratinocytes. Not to our surprise, removal of 6-4PPs from basal keratinocytes did not reduce the hyperplasia, resembling the responses observed when these lesions are removed from all cell types. Strikingly, by photoreactivation of CPDs only from basal keratinocytes, epidermal hyperplasia was almost absent. This indicates not only that CPDs and not 6-4PPs are the main trigger for UV-induced hyperplasia in epidermis, it also indicates that hyperplasia is brought about primarily by CPDs in the basal keratinocytes. The thickening of the epidermis is independent of lesions persisting in more differentiated keratinocytes.

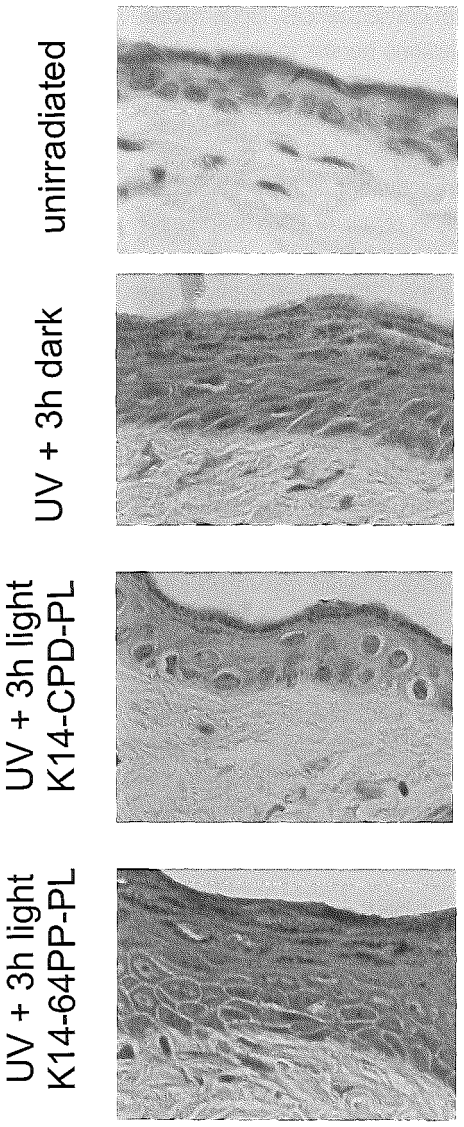

Figure 4
Effect of CPD or 64PP photoreactivation on UV-induced epidermal hyperplasia in the skin of photolyase transgenic mice.
The depilated back of K14 photolyase transgenic mice was exposed to UV-B light for four consecutive days (1.5 MED/day) and were either given 3 h of photoreactivating light or kept in the dark. As a control non-UV exposed animals were used. Animals were sacrificed 3 days after the last exposure and, except for the photoreactivation step, had been kept in the dark throughout the experiment. Skin biopsies were processed for haematoxilin and eosin staining.

Potential of photolyase mice

Previous work by our lab has proven transgenic photolyase mice to be a powerful tool to investigate the role of DNA lesions in the plethora of responses to UV. We showed that many of the adverse effects of UV light could be abolished with the aid of CPD photolyase.

We now provide evidence that DNA damage in basal keratinocytes is of crucial importance in the processes of apoptosis and hyperplasia in the skin. Although it is evident that basal keratinocytes are an important target for UV-induced carcinogenesis, whether DNA lesions in these cells are the prime cause of skin cancer remains to be elucidated. Since the process of carcinogenesis involves the interplay of many different processes and cell types, both in the dermis as well as the epidermis, the effect of damage removal from basal keratinocytes alone may not be that straightforward. Prolonged exposure of K14-photolyase mice to UV and photoreactivating light will not only shed light on the protection offered by photoreactivation in basal keratinocytes, moreover it will provide information on the way different cell types communicate in their response to damage.

Acknowledgements

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References

Armstrong, B.K. and Kricker, A. (2001) The epidemiology of UV induced skin cancer. J Photochem Photobiol B, 63, 8-18.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell, 40, 359-369.

Bootsma, D., Kraemer,K.H., Cleaver,J.E., Hoeijmakers,J.H.J. (2001) Nucleotide excision repair syndromes: xeroderma pigmentosum, Cockayne syndrome amd trichothiodystrophy. In Scriver, C.R., Beaudet,A.L., Sly,W.S., Valle,D. (ed.), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 677-703.

de Laat, W.L., Jaspers, N.G. and Hoeijmakers, J.H. (1999) Molecular mechanism of nucleotide excision repair. Genes Dev, 13, 768-785.

Epidemiology, C.f. (2000) Cancer Incidence in Sweden. Center for Epidemiology. Stockholm, Sweden.

Friedberg, E., Walker, G.C., Siede, W. (ed.). (1995) DNA repair and mutagenesis. ASM press, Washington DC.

Friedberg, E.C. (2003) DNA damage and repair. Nature, 421, 436-440.

Hill, L.L., Ouhtit, A., Loughlin, S.M., Kripke, M.L., Ananthaswamy, H.N. and Owen-Schaub, L.B. (1999) Fas ligand: a sensor for DNA damage critical in skin cancer etiology. Science, 285, 898-900.

Hoeijmakers, J.H. (2001) Genome maintenance mechanisms for preventing cancer. Nature, 411, 366-374.

Hogan, B., Beddington,R., Costantini,F. and Lacy,E. (1994) Manipulating the mouse embryo Section E: Production of transgenic mice. In. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 212-252.

Hwang, B.J., Ford, J.M., Hanawalt, P.C. and Chu, G. (1999) Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. Proc Natl Acad Sci U S A, 96, 424-428.

Hwang, B.J., Toering, S., Francke, U. and Chu, G. (1998) p48 Activates a UV-damaged-DNA binding factor and is defective in xeroderma pigmentosum group E cells that lack binding activity. Mol Cell Biol, 18, 4391-4399.

Mellon, I., Spivak, G. and Hanawalt, P.C. (1987) Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell, 51, 241-249.

Mitchell, D.L. (1988) The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem Photobiol, 48, 51-57.

Mori, T., Nakane, M., Hattori, T., Matsunaga, T., Ihara, M. and Nikaido, O. (1991) Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6-4)photoproduct from the same mouse immunized with ultraviolet-irradiated DNA. Photochem Photobiol, 54, 225-232.

Schul, W., Jans, J., Rijksen, Y.M., Klemann, K.H., Eker, A.P., de Wit, J., Nikaido, O., Nakajima, S., Yasui, A., Hoeijmakers, J.H. and van der Horst, G.T. (2002) Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice. Embo J, 21, 4719-4729.

Sugasawa, K., Okamoto, T., Shimizu, Y., Masutani, C., Iwai, S. and Hanaoka, F. (2001) A multistep damage recognition mechanism for global genomic nucleotide excision repair. Genes Dev, 15, 507-521.

van Hoffen, A., Natarajan, A.T., Mayne, L.V., van Zeeland, A.A., Mullenders, L.H. and Venema, J. (1993) Deficient repair of the transcribed strand of active genes in Cockayne's syndrome cells. Nucleic Acids Res, 21, 5890-5895.

Vassar, R., Rosenberg, M., Ross, S., Tyner, A. and Fuchs, E. (1989) Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. Proc Natl Acad Sci U S A, 86, 1563-1567.

Wassberg, C., Thorn, M., Johansson, A.M., Bergstrom, R., Berne, B. and Ringborg, U. (2001) Increasing incidence rates of squamous cell carcinoma of the skin in Sweden. Acta Derm Venereol, 81, 268-272.

Wehrli, P., Viard, I., Bullani, R., Tschopp, J. and French, L.E. (2000) Death receptors in cutaneous biology and disease. J Invest Dermatol, 115, 141-148.

Wood, R.D. (1996) DNA repair in eukaryotes. Annu Rev Biochem, 65, 135-167.

Yasui, A., Eker,A.P.M. (1997) DNA photolyases. In Nickoloff, J.A., Hoekstra,M.F. (ed.), DNA Damage and Repair: Biochemistry, Genetics and Cell Biology. Humana Press, Totowa, Vol. 2, pp. 9-32.

Chapter Five

Photoreactivation of cyclobutane pyrimidine dimers suppresses photocarcinogenesis

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UV light induces DNA damage, mainly cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). These two classes of photolesions are potentially mutagenic and thereby can induce skin cancer. In addition, UV light can suppress the immune system, and thus promote tumor persistence and progression. Two evolutionary highly conserved DNA repair mechanisms have evolved allowing removal of photolesions: nucleotide excision repair (NER) and photoreactivation. Interestingly, despite its presence in many different organisms, placental mammals do not contain photolyases, the enzyme responsible for photoreactivation, which suggests that they have lost this repair system in evolution. Previously we have supplemented mice with the *Potorous tridactylus* CPD lesion-specific photolyase. Here, we show that CPD-photolyase transgenic mice are protected to a large extent from developing UV-induced skin cancer: photoreactivation of CPD lesions resulted in an increased latency time of skin tumors as well as a substantial reduction in the number of tumor bearing animals and tumors per animal. Importantly, not only the mutation load is lower upon photoreactivation of CPDs, we also show that UV-induced immunosuppression is completely abolished, possibly allowing skin tumors to persist. Our data suggest that CPD lesions are an important trigger for UV-induced skin cancer and that placental mammals, despite the presence of NER, may benefit from a photoreactivation system.

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Introduction

Life on earth is constantly challenged by radiation from the sun. Whereas the UV-C component of sunlight is effectively absorbed by stratospheric ozone, UV-B and UV-A light reach the earth and penetrate the skin, causing extensive damage to cellular components such as lipids and DNA. Moreover, absorption of UV light damages the DNA, primarily resulting in dimer-formation at bipyrimidinic sites. These UV-induced DNA lesions can be found in two configurations: cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) (Mitchell, 1988).

A clear correlation between exposure to sunlight and the induction of skin cancer has been described, and UV-induced DNA lesions have been implicated as the major cause (Muir, 1987; Scotto, 1983). UV light induces distinct mutation patterns, mainly C>T and CC>TT transitions. These so-called UV signature mutations are often found in the *p53* tumor suppressor gene of squamous cell carcinomas, suggesting that UV-induced DNA damage in the *p53* gene is an important and early event in the multi-step process of skin carcinogenesis (Brash et al., 1991; Wikonkal and Brash, 1999; Ziegler et al., 1994; Ziegler et al., 1993).

Although mutations in crucial genes are required to initiate the process of carcinogenesis, they may not be sufficient. A critical role in UV-induced skin carcinogenesis is performed by the immune system. Fisher and Kripke (1977) were the first to show that UV-induced skin tumors are highly immunogenic and are immediately rejected when transplanted onto non-irradiated syngeneic mice, unless recipient animals were exposed to sub-carcinogenic doses of UV-B light prior to transplantation. This indicates that UV-B radiation has immunosuppressive effects, allowing skin tumors to persist. DNA damage has been shown an important factor in the initiation of UV-induced immunosuppression (Kripke, 1992).

During evolution, several DNA repair mechanisms have evolved to counteract the potentially life threatening consequences of photolesions in the DNA (Friedberg, 1995; Friedberg, 2003; Hoeijmakers, 2001). One of these mechanisms, enzymatic photoreactivation, is performed by photolyases that repair UV-induced DNA damage in a lesion-specific manner, using visible light as an energy source. Whereas photolyases are present in many organisms, placental mammals (including mice and man) lack photoreactivating activity, which suggests that the enzyme has been lost in evolution (for a review, see Yasui and Eker 1997).

Another evolutionary highly conserved repair mechanism that deals with UV-induced DNA lesions is nucleotide excision repair (NER). In contrast to the rather straightforward photoreactivation system, NER is a complex DNA repair mechanism with a substrate specificity that is not limited to photolesions but extends to other (DNA helix distorting) lesions. The reaction involves more than 30 proteins to sequentially recognize the lesion, unwind the DNA, excise a single-stranded DNA fragment of apprroximately 30 nucleotides containing the lesion, and fill in and close the resulting gap (de Laat et al., 1999; Friedberg, 1995; Hoeijmakers, 2001; Wood, 1996). In man, the relevance of UV-induced DNA damage and NER in the process of UV sensitivity and carcinogenesis is highlighted by the occurrence of three hereditary disorders associated with a NER defect: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (Bootsma, 2001). Patients with these disorders are highly sensitive to sunlight and, in the case of XP, show a more than 1000 fold increased risk at developing skin cancer.

Whereas the relation between UV exposure and skin cancer development is evident, the exact role of individual classes of photolesions, notably CPDs and 6-4PPs, in the process of carcinogenesis has never been established. To investigate the contribution of these photolesions to acute UV-mediated skin effects (e.g. sunburn) and mutagenesis, we previously generated transgenic mice, expressing a marsupial (*Potorous tridactylus* or rat kangaroo) CPD lesion-specific photolyase (Schul et al., 2002). We have shown that supplementing mice with the photoreactivation system allows light-dependent removal of CPDs (and not 6-4PPs) in the skin of UV-exposed mice, which in turn suppresses induction of acute skin effects such as apoptosis (sunburn cell formation), hyperplasia and erythema. Furthermore, we have shown a reduction in the UV-induced mutation frequency in the skin upon photoreactivation of CPDs. Although these experiments point to a crucial role for the CPD type of lesion in the induction of skin cancer, it remains to be established whether photoreactivation of CPDs indeed does prevent or delay the onset of skin carcinogenesis.
In the present study, we have investigated the induction of skin tumors by UV light in mice supplemented with a CPD lesion-specific photolyase. In addition, as suppression of the immune system has been shown a critical prerequisite in the process of skin carcinogenesis, we have determined to what extent photoreactivation of CPDs inhibits the immunosuppressive effect of UV light.

Materials and Methods

Mice

The generation of β-act-CPD photolyase mice was described previously. The *Potorous tridactylus* CPD photolyase gene is driven by the ubiquitous β-actin promoter and CMV enhancer. Animals are in a mixed FVB/c57bl6 background. For carcinogenesis experiments, mice were backcrossed into the hairless SKH:HR1 background to avoid regular shaving. Genotyping was performed as described previously. All animal experiments are performed in compliance with the Dutch law and are approved by the local animal ethical committee.

Carcinogenesis

Hairless CPD-PL mice (n=15) and their wild type littermates (n=8) aged 8 to 12 weeks were exposed daily to 500 J/m^2 UV-B (Philips TL-12 tubes) followed by three hours of photoreactivating light (GE Lightning Polylux XL F36W/840 lamps). Mice were followed in time and thoroughly screened weekly for the occurrence of skin abnormalities. Typically, carcinomas were expected to occur after three months of treatment. Mice were sacrificed when tumors >4 mm occurred. Biopsies of tumors were taken and processed for routine haematoxylin/eosin staining.

Immunosuppression

CPD-PL mice aged 8 to 12 weeks were shaven on the back one day prior to UV treatment. For UV treatment, a Bluepoint 2 source (Dr Hönle, München, Germany) was used. Two circular areas (6.3 cm^2 in total) were irradiated with 12 seconds (561 J/m^2) each day for 5 consecutive days. The mice were

skin-sensitized 4 days after the last UV exposure by topical application of 5% Picryl Chloride (PCl) to non-irradiated shaven abdomen, chest and feet. Four days later, duplicate ear measurements were performed with an engineer's micrometer (Mitutoyo digimatic 293561, Veenendaal, the Netherlands) and ears were challenged with 0.8% PCl in olive oil. Ear thickness was measured again 24 hours after the challenge and immune response of each mouse was expressed as the percentage of ear swelling.

Results

UV-induced carcinogenesis

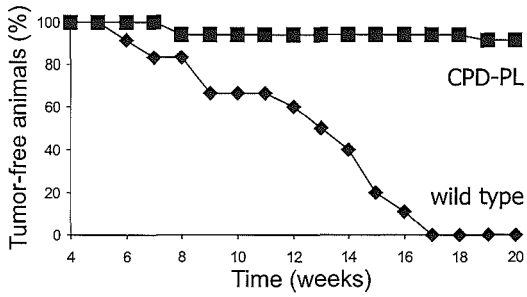
Chronic exposure of mice to UV light results in the formation of skin tumors, in particular squamous cell carcinomas (SCCs) in SKH1:HR1 mice (van Weelden et al., 1990). To investigate the contribution of CPDs to this process we determined whether photoreactivation of this particular type of lesion prevents the occurrence of skin cancer in UV-exposed CPD-PL mice. To avoid regular shaving, the CPD photolyase transgene was first crossed into a hairless SKH:HR1 background. Next, hairless CPD-PL mice and their wild type littermates were treated with a daily dose of 500 J/m^2 of UV-B light, followed by exposure to photoreactivating light for three hours. Previously, we have shown that this time window is sufficient for photolyase to remove the vast majority of the CPD lesions (Schul et al., 2002). Figure 1A shows the fraction of tumor-free mice, revealing a continuous decrease in the number of tumor-free wild type animals, starting 6 weeks after the first exposure to UV light and reaching a maximum after 17 weeks, when all the animals have developed one or more tumors. Figure 1B shows the tumor yield, presented as the average number of tumors per animal in time after the initial UV treatment. After the first tumors have appeared, an exponential increase in tumor yield is observed in wild type animals, resulting in an average of 13 tumors per animal after 20 weeks of treatment. As ethical reasons necessitated the withdrawal of animals with tumors larger than 4 mm from the experiment, the depicted number of tumors per animal probably is an underestimation of the actual tumor yield. Pathohistological analysis identified the tumors as skin carcinomas (data not shown).

Strikingly, in comparison to their wild type littermates, CPD-PL animals show a tremendous delay in the onset of UV-induced skin cancer. As shown in Figure 1, the fraction of tumor-free mice (panel A) remains high whereas the tumor yield (panel B) remains remarkably low. Only after 19 weeks of continuous UV treatment the first tumors start to appear. These data demonstrate a clear protective effect of photoreactivation of CPD lesions against skin tumor formation in mice.

Systemic immunosuppression in photolyase mice

Although the immunosuppressive potential of UV light has already been described years ago (Fisher, 1977), the existence of photolyase mice now enable a thorough investigation of the exact role of DNA lesions in this process. To this end, we have studied the suppressive effects of UV light on the contact hypersensitivity (CHS) response (as measured by the ear swelling test) in CPD photolyase transgenic mice. To exclude that integration of the CPD photolyase transgene affected the CHS response, we first compared the immune response in non-UV treated CPD-PL mice and their wild type littermates.

A



B

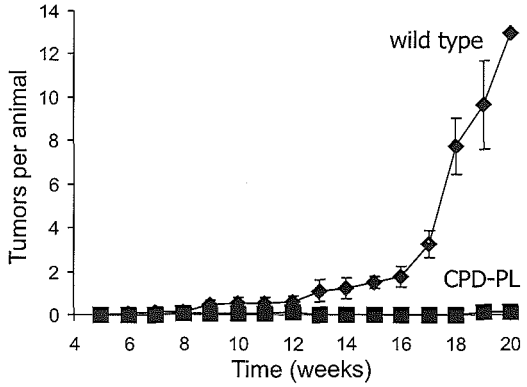


Figure 1
UV-induced skin carcinomas
Photolyase mice (n=15) and their wild type littermates (n=8) received daily UV-B treatments (500J/m²) followed by three hours of photoreactivating light. A. The fraction of tumor-free mice in time after the first UV treatment. B. The average yield of squamous cell carcinomas per mouse in time after the first UV treatment. Error bars indicate the SEM.

Irrespective of the presence of the photolyase transgene, application of picryl chloride (PCl; 0.8%) to the ears of animals that had previously been sensitized by topical application of picryl chloride (PCl; 5%) to the shaven abdomen, resulted in a swelling of approximately 40% (data not shown). Thus, CPD-PL mice display a wild type systemic (CHS) immune response.

Next, we studied the involvement of CPD lesions in the systemic immune suppressive effects of UV light. CPD-PL mice and their wild type littermates were locally exposed to UV-B light (12 seconds; 561 J/m² UV-B) for 5 consecutive days (for details, see Materials and Methods section). Following UV exposure, mice were either exposed to photoreactivating light for three hours or kept in the dark. Four days after the last UV treatment, all mice were sensitized by application of PCl (5%) to the shaven abdomen. Another four days later, the CHS response was provoked by challenging the ears with 0.8% PCl. The immune response of wild type mice is shown in figure 2A where the ear swelling measured in non UV-irradiated mice is set at 100%. As expected, treatment with UV results in a reduction of the immune response with approximately 40%, independent on whether animals were kept in the dark or exposed to photoreactivating light. This finding not only confirms that UV light has immunosuppressive effects, but also shows that exposure to photoreactivating light does not affect UV-induced immune

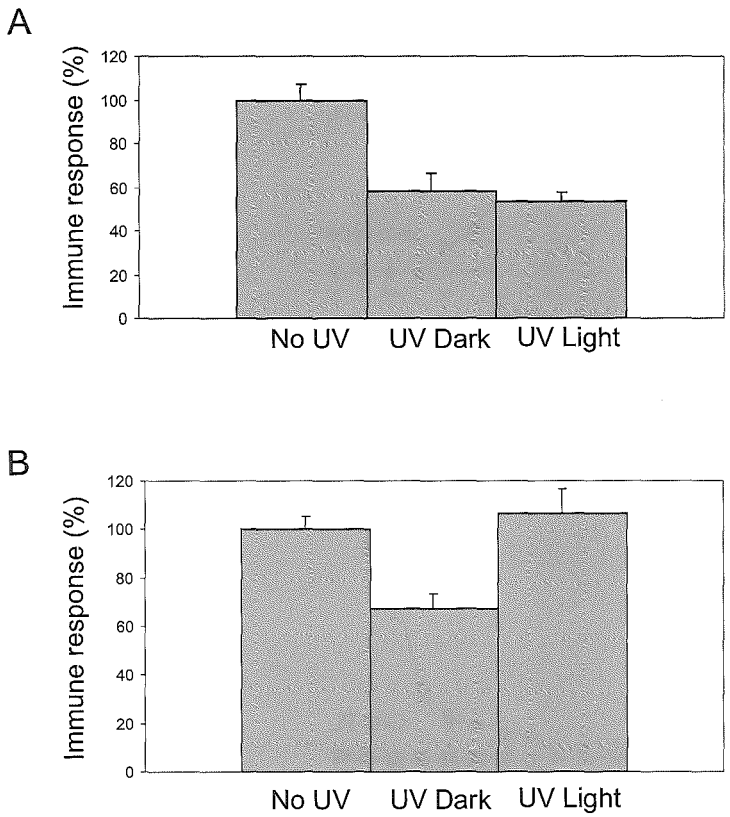


Figure 2
Systemic immunosuppression
Wild type (A) and CPD-PL (B) mice were irradiated with UV-B for 5 consecutive days. The mice were skin-sensitized 4 days after the last UV exposure by topical application of Picryl Chloride (PCl) to non-irradiated shaved abdomen, chest and feet. Four days after sensitization, both ears of the mice were challenged with 0.8% PCl in olive oil. 24 hours after the challenge, duplicate ear measurements were performed with an engineer's micrometer (Mitutoyo digimatic 293561, Veenendaal, the Netherlands). Earswelling is expressed as a percentage of the swelling observed in non-UV treated animals. 34 animals per genotype were used. Error bars indicate the SEM.

suppression, thus validating the use of photolyase mice as a tool to study the contribution of DNA lesions to immunosuppression.

As shown in figure 2B, when CPD-PL mice were exposed to UV light and subsequently kept in the dark, a clear suppression of the immune system similar to that observed in wild type animals is noticed. Importantly, upon removal of CPDs by exposing UV-treated CPD-PL mice to photoreactivating light, the immunosuppressive effects of UV light are completely abolished. Thus, unrepaired CPD lesion form a prime trigger for UV-mediated systemic immune suppression, which in turn may contribute to skin tumor progression.

Discussion

Changes in the environment as well as human behavior have resulted in an enhanced exposure of the human population to UV light and has promoted the incidence of skin carcinomas in the white population. It therefore is of great importance to understand the mechanisms behind the process of UV-induced carcinogenesis and, more importantly, to be able to prevent the occurrence of this malignancy. Although mammals are equipped with the NER machinery, it is not known whether this DNA repair system is capable of protecting human beings optimally from the sun. In particular, repair of CPDs from non-transcribed strands by NER is slow (Bohr et al., 1985; Mitchell, 1988). In the present study, using CPD photolyase transgenic mice (Schul et al., 2002), we have investigated the potential benefit of supplementing rodents with a CPD lesion-specific photolyase in preventing the development of UV-induced skin cancer. We show a tremendous profit of photoreactivation of CPDs in terms of skin cancer protection. Notably, even after 20 weeks of UV treatment, when the complete group of wild type mice has developed on average 13 skin carcinomas per animal, tumors in photolyase mice were scarcely observed. This observation fits nicely with our previous finding that photoreactivation of CPD lesions caused a near complete reduction in the formation of pre-carcinogenic lesions, measured by the number of mutant-p53 patches. Apparently, p53 patches measured 3 weeks after the start of UV exposure form a good predictive read-out for skin carcinogenesis (Chapter three).

The observed reduction in UV-induced skin cancer sensitivity of CPD photolyase transgenic mice may find its origin in various processes affected by the removal of CPD lesions, i.e. tumor induction and tumor promotion. Inevitably, the immediate consequence of the ability of basal keratinocytes in the skin of CPD-PL transgenic animals to rapidly reduce the DNA damage load should be a reduction in the UV-induced mutation frequency, and therefore UV-induced carcinogenesis. Indeed, we have previously shown that photoreactivation of CPD lesions causes a 40% decline in the UV-induced mutation load in the skin of CPD PL mice, two weeks after animals had been exposed to a single dose of UV (Chapter 3). Yet, mutation frequencies were still above background levels (as detected in non-exposed animals), which in part may be explained by the fact that photoreactivation of CPD lesions weakens the apoptotic response in UV-exposed basal keratinocytes (Schul et al., 2002), thereby potentially allowing more 6-4PP type of lesions to be converted into mutations. Apparently, since carcinogenesis is a multi-step process requiring mutations in multiple genes, the speed at which mutations accumulate in chronically UV-exposed CPD PL mice is sufficiently low to retard the appearance of tumors.

Carcinogenesis not only depends on the induction of mutations, but also requires the proper environment for pre-cancerous cells to develop into tumors. A prerequisite for the occurrence of carcinomas is suppression of the immune system; without immunosuppression tumors will be rejected (Kripke, 1974). In addition to the induction of mutagenic DNA lesions, UV light suppresses the immune system, allowing skin tumors to progress. Several chromophores in the skin have been implicated to trigger UV-mediated immunosuppression; the most important ones are DNA damage and the isomerization of *trans*-urocanic acid (UCA) to *cis*-urocanic acid. Here, using the contact hypersensitivity assay, we show a complete absence of UV-induced systemic immunosuppression upon removal of CPDs by photoreactivation. This finding suggests that CPD photolesions rather than UCA are the major initiator of UV-induced immune suppression. Still, we should not disregard the role of UCA in this process. Convincing evidence exists for a role of *cis*-UCA in immune suppression, as best illustrated by increased

tumor-yield in UV-exposed hairless mice that have been treated with UV-irradiated UCA (Reeve et al., 1989). Therefore, it is not excluded that immunosuppressive effects of UV light require the presence of both CPDs and isomerized UCA. We have previously generated transgenic mice expressing photolyase from a basal keratinocyte-specific promoter, K14 (Chapter four). With these K14 photolyase mice, we are able to specifically remove CPDs or 64PPs from basal keratinocytes, while lesions in other cell types such as fibroblasts and Langerhans cells, important players in the skin's immune system, persist longer. It will be of particular interest to investigate CHS responses in these animals upon photoreactivation of CPDs. The presence of lesions in a subset of cells only will provide the unique opportunity to establish the relative importance of the cell types involved in the immune response.

In conclusion, the present study uncovers a tremendous benefit for rodents in which the NER system has been supplemented with a CPD photolyase. Photoreactivation of CPD lesions protects the mouse from skin cancer not only by reducing the chances of mutations in crucial genes, but also by allowing the immune system to respond adequately to these malignancies. We should, however, be careful when extrapolating these data to man. Although repair of CPDs from inactive and non-transcribed DNA by NER in human cells is slow (approximately 60% repair in 24 hours), repair of CPDs in rodents in nearly absent (Bohr et al., 1985; Mitchell, 1988). Therefore, the protective effect of CPD photolyase may be milder in humans. However, previous work on the opossum *Monodelphis* suggests that CPD photolyase can protect against UV-induced skin cancer even in the presence of a functional NER. In the *Monodelphis* held in the dark, CPDs can be removed by NER with an efficiency similar to human NER resulting in approximately 60% repair within 15 hours (Applegate and Ley, 1987). Despite the ability to remove CPDs by NER, photoreactivation of CPDs in these animals strongly reduced the incidence of skin cancer (Ley et al., 1991). Thus, presence of photolyase genes can be beneficial and offer protection from UV-induced skin cancer not only to rodents, but also to organisms with relatively efficient NER.

Taken together, our data suggest that evolution may have taken an unfortunate turn in a time at which no evolutionary selection pressure on photolyase existed, e.g. in nocturnal times. Since it will not be possible to reverse evolution, we will have to take different action if we want to make use of protective capacities of photolyase during, or even after sun exposure. Previously, liposomes with photolyase enzymes were manufactured and were shown to be functional when applied to human skin (AGI dermatics). The use of these liposomes may compensate in part for the evolutionary loss of photolyase genes.

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References

Applegate, L.A. and Ley, R.D. (1987) Excision repair of pyrimidine dimers in marsupial cells. Photochem Photobiol, 45, 241-245.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell, 40, 359-369.

Bootsma, D., Kraemer,K.H., Cleaver,J.E., Hoeijmakers,J.H.J. (2001) Nucleotide excision repair syndromes: xeroderma pigmentosum, Cockayne syndrome amd trichothiodystrophy. In Scriver, C.R., Beaudet,A.L., Sly,W.S., Valle,D. (ed.), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 677-703.

Brash, D.E., Rudolph, J.A., Simon, J.A., Lin, A., McKenna, G.J., Baden, H.P., Halperin, A.J. and Ponten, J. (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci U S A, 88, 10124-10128.

de Laat, W.L., Jaspers, N.G. and Hoeijmakers, J.H. (1999) Molecular mechanism of nucleotide excision repair. Genes Dev, 13, 768-785.

Fisher, M.S.K., M. L. (1977) Systemic alteration induced in mice by ultraviolet light irradiation and its relationship to ultraviolet carcinogenesis. Proc Natl Acad Sci U S A, 74, 1688-1692.

Friedberg, E., Walker, G.C., Siede, W. (ed.). (1995) DNA repair and mutagenesis. ASM press, Washington DC.

Friedberg, E.C. (2003) DNA damage and repair. Nature, 421, 436-440.

Hoeijmakers, J.H. (2001) Genome maintenance mechanisms for preventing cancer. Nature, 411, 366-374.

Kripke, M.L. (1974) Antigenicity of murine skin tumors induced by ultraviolet light. J Natl Cancer Inst, 53, 1333-1336.

Kripke, M.L., Cox P.A., Alas L.G., Yarosh D.B. (1992) Pyrimidine dimers in DNA initiate systemic immunosuppression in UV-irradiated mice. Proc Natl Acad Sci U S A, 89, 7516-7520.

Ley, R.D., Applegate, L.A., Fry, R.J. and Sanchez, A.B. (1991) Photoreactivation of ultraviolet radiation-induced skin and eye tumors of Monodelphis domestica. Cancer Res, 51, 6539-6542.

Mitchell, D.L. (1988) The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem Photobiol, 48, 51-57.

Muir, C., Waterhouse, J., Mack, T., Powell, J., Whelan, S. (1987) Cancer incidence in five continents. In. International Agency for research on cancer, Lyon, Vol. 5.

Reeve, V.E., Greenoak, G.E., Canfield, P.J., Boehm-Wilcox, C. and Gallagher, C.H. (1989) Topical urocanic acid enhances UV-induced tumour yield and malignancy in the hairless mouse. Photochem Photobiol, 49, 459-464.

Schul, W., Jans, J., Rijksen, Y.M., Klemann, K.H., Eker, A.P., de Wit, J., Nikaido, O., Nakajima, S., Yasui, A., Hoeijmakers, J.H. and van der Horst, G.T. (2002) Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice. Embo J, 21, 4719-4729.

Scotto, J., Fears, T.R., Fraumeni, J.F. (1983) Incidence of Nonmelanoma Skin Cancer in the United States. Department of Health and Human Services, Washington.

van Weelden, H., van der Putte, S.C., Toonstra, J. and van der Leun, J.C. (1990) Ultraviolet B-induced tumors in pigmented hairless mice, with an unsuccessful attempt to induce cutaneous melanoma. Photodermatol Photoimmunol Photomed, 7, 68-72.

Wikonkal, N.M. and Brash, D.E. (1999) Ultraviolet radiation induced signature mutations in photocarcinogenesis. J Investig Dermatol Symp Proc, 4, 6-10.

Wood, R.D. (1996) DNA repair in eukaryotes. Annu Rev Biochem, 65, 135-167.

Ziegler, A., Jonason, A.S., Leffell, D.J., Simon, J.A., Sharma, H.W., Kimmelman, J., Remington, L., Jacks, T. and Brash, D.E. (1994) Sunburn and p53 in the onset of skin cancer. Nature, 372, 773-776.

Ziegler, A., Leffell, D.J., Kunala, S., Sharma, H.W., Gailani, M., Simon, J.A., Halperin, A.J., Baden, H.P., Shapiro, P.E., Bale, A.E. and et al. (1993) Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. Proc Natl Acad Sci U S A, 90, 4216-4220.

Chapter Six

Concluding remarks and perspectives

UV effects (De Fabo and Noonan, 1983; de Gruijl et al., 1993). Apart from the action spectrum, more direct evidence was obtained from patients with NER disorders. In these patients, repair of UV-induced DNA damage is impaired and, as a consequence, the patients suffer from photosensitivity (for a more detailed description, see Chapter 1, Bootsma 2001). In patients with xeroderma pigmentosum (one of the NER disorders), various responses including erythema, immunosuppression and carcinogenesis are observed at lower doses of UV than in healthy individuals due to reduced repair of lesions and, consequently, an increased remaining damage load. These findings are corroborated by more recent studies performed on NER deficient mouse models that mimic gene defects found in patients (Boonstra et al., 2001; Garssen et al., 2000; van der Horst et al., 1997). In addition to this genetic evidence for a role of DNA damage in UV responses, liposomes containing enzymes that enhance DNA repair have been applied to both mouse and human skin, which significantly alleviated the consequences of exposure to UV light (Gilchrest and Eller, 1999; Gilchrest, 1993; Kripke, 1992; Wolf, 1995; Yarosh et al., 1992; Yarosh, 2002). These studies have been most valuable and have provided crucial information regarding the effects of UV light.

Although convincing evidence is put forward with respect to a role of DNA damage in responses to UV exposure, the previous studies did not dissect the types of DNA damage that elicit such effects. UV light induces multiple types of DNA lesions. The majority (> 90%) of these lesions consists of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) (Mitchell, 1988). The number of CPDs induced by UV is approximately three-fold higher than the number of 6-4PPs. A minor fraction of the UV-induced DNA damage includes other DNA lesions such as oxidative damage and strand breaks. It has thus far not been possible to determine which of these lesions is the main cause of the responses inflicted by UV light.

To unequivocally determine the contribution of individual types of UV lesions, we have exploited a second natural repair mechanism that has evolved to remove UV-induced DNA lesions: photoreactivation (reviewed by Yasui and Eker (1997)). Photoreactivation is an enzymatic reaction performed by photolyases that revert DNA damage in a lesion-specific manner with the aid of visible light as a source of energy. Photoreactivation does not occur in placental mammals such as mice and man. We have generated mice expressing CPD photolyase from the *Potorous tridactylis* and/or 6-4PP photolyase from *Arabidopsis thaliana*, both driven by the ubiquitous β-actin promoter (Schul et al., 2002, this thesis). Exposing UV treated β-act-CPD-PL or β-act-6-4PP-PL animals to photoreactivating light results in removal of CPDs or 6-4PPs respectively. This now allows for the first time a systematic analysis of the effects of individual classes of unrepaired photolesions in the intact mouse.

Utilizing transgenic β-actin photolyase mice, we have shown a crucial role for CPDs in many of the adverse as well as protective responses to UV. Apoptosis, sunburn, epidermal hyperplasia, mutagenesis as well as suppression of the immune system are all strongly reduced or even undetectable upon removal of CPDs. Moreover, we found a tremendous protection from the development of UV-induced skin tumors upon photoreactivation of CPDs. In strong contrast to the data obtained with photoreactivation of CPDs, we show that enhanced removal of 6-4PPs by photoreactivation does not detectably alter the biological responses upon UV in mice.

Although experiments with various types of cultured cells (e.g. fibroblasts, keratinocytes, mouse embryonic stem cells) have revealed important and interesting data, such experimental systems are

hampered by the lack of cross talk between cell types, which is of crucial importance *in vivo*. We have addressed this issue by the generation of transgenic mouse lines, expressing the photolyase genes in basal keratinocytes only. In these keratin-14 promoter-driven photolyase animals, basal keratinocytes have been shown to be mostly free of lesions upon exposure to photoreactivating light, whereas lesions persist longer in other cell types of the skin such as Langerhans cells, fibroblasts and more differentiated keratinocytes due to the slower action of NER. The advantages of such a mouse model are demonstrated in Chapter 4, where we describe a reduced apoptotic response of fibroblasts upon photoreactivation of CPDs from basal keratinocytes. For the first time, we are now able to assign effects of UV light to individual types of DNA lesions in specific cell types. Our experiments have shown a prominent role of DNA damage in basal keratinocytes, rather than other cell types, in UV-induced apoptosis and hyperplasia.

Remaining questions

Our experiments with β-act-photolyase and K14-photolyase mice have shed light on the individual contributions of CPDs and 6-4PPs to UV responses, and have pointed towards CPDs as the major initiator of events. Despite these efforts, however, many questions remain unanswered. Whereas the effect of CPD lesions is unequivocally established, it is still unclear to what extent 6-4PPs have an effect on the biological effects. We did not observe diminished UV responses upon photoreactivation of 6-4PPs, suggesting that 6-4PPs are not the primary cause of any of the UV responses we have investigated. This may seem surprising as these lesions are more helix distorting and mutagenic than CPDs (Mitchell, 1988). However, the number of 6-4PPs induced by UV is approximately three-fold lower than the number of CPDs. Therefore, the effect of photoreactivation of 6-4PPs may be milder than that of CPDs. Still, assuming that the total number of lesions determines the biological outcome, one would expect that 6-4PP photoreactivation results in approximately one-third of the effect observed upon photoreactivation of CPDs. Yet, we do not see a tendency of reduced biological responses in UV exposed 6-4PP photolyase animals, although not all the assays described in this thesis allow detection of mild effects. The different biological effects exerted by the individual classes of photolesions may find its origin in the different capacity of NER to remove these lesions (Bohr et al., 1985; Mitchell, 1988; van Hoffen et al., 1993). As discussed in previous chapters, 6-4PP lesions are repaired efficiently by NER and, thus, providing animals with 6-4PP photolyase in addition to NER may have no effect on the outcome. To investigate the intrinsic capacity of 6-4PPs to induce biological responses, photolyase mice are currently crossed with NER deficient XPA mice. In the absence of NER, removal of CPDs and 6-4PPs will be completely dependent on photoreactivation. Whereas our experiments in mice with a functional NER have served to uncover the consequences of enhanced removal of specific lesions, experiments with XPA mice supplemented with photolyase will allow investigation of the consequences of specific persisting lesions.

As evident from the work presented in this thesis, the presence of the CPD photolyase gene in the mouse genome seems to be beneficial considering the protective effects we observe on acute and chronic responses. Whether this will hold true for the human situation, however, remains to be proven. In contrast to rodents, CPD removal by the global genome subpathway of NER (GG-NER, see Chapter 1) in humans is greatly enhanced by p53 dependent upregulation of the p48 subunit of the DNA

damage binding protein (DDB). As a consequence, the pronounced effect of enhanced removal of CPDs by photoreactivation as we have observed, may be less effective in the human situation. Evidence that a photoreactivation mechanism in addition to NER may be beneficial in animals other than rodents stems from work on the opossum *Monodelphis* (Applegate and Ley, 1987). In *Monodelphis*, CPDs are removed by NER with an efficiency similar to human NER. Despite the ability to remove CPDs by NER, photoreactivation of CPDs in these animals strongly reduced the incidence of skin cancer (Ley et al., 1991).
For a proper comparison of data obtained with mice, the generation of transgenic mice with a p53 responsive *p48* gene will be of tremendous assistance. Ideally, the NER properties of these "humanized" mice will be similar to the properties in man and will allow a more precise extrapolation of the data obtained in rodents.

If the protective properties of CPD photoreactivation, as observed in transgenic photolyase mice, hold true for all placental mammals, than why have we lost this gene during evolution? One can speculate that organisms do not require the process of photoreactivation since NER can remove the same DNA lesions. This may be true in the case of 6-4PPs, and indeed we do not observe any benefit of photoreactivation of this lesion in a NER proficient environment. However, NER is not able to cope with CPDs in non-transcribed strands. In addition, the increasing incidence of sunlight-induced skin cancer leads us to believe that NER is not able to offer full protection from the sun. Possibly, placental mammals have lost the photolyase genes during a time at which no selection pressure was present, e.g. in a nocturnal period. One may argue that presence of photolyase in the absence of light can be dangerous, since its affinity for DNA will disturb many important cellular processes such as transcription and replication. In concordance with this, we were unable to obtain mouse lines with high copy numbers of the photolyase transgene driven by the strong β-actin promoter (Chapters 2 and 3). In many organisms with photolyase, expression of the photolyase gene is strictly regulated, e.g. in a light-dependent or UV-dependent manner, ensuring expression only at times at which it is required, thereby minimizing the disturbance of cellular processes.
Along the same line, the difference in GG-NER capacity between humans and rodents may be explained from an evolutionary point of view. Rodents are nocturnal animals and, as a consequence, are not exposed to UV. Without regular exposure to UV as present in sunlight, selection pressure upon the presence of the p48 regulatory element is absent. Therefore, this may have been lost in rodents. Alternatively, man may have gained this element to cope with UV-induced lesions in their global genome.

We have shown that CPD type of DNA lesions in basal keratinocytes are responsible for some acute responses of the skin. However, whether DNA damage in basal keratinocytes is solely responsible for the induction of carcinogenesis, or whether interplay with other cell types is required remains to be elucidated. For instance, suppression of the immune system most likely involves cross-talk between multiple cell types including keratinocytes and Langerhans cells residing in the epidermis. Contact hypersensitivity studies on K14 photolyase mice will reveal whether DNA lesions in keratinocytes are required for immunosuppression, or whether damage in e.g. Langerhans cells is sufficient. A detailed comparison of β-actin photolyase mice with K14 photolyase mice may uncover to what

extent basal keratinocytes affect other cell types. Furthermore, analysis of gene expression profiling in UV irradiated (micro-dissected) skin of these animals by micro-array technology will be a powerful approach to uncover the complex signaling pathways involved. The potency of this approach was recently demonstrated by work in our lab showing CPD-dependent gene expression profiles in dermal fibroblasts isolated from β-act-CPD-PL mice.

Future perspectives

Apart from studies with NER deficient mice, our set of transgenic photolyase animals may prove to be a valuable tool to unravel other pathways involved with the integrity of the genome, e.g. the translesion synthesis pathway. Photolesions that persist in the DNA upon entry into S-phase represent a block for the replication machinery. To overcome this problem, cells perform translesion synthesis (Baynton and Fuchs, 2000; Friedberg and Gerlach, 1999; Hubscher et al., 2000; Woodgate, 1999). When the DNA polymerase encounters a lesion during replication, specialized translesion synthesis polymerases take over and insert a base opposite the lesion. An extensive family of translesion polymerases has been described. Polymerases that can bypass UV-induced lesions include polymerase η (*hHR30A*, the gene mutated in XPV patients) and polymerase ι (*hHR30B*). Whereas pol η is fairly accurate, pol ι is rather sloppy and often inserts the wrong base, resulting in the induction of mutations (Masutani et al., 2000; Masutani et al., 1999; Tissier et al., 2000). Up till now, it is not clear how these polymerases deal with CPDs and 6-4PPs at specific bipyrimidinic sites. Experiments with photolyase transgenic mice may shed light on this matter. Currently, mouse models with a defect in the *pol η* and *pol ι* gene are being generated. Supplementing these mice with the photolyase transgenes by intercrossing the animals will allow a thorough analysis of lesion-specific actions of these polymerases. Whereas UV-induced mutation frequencies will reveal information on the ability of the polymerases to bypass CPDs and 6-4PPs, the mutation spectrum will shed light on the sequence-specificity (CC, CT, TC and TT dimers) of these polymerases.

Other unresolved questions, which photolyase transgenic mice may help elucidate, include the etiology of photoaging. Photoaging occurs upon chronic exposure to UV light and is characterized by wrinkles, a loss in skin tone and resilience. Reactive oxygen species (ROS) induced by UV-A and UV-B are considered to be the main cause of the alterations observed in photoaged skin (Scharffetter-Kochanek et al., 1993; Tanaka et al., 1993). The generation of ROS via UV requires the absorption of photons by endogenous photosensitizer molecules (e.g. *trans*-UCA, (Hanson and Simon, 1998)). Although a direct role for UV-induced CPDs or 6-4PPs in the process of photoaging is currently not a favored model, a role for these photolesions cannot yet be excluded. UV-induced photolesions may indirectly affect photoaging by e.g. inducing a hyperproliferative response of keratinocytes accompanied by increased cellular metabolism and, as a consequence, higher production of ROS. Chronic exposure of photolyase transgenic animals may facilitate research on the effect of remaining CPDs and 6-4PPs on photoaging in the skin.

In addition to its value in understanding the contribution of photolesions in the development of carcinomas, experiments with transgenic photolyase mice may shed some light on the etiology of malignant melanomas. Whereas a role for UV-induced DNA lesions in squamous cell carcinomas has

been well established (see Chapter 1), for malignant melanoma the situation is unclear. Upon UV exposure mice develop skin carcinomas, they do not develop melanomas. Therefore; it has proven difficult to establish an action spectrum to ascertain the wavelength-dependence of melanomas. Although exposure to solar radiation increases incidence rates of melanoma in the human population, this correlation is only minor compared to the correlation of solar radiation with carcinoma induction (reviewed by Armstrong and Kricker (2001)). However, when solar UV-A is taken into account, higher correlations are found between melanomas and exposure, suggesting that these wavelengths act on melanoma induction. It is not known whether UV-induced DNA lesions influence this process. Recently, animal models that are prone to develop melanoma have been generated (reviewed by Noonan (2003)). In melanoma research, transgenic photolyase mice may prove to be useful in two different ways. First of all, breeding photolyase mice with melanoma-prone animals (e.g. the INK4a mouse model) will enable studies on the role of CPDs and 6-4PPs in the formation of these tumors. Secondly, mice mainly develop squamous cell carcinomas upon UV radiation due to remaining CPDs. Removal of CPDs from basal keratinocytes by photoreactivation of K14-CPD photolyase mice might delay the induction of carcinomas. This would allow these animals to endure UV-B treatment for a prolonged time period and at a higher dose and will reveal whether UV-B can cause melanomas, solving longstanding questions regarding the etiology of melanoma.

Advantage of transgenic photolyase mice

The approach we have taken, i.e. the introduction of photolyase transgenes in mice, offers many advantages over previous studies on the role of DNA lesions in UV responses. First of all, studies with our transgenic animals allow removal of individual types of DNA damage, enabling investigation of CPDs and 6-4PPs separately. Although this could already be investigated in part by application of photolyase or T4N5 liposomes to skin, these methods are unsuitable for chronic experiments such as carcinogenesis. Also, a transgenic approach ensures presence of the enzyme in all cell types of interest and, as opposed to topical application to skin, is not dependent on the uptake by the skin and potential artifacts in application of the liposomes, reducing the variability within en between experiments.
In addition to the ubiquitous presence of photolyase, the generation of transgenic mice is especially valuable in elucidation of cell type specific responses. The K14-promoter driven photolyase mice are an elegant example of this approach. Other promoters that would be of particular interest with respect to photolyase include those specific for fibroblasts, Langerhans cells and differentiated keratinocytes. Alternatively, the generation of a conditional photolyase transgenic mouse model can be considered. This allows presence or absence of the photolyase gene at any given time and in any given cell type.
The advantage of photolyase transgenic animals is especially evident from the view of mouse genetics. We can cross these animals into various other genetic backgrounds, as discussed above, such as p48 transgenic mice, melanoma-prone or NER-deficient mice. In addition to the totally NER deficient XPA mouse, analysis of TC-NER (CSB) or GG-NER (XPC) deficient animals with photolyase will provide information on the consequences of lesions on inactive DNA and non-transcribed strands of active DNA versus transcribed strands of active genes.

Final Considerations

In many cases, NER is sufficient for protection against UV-induced carcinogenesis. However, in cases of excessive exposure e.g. due to sunbathing or a reduced ozone layer or in cases of inherited disorders affecting the DNA repair capacity, photoreactivation may prove to be extremely valuable. Since we are not able to reverse evolution, we will need to take different action if we desire to benefit from photoreactivation. At present, photolyase is commercially available in liposomes. Application of these liposomes on human and mouse skin has been shown to remove CPDs upon exposure to light, proving that these liposomes may be used to protect us from solar UV radiation. Taking this into account, these photolyase mice might ultimately lead to the complete elucidation of the mechanisms by which UV radiation exerts its harmful effects, and provide a basis for therapeutic and prophylactic strategies to combat skin cancer.

References

Applegate, L.A. and Ley, R.D. (1987) Excision repair of pyrimidine dimers in marsupial cells. Photochem Photobiol, 45, 241-245.

Armstrong, B.K. and Kricker, A. (2001) The epidemiology of UV induced skin cancer. J Photochem Photobiol B, 63, 8-18.

Baynton, K. and Fuchs, R.P. (2000) Lesions in DNA: hurdles for polymerases. Trends Biochem Sci, 25, 74-79.

Berg, R.J., Ruven, H.J., Sands, A.T., de Gruijl, F.R. and Mullenders, L.H. (1998) Defective global genome repair in XPC mice is associated with skin cancer susceptibility but not with sensitivity to UVB induced erythema and edema. J Invest Dermatol, 110, 405-409.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell, 40, 359-369.

Boonstra, A., van Oudenaren, A., Baert, M., van Steeg, H., Leenen, P.J., van der Horst, G.T., Hoeijmakers, J.H., Savelkoul, H.F. and Garssen, J. (2001) Differential ultraviolet-B-induced immunomodulation in XPA, XPC, and CSB DNA repair-deficient mice. J Invest Dermatol, 117, 141-146.

Bootsma,D., Kraemer,K.H., Cleaver,J.E., Hoeijmakers,J.H.J. (2001) Nucleotide excision repair syndromes: xeroderma pigmentosum, Cockayne syndrome amd trichothiodystrophy. The Metabolic and Molecular Bases of Inherited Disease. Scriver,C.R., Beaudet,A.L., Sly,W.S., Valle, D.(eds)

De Fabo, E.C. and Noonan, F.P. (1983) Mechanism of immune suppression by ultraviolet irradiation in vivo. I. Evidence for the existence of a unique photoreceptor in skin and its role in photoimmunology. J Exp Med, 158, 84-98.

de Gruijl, F.R., Sterenborg, H.J., Forbes, P.D., Davies, R.E., Cole, C., Kelfkens, G., van Weelden, H., Slaper, H. and van der Leun, J.C. (1993) Wavelength dependence of skin cancer induction by ultraviolet irradiation of albino hairless mice. Cancer Res, 53, 53-60.

de Gruijl, F.R. and van der Leun, J.C. (1982) Systemic influence of pre-irradiation of a limited skin area on UV-tumorigenesis. Photochem Photobiol, 35, 379-383.

De Gruijl, F.R. and Van Der Leun, J.C. (1983) Follow up on systemic influence of partial pre-irradiation on UV-tumorigenesis. Photochem Photobiol, 38, 381-383.

Friedberg, E., Walker, G.C., Siede, W. (ed.). (1995) DNA repair and mutagenesis. ASM press, Washington DC.

Friedberg, E.C. and Gerlach, V.L. (1999) Novel DNA polymerases offer clues to the molecular basis of mutagenesis. Cell, 98, 413-416.

Garssen, J., van Steeg, H., de Gruijl, F., de Boer, J., van der Horst, G.T., van Kranen, H., van Loveren, H., van Dijk, M., Fluitman, A., Weeda, G. and Hoeijmakers, J.H. (2000) Transcription-coupled and global genome repair differentially influence UV-B-induced acute skin effects and systemic immunosuppression. J Immunol, 164, 6199-6205.

Gilchrest, B.A. and Eller, M.S. (1999) DNA photodamage stimulates melanogenesis and other photoprotective responses. J Investig Dermatol Symp Proc, 4, 35-40.

Gilchrest, B.A., Zhai, A., Eller, S.E., Yarosh, D.B., Yaar, M. (1993) Treatment of human Melanocyte and S91 Melanoma cells with the DNA repair enzyme T4 Endonuclease V Enhances Melanogenesis After Ultraviolet Irradiation. J Invest Dermatol, 101, 666-672.

Hanson, K.M. and Simon, J.D. (1998) Epidermal trans-urocanic acid and the UV-A-induced photoaging of the skin. Proc Natl Acad Sci U S A, 95, 10576-10578.

Hoeijmakers, J.H. (2001) Genome maintenance mechanisms for preventing cancer. Nature, 411, 366-374.

Hubscher, U., Nasheuer, H.P. and Syvaoja, J.E. (2000) Eukaryotic DNA polymerases, a growing family. Trends Biochem Sci, 25, 143-147.

Kripke, M.L. (1974) Antigenicity of murine skin tumors induced by ultraviolet light. J Natl Cancer Inst, 53, 1333-1336.

Kripke, M.L., Cox P.A., Alas L.G., Yarosh D.B. (1992) Pyrimidine dimers in DNA initiate systemic immunosuppression in UV-irradiated mice. Proc Natl Acad Sci U S A, 89, 7516-7520.

Ley, R.D., Applegate, L.A., Fry, R.J. and Sanchez, A.B. (1991) Photoreactivation of ultraviolet radiation-induced skin and eye tumors of Monodelphis domestica. Cancer Res, 51, 6539-6542.

Masutani, C., Kusumoto, R., Iwai, S. and Hanaoka, F. (2000) Mechanisms of accurate translesion synthesis by human DNA polymerase eta. Embo J, 19, 3100-3109.

Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K. and Hanaoka, F. (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. Nature, 399, 700-704.

Mitchell, D.L. (1988) The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem Photobiol, 48, 51-57.

Natali, P.G. and Tan, E.M. (1973) Experimental skin lesions in mice resembling systemic lupus erythematosus. Arthritis Rheum, 16, 579-589.

Noonan, F.P.D., J. Merlino, G. De Fabo, E.C. (2003) Animal models of melanoma: an HGF/SF transgenic mouse model may facilitate experimental access to UV initiating events. Pigment Cell Res, 16, 16-25.

Norris, P.G., Morris, J., McGibbon, D.M., Chu, A.C. and Hawk, J.L. (1989) Polymorphic light eruption: an immunopathological study of evolving lesions. Br J Dermatol, 120, 173-183.

Scharffetter-Kochanek, K., Wlaschek, M., Briviba, K. and Sies, H. (1993) Singlet oxygen induces collagenase expression in human skin fibroblasts. FEBS Lett, 331, 304-306.

Schul, W., Jans, J., Rijksen, Y.M., Klemann, K.H., Eker, A.P., de Wit, J., Nikaido, O., Nakajima, S., Yasui, A., Hoeijmakers, J.H. and van der Horst, G.T. (2002) Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice. Embo J, 21, 4719-4729.

Smit, N.P., Vink, A.A., Kolb, R.M., Steenwinkel, M.J., van den Berg, P.T., van Nieuwpoort, F., Roza, L. and Pavel, S. (2001) Melanin offers protection against induction of cyclobutane pyrimidine dimers and 6-4 photoproducts by UVB in cultured human melanocytes. Photochem Photobiol, 74, 424-430.

Tanaka, H., Okada, T., Konishi, H. and Tsuji, T. (1993) The effect of reactive oxygen species on the biosynthesis of collagen and glycosaminoglycans in cultured human dermal fibroblasts. Arch Dermatol Res, 285, 352-355.

Tissier, A., Frank, E.G., McDonald, J.P., Iwai, S., Hanaoka, F. and Woodgate, R. (2000) Misinsertion and bypass of thymine-thymine dimers by human DNA polymerase iota. Embo J, 19, 5259-5266.

van der Horst, G.T., van Steeg, H., Berg, R.J., van Gool, A.J., de Wit, J., Weeda, G., Morreau, H., Beems, R.B., van Kreijl, C.F., de Gruijl, F.R., Bootsma, D. and Hoeijmakers, J.H. (1997) Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. Cell, 89, 425-435.

van Hoffen, A., Natarajan, A.T., Mayne, L.V., van Zeeland, A.A., Mullenders, L.H. and Venema, J. (1993) Deficient repair of the transcribed strand of active genes in Cockayne's syndrome cells. Nucleic Acids Res, 21, 5890-5895.

Wolf, P., cox P., Yarosh D.B., Kripke M.L. (1995) Sunscreens and T4N5 liposomes differ in their ability to protect against ultraviolet-induced sunburn cell formation, alterations of dendritic epidermal cells, and local suppression of contact hypersensitivity. J Invest Dermatol, 104, 287-292.

Woodgate, R. (1999) A plethora of lesion-replicating DNA polymerases. Genes Dev, 13, 2191-2195.

Yarosh, D., Alas, L.G., Yee, V., Oberyszyn, A., Kibitel, J.T., Mitchell, D., Rosenstein, R., Spinowitz, A. and Citron, M. (1992) Pyrimidine dimer removal enhanced by DNA repair liposomes reduces the incidence of UV skin cancer in mice. Cancer Res, 52, 4227-4231.

Yarosh, D.B. (2002) Enhanced DNA repair of cyclobutane pyrimidine dimers changes the biological response to UV-B radiation. Mutat Res, 509, 221-226.

Yasui, A., Eker,A.P.M. (1997) DNA photolyases. In Nickoloff, J.A., Hoekstra,M.F. (ed.), DNA Damage and Repair: Biochemistry, Genetics and Cell Biology. Humana Press, Totowa, Vol. 2, pp. 9-32.

Samenvatting

De mens is opgebouwd uit vele miljarden cellen. Er bestaan ongeveer 200 verschillende soorten cellen, zoals bloedcellen, zenuwcellen, spiercellen en huidcellen. Verschillende celtypes samen kunnen een orgaan vormen, zoals bijvoorbeeld de nieren of de hersenen. In elke cel bevindt zich DNA, de drager van het erfelijk materiaal. DNA bevat alle informatie die nodig is voor het functioneren van een cel, en daarmee ook voor het functioneren van de organen.

DNA schade en herstel

Het is van groot belang het DNA in een goede staat te houden. De integriteit van het DNA wordt echter continu bedreigd door reacties binnen de cel, maar ook door chemische stoffen en straling. Beschadigingen van het DNA kunnen worden omgezet in permanente veranderingen in het DNA (mutaties) wat uiteindelijk kan leiden tot een afwijkend gedrag van de cel; de cel kan bijvoorbeeld ongeremd gaan groeien en zich ontwikkelen tot een kwaadaardige tumor.

Om het DNA intact te houden zijn planten en dieren uitgerust met verschillende DNA herstel mechanismen. Eén van deze DNA herstel mechanismen, fotoreactivatie genaamd, wordt uitgevoerd door een ingenieus enzym (fotolyase) dat specifieke beschadigingen van het DNA herkent en deze repareert. Alhoewel fotolyase bij veel planten en dieren voorkomt is dit enzym bij mensen en andere placentale zoogdieren zoals muizen afwezig.

Zonlicht

Blootstelling aan zonlicht kan veel schadelijke gevolgen hebben. Deze gevolgen variëren van zonnebrand en veroudering van de huid (rimpels) tot zelfs huidkanker. Met name de ultraviolette (UV) straling uit de zon wordt voor deze schadelijke gevolgen verantwoordelijk gehouden. De laatste jaren is een toename in het aantal patienten met huidkanker waargenomen. Dit komt mede door meer zonvakanties en het gebruik van zonnebanken, maar kan ook veroorzaakt worden door de dunner wordende ozon-laag in de atmosfeer. Deze laag filtert namelijk een groot deel van het schadelijk UV uit het zonlicht zodat het de aarde niet bereikt.

Wanneer UV straling het DNA van de huidcellen bereikt kan dit leiden tot 2 types schade: CPDs (cyclobutaan pyrimidine dimeer) en 64PPs (6-4 photoproduct). Het CPD-fotolyase enzym is in staat om CPDs te verwijderen, terwijl het 64PP-fotolyase enzym 64PPs verwijdert. Zoals gezegd hebben mensen geen fotolyase. Toch zijn mensen in staat om deze schades met een ander (maar helaas minder efficiënt) mechanisme te verwijderen. Mensen bij wie door een erfelijke aandoening dit mechanisme niet goed functioneert blijken dan ook uitermate gevoelig voor zonlicht en hebben een 2000 maal grotere kans op het ontwikkelen van huidkanker. Er bestaan aanwijzingen dat de door UV geïnduceerde DNA schades ten grondslag liggen aan veel van de ongewenste gevolgen van blootstelling aan zonlicht.

Dit proefschrift

Het onderzoek dat in dit proefschrift beschreven staat betreft de rol van de twee UV-geïnduceerde DNA schades (CPDs en 64PPs) in de schadelijke gevolgen van zonlicht. Hierbij hebben we ons met name gericht op de ontwikkeling van huidkanker.

Omdat deze studies niet uitgevoerd kunnen worden met mensen hebben we gebruikt gemaakt van zogenaamde muismodellen. Zoals boven beschreven staat is bij mensen en muizen het fotolyase enzym afwezig. Om de rol van CPDs en 64PPs te bestuderen hebben we transgene muizen gemaakt: muizen waarbij we een stukje extra DNA hebben ingebouwd. Dit stukje DNA zorgt ervoor dat er in de cellen CPD-fotolyase enzymen of 64PP-fotolyase enzymen worden aangemaakt, zodat de muizen nu heel efficiënt de ene of de andere schade kunnen repareren.

Resultaten en Conclusies

We zijn er in geslaagd om zowel CPD-fotolyase transgene muizen als 64PP-fotolyase transgene muizen te maken. De muizen blijken de fotolyase enzymen te bezitten en, cruciaal voor dit onderzoek, in de cellen van de muizen kunnen we nu inderdaad specifiek CPDs of 64PPs weghalen nadat we de dieren hebben blootgesteld aan UV licht. Dit stelde ons in staat om de rol van de individuele types schade te gaan onderzoeken.

We hebben aangetoond dat de acute gevolgen van UV straling (zoals bijvoorbeeld cel-dood, zonnebrand en het dikker worden van de huid) kunnen worden voorkomen wanneer de CPDs verwijderd worden door fotolyase. Verwijdering van 64PPs heeft geen effect op de acute gevolgen van UV licht. Deze resultaten laten zien dat CPD schades, en niet 64PPs, een belangrijke oorzaak zijn van deze processen.

Vervolgens hebben we de ontwikkeling van huidtumoren bestudeerd. Een eerste stap in de ontwikkeling van huidkanker is, zoals hierboven genoemd, het ontstaan van mutaties in het DNA. We hebben de muizen blootgesteld aan UV licht en vervolgens het aantal mutaties in de huid van deze dieren gemeten. Hieruit bleek dat de 64PP-fotolyase muizen evenveel mutaties hebben als muizen zonder fotolyase, de dieren lijken dus niet te profiteren van dit fotolyase enzym. De CPD-fotolyase transgene muizen hebben echter minder mutaties dan muizen zonder dit enzym, dit wil dus zeggen dat verwijdering van CPDs resulteert in een verlaging van de mutatie frequentie.

Wanneer mutaties de eerste stap naar tumoren zijn, suggereren deze resultaten dat verwijdering van CPDs waarschijnlijk ook leidt tot minder kanker. Het optreden van mutaties is echter niet voldoende voor het ontstaan van kanker; het immuun systeem is namelijk zeer goed in staat om huidtumoren in een vroeg stadium te herkennen en op te ruimen, vergelijkbaar met het opruimen van andere ongewenste dingen in het lichaam zoals bepaalde soorten bacteriën. Huidtumoren krijgen toch de kans om zich te ontwikkelen omdat UV licht niet alleen mutaties veroorzaakt; UV licht is tevens in staat het immuun systeem te onderdrukken. Met behulp van de fotolyase muizen hebben we nu kunnen aantonen dat CPDs deze onderdrukking van het immuunsysteem kunnen veroorzaken, en dat verwijdering van CPDs door fotolyase deze onderdrukking teniet doet.

Ten slotte hebben we de muizen dagelijks blootgesteld aan een kleine hoeveelheid UV licht om te kijken naar het ontstaan van huidtumoren. Hiermee hebben we kunnen aantonen dat CPDs verantwoordelijk zijn voor het ontstaan van huidkanker en dat de CPD-fotolyase muizen in zeer sterke mate beschermd zijn tegen huidkanker.

In dit onderzoek hebben we niet alleen gevonden dat DNA schade, en dan met name de CPD, verantwoordelijk is voor het ontstaan van schadelijke effecten van zonlicht, we hebben tevens sterke aanwijzingen dat mensen en dieren waarbij het fotolyase niet aanwezig is zouden kunnen profiteren van de DNA herstel capaciteiten van dit enzym. Met name gezien het toenemende aantal patiënten met huidkanker door steeds meer blootstelling aan UV licht lijkt het aanbrengen van dit enzym op de huid een goede bescherming te kunnen bieden.

List of abbreviations

6-4PP	pyrimidine-(6,4)-pyrimidone photoproduct
8-HDF	8-hydroxy-5-deazariboflavin
A	adenine
Å	ångstrom
A. thaliana	*Arabidopsis thaliana*
β-act	β-actin
BCC	basal cell carcinoma
BER	base excision repair
C	cytosine
CHS	contact hypersensitivity
CPD	cyclobutane pyrimidine dimer
CS	Cockayne syndrome
CSA/CSB	Cockayne syndrome A/B protein
CMV	cytomegalovirus
DDB	DNA damage binding protein
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
ERCC1	human excision repair cross complementing gene 1
FAD	flavin adenine dinucleotide
GG-NER	global genome NER
HHR23B	human homolog of *S. cerevisiae* repair protein RAD23B
HPLC-MS/MS	high performance liquid chromatography-tandem mass spectrometry
kb	kilobase
kD	kilodalton
K14	keratin-14
LC	Langerhans cell
MDF	mouse dermal fibroblast
MED	minimal erythemal dose
MMR	mismatch repair
MTHF	5,10-methenyl tetrahydrofolate
NER	nucleotide excision repair
nm	nanometer
NMSC	non-melanoma skin cancer
P. tridactylis	*Potorous tridactylis*
PCL	picryl chloride
PL	photolyase
ROS	reactive oxygen species
RNApolII	RNA polymerase II
RPA	replication protein A
SCC	squamous cell carcinoma
T	thymine
T4N5	T4 endonuclease V
TC-NER	transcription coupled NER
TBP	TATA-box binding protein
TFIIH	transcription factor IIH
TTD	trichothiodystrophy
TUNEL	TdT-mediated dUTP Nick-End Labeling
UCA	urocanic acid
UDS	unscheduled DNA synthesis
UV	ultraviolet
XP	xeroderma pigmentosum
XPA to –G	xeroderma pigmentosum group A to G protein
XPV	xeroderma pigmentosum variant

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