The DNA Damage Signal for Mdm2 Regulation, Trp53 Induction, and Sunburn Cell Formation *In Vivo* Originates from Actively Transcribed Genes

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The stratum corneum and DNA repair do not completely protect keratinocytes from ultraviolet B. A third defense prevents cells with DNA photoproducts from becoming precancerous mutant cells: apoptosis of ultraviolet-damaged keratinocytes (''sunburn cells''). As signals for ultraviolet-induced apoptosis, some studies implicate DNA photoproducts in actively transcribed genes; other studies implicate non-nuclear signals. We traced and quantitated the *in vivo* DNA signal through several steps in the apoptosis-signaling pathway in haired mice. Homozygous inactivation of *Xpa*, *Csb*, or *Xpc* nucleotide excision repair genes directed the accumulation of DNA photoproducts to specific genome regions. Repair-defective $Xpa^{-/-}$ mice were 7–10-fold more sensitive to sunburn cell induction than wild-type mice, indicating that 86–90% of the ultraviolet B signal for keratinocyte apoptosis involved repairable photoproducts in DNA; the remainder involves unrepaired DNA lesions or nongenomic targets. $Csb^{-/-}$ mice, defective only in excising photoproducts from actively transcribed genes, were as sensitive as $Xpa^{-/-}$, indicating that virtually all of the DNA signal originates from photoproducts in active genes. Conversely, $Xpc^{-/-}$ mice, defective in repairing the untranscribed majority of the genome, were as resistant to apoptosis as wild type. Sunburn cell formation requires the Trp53 tumor suppressor protein; 90–96% of the signal for its induction *in vivo* involved transcribed genes. Mdm2, which regulates the stability of Trp53 through degradation, was induced *in vivo* by low ultraviolet B doses but was suppressed at erythemal doses. DNA photoproducts in actively transcribed genes were involved in ≈89% of the Mdm2 response. ***Key words: apoptosis/Cockayne syndrome/mdm2 protein/MeSH/protein p53/ultraviolet rays/xeroderma pigmentosum. J Invest Dermatol 117:1234–1240, 2001***

Skin irradiated with ultraviolet (UV) B generates characteristic ''sunburn cells''—keratinocytes with pyknotic nuclei and intense eosinophilic staining. Sunburn cells arise in the epidermis of many mammalian species, including humans (Danno and Horio, 1987; Young, 1987). They are produced weakly after UVA irradiation (Rosario *et al*, 1979; Lavker and Kaidbey, 1997), whereas UVC is ineffective due to absorption by keratin (Freeman *et al*, 1989). These curiosities became of potential importance for human skin cancer after the demonstration that individual sunburn cells contain the DNA double-strand breaks typical of cells undergoing apoptosis (Ziegler *et al*, 1994; Brash *et al*, 1996) and that *Trp53* knockout mice exhibit an approximately 7-fold deficiency in sunburn cell production (Ziegler *et al*, 1994). The effect of a point mutation on apoptosis depends on the particular amino acid substituted (Li *et al*, 1996; Rowan *et al*, 1996; Henseleit *et al*, 1997). The involvement of *Trp53* in sunburn cell formation provides a connection to cancer, as *TP53* mutations are present in most human nonmelanoma skin tumors or precancers (actinic keratoses) and *Trp53* mutations are present in most murine skin squamous cell carcinomas induced by UVB (Brash *et al*, 1991, 1996; Nataraj *et al*, 1995; Dumaz *et al*, 1997).

The involvement of apoptosis, rather than cell cycle arrest, is consistent with the existence of a *Trp53*-dependent ''cellular proofreading'' mechanism that removes aberrant cells rather than restoring them to normal (Brash, 1996). For example, infection of murine cells with dominant oncogenes from DNA tumor viruses typically leads to both cell proliferation and *Trp53*-dependent

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Abbreviations: CSB, Cockayne syndrome complementation group B; XPA, xeroderma pigmentosum complementation group A; XPC, xeroderma pigmentosum complementation group C; *TP53*, human p53 tumor suppressor gene; *Trp53*, murine p53 gene; TP53, human p53 protein; Trp53, murine p53 protein

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apoptosis; tumors arise only when apoptosis is inactivated by inactivating the *Trp53* gene (Morgenbesser *et al*, 1994; Pan and Griep, 1994; Symonds *et al*, 1994). In skin, apoptosis of keratinocytes after UVB has the important function of preventing the appearance of mutant cells. A 3.6-fold reduction in apoptosis, due to a defective *FasL* gene, results in a much larger increase in the frequency of UV-induced mutations in the epidermis (Hill *et al*, 1999). Other gene products now known to participate in UV-induced apoptosis include the apoptosis agonists and antagonists p53IAP1, BCL2, and BCL-xL (Pena *et al*, 1997; Rodriguez-Villanueva *et al*, 1998; Gillardon *et al*, 1999; Muller-Rover *et al*, 2000; Oda *et al*, 2000); the TP53 homolog P63 (Liefer *et al*, 2000); and members of two of the three MAPK signal transduction pathways, ERK, JNK, and the JNK target FOS (Schreiber *et al*, 1995; Peus *et al*, 1999; Tournier *et al*, 2000).

The signal that initiates UVB-induced apoptosis has been intensely sought. For keratinocytes, convincing *in vivo* experimental evidence implicates UVB photoproducts in the DNA as one signal. First, the number of sunburn cells in opossum skin decreases 5-fold if, immediately after UVB irradiation, cyclobutane pyrimidine dimers are specifically removed by using visible light to activate photoreactivating enzyme (Ley and Applegate, 1985). Second, *Xpa* knockout mice, which are defective in one of the initial damage recognition/verification steps of DNA nucleotide excision repair and thus accumulate cyclobutane dimers and pyrimidine-pyrimidone (6–4) photoproducts throughout the genome, induce 2–3-fold more sunburn cells than do their wild-type littermates at the same dose (Miyauchi-Hashimoto *et al*, 1996; Okamoto *et al*, 1999).

A clue to the localization of this DNA-based apoptosis signal came from experiments with UVC-irradiated immortalized fibroblasts derived from patients with a point mutation in the *CSB* gene (Cockayne's syndrome complementation group B). These cells are defective in excision repair of the transcribed strand of active genes, a small fraction of the genome. In these fibroblasts, TP53 protein and apoptosis were induced at one-third the dose required for normal cells, although the dose was still 3-fold higher than that required in XPA cells (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). The response may be mediated via MDM2, which targets TP53 for degradation, as MDM2 was induced in control but not XPA or CSB patient fibroblasts (Conforti *et al*, 2000). These results suggest that a major portion of the signal for TP53 induction and apoptosis requires a signal from photoproducts in active genes. This signal appears to originate when RNA polymerase II is stalled at a DNA photoproduct (Ljungman, 2000). Similar experiments in hairless mice indicate the presence of such apoptosis signals *in vivo*; these may prevent mutations after spontaneous DNA lesions (van Oosten *et al*, 2000; Wijnhoven *et al*, 2000). This pathway differs from apoptosis induced by agents such as ionizing radiation, for which DNA double-strand breaks are a signal (Nelson and Kastan, 1994).

In contrast, a number of *in vitro* studies indicate the involvement of non-nuclear UV-induced signals such as ligand-independent activation of membrane receptors by UVC or UVB (Devary *et al*, 1993; Sachsenmaier *et al*, 1994; Rosette and Karin, 1996; Aragane *et al*, 1998; Sheikh *et al*, 1998; Kulms *et al*, 1999). *In vivo*, on the other hand (see *Discussion*), apoptosis can be reduced by blocking receptors or ligands, as if a ligand–receptor interaction transmits an apoptosis signal that originates elsewhere. Yet, introducing ligand does not itself substitute for UVB. We therefore sought to determine in keratinocytes *in vivo*: (i) the fraction of the signal for UVB-induced apoptosis that requires DNA photoproducts; (ii) the extent to which this signal regulates two key molecular components of the UV apoptosis pathway, Trp53 and Mdm2; and (iii) whether this DNA photoproduct signal arises from the small proportion of genes that are actively transcribed. We focused on haired mice, because hairless mice exhibit abnormal follicle development, including cysts, and have a thickened epidermis (Sundberg, 1994). Moreover, the use of haired mice allows us to compare our results directly with previous studies in which *Trp53* knockout mice in a similar genetic background were essential for UV-induced apoptosis (Ziegler *et al*, 1994).

MATERIALS AND METHODS

Experimental design Mice defective in nucleotide excision repair of a genomic region critical to signaling UVB apoptosis will accumulate unrepaired photoproducts in those regions. Such mice are predicted to generate sunburn cells at lower UVB doses than wild-type cells. In contrast, an apoptosis pathway initiated by membrane events would be unaffected by a DNA repair deficit. $Csb^{-/-}$ mutant mice carry homozygously inactivated homologs of the human *CSB* gene, mimicking a protein-truncation mutation found in a human Cockayne syndrome patient. The mice are defective only in transcription-coupled excision repair, leading to a defect in repairing the transcribed strand (but not the coding strand) of the subset of genes being actively transcribed in any particular differentiated cell type (van der Horst *et al*, 1997). After UVB irradiation, these mice accumulate cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6–4) photoproducts in the transcribed strand of active genes. $Xpc^{-/-}$ mice are inactivated for the murine homolog of the human *XPC* gene, mutations which underlie xeroderma pigmentosum complementation group C. These mice are defective in global excision repair (Cheo *et al*, 1997) and thus accumulate UVB photoproducts in the majority of the DNA, including nontranscribed genes, the nontranscribed strand of active genes, and extragenic regions. $Xpa^{-/-}$ mice, inactivated for the murine homolog of the human *XPA* gene, are defective in one of the initial incision steps of DNA excision repair and are defective in both transcription-coupled and global excision repair pathways. After UVB irradiation, they accumulate photoproducts throughout the genome (de Vries *et al*, 1995).

Quantitation of relative biologic effects If the dose–response of apoptosis due to DNA or membrane signals is not linear, the relative importance of these signals will depend on the particular dose chosen. For this reason, photobiologists quantitate and compare the underlying molecular causes (x-axis of a dose–response) rather than the resulting biologic response (y-axis). Causes cannot be compared at a single dose. Dose–responses for modified (e.g., knockout) organisms and unmodified organisms are compared to determine the "dose modification factor"—the amount by which the dose must be increased in, for example, the wild-type mouse to give the same level of apoptosis seen in the knockout (Harm, 1976). The "modifiable sector" (i.e., the fraction of the dose modifiable by the knockout) is then calculated as 1–1/(dose modification factor). For excision repair knockouts and apoptosis, this number (the "excision repairable sector") represents the fraction of the UVB dose (and thus the fraction of DNA photoproducts) that leads to apoptosis and is modifiable by excision repair of the type affected by the knockout. Because the same biologic effect is compared (at different doses) in the knockout and wild type, the same amount of relevant DNA damage is present in each. Therefore, the excision repairable sector figure applies to both organisms.

Comparing equal levels of apoptosis at different UVB doses, rather than asking whether repair-defective knockouts induce more apoptosis at the doses used for wild type, has a second advantage in addition to correcting for nonlinear dose–responses. The latter approach would bias the results toward a DNA-mediated apoptosis mechanism by effectively flooding the repair-defective keratinocyte with unrepaired DNA photoproducts. We therefore use the dose-modification factor to calculate the fraction of UVB-induced apoptosis that is due to excision-repairable DNA photoproducts in particular genomic regions.

Animals and UV irradiation Mice inactivated in the *Xpa*, *Xpc*, or *Csb* genes were as described (de Vries *et al*, 1995; Cheo *et al*, 1997; van der Horst *et al*, 1997). The genotype of progeny were determined in polymerase chain reaction amplifications specific for the inactivated alleles (neomycin cassette) and for the wild-type allele. Animals were used for experiments at age 6–9 wk, with individual animals excluded if there was extensive hair regrowth 40 h after shaving, indicative of being in the hair cycle. Groups of wild-type and homozygous-mutant littermates were shaved on the back under Ketamine–Rompun–Atropine anesthesia (AUV, Inc., Cuyk, the Netherlands) approximately 24 h before the experiment. The next morning, between 9 and 10 a.m., mice in both groups were irradiated from broadband Philips FS40 sunlamps (250–400 nm). The UVB output of the lamp was measured prior to each session by a UVX meter (UV Products, Upland, CA). During irradiation, the animals were allowed to move freely but were prevented from standing upright by a stainless steel wire mesh with 4 cm × 1 cm openings at a height of 3 cm. Twenty-four hours later, the animals were

killed by cervical dislocation. Skin areas (1 cm × 2 cm) were taken from the mid-dorsal region and fixed in 10% neutral buffered formalin, processed, and embedded in paraffin. The experimental protocol was reviewed and approved by the RIVM Animal Committee.

***In vivo* detection of sunburn cells (apoptotic keratinocytes)** Five micrometer thick sections were cut and every third section stained with hematoxylin–eosin. Sunburn cells were identified under a light microscope at ×150 magnification based on their characteristic morphology: pyknotic, darkly basophilic nuclei, eosinophilic cytoplasm, and intercellular gap (halo) formation (Ziegler *et al*, 1994). In contrast to previous work (Ziegler *et al*, 1994; Brash *et al*, 1996), scoring in this study also included cells with eosinophilic cytoplasm but no nuclei, which time-course studies indicated are late-stage apoptotic cells (D.E.B, unpublished observations). At a fixed time-point, here 24 h, this inclusion has the effect of increasing the number of sunburn cells scored at the higher doses and results in a curvilinear dose–response. Sunburn cells were counted on six nonadjacent sections and the length of each epidermal section was measured. The apoptosis frequency was expressed as number of sunburn cells per centimeter of epidermis. The apoptosis frequency was averaged for each mouse and these averages were used in computing the mean and SEM.

Trp53 protein induction Twenty-four hours after UVB irradiation, skin was isolated from three to four mice per genotype and from their wild-type littermates. Skin samples were divided in half to create duplicate samples, embedded in paraffin, and immunostained with CM5 antibody for Tpr53 protein (Midgley *et al*, 1995; van Kranen *et al*, 1995). $Xpc^{-/-}$ mice were examined in an experiment performed separately from $Xpa^{-/-}$ and $Csb^{-/-}$, with each experiment containing internal wild-type controls. Cells were categorized according to the intensity of immunopositivity as minimal (light brown), medium (brown), and strong (dark brown). Two 2.5 mm sections were scored for each mouse, with two to four mice per point, and the level of Trp53 induction was expressed as the number of strongly immunopositive basal cells per centimeter of epidermis. The Trp53 induction frequency was averaged for each mouse and these averages were used in computing the mean and SEM.

Mdm-2 protein induction Paraffin-embedded sections adjacent to those analyzed for Trp53 were immunostained for Mdm-2 using similar methods, including antigen retrieval. SMP14 mouse monoclonal antibody for Mdm-2 protein (Santa Cruz Biotech, Santa Cruz, CA) was diluted 1:50 in MoM diluent (Vector, Burlingame, CA), which allows mouse monoclonal antibodies to be used on mouse tissues. Immunopositivity was scored in four to six sections per mouse, with two to four mice per point, and the level of Mdm-2 induction was expressed as immunopositive cells per centimeter of epidermis. The Mdm-2 induction frequency was averaged for each mouse and these averages were used in computing the mean and SEM.

RESULTS

Sunburn cell production requires a signal from DNA photoproducts In wild-type mice, increasing doses of UVB radiation led to an increased frequency of sunburn cells (apoptotic keratinocytes) on the dorsal skin, as expected (Ziegler *et al*, 1994) (**Fig 1**). The doses used here are physiologically relevant, as the maximum (1250 J per m^2) corresponds to approximately 1 minimal erythemal dose. The dose–response, including both high and low UVB doses, makes it clear that $Xpa^{-/-}$ mice generated sunburn cells at 7–10-fold lower doses than wild-type (**Fig 1**). This control is consistent with qualitative results (Miyauchi-Hashimoto *et al*, 1996; van Oosten *et al*, 2000). It indicates that the apoptosis signal originates from a site susceptible to excision repair, i.e., from photoproducts in DNA. Quantitatively, the result indicates that, *in vivo*, the excision repairable sector for apoptosis is 0.86–0.90 (Harm, 1976); i.e., 86–90% of the signal for UVB-induced apoptosis in keratinocytes involves excision-repairable photoproducts in the DNA. The remaining 10–15% of the apoptosis signal arises from DNA photoproducts that are ineffectively repaired, or from a non-DNA source.

The DNA photoproduct signal *in vivo* originates from actively transcribed genes The $Csb^{-/-}$ mutant mice, although lacking excision repair only in DNA regions that are being actively transcribed, and on only one strand of such regions, induce sunburn cells with a dose–response nearly identical to that observed in $Xpa^{-/-}$ animals (**Fig 2**). This result indicates that the entire DNA signal for UVB-induced apoptosis comes from the minor population of cyclobutane dimers and (6–4) photoproducts in actively transcribed genes.

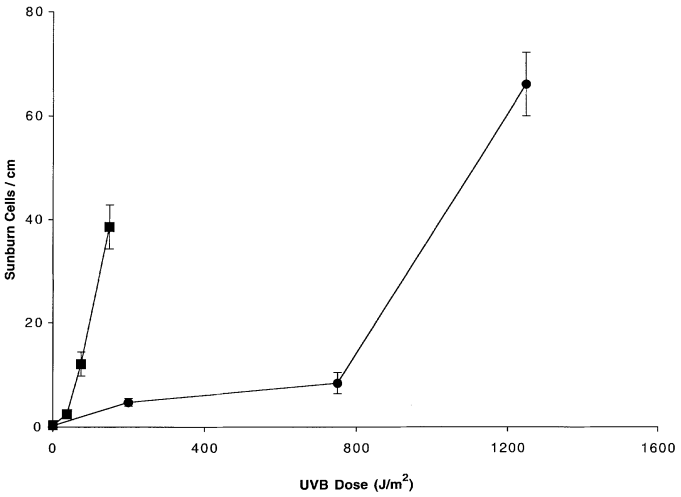

Figure 1. Hypersensitivity to UVB induction of apoptotic keratinocytes ("sunburn cells") in the dorsal epidermis of $Xpa^{-/-}$ mice compared with wild-type littermates. After shaving, haired mice were irradiated with the indicated fluences of UVB. 24 h later the frequency of sunburn cells was scored according to criteria described in *Materials and Methods*. $Xpa^{-/-}$ mice are defective in excision repair of DNA photoproducts throughout the genome. Circles, wild-type; squares, $Xpa^{-/-}$. Error bars, mean ± SEM (n = 3–4 animals per point).

The majority of the genome can accumulate DNA photoproducts without inducing apoptosis If actively transcribed genes are the sole source of the DNA signal for sunburn cell formation, then increasing the accumulation of DNA photoproducts in nontranscribed regions of the genome should not increase sunburn cell induction compared with the wild type. **Figure 3** shows that $Xpc^{-/-}$ mice indeed induce sunburn cells at the same doses and same frequencies as their wild-type littermates.

The signal for Trp53 induction *in vivo* also originates from actively transcribed genes UVB-induced apoptosis in skin requires induction of the Trp53 tumor suppressor protein (Ziegler *et al*, 1994). We therefore determined whether, as seen in fibroblasts *in vitro*, the signal for Trp53 induction also arises from DNA photoproducts in the transcribed strand of active genes. **Figure 4*A*** shows that $Xpa^{-/-}$ and $Csb^{-/-}$ mice induced Trp53 protein at 10–25-fold lower UVB doses than wild-type mice. Thus, 90–96% of the DNA signal for UVB induction of Trp53 involves excision-repairable DNA photoproducts in the transcribed strand of active genes. **Figure 4*B*** shows that $Xpc^{-/-}$ mice induce Trp53 in the same range of UVB doses as do wild-type animals, rather than in the lower dose range typical of $Xpa^{-/-}$ and $Csb^{-/-}$ mice.

Mdm2 is suppressed by erythemal UVB doses, with the UVB signal originating from actively transcribed genes The steady-state level of Trp53 is primarily determined by its degradation rate. This, in turn, is regulated by Mdm2, which targets Trp53 for degradation by the ubiquitin conjugation system (Ljungman, 2000). In cultured human fibroblasts, MDM2 was induced by UVC in control and XPC cells, but not in XPA or CSB patient cells (Conforti *et al*, 2000). Whereas the resulting elevation of TP53 in XPA and CSB cells would correlate with their sensitivity to UV-induced apoptosis, it is somewhat paradoxical that induction would be least in the most UV-sensitive genotypes. We therefore determined the dose–response for UVB induction of Mdm2 *in vivo*. **Figure 5** shows that DNA damage from

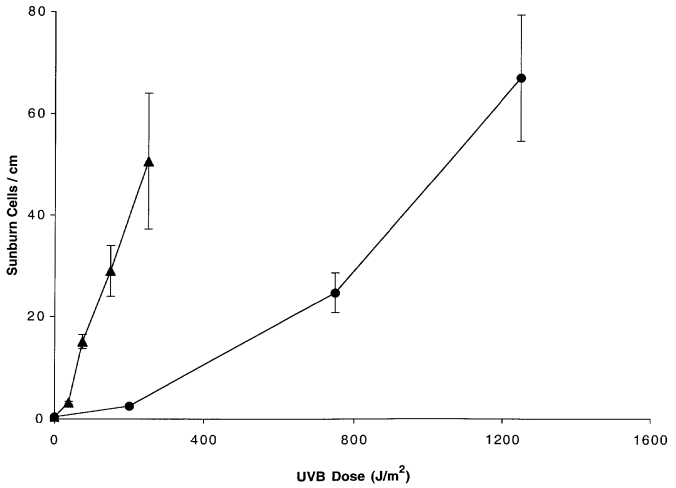

Figure 2. Hypersensitivity to UVB induction of apoptotic keratinocytes in $Csb^{-/-}$ mice. $Csb^{-/-}$ mice are defective in excision repair of DNA photoproducts specifically on the transcribed strand of actively transcribed genes. Circles, wild-type; triangles, $Csb^{-/-}$. Error bars, mean ± SEM (n = 3–4 animals per point).

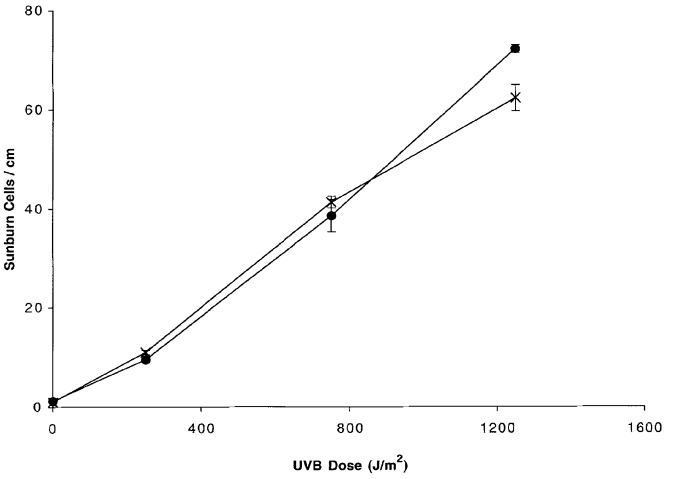

Figure 3. Normal UVB induction of apoptotic keratinocytes in $Xpc^{-/-}$ mice. $Xpc^{-/-}$ mice are defective in global excision repair of DNA photoproducts, resulting in the accumulation of DNA photoproducts in inactive genes, the nontranscribed strand of active genes, and in extragenic regions. Circles, wild-type; crosses, $Xpc^{-/-}$. Error bars, mean ± SEM (n = 3–4 animals per point).

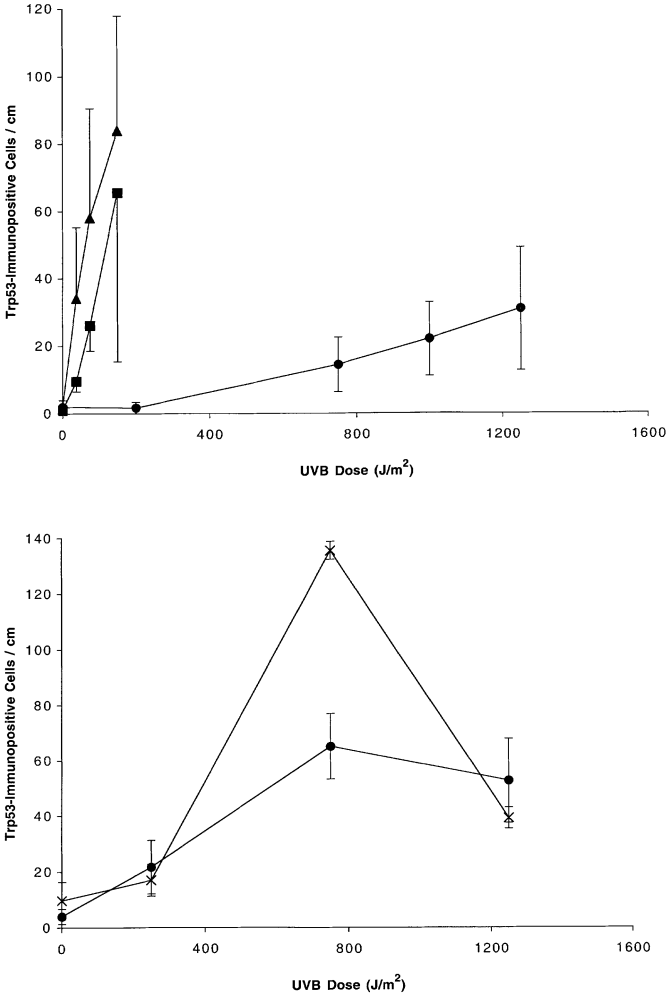

Figure 4. Hypersensitivity for UVB induction of the Trp53 tumor suppressor protein in the epidermis of $Xpa^{-/-}$ and $Csb^{-/-}$ mice. Cells expressing Trp53 protein 24 h after UVB irradiation ranged in immunopositivity from minimal (light brown) to medium (brown) or strong (dark brown). The level of Trp53 induction is expressed as the number of strongly immunopositive basal cells per centimeter of epidermis. (*A*) Wild-type, $XPA^{-/-}$; and $CSB^{-/-}$ mice. Circles, wild-type; squares, $XPA^{-/-}$; triangles, $CSB^{-/-}$. (*B*) $Xpc^{-/-}$ mice were similarly examined in an experiment performed separately and compared with $Xpc^{+/+}$ littermate controls. Circles, wild-type; crosses, $Xpc^{-/-}$. Error bars, mean ± SEM (n = 2–4 animals per point).

suberythemal doses of UVB induced Mdm2, but erythemal doses suppressed Mdm2 below its basal level. For the UV-sensitive $Xpa^{-/-}$ and $Csb^{-/-}$ mice, this pattern occurred at lower doses than in the wild-type and $Xpc^{-/-}$ mice. Half-inhibition occurred at a 9.1-fold lower UVB dose in $Csb^{-/-}$ than in wild-type mice, indicating that approximately 89% of the UVB signal for Mdm2 inhibition involves DNA photoproducts actively transcribed genes. The unusual response in the fibroblast experiments most likely reflected the use of the same UV dose in all four genotypes; this dose evidently led to suppression of Mdm2 in the UV-sensitive genotypes but induction of Mdm2 in the UV-resistant genotypes.

DISCUSSION

The DNA photoproduct signal for sunburn cells Leaving unrepaired UVB photoproducts at specific DNA regions *in vivo* revealed that 90% or more of the signal to induce sunburn cells involved DNA photoproducts in the template strand of actively transcribed genes (**Figs 1** and **2**). This result is consistent with observations on apoptosis at a single dose in hairless strains (van Oosten *et al*, 2000). The *in vivo* result is more striking than the analogous findings in human fibroblasts *in vitro*, in which a 3-fold difference remained between the sensitivity of XPA cells and CSB cells (Ljungman and Zhang, 1996). The smaller effect in humans could result from eventual repair of active genes by global excision repair, which, at least for cyclobutane pyrimidine dimers, is more active in human cells than murine cells. Other differences may also be significant. The higher proliferating fraction of cells *in vitro* (nearly 100% rather than the 5% *in vivo*) may lead to modes of apoptosis not represented *in vivo*. The ligands available to cell surface receptors also differ *in vitro*. Finally, cultured cell lines are typically immortalized using viral oncogenes; immortalization involves suppression of spontaneous apoptosis (Zindy *et al*, 1998) and many gene products inactivated by DNA viral oncogenes—such as the RB and TP53 tumor suppressors—influence apoptosis. Our study examined UVB-induced apoptosis in keratinocytes, the precursor of sunburn cells, in their natural epidermal environment.

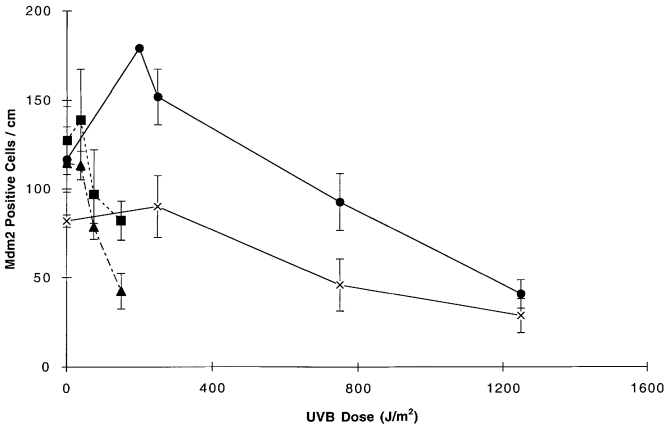

Figure 5. Induction and suppression of Mdm2 by UVB, signaled by DNA photoproducts in actively transcribed genes. Keratinocytes expressing Mdm2 protein 24 h after UVB irradiation were visualized by immunohistochemistry of paraffin sections adjacent to those scored for Trp53. Circles, wild-type; squares, $XPA^{-/-}$; triangles, $CSB^{-/-}$; crosses, $Xpc^{-/-}$. Error bars, mean ± SEM (n = 2–4 animals per point).

The DNA signal for upstream molecular events An effector of the signaling pathway for UVB-induced apoptosis is Trp53, which is induced by UV and is required for sunburn cell induction (Ziegler *et al*, 1994). Induction of Trp53 was even more dependent on DNA photoproducts in actively transcribed genes (90–96%) than was apoptosis itself (**Fig 4**). This dependence was again larger than seen *in vitro* (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). The pro-apoptotic effect of TP53 is known to be antagonized by an earlier anti-apoptotic effect, evidently due to the ability of TP53 to accelerate the recovery of mRNA synthesis inhibited by UV irradiation (McKay *et al*, 2000). Thus TP53 both initiates apoptosis and serves to self-limit the cause of its induction. In a Trp53 knockout mouse, apoptosis will be blocked. But in cells with partially active TP53, due to point mutations or partial inactivation by the human papillomavirus protein E6, the anti-apoptotic effect can prevail at certain doses and times (McKay and Ljungman, 1999).

The stability of Trp53 in S and G_2/M phases of the cell cycle is regulated by Mdm2, which targets it for degradation by the proteosome. Mdm2 may inhibit Trp53 in other ways as well; its binding to Trp53 competes with components of transcription factor TFIID and it may direct Trp53 nuclear export. Mdm2 has been reported to be suppressed by UV irradiation (Ljungman, 2000). *In vivo*, we found that low UVB doses induced Mdm2, whereas higher doses suppressed it (**Fig 5**). This biphasic dose–response removes certain anomalies in experiments that used a single UVB dose (Conforti *et al*, 2000). Eighty-nine percent of the signal for UVB suppression of Mdm2 arose from DNA photoproducts in actively transcribed genes. It is therefore likely that the order of events in signaling UVB-induced apoptosis in keratinocytes *in vivo* is primarily: blocked transcription → Mdm2 suppression → Trp53 induction → apoptosis.

Though DNA photoproducts block DNA replication and are mutagenic anywhere in the genome, the keratinocyte monitors only a minority of it for regulating Mdm2, Trp53, and apoptosis. The magnitude of this choice is highlighted by comparing similarly acting mouse strains. In $Xpc^{-/-}$ mice, which were irradiated with the same doses as wild types, keratinocytes are flooded with unrepaired photoproducts throughout most of the genome. Yet, there is no increase in the apoptosis pathway (**Figs 3–5**). As DNA replication occurs throughout the genome, the *Xpc* result also implies that DNA replication *per se* is not an essential factor in sunburn cell induction. The fact that *Csb* mice are sensitive, rather than resistant, to induction of Mdm2, Trp53, and apoptosis implies that the *Csb*-encoded coupling factor for transcription-coupled repair does not transmit the apoptosis signal; e.g., by coupling transcription to Trp53.

The role of non-DNA signals A large literature indicates a role for direct UV activation of cell surface receptors in UV signaling. Therefore, it is important to consider how the present findings relate to this data. The majority of the literature refers to the ''UV response'', the induction of JUN and FOS, and will not be discussed here except to note that most studies used UVC fluences in the vicinity of 40 J per m^2, which leaves 2% of the cells surviving (Wilkinson *et al*, 1970); occasionally, doses in the range 200–5000 J per m^2 were used. For apoptosis, our study shows that 90% of the signal induced by physiologic levels of UVB *in vivo* requires DNA photoproducts. Thus, an isolated receptor-initiated pathway could only contribute 10% of the signal. A cell surface event could nevertheless control 90% of apoptosis if: (i) it occurs downstream of the DNA photoproduct signal, or (ii) two signals are required for apoptosis, one from DNA photoproducts and the second from a non-DNA source. Which is the case?

In cell lines, UVC and UVB cause Fas receptors to aggregate and, as judged by recruitment of the apoptosis-related FADD adaptor protein, be activated (Rehemtulla *et al*, 1997; Aragane *et al*, 1998; Kulms *et al*, 1999). Apoptosis is reduced by 30–40% by inhibiting aggregation via lowering the membrane temperature; by inhibiting FADD with a dominant negative mutant; or by inhibiting FADD apoptosis initiator caspase 8. Similarly, dominant-negative inhibitors of tumor necrosis factor (TNF)-α receptor or anti-TNF-α antibody block UVB-induced apoptosis partially or completely (Schwarz *et al*, 1995; Sheikh *et al*, 1998). Downstream, MAPK members such as JUN, p38, and 14-3-3 regulators of p38 appear to be involved (Verhiej *et al*, 1996; Shimizu *et al*, 1999; Assefa *et al*, 2000; Xing *et al*, 2000), at least at high UV doses. The effect of UV on the Fas and TNF-α receptors was proposed to be direct, rather than involving receptor–ligand interactions, because neutralizing antibodies specific for the receptor do not prevent apoptosis (Aragane *et al*, 1998; Sheikh *et al*, 1998). Removing DNA photoproducts with photolyase reduces apoptosis by 50%. A partial additivity of photolyase and low temperature treatments suggested two independent pathways, of which the DNA damage pathway slightly predominates (Kulms *et al*, 1999).

A caveat to most of these experiments, including all of those indicating direct effects of UVB on receptors, is that they employed cell lines such as HeLa or HaCaT, which are known to be TP53 defective. As *Trp*53 knockout cells have 15% of the UV-induced apoptosis seen in normal cells (Ziegler *et al*, 1994), the direct effect of UV on receptors may be responsible for the 15% residual apoptosis, consistent with 90% originating from DNA photoproducts. Cells having normal Trp53 (primary murine fibroblasts or keratinocytes) do not show a requirement for the receptor-associated caspase 8 for UV-induced apoptosis *in vitro* (Varfolomeev *et al*, 1998; Tournier *et al*, 2000). Yet, they do still require one of the three MAPK signal transduction pathways, JNK (Tournier *et al*, 2000), and another, ERK, may be protective (Peus *et al*, 1999). Thus, in normal cells a receptor effector pathway other than a death receptor-adapter is indicated (Varfolomeev *et al*, 1998).

In vivo, the largest effect of receptor signaling on UV-induced apoptosis is reported for mice with mutant Fas ligand (C3H/HeJ gld/gld). In these animals, sunburn cells are reduced 3.6-fold (Hill *et al*, 1999). Thus, in contrast to the *in vitro* situation, FasL is a major component of UVB apoptosis. The Fas–FasL system has the potential for being an ''and'' gate, one arm of which is a DNA-surface loop: Fas receptor is induced by UVB and other DNA damaging agents via Trp53 (Bennett *et al*, 1998; Muller *et al*, 1998; Owen-Schaub *et al*, 2000) and FasL is induced by UVB via an unknown pathway (Leverkus *et al*, 1997; Hill *et al*, 1999). In UVB-irradiated T lymphocytes, FasL induction proceeds via the NF-κB and JNK-Fos/Jun pathways (Kasibhatla *et al*, 1998). Similarly, inactivating the TNF-α receptor *in vivo* by gene knockout or antibody binding leads to a 40% reduction in sunburn cell formation (Schwarz *et al*, 1995; Zhuang *et al*, 1999). Blocking

TNF-α release with pentoxifylline also reduces sunburn cell formation (Schwarz *et al*, 1997), ruling out a direct effect of UVB on the receptor. Importantly, injecting TNF-α does not itself lead to sunburn cells (Schwarz *et al*, 1995; Zhuang *et al*, 1999), implying that a second UVB-related signal is needed.

Existing data for cells with intact TP53 therefore do not conflict. They suggest that both a nuclear signal and a receptor-mediated signal are required to induce apoptosis after UVB. The latter appears to involve ligand-receptor binding; the former clearly originates from DNA photoproducts in actively transcribed genes.

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