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p53-Dependent DNA repair and apoptosis respond differently to high- and low-dose ultraviolet radiation

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Summary p53 plays an essential part in the maintenance of the cellular genetic stability after a DNA-damaging event such as ultraviolet (UV) radiation. Following UV radiation, the amount of p53 protein is elevated. The increased p53 is believed to induce cell cycle arrest, promote nucleotide excision repair (NER) and apoptosis. To study if cells respond differently to high- and low-dose UV radiation, we examined the DNA repair efficiency and apoptosis rate of human and murine fibroblasts after UV radiation. Using a host cell reactivation assay, we found that NER was increased after low doses but not after high doses of UV radiation. In contrast, apoptosis occurred only after the cells received high doses (over 200 J/m^2), but not low doses of UVB. The induction of both NER and apoptosis was observed only in p53$^{+/+}$ murine fibroblasts, not in p53$^{-/-}$ cells, indicating that both stress response mechanisms are dependent on wild-type p53 function. UV radiation induced the expression of p53 protein in a dose-dependent manner up to 400 J/m^2. In contrast, p21$^{waf1/cip1}$ was induced only after low doses and bax only after high doses of UV radiation, supporting the roles of p21$^{waf1/cip1}$ and bax in NER and apoptosis, respectively. Taken together, these results indicate that cellular stress response to UV radiation depends on UV dose, DNA repair after low doses and apoptosis after high doses, and that both mechanisms are dependent on wild-type p53 function.

Experimental and epidemiological data strongly implicate ultraviolet (UV) radiation as an important factor in the development of skin cancers. The depletion of the ozone layer is expected to lead to a significant increase in the incidence of skin cancers. For every 1% reduction in the ozone layer, the incidence of skin cancers will increase by 2–4%.[1] The UV spectrum can be divided into three parts by wavelength: UVA (320–400 nm), UVB (290–320 nm) and UVC (200–290 nm). UVC radiation is absorbed efficiently by the ozone layer and does not reach the surface of the earth. Studies on the carcinogenic effect of UVA and UVB indicate that UVB is responsible for most of the carcinogenic effects of sun exposure.[2] UV radiation induces the formation of cyclobutane pyrimidine dimers and (6-4) photoproducts which may lead to mutations and cancer development if the DNA damage is not removed from the genome.[3]

In the past few years, many studies have suggested that mutations of the p53 tumour suppressor gene are involved in the development of skin cancers.[4] Mutations of the p53 gene occur in most human squamous cell carcinomas,[5,6] UVB-induced mouse squamous cell carcinomas,[7] human basal cell carcinomas[8] and skin tumours (including squamous cell carcinoma and basal cell carcinoma) in DNA-repair-deficient xeroderma pigmentosum (XP) patients.[9] The mutations are predominantly UV-specific C → T or CC → TT transitions.[4] To confirm the role of p53 in the pathogenesis of UV-induced skin cancer, we previously exposed p53 transgenic mice that contain mutant alleles of the p53 gene to UVB radiation and found that p53 transgenic mice are predisposed to UV-induced squamous cell carcinomas.[10] These data suggest that p53 inactivation leads to a selective advantage, and thus wild-type p53 possibly plays an important part in protecting the genome against UV damage.

Many studies indicate that p53 plays a vital part in mediating the cellular response to UV radiation. There are two mechanisms for a cell to remove DNA damage: repairing the DNA damage or inducing apoptosis. There is evidence to suggest that p53 is involved in both DNA repair and apoptosis.

G1 arrest is induced when p53 transcriptionally upregulates p21$^{waf1/cip1}$, a potent inhibitor of cyclin-dependent kinase.[11–13] It is assumed that p53

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induces cell cycle arrest to provide extra time for the cell to repair DNA damage before the passage of the damaged DNA to daughter cells. Recent studies suggest that p53 may directly participate in nucleotide excision repair (NER). Wild-type p53 binds to and modulates XPB and XPD, two components of the TFIIH transcription unit, which possesses helicase, ATPase and kinase activity.[14,15] Another direct link between p53 and NER is replication protein A, a factor that is essential for the first step of DNA replication.[16,17] p53 also binds to DNA strand breaks, possibly to recruit repair proteins.[18] Direct evidence of the role of p53 in DNA repair comes from the observation that NER is reduced in cells with abnormal p53 functions. Smith *et al.*[19] showed that the disruption of normal p53 function in human colon carcinoma RKO cells with either the human papillomavirus E6 oncoprotein or a mutant p53 transgene results in reduced repair of UV-induced DNA damage. Using radioimmunoassay and host cell reactivation assay, we have demonstrated that NER is reduced in the skin cells from p53 transgenic mice and p53 knockout mice both *in vitro* and *in vivo*,[20,21] further supporting the role of p53 in NER.

Another important role of the p53 protein in maintaining the genomic stability of the cells is to trigger apoptosis after UV irradiation. Apoptosis is a self-protective mechanism by which cells possessing significant DNA damage can be deleted. It has been shown that apoptosis of cultured cells induced by ionizing radiation,[22,23] anticancer drugs[24,25] or growth factor deprivation,[26] is dependent on wild-type p53 function. Furthermore, p53-dependent apoptosis suppresses tumour growth and progression *in vivo*.[27] Ziegler *et al.*[28] showed that the rate of apoptosis induced by UV radiation is significantly reduced in the keratinocytes of p53 knockout mice. It is suggested that p53 regulates the expression of bcl-2/bax in the process of inducing apoptosis.[29] Bcl-2 protein is thought to prevent most types of apoptotic cell death, whereas bax protein heterodimerizes with bcl-2 and promotes apoptosis.[30] p53 is found to be a direct transcriptional activator of the human bax gene.[31–33]

DNA repair and apoptosis are important cellular responses to UV radiation. We have hypothesized that low- vs. high-dose UV radiation may preferentially trigger one or the other mechanism. We therefore explored the effect of low- and high-dose UV radiation on NER and apoptosis. In this paper, a UV dose which does not affect cell survival is considered as a low UV dose. Our results indicate that low-dose UV radiation preferentially triggers DNA repair while high-dose UV radiation induces apoptosis, and that both mechanisms are dependent on wild-type p53 function.

Materials and methods

Cell culture

Human dermal fibroblasts were obtained from the Tissue Bank of Vancouver Hospital and Health Sciences Centre. The cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

Dermal fibroblasts of $p53^{+/+}$ and $p53^{-/-}$ mice (Genpharm, Mountainview, CA, U.S.A.) were isolated from 4-week-old mice. The mice were killed by cervical dislocation and a 2 × 2 cm skin biopsy was dissected from the dorsal area. The hair was removed and the skin biopsy was disinfected with 2·5% betadine for 1 min, followed by 1 min in 70% ethanol, and washed with phosphate-buffered saline (PBS) twice. The skin tissue was then minced and incubated in DMEM containing 200 units/mL collagenase (Sigma, St Louis, MO, U.S.A.) at 37 °C for 6 h. The digested skin tissue was centrifuged at 1000 ***g*** for 10 min and the pellet washed twice with prewarmed DMEM. The cells were resuspended in DMEM containing 10% FBS and incubated at 37 °C in a 5% CO_2 atmosphere.

Ultraviolet irradiation

Cultured cells were exposed to UVB (290–320 nm), using a bank of four unfiltered FS40 sunlamps (Westinghouse, Bloomfield, NJ, U.S.A.). The intensity of the UV light was measured by an IL 700 radiometer fitted with a WN 320 filter which is specific for UVB wavelengths, and an A127 quartz diffuser (International Light, Newburyport, MA, U.S.A.). The same UVB source was used for all the experiments.

Western blot analysis

Cells were washed with PBS three times, and lysed in triple-detergent buffer (50 mmol/L Tris–HCl (pH 8·0), 150 mmol/L NaCl, 0·02% sodium azide, 0·1% sodium dodecyl sulphate (SDS), 100 μg/mL phenylmethylsulphonyl fluoride, 1 μg/mL aprotinin, 1% Nonidet P-40, 0·5% sodium deoxycholate) for 30 min. The lysate was centrifuged at 12,000 ***g*** for 10 min and the supernatant collected. The protein concentration was determined by

the DC Protein Assay (BioRad, Hercules, CA, U.S.A.). One hundred micrograms of protein were separated on a 12% polyacrylamide/SDS gel, and electroblotted on to a nitrocellulose filter. The filter was blocked with 5% milk overnight, hybridized to anti-p53 Cm1 antibody (Dimension, Mississauga, Ontario, Canada), anti-$p21^{cip1}$ antibody (Santa Cruz, Santa Cruz, CA, U.S.A.) or anti-bax antibody (Santa Cruz) for 1 h, and then hybridized with horseradish-peroxidase-labelled secondary antibody for 1 h at room temperature. The signals were detected with an ECL–Western chemiluminescence detection kit (Amersham, Quebec, Canada). The intensity of the bands was quantified with a densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Chloramphenicol acetyltransferase assay

The plasmid $pCMV_{cat}$ (a kind gift of Dr L.Grossman, The Johns Hopkins University, Baltimore, MD, U.S.A.) contains a gene encoding for chloramphenicol acetyltransferase (CAT), under the transcriptional control of the immediate early promoter of the human cytomegalovirus. Transfection of the UVC-damaged plasmid $pCMV_{cat}$ DNA into the fibroblasts was performed using a liposome-mediated procedure as described.[21] The assay was performed in triplicate. Controls included transfection with undamaged plasmid DNA and mock transfection without plasmid DNA.

Enzyme-linked immunosorbent apoptosis assay

Cells were seeded at a density of 2×10^4/well in a 96-well plate, grown for 24 h, and exposed to 0, 50, 100, 200, 400 and 800 J/m^2 of UVB. After 24 h, an ELISA was performed using a cell death detection $ELISA^{plus}$ kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells were incubated in 200 μL lysis buffer for 30 min at room temperature. The lysate was centrifuged at 2000 ***g*** for 10 min, and 150 μL of supernatant were collected, of which 20 μL were incubated with anti-histone–biotin and anti-DNA peroxidase at room temperature for 2 h. After washing with incubation buffer three times, 100 μL of substrate solution (2,2′-azino-di(3-ethylbenzthiazolin-sulphonic acid)) was added to each well for 15–20 min. The photometric analysis was carried out in a Titertek Multiskan (Eflab Oy, Helsinki, Finland) at 405 nm.

Apoptosis assay by DNA fragmentation

Cells were seeded at 1×10^5/35 mm dishes, and labelled with [methyl-^{3}H]thymidine to a final concentration of 1 μCi/mL for 24 h. The cells were washed three times with PBS, and then exposed to UVB irradiation. After 24 h, attached and detached cells were pooled and washed three times with ice-cold PBS, and analysed for DNA fragmentation.[34] Briefly, the cells were incubated in lysis buffer [5 mmol/L Tris–HCl (pH 8·0), 5 mmol/L ethylenediamine tetraacetic acid, 0·5% Triton X-100] on ice for 2 h. The lysate was then centrifuged for 30 min at 12,000 ***g*** to separate high molecular weight and low molecular weight (fragmented) DNA. The radioactivity of the supernatant (low molecular weight DNA) and the pellet (high molecular weight DNA) was quantified separately by liquid scintillation counting. The percentage of fragmented DNA was determined as (radioactivity of supernatant (d.p.m.)/radioactivity of supernatant and pellet (d.p.m.))$\times$100%.

Cell survival assay

Human fibroblasts were seeded at 2×10^4/well in a 96-well plate and incubated in DMEM containing 10% FBS. After 24 h, the cells were exposed to UVB at 0, 50, 100, 200, 400 and 800 J/m^2. The cells were then refed with fresh DMEM. The cell survival rate was determined using the sulphorhodamine B (SRB) assay.[35] Briefly, 72 h after UV irradiation, the cells were fixed with 10% trichloroacetic acid for 1 h at 4 °C, washed five times with water, air dried, and stained with 0·4% SRB in 1% acetic acid for 30 min. Cells were then washed with 1% acetic acid and air dried. One hundred microlitres of Tris base (pH 10·5) were added to each well for 30 min and the optical density (OD) reading was performed using a Titertek Multiskan (Eflab Oy) at 550 nm. The percentage of survival of UV-irradiated cells was determined by comparing the OD reading with that of non-irradiated cells.

Results

p53-dependent induction of nucleotide excision repair by low-dose ultraviolet radiation

Using p53 transgenic mouse models, we previously reported that NER is regulated by p53.[20,21] UV irradiation has been shown to induce p53 expression.[36–38] To examine if p53 induction by UV radiation affects NER, human dermal fibroblasts were exposed to 0, 50, 100 and 200 J/m^2 of UVB, and transfected with an undamaged or a UV-damaged plasmid DNA harbouring a CAT

reporter gene 24 h after UV radiation. Excision repair of the damaged DNA was then determined as a function of reactivation of CAT enzyme activity. The percentage of CAT activity was expressed as a ratio of net d.p.m. damage dose/net d.p.m. undamaged plasmid DNA. Cells with greater capacity to repair UV damage should demonstrate a higher percentage of CAT activity compared with cells transfected with undamaged plasmid DNA. Figure 1 shows that cells exposed to 50 J/m^2 of UVB had 80% of repair compared with 34% in the control cells. However, after exposure to 100 J/m^2, the NER was almost the same as in control cells. The NER was reduced in the cells that received 200 J/m^2 of UVB. The difference in NER after UV irradiation is not due to the transfection efficiency, because the CAT activity of cells transfected with undamaged DNA is similar after exposure to 50 J/m^2 and 100 J/m^2. However, high doses of UVB ($\geq$ 200 J/m^2) caused cell death and the transfection efficiency was greatly reduced. Virtually all the cells died after exposure to 400 J/m^2 of UVB and lipofectin transfection, and therefore the CAT assay was not performed at doses $\geq$ 400 J/m^2.

To give further confirmation of the role of p53 in NER, murine p53$^{+/+}$ and p53$^{-/-}$ dermal fibroblasts were exposed to UVB and transfected with UV-damaged pCMV$_{cat}$ DNA. The ability to repair the DNA damage of the cells was determined by assaying for CAT activity 40 h after transfection. The CAT activity was doubled in UV-irradiated p53$^{+/+}$ fibroblasts compared with non-irradiated controls (Fig. 2). In p53$^{-/-}$ fibroblasts, UV radiation did not enhance DNA repair (Fig. 2), indicating that the induction of NER by UV radiation is p53-dependent.

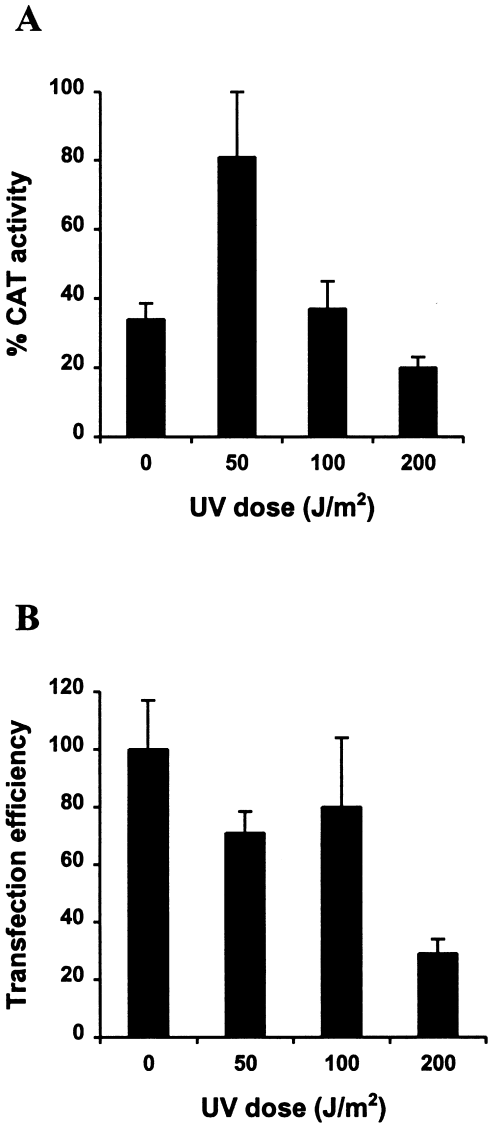

Figure 1. Nucleotide excision repair of human fibroblasts after ultraviolet (UV) irradiation. Cells were irradiated with UVB at various doses and transfected with UVC (254 nm) damaged pCMV$_{cat}$ plasmid DNA. The chloramphenicol acetyltransferase (CAT) activity was assayed 40 h later and compared with cells transfected with undamaged pCMV$_{cat}$ plasmid DNA (A). The transfection efficiency of UV-irradiated cells was measured by comparing the CAT activity of the cells transfected with undamaged plasmid DNA with the CAT activity of control non-irradiated cells (B).

p53-dependent induction of apoptosis by high-dose ultraviolet radiation

Cells undergo apoptosis if an extraordinary amount of DNA damage occurs. UV radiation has been known to induce apoptosis.[28] To investigate the effect of different doses of UV radiation on apoptosis, human dermal fibroblasts were irradiated with 0, 50, 100, 200, 400 and 800 J/m^2 of UVB. The apoptosis rate was determined by a sensitive ELISA. As shown in Figure 3(A), cells underwent apoptosis only after exposure to high doses of UVB in a dose-dependent manner up to 400 J/m^2. The percentage of cell survival of UV-irradiated cells was determined by SRB assay. Figure 3(B) shows that low doses of UVB ($\leq$ 100 J/m^2) had little effect on cell survival while UV doses $\geq$ 200 J/m^2 induced cell death in a dose-dependent manner up to 400 J/m^2. At 800 J/m^2, UVB did not induce more apoptosis (Fig. 3A) or further reduce cell survival (Fig. 3B).

To confirm the role of p53 in UV-induced apoptosis, murine p53$^{+/+}$ and p53$^{-/-}$ dermal fibroblasts were labelled with ^{3}H-thymidine and exposed to 100, 200 and 400 J/m^2 of UVB. The fragmented DNA of the apoptotic cells was assayed by centrifugation.[34] After

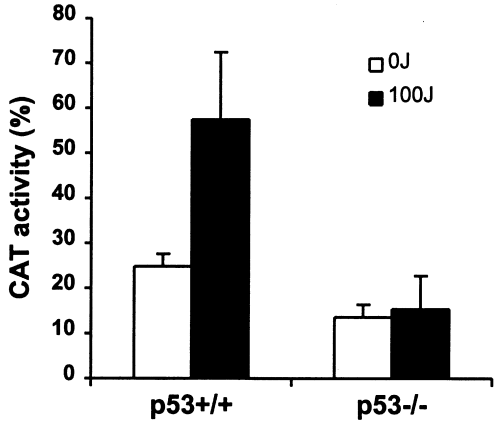

Figure 2. p53-dependent induction of nucleotide excision repair by ultraviolet (UV) radiation in murine fibroblasts. The cells were irradiated with UVB (100 J/m^2), and transfected with UVC (254 nm) damaged (350 J/m^2) pCMV$_{cat}$ 24 h later. The chloramphenicol acetyltransferase (CAT) activity was assayed 40 h after transfection.

low-dose UV radiation ($\leq$ 100 J/m^2), apoptosis was not significant in either p53$^{+/+}$ or p53$^{-/-}$ cells (Fig. 4). After the cells were exposed to high doses of UV radiation ($\geq$ 200 J/m^2), apoptosis occurred in p53$^{+/+}$ cells in a dose-dependent manner, while p53$^{-/-}$ cells did not undergo apoptosis (Fig. 4).

Dose-dependent induction of p53, p21$^{waf1/cip1}$ and bax after ultraviolet radiation

As cells respond differently to high and low doses of UVB irradiation, we examined by Western blot analysis the effects of different doses of UV radiation on the expression of p53, p21$^{waf1/cip1}$ and bax proteins, which are important in promoting NER and/or apoptosis (Fig. 5A). The expression of p53 protein was induced by UVB irradiation in a dose-dependent manner with a maximum induction of 15-fold at 400 J/m^2 (Fig. 5A,B). No further increase in the level of p53 was found with 800 J/m^2 of UVB. p21$^{waf1/cip1}$ expression was also induced after UV radiation, with a maximum induction of 3·3-fold at 200 J/m^2 (Fig. 5A,C). Higher doses of UV radiation reduced the expression of p21$^{waf1/cip1}$. At 800 J/m^2, the expression of p21$^{waf1/cip1}$ was undetectable. UVB also induced bax expression, but only at high doses (Fig. 5A). Bax expression was increased by 4·6-fold after 800 J/m^2 of UV radiation, while low doses of UV radiation ($\leq$ 200 J/m^2) had little effect on bax expression (Fig. 5D).

Discussion

Cellular stress response is crucial in preserving the genomic stability after a DNA-damaging event. p53 is the key element in stress response and is involved in the decision process which determines the fate of the cells after UV radiation-induced DNA damage. After a DNA-damaging event, the p53 protein is elevated. p53 has been shown to be involved in both enhancing NER and promoting apoptosis after UV radiation.[19–21,28,39] In this study, we have demonstrated that cells respond differently to high- and low-dose UV radiation. Cells exposed to low doses of UVB exhibited increased NER of UV-damaged CAT reporter gene (Fig. 1). The inducible DNA repair response by UV radiation is p53-dependent

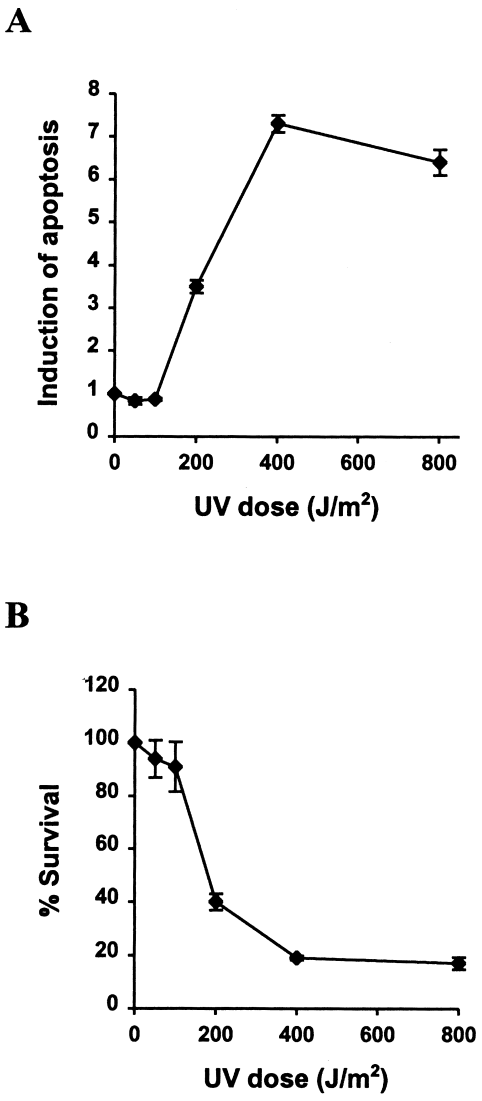

Figure 3. Ultraviolet (UV)-induced apoptosis in human fibroblasts. Cells were irradiated with UVB at various doses. The apoptosis rate was assayed with ELISA death detection kit^{plus} 24 h after UV radiation (A), and the cell survival was determined by sulphorhodamine B assay (B) (see Materials and methods).

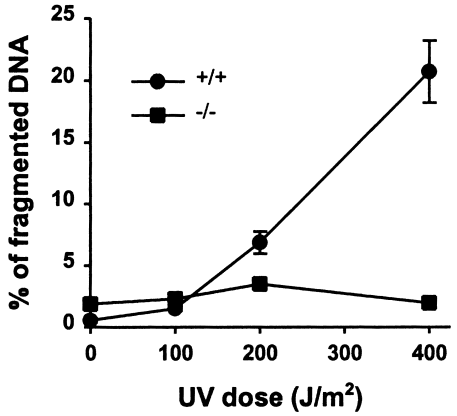

Figure 4. p53-dependent apoptosis of murine fibroblasts induced by ultraviolet (UV) irradiation. Cells were irradiated with UVB at 0, 100, 200 and 400 J/m^2, and the apoptosis rate was assayed by measuring the percentage of fragmented DNA.

(Fig. 2). Our data support the observations that wild-type p53 is required for heat shock and UV irradiation-enhanced repair of DNA damage.[19,40] However, as the UV dose increases, the capacity to repair the DNA damage is gradually reduced. As the NER activity diminishes after exposure to high doses of UV radiation, cells trigger apoptosis to remove the damaged DNA (Fig. 3). UV radiation-induced apoptosis is also dependent on p53 function (Fig. 4). It appears that NER requires low levels of p53 induction, while a large amount of p53 is required for triggering apoptosis, as p53 induction is directly proportional to UV dose up to 400 J/m^2, including the doses inducing DNA repair and apoptosis. Interestingly, 800 J/m^2 of UVB did not further increase the p53 level (Fig. 5A) and apoptosis (Fig. 3A), nor further reduce cell survival (Fig. 3B). This correlation between p53 expression, apoptosis induction and cell survival supports the important role of p53 in mediating cellular stress response to UV radiation.

p21$^{waf1/cip1}$ was induced only at low doses of UV radiation (Fig. 5A,C). The induction of p21$^{waf1/cip1}$ may induce cell cycle arrest and ensure that the DNA repair programmes take place.[12–14] p21$^{waf1/cip1}$ induction may also enhance NER. McDonald *et al.*[41] showed that repair of UV-damaged reporter DNA is decreased in p21$^{waf1/Cip1-/-}$ cells, and reintroduction of wild-type p21$^{waf1/cip1}$ restored the repair capacity of the p21$^{waf1/Cip1}$ deficient cells. However, the role of p21$^{waf1/cip1}$ in NER is controversial. Pan *et al.*[42] reported that p21$^{waf1/cip1}$ inhibited DNA replication and NER in *in vitro* cell extracts. Different assay methods may account for the discrepancy. The advantage of the host cell reactivation assay is that it measures the cellular responses to environmental stress rather the mere

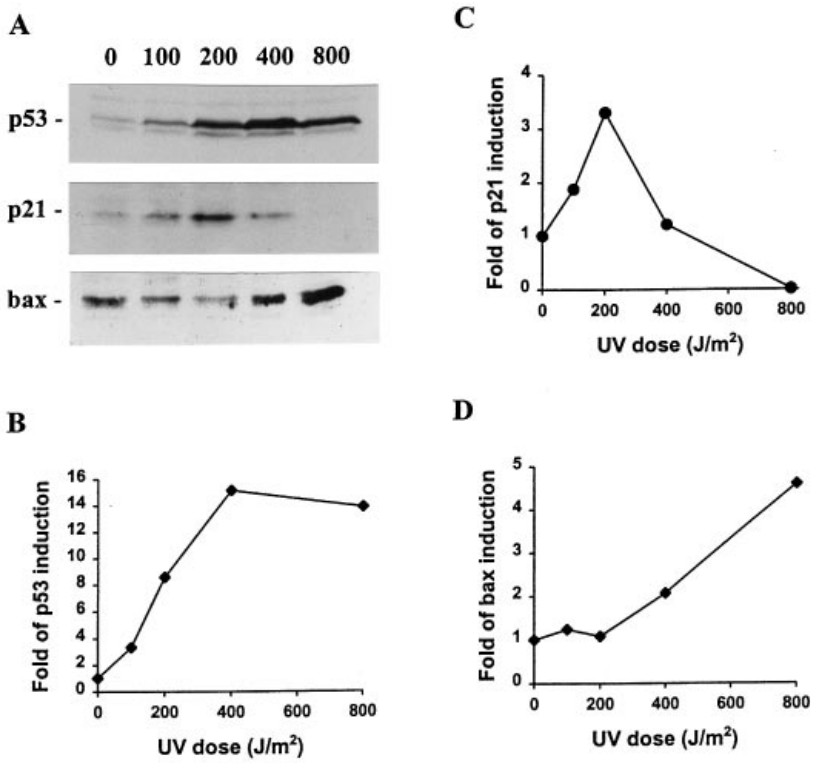

Figure 5. Dose–response induction of p53, p21$^{waf1/Cip1}$ and bax in human fibroblasts by ultraviolet (UV) radiation. Cells were irradiated with UVB at various doses and proteins extracted 24 h later. Fifty micrograms of protein per lane were subjected to Western blot analysis (A). The intensity of the bands was quantified with a densitometer. (B) p53; (C) p21$^{waf1/Cip:1}$ (D) bax.

interactions of molecules *in vitro* as the free-cell extract system.

Wild-type p53 function is necessary for many types of programmed cell death, including apoptosis induced by UV radiation. p53 upregulates the expression of bax to execute the death programme. Merchant *et al.*[32] showed that using a temperature-sensitive p53 mutant, bax levels rise when wild-type p53 activity is expressed. Yin *et al.*[33] elegantly showed that in a transgenic mouse brain tumour model, inactivation of p53 caused a dramatic acceleration of tumour growth due to a reduction in apoptosis of over 90%, p53-dependent expression of bax was induced in slow-growing apoptotic tumours, and apoptosis reduced by 50% and tumour growth accelerated in bax-deficient mice. Bax is now considered as a tumour suppressor.[33] Bax induction was observed only at high doses of UV radiation which induced apoptosis (Fig. 5A,D), confirming the role of bax in UV-induced apoptosis.

It appears that p53 is a key element in regulating cellular response after UV irradiation. It is still unclear as to how different amounts of p53 trigger different stress-response programmes. The subcellular localization of p53 after different doses of UV radiation may be responsible for triggering different programmes. At low doses, the p53 staining shifted from perinuclear to cytoplasmic to nuclear and back to perinuclear within 24 h in a cyclic manner.[43] NER involves more than 30 proteins and the exact relationship between p53 and the repair enzymes is not known. The shifting of p53 subcellular localization suggests that p53 may participate in more than one step in NER. It has been shown that p53 binds to XPB, XPD,[14,15] replication protein A[16] and DNA strand breaks,[17] supporting the multistep involvement of p53 in NER. In contrast, at high doses of UVB irradiation, p53 expression was moderately to intensely nuclear with diffuse cytoplasmic staining.[43] This pattern of p53 staining is in agreement with the apoptosis scheme, as apoptosis is a cell death programme that initiates in the nucleus.

In summary, our data support the notion that p53 is a guardian of the genome. p53 participates in both repair of UV-damaged DNA and the induction of apoptosis. When cells are exposed to low doses of UV radiation, the moderate induction of p53 upregulates NER and $p21^{waf1/cip1}$ which initiates cell cycle arrest to allow DNA repair. However, when cells are exposed to high doses of UV radiation, p53 protein is drastically increased which upregulates bax and triggers apoptosis.

Acknowledgments

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