

Ultraviolet-B Irradiation Alters the Cell Cycle Machinery in Murine Epidermis *In Vivo*

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Ultraviolet radiation of mouse skin leads to epidermal hyperplasia, inflammation, and subsequent tumor development. In this study we determined to what extent the cell cycle machinery is altered during epidermal proliferation after ultraviolet B radiation. A minimal erythema dose, 90 mJ per cm², increased the protein expression of the G1 phase cyclins, cyclin D1 and E, by 12 h. The majority of epidermal cells entered S phase between 18 and 24 h as determined by 5'-bromo-2'-deoxyuridine incorporation, proliferating cell nuclear antigen, and cyclin A immunohistochemistry. An increase in cyclin-dependent kinase 2 (cdk-2) protein expression occurred after 12 h, but no changes in cdk-4 or cdk-6 protein levels were observed. The increase in cyclin D1, E, and A protein expression was associated with an increase in

cyclin D1-cdk-4, cyclin E-cdk-2, and cyclin A-cdk-2 complex formation. p53 protein expression was elevated through 48 h, and the cdk inhibitor protein p21^{Cip1/WAF1} was elevated 6-fold to 7.5-fold between 12 and 24 h. The elevated p21^{Cip1/WAF1} protein contributed to an enhanced association with cdk-2 and cdk-4 at 3–24 h and 6–24 h post-ultraviolet B irradiation, respectively. These data indicate that 90 mJ per cm² of ultraviolet B irradiation induces a DNA damage response, by increasing p53 and p21^{Cip1/WAF1} protein expression, but also induces a rapid and sustained increase in S phase by 18 h. **Key words: hairless mice/immunoblotting/immunohistochemistry/immunoprecipitation/p53/proliferation/UVB. *J Invest Dermatol* 117:1171–1178, 2001**

Control of mammalian cell proliferation is regulated by a network of proteins that act synergistically during the cell cycle to ensure proper cell division. Progression through the cell cycle is dependent upon the activation of the cyclin-dependent kinases (cdk) (MacLachlan *et al*, 1995). Cdk regulate the progression of the cell cycle machinery through multisubunits that have stimulatory and inhibitory action. The stimulatory subunits, referred to as cyclins, are expressed in different phases of the cell cycle and regulate the activity of cdk through their accumulation and degradation (reviewed in Morgan, 1995; 1996). The transition from G1 to S phase is partly dependent upon the accumulation of the D-type cyclins D1, D2, and D3 and their association with cdk-4 and cdk-6 (Xiong *et al*, 1992; Kato *et al*, 1993; Meyerson and Harlow, 1994), and the increase in cyclin E and its association with cdk-2 (Dulic *et al*, 1992). As cells approach S phase, cyclin A protein increases and becomes associated with cdk-2 (Rosenblatt *et al*, 1992). Further regulation of the cell cycle is mediated through the activation of the catalytic subunit of the cdk, which is controlled by the cyclin-dependent activating kinase that phosphorylates tyrosine residues on cdk-2 and cdk-4 (Gu *et al*, 1993; Kato *et al*, 1994). The activation of the cdk is also controlled by the interaction of the cdk inhibitor (cki) proteins, which can bind to the catalytic subunit of

the cdk and inhibit their activity. There are two classes of cki based on their sequence homology. One group is the INK4 family, consisting of p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}, which have an ankyrin repeat domain (Hunter and Pines, 1994; Harper and Elledge, 1996). These proteins are G1 phase inhibitors and specifically bind to cdk-4 and cdk-6 and prevent their association to the D-type cyclins (Sherr and Roberts, 1995; Harper and Elledge, 1996). The second group consists of the cdk interacting protein (Cip)/kinase inhibitor protein (Kip) family, p21^{Cip1/WAF1}, p27^{Kip1}, and p57^{Kip2}. p21^{Cip1/WAF1}, which is regulated by p53 (el-Deiry *et al*, 1993), binds to cdk-2, cdk-4, or cdk-6 and is decreased when cells progress from quiescence into G1 (Sherr and Roberts, 1995; Harper and Elledge, 1996). p27^{Kip1}, which is usually low in proliferating cells and highest during quiescence, is induced by transforming growth factor β to arrest cells in G1 (Harper and Elledge, 1996) by inhibiting the kinase activity of cdk-2, cdk-4, and cdk-6 (Sherr and Roberts, 1995; Harper and Elledge, 1996).

Ultraviolet radiation (UV) is the leading cause of human cutaneous tumors and can lead to the development of murine cutaneous neoplasms. As an initiator, UVB radiation has been shown to induce the formation of cyclobutane pyrimidine dimers (CPD), (6–4)-pyrimidine pyrimidone photoproducts [(6–4)PD], and single strand breaks (Olsen *et al*, 1989; Vink *et al*, 1993; Berg *et al*, 1995; Friedberg *et al*, 1995; Berton *et al*, 1997). To maintain genomic integrity the cell has cell cycle checkpoints to provide time for repair of DNA damage. In response to the UVB-radiation-induced damage there is an increase in p53 protein expression (Ziegler *et al*, 1994; Berg *et al*, 1996; Inohara *et al*, 1996; Berton *et al*, 1997; de Laat *et al*, 1997), due to increased post-translational stability (Kastan *et al*, 1991; Lu and Lane, 1993). p53 can act as a

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Abbreviations: cdk, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen.

transcription factor for various genes like cki protein p21^{Cip1/WAF1} (el-Deiry *et al*, 1993; Inohara *et al*, 1996; Ouhtit *et al*, 2000), the growth inhibitor and excision repair protein GADD45 (Banrud *et al*, 1995), the apoptosis inducing protein Bax (Miyashita *et al*, 1994; Ouhtit *et al*, 2000), and the p53 regulating protein mdm2 (Perry *et al*, 1993; Chen *et al*, 1994). *In vitro*, UVB irradiation is capable of inducing a cell cycle arrest in G1 phase, thereby delaying S phase (Banrud *et al*, 1995; Petrocelli *et al*, 1996). This G1 arrest has been associated with suppression of G1 cyclin associated kinase activity by p21^{Cip1/WAF1}, but not the suppression of cyclin-cdk complex formation (Petrocelli *et al*, 1996).

Previously we have shown that after chronic exposure of murine skin to UVB irradiation there is a direct correlation between the number of proliferating cells and subsequent increase in epidermal hyperplasia leading to UVB-irradiation-induced tumorigenesis (Berton *et al*, 1997). The purpose of this study was to determine *in vivo* how one minimal erythema dose of UVB irradiation to the dorsal surface of SKH-1 hairless mice affects the keratinocyte cell cycle machinery in response to DNA damage. In this paper we report that there is an increase in p53 protein and p21^{Cip1/WAF1} protein expression up to 48 h after irradiation; however, no cell cycle arrest was observed. Instead, we detected an increase in protein expression of the G1 cyclins, cyclin D1 and E, and the association of these cyclins with their cdk. The increase in G1 phase cyclins corresponded to a large increase in the number of epidermal cells entering S phase after 18 h as measured by 5'-bromo-2'-deoxyuridine (BrdU) incorporation, proliferating cell nuclear antigen (PCNA), and cyclin A immunohistochemistry.

MATERIALS AND METHODS

Animals and UVB irradiation treatments Female hairless SKH-1-hrBr (SKH-1) mice were obtained from Charles River Laboratories (Wilmington, MA) at 3–4 wk of age, and were used at 8 wk of age.

Upon arrival, the mice were housed in climate-controlled quarters (22°C ± 1°C at 50% humidity), with a 12 h light/dark cycle under yellow fluorescent lights. The mice were UV irradiated with 12 Westinghouse FS20 sunlamps in a radiation chamber of our design and manufactured to maximize uniformity of incident fluence (Starch Art, Smithville, TX; Mitchell *et al*, 1999). According to the manufacturer's specifications the broad-band range for the UV lamps was between 260 and 400 nm, with 3%–5% of the radiation in the UVC region, 75%–77% of the radiation in the UVB region, and 20% in the UVA region, with a peak wavelength at 297 nm. The transparent plastic lid that covers the mice in the radiation chamber filters out the small amount of UVC radiation emitted from these lamps. This UV-transparent cover absorbs >90% of the radiation below 285 nm. The fluence was measured on a rotating platform 20 cm from the dorsal surface of the mice with an IL-1400 A Radiometer/Photometer coupled to an SEL 240/UV-B-1/TD detector containing a 280 nm Sharp Cutoff Filter (International Light, Newburyport, MA). This filter does not measure any radiation below 280 nm as described by the manufacturer. The UVB radiation fluence rate was determined to be 0.38 J per m² per s. The minimal erythema dose was determined to be 90 mJ per cm², which is sufficient to induce mild erythema and DNA damage (Berton *et al*, 1997).

Immunohistochemical staining and analysis For detection of BrdU incorporation three mice for each time point were injected intraperitoneally with a filter sterilized solution of 2.0% BrdU (Sigma) at 0.1 mg per g body weight in phosphate-buffered saline (PBS). After 1 h the mice were euthanized at the indicated time points after UVB irradiation, and three to six 5.0 mm × 2.0 cm sections were excised from the dorsal surface and fixed in 10% neutral buffered formalin. Four-micron paraffin sections from each mouse were stained with the following antibodies: a 1:50 dilution of rat anti-BrdU (Becton Dickinson), a 1:35 dilution of rabbit anti-p53 (NCL-p53-CMP, Novocastra Laboratories, Newcastle, U.K.), a 1:800 dilution of murine anti-PCNA (Coulter Immunology), and a 1:100 dilution of rabbit anticyclin A (C-19), rabbit anti-cdk-2 (M-22), and rabbit anti-p21 (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA). The antigens were visualized with 3,3'-diaminobenzidine (Sigma) through an avidin-biotin horseradish peroxidase (ABC; Vectastain Elite ABC kit; Vector

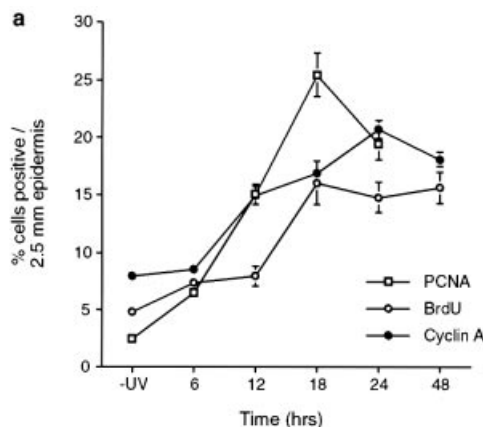
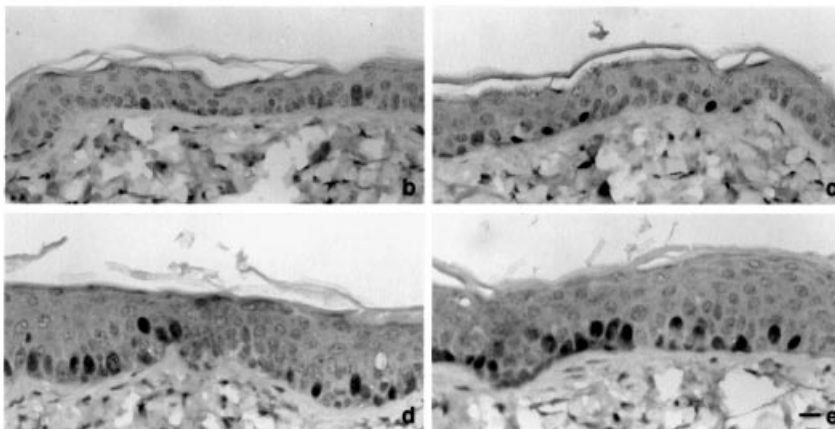


Figure 1. BrdU incorporation and cyclin A immunoreactivity are similar after UVB irradiation. SKH-1 hairless mice were UVB irradiated with 90 mJ per cm² and then euthanized at the indicated time points. The dorsal surface was isolated, fixed in 10% neutral buffered formalin, and prepared for immunohistochemistry. Four-micron sections were cut and probed with an anti-BrdU or an anticyclin A antibody as described in the text. (a) Open circles, BrdU incorporation; dosed circles, cyclin A immunostaining. Open squares, PCNA immunostaining (mean ± SEM; n = 18, for the number of cells in duplicate areas of 2.5 mm of epidermis from four dorsal sections of four mice at 200× for each time point). (b)–(e) The immunostaining pattern of cyclin A in (b) unirradiated epidermis, and UVB-irradiated epidermis after (c) 12 h, (d) 24 h, and (e) 48 h. Scale bar: 30 μm.



Laboratories) linked to a rabbit biotinylated anti-rat IgG for BrdU, or goat biotinylated anti-rabbit IgG for cyclin A, cdk-2, p21, and p53, or a goat biotinylated anti-mouse IgM for PCNA. For each slide the numbers of positive cells and hematoxylin-stained cells were counted as previously described (Berton *et al.*, 1997, 1998).

Western blotting Crude epidermal whole cell lysate was collected as previously described (Berton *et al.*, 1998), where the dorsal skin was removed and the epidermis was scraped onto a glass plate on ice. This method has been shown to be effective in removing the epidermis and leaving the dermis and fat pad (Rodriguez-Puebla *et al.*, 1998). The crude epidermal whole cell lysate was lysed in Nonidet P-40 buffer [50 mM Tris (hydroxymethyl)-aminomethane and 150 mM NaCl, 1.0% Nonidet P-40, pH 7.5, containing 1.0 mM sodium fluoride, 0.1 mM sodium vanadate, 2.0 mM phenylmethylsulfonyl fluoride, 1.0 µg per ml of leupeptin, aprotinin, and pepstatin] and the lysates were aliquoted and snap-frozen in liquid nitrogen. At the time of the assay, epidermal lysates were thawed at 4°C and quantitated by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). The protein lysates (40–50 µg) were electrophoresed through a 10% or 12% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membrane by wet transfer. Equivalent protein loading was assessed and the nitrocellulose was subjected to Western blotting as previously described (Berton *et al.*, 1998). Nonspecific binding sites were blocked with 5.0% nonfat dry milk in 0.1% Tween 20 (Sigma) in Tris-buffered saline (T-TBS) for 1 h at room temperature. The membranes were incubated with 1 µg of primary antibody per ml of 5.0% nonfat dry milk in 0.1% T-TBS for 1 h at room temperature. The primary antibodies were cyclin D1 (C-19, rabbit polyclonal IgG), cyclin E (M-20, rabbit polyclonal IgG), cyclin A (C-19, rabbit polyclonal IgG), p21 (C-19, rabbit polyclonal IgG), p27 (C-19, rabbit polyclonal IgG), cdk-2 (M-2, rabbit polyclonal IgG), cdk-4 (C-22, rabbit polyclonal IgG), and cdk-6 (C-21, rabbit polyclonal IgG) (Santa Cruz Biotechnology). After washing the membrane was incubated for 1 h at room temperature with a secondary donkey antirabbit horseradish peroxidase antibody (Amersham) at a 1:2500 dilution in 5.0% nonfat dry milk in 0.1% T-TBS. For detection the membrane was incubated with a chemiluminescence detection kit according to the manufacturer's directions (ECL kit, Amersham). Protein bands were quantitated by a Bio-Image Visage 60 (Bio-Image Products, Ann Arbor, MI).

Immunoprecipitation Cyclin-cdk and cdk-cki complex formation was detected by adding 250 µg epidermal protein lysate to anti-cdk-2, anti-cdk-4, anti-cdk-6, anticyclin E, anticyclin A, or anti-p21 bound to protein G-agarose beads (Life Technologies, Gaithersburg, MD). The mixture was incubated overnight at 4°C with constant rotation. After extensive washing the mixture was heated to 95°C for 5 min in sample loading buffer, electrophoresed on a 10% or 12% sodium dodecyl sulfate polyacrylamide gel, and transferred to nitrocellulose membrane by wet transfer. Detection of cyclin-cdk and cdk-cki complex formation was performed by immunoblotting as described above.

RESULTS

BrdU incorporation and cyclin A immunoreactivity kinetics after UVB irradiation UV radiation has been shown to increase the mitotic rate of keratinocytes, leading to subsequent hyperplasia and an increase in the number of suprabasal cells in the epidermis of mice (Olsen, 1988; Olsen and Kirkhus, 1989; Berg *et al.*, 1996; Berton *et al.*, 1997). In this study, we wanted to determine the changes in the cell cycle machinery of keratinocytes after UVB irradiation. As shown in **Fig 1(a)**, we observed an increase in the number of basal cells incorporating BrdU within 12 h after UVB irradiation. By 18 h BrdU incorporation was maximal and remained constant through 48 h. To confirm these results immunohistochemical staining against the mitotic cell cycle protein, cyclin A, and PCNA were performed. It is known that cyclin A protein levels increase when cells enter S phase and remain elevated through G2 phase (Rosenblatt *et al.*, 1992). The immunoreactivity to cyclin A was 2-fold after 12 h, increased to 3-fold after 24 h, and remained elevated through 48 h (**Fig 1a**). The location of cyclin A immunoreactivity and the cells incorporating BrdU were confined to the basal layer of the epidermis (**Fig 1b–e**). PCNA is a subunit of DNA polymerase δ and participates in DNA replication and repair (reviewed in Warbick, 2000). PCNA-positive cells were confined to the layer

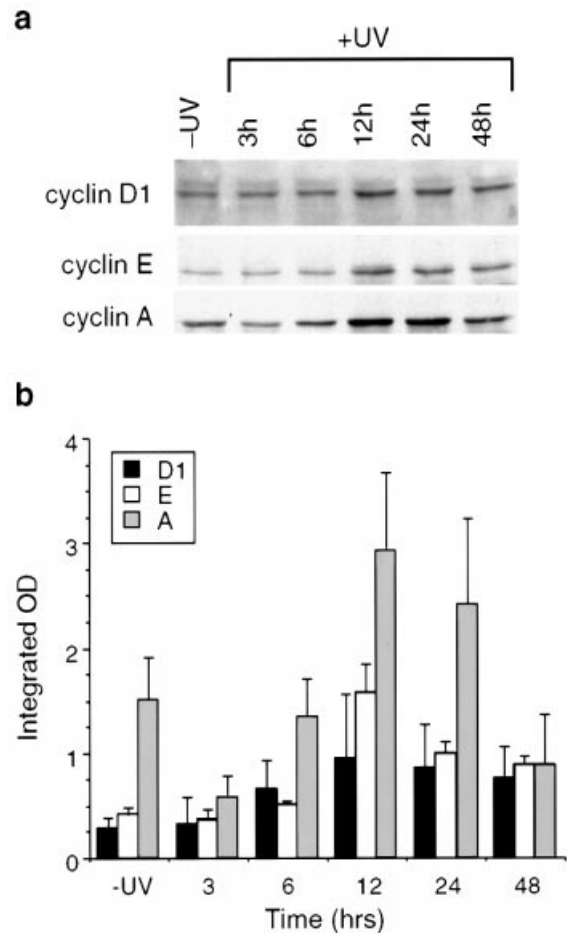


Figure 2. Protein expression of cyclin D1, cyclin E, and cyclin A after UVB irradiation. SKH-1 hairless mice were UVB irradiated and then euthanized; epidermal protein was isolated, electrophoresed, and probed as described in the text. (a) A representative Western blot for cyclin D1, cyclin E, and cyclin A. (b) Black bars, cyclin D1; open bars, cyclin E; Gray bars, cyclin A. (Mean \pm SD of three blots from separate experiments.)

up to 18 h; thereafter they appeared in the basal and suprabasal compartments. In agreement with our BrdU immunohistochemistry, PCNA staining was greatest at 18 h, where 25% of the cells were PCNA positive, a 10-fold increase over unirradiated epidermis (**Fig 1a**). The BrdU incorporation and PCNA immunohistochemistry data indicate that DNA synthesis was maximal 18 h after UVB irradiation.

UVB irradiation increases cyclin D1, cyclin E, cyclin A, and cdk-2 protein expression To corroborate the induction of S phase, whole cell epidermal protein lysates from sham-treated and UVB-irradiated SKH-1 mice were analyzed by immunoblot for cyclin and cdk protein expression. As shown in **Fig 2(a, b)** there was a 3.2-fold increase in cyclin D1 and a 3.7-fold increase in cyclin E protein levels by 12 h after UVB irradiation. By 24 h both cyclin D1 and cyclin E protein levels began to decrease. The cyclin A immunoblotting results corroborated the cyclin A immunostaining (compare **Fig 1a** and **Fig 2a, b**), where we observed a 2.0-fold increase in cyclin A protein between 12 and 24 h in UVB-irradiated epidermis followed by a decrease to unirradiated levels by 48 h. These results indicate that the majority of the keratinocytes were cycling through G1 and S phase between 12 and 24 h and into G2 by 24 h. Further analysis of the cell cycle machinery was performed by immunoblotting for cdk-2, cdk-4, and cdk-6 expression, and by immunohistochemistry for cdk-2 expression.

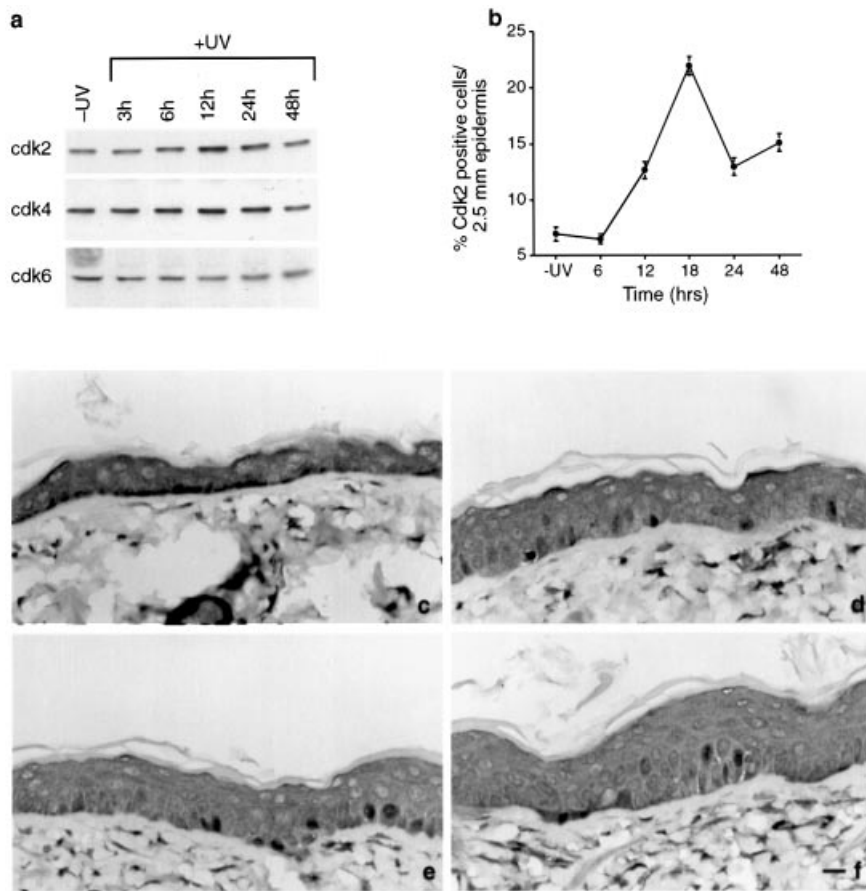


Figure 3. Cdk-2, cdk-4, and cdk-6 protein expression, and cdk-2 immunohistochemistry after UVB irradiation. SKH-1 hairless mice were UVB irradiated and then euthanized at the indicated time points; epidermal protein was isolated, electrophoresed, and probed as described in the text. For immunohistochemistry 4 μ m dorsal sections were cut and probed with an anti-cdk-2 antibody as described in the text. (a) A representative blot for cdk-2, cdk-4, and cdk-6 Western blotting. (b) The percentage of cells positive for cdk-2 (mean \pm SEM; $n = 12$, for the number of cells in duplicate areas of 2.5 mm of epidermis from four sections of three mice at 200 \times for each time point). (c)–(f) cdk-2 immunostaining in (c) unirradiated epidermis, and UVB-irradiated epidermis after (d) 12 h (e) 18 h, and (f) 48 h. Scale bar: 30 μ m.

Although there were no changes in cdk-4 or cdk-6 protein levels, cdk-2 protein expression was elevated 2.0-fold as measured by immunohistochemistry and 1.3-fold as measured by immunoblotting at 12 and 24 h (Fig 3a, b). Cdk-2 immunopositive cells were confined to the basal layer and its expression was maximal (3.2-fold) at 18 h (Fig 3b, e).

UVB irradiation increases cyclin–cdk association but not phosphorylation status An integral part of the progression of cells through the cell cycle is the interaction of the cyclins and cdk, and their phosphorylation status. To further characterize the rapid S phase progression induced by UVB irradiation, we assessed the cyclin–cdk complex formation of various cell cycle components following immunoprecipitation and assessed their ability to phosphorylate histone H1 and pRb in a kinase assay. Three hours after UVB irradiation we observed a decrease in the formation of a cdk-4 and cyclin D1 complex compared to unirradiated skin, and between 6 and 48 h the cdk-4–cyclin D1 association was equivalent to unirradiated skin (Fig 4). When we immunoprecipitated cdk-6 from epidermal lysates and immunoblotted for cyclin D1 we observed no change in complex formation at the indicated time points in UVB-irradiated epidermis compared to unirradiated epidermis. On the other hand, an association between cyclin E and cdk-2 was observed 6 h after UVB irradiation and remained elevated through 24 h. The cyclin A–cdk-2 complex increased early, within 3 h after UVB irradiation, and remained elevated through 48 h (Fig 4). The multiple bands detected by Western blot in these immunoprecipitations may indicate nonspecific binding to unrelated protein or changes in the phosphorylation status of these complexes. To clarify whether there was a change in phosphorylation status of these proteins, kinase assays were performed with cdk-2, cdk-4, and cdk-6. We could not detect significant differences in histone H1 or pRb phosphorylation after

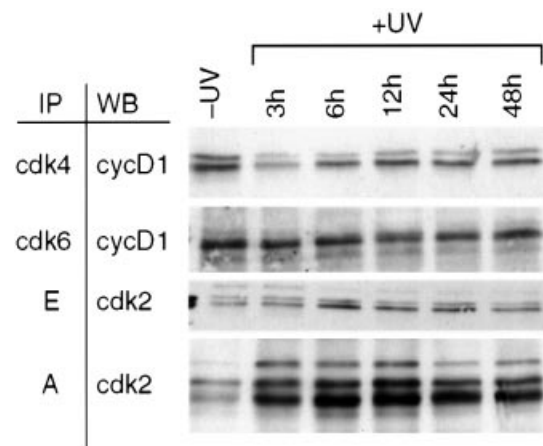


Figure 4. UVB irradiation induces cdk-4–cyclin D1 and cdk-2–cyclin A association. SKH-1 hairless mice were UVB irradiated and then euthanized at the indicated time points. 250 μ g of epidermal protein was isolated and added to anti-cdk-4, anti-cdk-6, anti-cyclin E, or anti-cyclin A, bound to protein G-agarose beads, electrophoresed, and probed as described in the text.

UVB irradiation compared to unirradiated mouse skin (not shown). This is probably a result of the use of total epidermis, which contains a large fraction of nonproliferating cells.

p53 immunoreactivity is elevated after UVB irradiation In response to UVB-irradiation-induced DNA damage we previously observed an increase in p53 protein expression (Berton *et al*, 1997). Here we have determined the

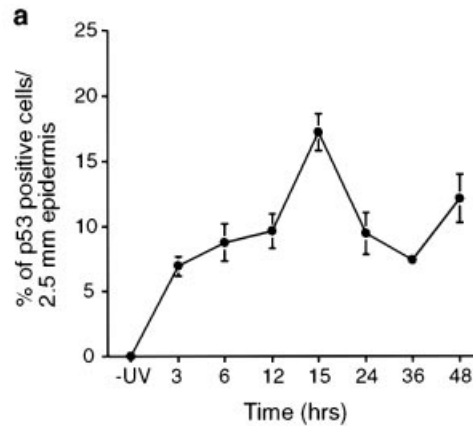
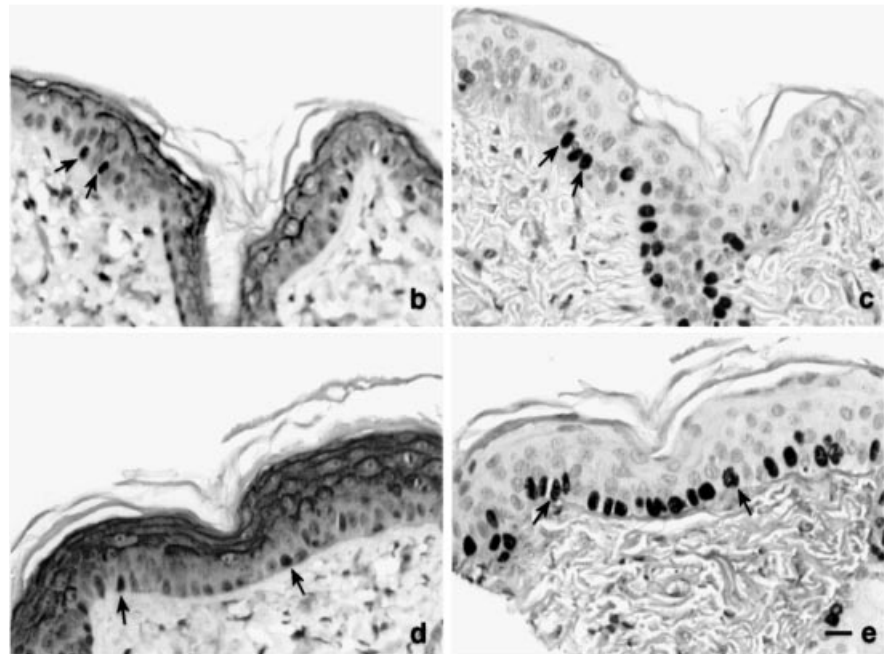


Figure 5. The percentage of p53 immunopositive cells, and p53 and BrdU staining in consecutive sections after UVB irradiation. SKH-1 mice were UVB irradiated and then euthanized at the indicated time points. For immunohistochemistry 4 μ m dorsal sections were cut and probed with an anti-p53 antibody, and then a consecutive section was probed with an anti-BrdU antibody as described in the text. (a) The percentage of positive cells for p53 immunoreactivity (mean \pm SEM; $n = 18$, for the number of cells in duplicate areas of 2.5 mm of epidermis from three sections of three mice at 200 \times). (b) p53 immunostaining and (c) BrdU incorporation after 12 h of UVB irradiation. (d) p53 immunostaining and (e) BrdU incorporation after 48 h of UVB irradiation. Arrows indicate the cells that are both p53 and BrdU positive. Scale bar: 40 μ m.



temporal pattern of p53 protein expression after UVB irradiation by immunostaining formalin-fixed skin. Cells positive for p53 were evident as early as 3 h after UVB irradiation. By 15 h there was a 2-fold increase in the number of p53 immunopositive cells in the basal layer of the epidermis (**Fig 5a**). Our pattern of p53 induction was similar to that reported by Ouhitit *et al* (2000), where they used a dose of UVB radiation 3-fold greater than reported here.

p53 and BrdU are expressed within the same cell 12–48 h after UVB irradiation As there was an increase in p53 and BrdU immunoreactivity after UVB irradiation in the basal layer of the epidermis between 12 and 48 h, and p53 is expressed during UVB-irradiation-induced DNA damage, we wanted to determine if cells undergoing DNA synthesis were also immunoreactive for p53. Immunohistochemistry on consecutive sections showed that two cells out of 1000 in the basal layer were positive for both p53 and BrdU at 12 and 48 h after UVB irradiation (**Fig 5b–d**).

p21 protein levels and p21–cdk association are elevated after UVB irradiation To extend these findings and to understand the involvement on downstream effects of the increase in p53 protein expression in response to DNA damage, we analyzed the expression of cki by Western blot and immunohistochemistry. Of

the two families of cki only the p21^{Cip1/WAF1} and p27^{Kip1} of the Cip/Kip family could be detected by immunoblotting in normal or UVB-irradiated murine epidermis. We assessed the expression level of p21^{Cip1/WAF1} by immunohistochemistry and Western blot analysis. By Western blot we detected a 6-fold increase in p21^{Cip1/WAF1} protein expression by 6 h, and by 24 h p21^{Cip1/WAF1} protein levels were elevated 7.5-fold (**Fig 6a, c**). When we stained normal epidermis with the p21^{Cip1/WAF1} antibody immunoreactivity was absent; however, 6 h after UVB irradiation approximately 14% of the cells in the epidermis were immunopositive, and after 24 h 25% of the epidermal cells were immunopositive (**Fig 7e**). This immunoreactivity was confined to the basal layer up to 12 h, but was located in both the basal and spinous layers 24 h post-UVB irradiation (**Fig 7a–d**). These results are similar to those reported by Ouhitit *et al* (2000). Analysis of p27^{Kip1} protein levels in the epidermis revealed a 2-fold increase between 3 and 24 h after UVB irradiation, but by 48 h the amount of protein decreased to unirradiated levels (**Fig 6a, c**). To determine if the increase in p21^{Cip1/WAF1} protein affected cyclin–cdk complex formation, we immunoprecipitated either cdk-2 or p21^{Cip1/WAF1} from epidermal protein lysates and immunoblotted for either p21^{Cip1/WAF1} or cdk-4. As shown in **Fig 6(b)**, there was

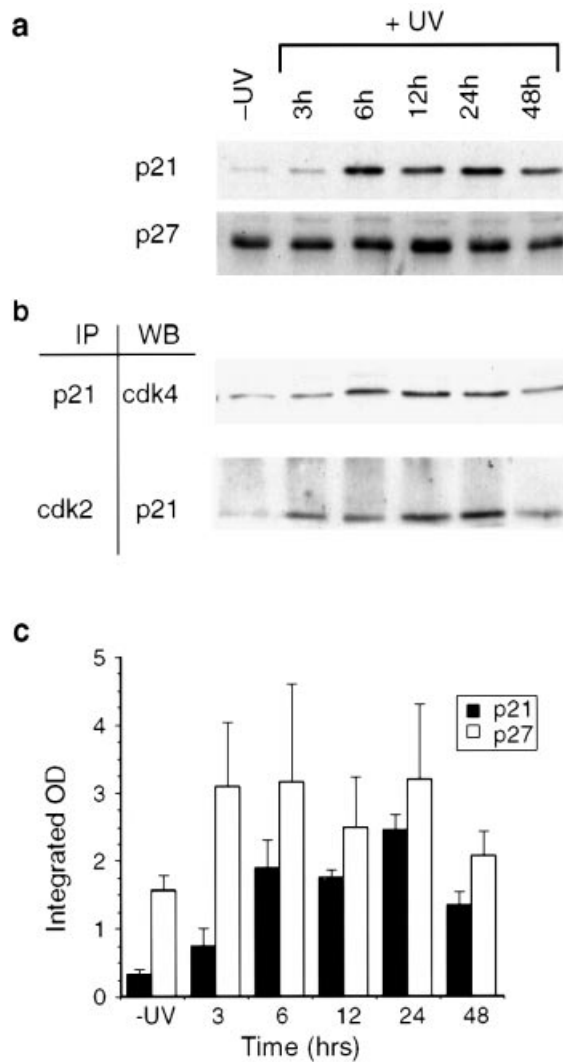


Figure 6. UVB irradiation induces p21 protein expression, and cdk-2-p21 and cdk-4-p21 association. SKH-1 mice were UVB irradiated and then euthanized at the indicated time points; epidermal protein was isolated, electrophoresed, and probed as described in the text. For the immunoprecipitations 250 μ g of epidermal protein was added to either anti-cdk-2 or anti-p21 bound to protein G-agarose beads, electrophoresed, and immunoblotted as described in the text. (a) Western blotting for p21 and p27. (b) An immunoprecipitation with p21 or cdk-2 followed by immunoblotting with cdk-4 or p21. (c) The mean \pm SD of three blots from separate experiments. Black bars, p21; open bars, p27.

an increase in the association between p21^{Cip1/WAF1}-cdk-4 between 6 and 24 h and an increase in the association between cdk-2-p21^{Cip1/WAF1} as early as 3 h, with the maximum association between 12 and 24 h after UVB irradiation.

DISCUSSION

Previous studies have shown that UVB radiation can cause the formation of DNA lesions, which is a primary factor in the development of skin cancer in mice (Kanjilal *et al*, 1993; Sato *et al*, 1993; Vink *et al*, 1993; Tornaletti and Pfeifer, 1994; van Kranen *et al*, 1995; Berton *et al*, 1997). We previously have shown that tumor development is more directly correlated with an increase in epidermal proliferation than with the absolute amount of DNA photodamage (Berton *et al*, 1997). In this study we determined how the mitogenic effect of a single erythemic dose of UVB radiation

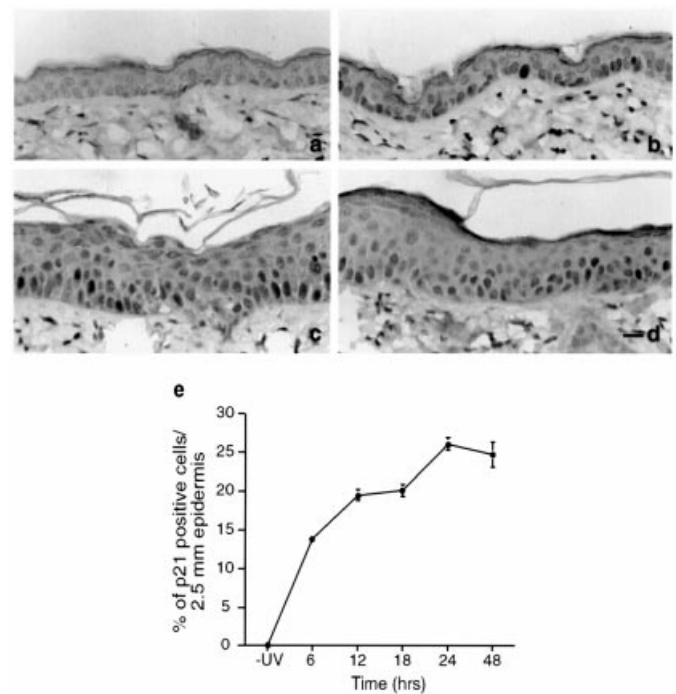


Figure 7. p21 immunostaining after UVB irradiation. SKH-1 mice were UVB irradiated and then euthanized at the indicated time points; the dorsal skin was prepared for immunohistochemistry with anti-p21 antibody as described in the text. (a)-(d) Representative areas were photographed to show localization: (a) unirradiated mouse skin; (b) 12 h, (c) 24 h, and (d) 48 h after UVB irradiation, respectively. (e) Mean \pm SEM ($n = 12$, for the number of cells in duplicate areas of 2.5 mm of epidermis from four sections of three mice at 200 \times for each time point). Scale bar: 30 μ m.

could alter the cell cycle machinery of epidermal keratinocytes after the induction and during the time of photodamage repair *in vivo*. In this report our data indicate that a pulse of BrdU 30 min prior to sacrifice preferentially labels cells that are undergoing DNA replication and that the maximum point of DNA replication is 18 h post-UVB radiation as determined by BrdU incorporation and PCNA immunohistochemistry. The overexpressions of cyclins D1 and E have been correlated with an increase in G1 to S phase transition by shortening G1 (Ohtsubo and Roberts, 1993; Quelle *et al*, 1993; Poon *et al*, 1996). Our data support this evidence: we observed a maximal increase in cyclin D1 and cyclin E 12 h post-UVB irradiation. As cells approach and enter S phase the increase in cell cycle progression becomes a concerted effort between the association of cyclin E and cyclin A with cdk-2 between 12 and 24 h post-UVB irradiation. In addition, the prolonged BrdU incorporation and the extended expression of the cell cycle components provide evidence that the UVB radiation dose used is mitogenic and that the cells in the epidermis are asynchronous with respect to cell cycling.

Evidence to support the mitogenic response of UVB irradiation comes from studies in hr/hr hairless mice. An increase in S phase was observed as early as 12 h after treatment with a suberythemic dose of 12.5 mJ per cm² or an erythemic dose of 25 mJ per cm² (Olsen, 1988; Olsen and Kirkhus, 1989), whereas a high dose (130 mJ per cm²) given to SKH-1 mice induced S phase only after 48 h (de Laat *et al*, 1997). *In vitro*, however, Petrocelli *et al* (1996) have shown that UVB-irradiated neonatal rat keratinocytes do not have elevated cyclin or cdk protein levels when cells enter S phase at 18 h. Instead they showed that cell cycle progression appears to be dependent on the elevated kinase activity of cyclin D1-cdk-4, cyclin E-cdk-2, and cyclin A-cdk-2. Although we could not detect any difference in activity of our cyclin-cdk complexes between

UVB-irradiated and normal epidermis, our results suggest that there is a positive correlation between entry into S phase and the association of cyclin-cdk and cyclin protein expression. The data presented here indicate that the alteration of the cell cycle machinery of epidermal keratinocytes *in vivo* with a dose of 90 mJ per cm² UVB irradiation reflects the changes that occur prior to UVB-irradiation-induced tumorigenesis as shown by Balasubramanian *et al* (1999). In their study they have shown that chronically UVB-irradiated mouse epidermis (180 mJ per cm², three times a week) between 4 and 16 wk has elevated cyclin D1 and cyclin E, cdk-2, and p21^{Cip1/WAF1} protein expression. Moreover, as shown in this study, they observed no changes in cdk-4, cdk-6, or p27^{Kip1} protein levels (Balasubramanian *et al*, 1999).

The transactivation of p21^{Cip1/WAF1} by p53 allows p21^{Cip1/WAF1} to participate in cell cycle arrest and DNA repair (el-Deiry *et al*, 1993; Inohara *et al*, 1996). This has been shown *in vitro* by Petrocelli *et al* (1996) and Poon *et al* (1996), who showed that UVB or UVC irradiation induces cell cycle arrest through the inhibitory action of p21^{Cip1/WAF1} when bound to cdk-2 and cdk-4. Chen *et al* (1996) and Flores-Rozas *et al* (1994) also showed that p21^{Cip1/WAF1} binds to the PCNA to assist in DNA repair and replication. Our results are in agreement with those of Inohara *et al* (1996), who found an increase in p53 and p21^{Cip1/WAF1} immunostaining in chronically sun-exposed normal skin. p21^{Cip1/WAF1} can also act as an assembly factor for cyclin D-cdk complexes and enhance cell cycle progression (LaBaer *et al*, 1997), and participate in the formation of p21-cyclin D/cdk-PCNA quaternary complexes to assist in DNA replication and repair (Xiong *et al*, 1992). The elevated and sustained PCNA, p53, and p21^{Cip1/WAF1} protein expression up to 48 h post-UVB irradiation coupled with the increase in p21^{Cip1/WAF1}-cdk-2 and -cdk-4 association during S phase is suggestive of ongoing DNA repair synthesis, which does not preclude replicative DNA synthesis. This is supported by two observations. Previously we showed that 30% and 75% of the (6-4)PD and CPD still remained 18 h after UVB irradiation (Berton *et al*, 1997), which corresponds to the maximal p53 protein expression as determined by immunohistochemistry in this report. Second, we detected two out of 1000 basal cells that were immunoreactive for both BrdU and p53 between 12 h and 48 h after UVB irradiation. It would be of interest to label cells simultaneously for BrdU and p53 as well as PCNA or p21^{Cip1/WAF1} to exactly determine the relationship between these proteins in the basal compartment *in vivo*. Serial sections are the only possible method at the moment, however, as the immunohistochemistry protocols for each of the above antigens are not compatible. The alternative would be to do these experiments with primary keratinocytes *in vitro*.

Additional evidence suggests alternative roles for the increase in p21^{Cip1/WAF1} expression. For instance, an increase in p21^{Cip1/WAF1} is correlated with apoptosis (el-Deiry *et al*, 1994), whereas its overexpression has been shown to prevent apoptosis (el-Deiry *et al*, 1994; Kobayashi *et al*, 1995; Gorospe *et al*, 1996; Erhardt and Pittman, 1998). We did not assess apoptosis in this study, but a UVB radiation dose of 100 mJ per cm² or 250 mJ per cm² induces apoptosis by 24 h in SKH-1 mice as determined by TUNEL staining (Kane and Maytin, 1995; Ouhtit *et al*, 2000). Coexpression of p21^{Cip1/WAF1} and keratin 10 by immunostaining was observed in the differentiating cells of human epidermis after exposure to solar UVB radiation (Tron *et al*, 1996). Although we have not analyzed keratin 10 in this report, we do show that p21^{Cip1/WAF1} is expressed in the stratum spinosum at later time points. Finally, evidence to support the role of p21^{Cip1/WAF1} in differentiating cells was observed in p21^{-/-} mice. These mice have normal development but their keratinocytes exhibit a reduction in the differentiation markers keratin 1, loricrin, and involucrin compared to their wildtype litter mates (Missero *et al*, 1996). Taken together, our results, and those of others, suggest multiple roles for p21^{Cip1/WAF1} in the epidermis.

In summary, our observations indicate that UVB irradiation induces a rapid entry into S phase by 18 h, which is partially regulated by the cyclin-cdk association and the increase in cyclin D1, cyclin E, and cyclin A protein levels. In addition, the p53 and BrdU coimmunostaining in a few basal cells suggests that p53 is involved in DNA repair synthesis during S phase. Based on these data, it is likely that, after chronic UVB irradiation where p53 mutations can occur in human skin (Jonason *et al*, 1996), the ability of p53 to participate in transactivation of target genes is impaired, thus contributing to genomic instability. In addition, the timing of the expression of p21^{Cip1/WAF1} in UVB-irradiated epidermis suggests that it may participate in DNA repair, apoptosis, cell cycle progression, and/or differentiation. In conclusion, these data suggest that UVB-irradiation-induced hyperplasia and tumorigenesis is partly mediated by the upregulation of cyclin D1, E, A, and cdk-2 and the association of cyclin D1-cdk-4.

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