Role of p53 in UVB-Induced Apoptosis in Human HaCaT Keratinocytes

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Apoptosis represents an active form of cell death that is involved in the control of tissue homeostasis and in the deletion of DNA-damaged cells. Because the product of the tumor suppressor gene p53 has been demonstrated to be crucial for the induction of apoptosis in certain cell types, the present study was aimed at elucidating its role in ultraviolet-induced apoptosis in HaCaT keratinocytes. After *in vitro* ultraviolet B irradiation, p53 protein levels were noted to increase prior to the induction of apoptosis in a time- and concentration-dependent fashion. This increase could not be inhibited by the protein synthesis inhibitor cycloheximide. Because HaCaT keratinocytes are known to bear two p53 point mutations and because it is unclear whether p53 in HaCaT cells is still functional regarding induction of apoptosis, HaCaT cells were stably transfected with wild-type p53 cDNA inserted into the expression vector pCMV-Neo-Bam in sense (pC53-SN3) and anti-sense (pC53-ASN) direction. After selection with geniticin, growing colonies were screened for the presence of the transfected cDNA constructs by polymerase chain reaction. Cell clones bearing the anti-sense product were further analyzed for p53 expression by western blotting. Clones showing reduced p53 protein levels were irradiated with ultraviolet B light, and there was a clear reduction of apoptosis in the pC53-ASN bearing cell clones compared with the parental HaCaT cells. These studies demonstrate that blocking mutated p53 can partially block apoptosis in HaCaT keratinocytes and furthermore can confirm the key role for p53 in ultraviolet-induced apoptosis in human keratinocytes. Moreover, HaCaT keratinocytes and their p53-transfectants provide a convenient model that allows for further detailed analyses of apoptosis-associated biochemical and molecular events in human keratinocytes. *Key words: cell death/p53 transfection/protein synthesis inhibition. J Invest Dermatol 109:722–727, 1997*

The term "apoptosis" was introduced in 1972 by Kerr *et al* to describe a distinct form of cell death that differs with regard to both morphologic features and associated biochemical events from necrosis. Furthermore, necrosis is always associated with pathologic processes, whereas apoptosis represents an active and physiologic process leading to the elimination of single cells without affecting neighboring cells. Functions of apoptosis include the control of cell population dynamics and the elimination of cells with damaged DNA (Kerr and Harmon, 1991). Currently, a "re-discovery" regarding the role of apoptosis in skin biology is taking place (Polakowska and Haake, 1994). There is morphologic evidence for apoptosis of keratinocytes in both diseased (Paus *et al*, 1993) and normal skin where keratinocytes undergoing terminal differentiation display several morphologic and biochemical features of apoptosis (McCall and Cohen, 1991). We have recently examined various agents capable of inducing apoptosis in hematopoietic cells regarding their ability to induce apoptosis in HaCaT keratinocytes and, among them, only ultraviolet B (UVB) light was able to cause apoptosis in a concentration- and time-dependent manner (Henseleit *et al*, 1996). The present study was aimed at elucidating the role of the tumor suppressor gene p53 in UVB-induced apoptosis.

Manuscript received July 17, 1997; revised August 15, 1997; accepted for publication August 15, 1997.

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Abbreviations: ASN, anti-sense; CDDE, cell death detection enzyme-linked immunosorbent assay; SN, sense; WT, wild-type.

The nuclear phosphoprotein p53 was originally isolated from SV 40-transformed cells complexed with the SV 40 oncogene product, the large T antigen (Lane and Crawford, 1979). p53 was first classified as a tumor antigen, later as an oncogene, and, more recently, as a tumor suppressor gene and a "guardian of the genome" (Levine *et al*, 1991; Lane, 1992). Furthermore, p53 has been suggested to play a role in the induction of apoptosis, particularly irradiation-induced apoptosis (Yonish-Rouch *et al*, 1991; Lowe *et al*, 1993; Lu and Lane, 1993). The current concept regarding the possible role of p53 in this process is that p53 accumulates after DNA damage by a still unknown mechanism and that it mediates an arrest of the cell cycle at G1 to allow for repair of the damage (Lane, 1992). In the case of an irreversible damage, p53 may trigger cell death by induction of apoptosis. Wild-type (WT) p53 is a sequence-specific DNA-binding protein that regulates the transcription of genes containing p53-binding sites (Kern *et al*, 1991; Bargonetti *et al*, 1991). Targets for positive transcriptional regulation include $p21^{WAF1/CIP1}$ (El-Deiry *et al*, 1994), GADD45 (Zhan *et al*, 1994), and MDM2 (Barak *et al*, 1993). Both $p21^{WAF1/CIP1}$ and GADD45 seem to be involved in the p53 response to DNA damage as described above (El-Deiry *et al*, 1994; Zhan *et al*, 1994). By contrast, MDM2 is a gene upstream from p53 and appears to function as a negative feedback regulator of p53 (Wu *et al*, 1993). Genes whose transcription can be repressed by p53 include bcl-2 that in turn inhibits apoptosis (Hockenberry, 1992; Miyashita *et al*, 1994).

The loss of p53 function by mechanisms such as mutations or binding to viral proteins increases the risk of development of certain types of cancer (Hollstein *et al*, 1991). Regarding UV-induced skin tumors, p53 is considered to play an important role in their pathogenesis (McNutt *et al*, 1994). Increasing levels of p53 protein were detected

in vivo in human keratinocytes after UV irradiation (Campbell *et al*, 1993; Hall *et al*, 1993). A link between UV irradiation, p53, and the induction of apoptosis in keratinocytes has recently been established from studies with mice exhibiting different p53 genotypes (Ziegler *et al*, 1994). In these mice, a correlation has been found between the decrease in the number of "sunburn cells," which are supposed to represent apoptotic keratinocytes, and the decrease of copy numbers of the p53 gene (Ziegler *et al*, 1994).

This study was attempted to confirm previous findings on p53 expression and induction of apoptosis in keratinocytes after UVB-irradiation *in vivo* in a convenient *in vitro* model, the human, nontumorigenic HaCaT cell line that behaves phenotypically like normal keratinocytes regarding patterns of growth and differentiation (Boukamp *et al*, 1988). Further evidence for a causal role of p53 in UVB-induced apoptosis was obtained with these cells via stable transfection with vectors for WT p53 in sense and anti-sense orientation. This model thus provides an elegant tool for exploring the exact biochemical events involved in p53-associated, UV-induced keratinocyte apoptosis.

MATERIALS AND METHODS

Cells Human HaCaT keratinocytes (kindly provided by Dr. N.E. Fusenig, DKFZ, Heidelberg, Germany) were seeded at 10^4 cells per cm^2 in 150-mm cell culture dishes (Falcon, Heidelberg, Germany). Dulbecco's Modified Eagle's Medium (Life Technologies, Eggenstein, Germany) supplemented with 5% fetal calf serum (Life Technologies), 2 mM glutamine (Biochrom, Berlin, Germany), and 100 U penicillin/streptomycin (Biochrom) per ml, served as cell culture medium. Cells were passaged every 7 d in a 1:10 split. Experiments were performed at confluency of keratinocytes, unless stated otherwise.

UVB irradiation UVB irradiation was performed as described previously (Henseleit *et al*, 1996). Briefly, the cell culture medium of confluent HaCaT keratinocytes was replaced by phosphate-buffered saline (PBS; Life Technologies). Subsequently, the HaCaT cells were exposed to various concentrations of UVB light emitted by a Philips TL 20 W/12 (Eindhoven, Netherlands) light source with an emission in the UVB range (290–320 nm) displaying a peak at 313 nm. UV dosimetry was performed with a Waldmann UV-meter (Waldmann, Villingen-Schwenningen, Germany). After removal of PBS and re-addition of the cell culture medium, the keratinocytes were incubated for various time intervals and harvested by trypsinization including those floating in the medium. PBS-treated cells without UVB irradiation served as controls.

DNA fragmentation Internucleosomal DNA fragmentation was determined by means of a cell death detection enzyme-linked immunosorbent assay (CDDE) (Boehringer, Mannheim, Germany), as described previously (Henseleit *et al*, 1996; Schadendorf *et al*, 1996; Dimmeler *et al*, 1997). Briefly, cytoplasmic fractions of 10 μl aliquots of a sample (10^6 cells per ml) were prepared according to the manufacturer's instructions. The assay is based on the measurement of cytoplasmic histon-associated DNA fragments (mono- and oligonucleosomes) contained in the samples that bind to an immobilized anti-histon antibody. The DNA-part of the nucleosomes is detected by an anti-DNA-peroxidase. The reaction is visualized with 2,2′-azino-dio-[3-ethylbenzthiazoline sulfonate] and measured photometrically. Results were expressed as absorbance ($A_{405\ nm}$) minus blank or as enrichment factor (absorbance of the sample divided by absorbance of the corresponding control).

Western blot analysis Cells were harvested as described above, resuspended in PBS (Ca^{2+} and Mg^{2+} free; Life Technologies) containing 1 mM phenylmethylsulfonyl fluoride (Merck, Darmstadt, Germany) and homogenized by sonification for 30 s. Samples were solubilized by addition of sample buffer yielding final concentrations of 2% sodium dodecylsulfate, 10% glycerol, 0.05% bromphenolblue, 5% mercaptoethanol, and 50 mM Tris (Sigma, Deisenhofen, Germany) and heated at 95°C for 5 min. Samples (50 μg per lane) were then loaded on polyacrylamide gels (10%). After separation, proteins were transferred on polyvinyldifluoridine membranes (DuPont, Boston, MA) that were afterwards preincubated with a 3% solution of non-fat milk powder (Biorad, München, Germany), followed by an incubation with monoclonal antibody DO-1 against p53 (Oncogene, Uniondale, NY) in a 1:50 dilution for 2 h at room temperature. Subsequently, the blot membrane was treated with alkaline phosphatase conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) in a 1:2000 dilution for 1 h at room temperature visualized on X-ray films (DuPont), using the enhanced chemiluminescence method (Oncogene).

Inhibition of protein synthesis In order to evaluate the effect of inhibition of protein synthesis, HaCaT cells were incubated for 4 or 24 h with different concentrations (0.1–10 μg per ml) of cycloheximide (Sigma) and 1 or 5 μCi ^{3}H-phenylalanine (Amersham, Braunschweig, Germany) per ml. After an incubation time of 24 h with 10 μg cycloheximide per ml, incorporation of ^{3}H-phenylalanine was almost completely inhibited (data not shown) so that these conditions were used to inhibit protein synthesis in the following experiments.

Immunoelectron microscopy Cells were processed for immunoelectron microscopy according to Kolde and Broeker (1986); with slight modifications. Briefly, cells were fixed in Nakane's fixative (periodate-lysine-paraformaldehyde; Sigma) and permeabilized with 0.2% saponin (Sigma). Unspecific protein binding was inhibited by addition of 1% bovine serum albumin (Sigma). Cells were then incubated with monoclonal antibody DO-1 directed against p53, followed by an incubation with peroxidase-conjugated rabbit anti-mouse immunglobulins (Dako, Hamburg, Germany). After visualization of the reaction with diaminobenzidine tetrahydrochloride substrate (Sigma), cells were post-fixed in 1.33% osmium tetroxide (Fluka, Buchs, Switzerland) and embedded in Araldite (Serva, Heidelberg, Frankfurt, Germany). The semi-thin or ultra-thin sections were counterstained with Richardson's solution or uranyl citrate and lead acetate, respectively, and examined by light or electron microscopy (Zeiss electron microscope 9S, Zeiss, Oberkochen, Germany).

Transfection with pC53-SN3 and pC53-ASN pC53-SN3 and pC53-ASN were obtained by insertion of the 1.8-kb WT p53 cDNA in sense or anti-sense direction into the expression vector pCMV-Neo-Bam (Baker *et al*, 1990). These constructs were kindly provided by Dr. B. Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD). The expression vector pCMV-Neo-Bam was derived from plasmid BCMGNeo-Il2 and contains two independent transcription units, a cytomegalovirus promoter/enhancer for expression of the recombinant DNA and a herpes simplex virus thymidine kinase promoter/enhancer upstream of the neomycin resistance gene, allowing for selection of transfected cells with geneticin (Karasuyama *et al*, 1989). For transfection of the constructs into HaCaT keratinocytes, the plasmids were linearized with the restriction enzyme HindIII (USB, Cleveland, OH), gel-purified, and 1 μg DNA in 100 μl Optimem cell culture medium (Life Technologies) was mixed with 5 μl LipofectAMINE (Life Technologies) in 100 μl Optimem. After an incubation of 45 min at room temperature, this mixture was added to HaCaT cells at 50–80% confluency in 35-mm dishes filled with 0.8 ml Optimem and was present for 18 h. After replacement with normal cell culture medium, selection for geneticin (0.7 μg per ml; Life Technologies) resistance was initiated 48 h after transfection. Geneticin-resistant colonies were counted 3 wk later. Individual colonies were picked, cultured, and screened for the presence of pC53-SN3 or pC53-ASN at their second passage by polymerase chain reaction (PCR) from isolated genomic DNA using the oligonucleotides p53 up (5′-GGTGCCCTATGAGCCGCCTG-3′) and p53 down (3′-GGGGTCGGTTTCTTCTTTGGTG-5′) (Lamb and Crawford, 1985). The amplified DNA fragments are 1258 bp for the endogenous p53 gene and 317 bp for the transfected cDNA construct. Conditions of the 35-cycle PCR were 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min in standard PCR buffer conditions (Saiki *et al*, 1985).

RESULTS

Induction of p53 protein expression and apoptosis by UVB irradiation In a first set of experiments, confluent HaCaT keratinocytes were exposed to UVB light in a concentration range between 0 and 0.5 J per cm^2, harvested after 24 h, and p53 protein expression was analyzed by immunoblot analysis. A representative immunoblot is depicted in **Fig 1*A*** demonstrating a maximal accumulation of p53 at a concentration of 0.1 J UVB per cm^2. A further increase in the UVB concentration yielded a decrease of p53 protein levels, with no more p53 detectable at a concentration of 0.5 J per cm^2. In order to elucidate the time course of p53 expression, HaCaT cells were harvested at various time points after exposure to 0.1 J UVB per cm^2. Maximal expression of p53 was detected 24 h after irradiation and high protein levels were still present 48 h after irradiation (**Fig 1*C***).

In parallel to p53 expression, induction of apoptosis by UVB light was assessed by determination of internucleosomal DNA fragmentation and typical morphologic changes (not shown) that revealed a similar concentration- (**Fig 1*B***) and time-dependent (**Fig 1*D***) response due to UVB irradiation, reaching maximal values at a concentration of 0.25 J UVB per cm^2.

Morphologic correlation between induction of apoptosis and p53 expression In order to investigate whether the same cells undergoing apoptosis express enhanced levels of p53 protein, HaCaT keratinocytes were irradiated with UVB light (0–0.2 J per cm^2) and

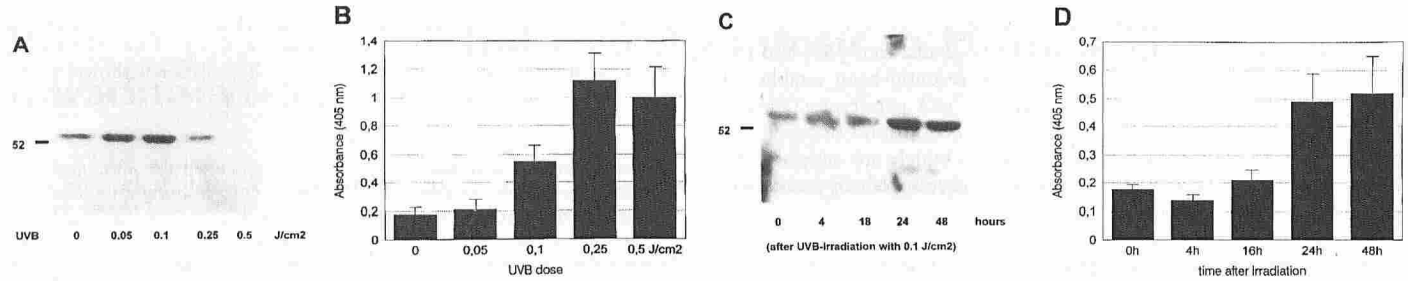

Figure 1. UVB irradiation induces p53 protein expression and DNA fragmentation in HaCaT keratinocytes. p53 protein expression was analyzed by immunoblotting (*A*, *C*). Internucleosomal DNA fragmentation (*B*, *D*) was determined in parallel by a CDDE. For concentration-response studies (*A*, *B*), cells were irradiated with various doses of UVB light as indicated and harvested after 24 h. For time-response studies (*C*, *D*), cells were exposed to 0.1 J UVB light per cm^2 and harvested after the given time intervals. Results of the CDDE are expressed as mean ± SEM of three independent experiments.

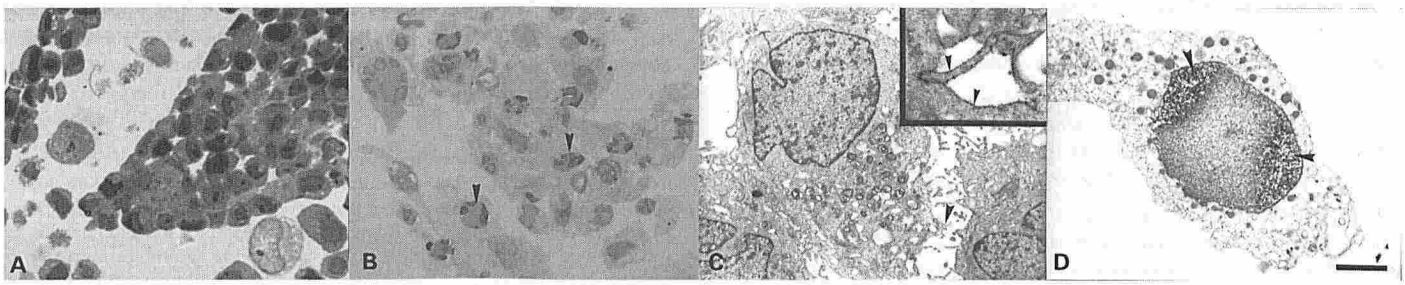

Figure 2. Light and electronmicroscopic demonstration of p53 protein in HaCaT keratinocytes. HaCaT cells either untreated (*A*, *C*) or irradiated with 0.15 J UVB per cm^2 (*B*, *D*) were harvested 24 h after irradiation and immunostained for p53 as described in *Material and Methods*. Semi-thin (*A*, *B*) and ultra-thin (*D*, *E*) sections were prepared and analyzed by light- or electron microscopy, respectively. Note the typical apoptotic morphology and the nuclear staining for p53 in several irradiated HaCaT keratinocytes (two of them are marked by *arrowheads*) in (*B*) and confirmed at the ultrastructural level in (*D*). The faint membrane staining of nonirradiated cells is demonstrated in (*C*) (see inset) and marked by *arrowheads*. *Scale bar*, (*A*, *B*) 12 μm, (*D*, *E*) 2 μm (*inset* 0.5 μm).

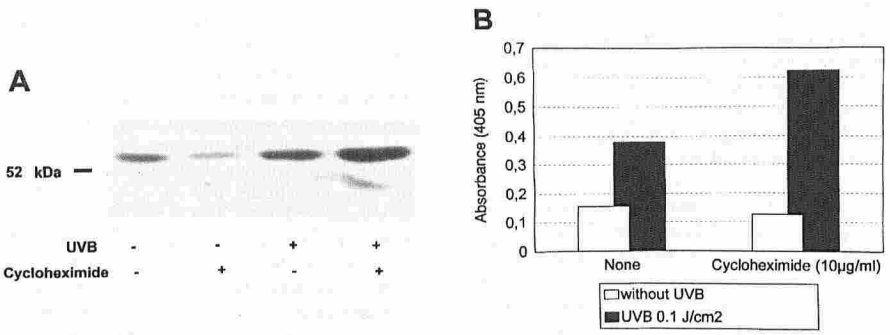

Figure 3. Effect of the protein synthesis inhibitor cycloheximide on p53 expression and apoptosis. HaCaT keratinocytes were treated with cycloheximide (10 μg per ml) or vehicle control 24 h prior to irradiation with 0.1 J UVB per cm^2. p53 protein expression was analyzed by immunoblotting (*A*). In parallel, the induction of apoptosis was determined by means of the CDDE (*B*).

harvested 24 h later. Cells were immunostained for p53 and further processed for preparation of semi-thin or ultra-thin sections. Light microscopical analysis of semi-thin sections revealed that an increase in the UVB concentration resulted in an enhanced number of cells displaying morphologic characteristics of apoptosis, e.g., condensation of cytoplasm and chromatin, and fragmentation of the nucleus. In parallel, an increased, mainly nuclear staining for p53 could be observed that was mostly confined to cells with apoptotic morphology (**Fig 2*B***). Ultrastructural analysis of these cells by immunoelectron microscopy confirmed these results (**Fig 2*D***). In nonirradiated HaCaT cells, there was only occasionally a faint staining of the nucleus (**Fig 2*A***), but in addition, a discrete staining of the cell membrane was detectable on the ultrastructural level (**Fig 2*C***).

Inhibition of protein synthesis by cycloheximide In an attempt to analyze the mechanism of p53 accumulation in HaCaT keratinocytes after UVB irradiation, HaCaT keratinocytes were preincubated for 24 h with the protein synthesis inhibitor cycloheximide (10 μg per ml) and then irradiated with UVB light (0.1 J per cm^2). Cells were harvested 24 h later and analyzed for p53 expression by western blotting. As shown in **Fig 3*A***, cycloheximide did not inhibit the UVB-induced increase of p53 protein, and even slightly enhanced p53 expression induced by UVB.

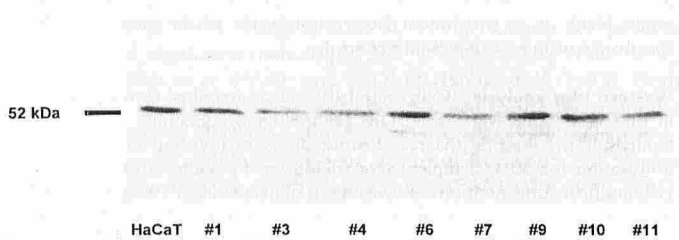

Figure 4. Effect of pC53-ASN transfection on p53 protein expression. p53 protein expression was compared in the parental HaCaT cells *versus* eight stable transfectants containing pC53-ASN (1–11) by western blot analysis.

Because the induction of apoptosis has been reported to depend on protein synthesis in various cell types, the induction of apoptosis by UVB light was examined in the presence of cycloheximide (up to 10 μg per ml). Neither UVB-induced enhancement of DNA fragmentation (**Fig 3*B***) nor morphologic apoptotic changes (not

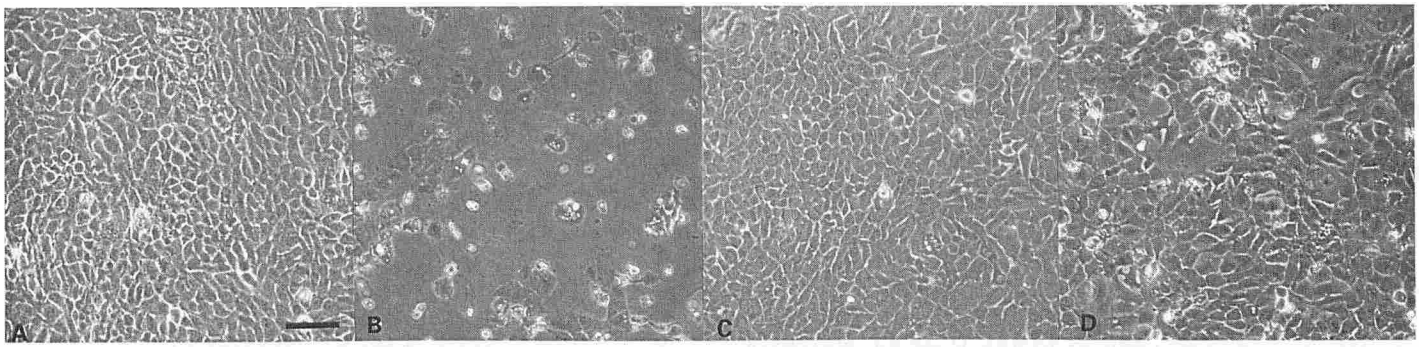

Figure 5. Comparison of morphologic features of parental *versus* pC53-ASN transfected HaCaT keratinocytes after UVB irradiation. Light microscopic morphology of parental HaCaT cells (*A*, *B*) or HaCaT clone 4 (*C*, *D*), either untreated (*A*, *C*) or 24 h after irradiation with 0.15 J UVB light per cm^2 (*B*, *D*). *Scale bar*, 20 μm.

shown) were inhibited by cycloheximide. As already observed with p53 accumulation, cycloheximide even caused an enhancement of DNA fragmentation (**Fig 3*B***).

Thus, both UVB-induced p53 accumulation and UVB-induced apoptosis are apparently not dependent on new protein synthesis.

Transfection of HaCaT keratinocytes with pC53-SN3 and pC53-ASN To directly prove that p53 plays a key role in UVB-induced apoptosis, HaCaT keratinocytes were stably transfected with the expression vector pCMV-Neo-Bam containing WT p53 cDNA in sense (pC53-SN3) and anti-sense (pC53-ASN) direction. After transfection with pC53-SN3, about 10-fold fewer geneticin-resistant colonies were detectable than after transfection with pC53-ASN. PCR analysis of the clonal lines revealed that those established from cells transfected with pC53-SN3 did not express the exogenous WT p53 sequences, whereas all clones derived from the pC53-ASN transfection did express the exogenous p53 gene (data not shown).

From about 40 isolated stable transfectants containing pC53-ASN, p53 protein expression was analyzed in eight transfectants (1, 3, 4, 6, 7, 9, 10, 11) by western blot (**Fig 4**) Four clones showed clearly reduced p53 protein levels as compared with the parental line. Two of those clones (4 and 7) were chosen for further study.

Induction of apoptosis by UVB irradiation in pC53-ASN transfected HaCaT keratinocytes The p53 anti-sense transfected HaCaT clones 4 and 7 and the parental HaCaT cells were irradiated with 0.15 J UVB light per cm^2 at the day of confluency. Twenty-four h after irradiation, morphology and DNA fragmentation were analyzed. The morphologic appearance of the untreated anti-sense clones and parental cells was very similar (**Fig 5*A*,*C***). Upon UVB irradiation, however, cell damage was markedly reduced in clone 4 (and 7, not shown) as compared with the parental cells (**Fig 5*B*,*D***). UVB-induced DNA fragmentation in both anti-sense clones was reduced by H ≈ 50% (**Fig 6**). In addition, HaCaT-ASN 4 and HaCaT-ASN 7 revealed both reduced morphologic changes typical for apoptosis and reduced p53 protein accumulation (not shown).

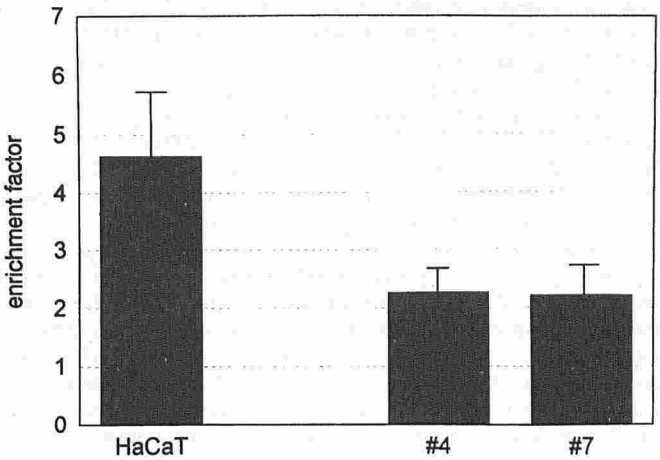

Figure 6. Effect of pC53-ASN transfection on UVB-induced DNA fragmentation. Twenty-four h after UVB irradiation (0.15 J per cm^2) of parental HaCaT cells or pC53-ASN transfected HaCaT clones (4, 7), internucleosomal DNA fragmentation was determined by the CDDE. Results are expressed as mean ± SEM of the enrichment factor (absorbance of the irradiated sample/absorbance of the nonirradiated corresponding control); n = 3.

DISCUSSION

A growing body of evidence suggests that p53 plays a crucial role particularly in irradiation-induced apoptosis (Lowe *et al*, 1993; Lu and Lane, 1993; Campbell *et al*, 1993; Hall *et al*, 1993; Ziegler *et al*, 1994). In the present investigation, analysis of p53 protein expression in HaCaT cells revealed a constitutive p53 expression that increased after UVB irradiation. The basal p53 expression in untreated HaCaT cells is due to the fact that HaCaT keratinocytes bear a mutant form of p53 that has a more extended protein half-life than WT p53 (Lehman *et al*, 1993). The p53 mutations in HaCaT cells found at dipyrimidine sites in codon 179 of exon 5 and codons 281 and 282 of exon 8 are typical of UV-induced damage and have been implicated in the mechanism of immortalization of this cell line (Lehman *et al*, 1993). Until now, it was not clear whether the mutated p53 protein of HaCaT keratinocytes is still functional.

A similar UV-induced accumulation of p53 protein has also been shown for the expression of WT p53 of human keratinocytes *in vivo*, although the concentration- and time-dependent response differs from the present *in vitro* findings (Hall *et al*, 1993; Healy *et al*, 1994). The accumulation of p53 was mainly detectable in the nuclei of HaCaT cells, as is the case for WT p53 in almost all other cells and tissues studied (Vojtesek *et al*, 1992). The p53 staining of the plasma membranes in untreated HaCaT cells was unexpected, but there is support for this phenomenon from investigations in hepatocellular carcinomas where a similar staining was detected depending on the antibody used (Zhao *et al*, 1994). In this context, it seems interesting to note that the UV response of mammalian cells is suggested to be initiated at or near the plasma membrane (Devary *et al*, 1992). On the other hand, WT p53 is supposed to exert most of its function in the nucleus, whereas nuclear exclusion results in inactivation of p53 function (Moll *et al*, 1992).

With the parallel assessment of p53 expression and apoptosis in HaCaT keratinocytes in the present study, it was possible to demonstrate an increase of both parameters after UVB irradiation. Similar findings have been reported with *in vitro* studies of other cells bearing WT p53 (Lu and Lane, 1993). Overall, our data indicate that p53 may be involved in UVB-induced apoptosis in HaCaT keratinocytes despite the mutations in the p53 gene of these cells.

Our data also suggest that the induction of p53 and apoptosis by UVB light is independent of de novo protein synthesis. Regarding p53, it has previously been shown for keratinocytes *in vivo* that p53 protein increased upon UVB irradiation without any change in p53 mRNA expression (Healy *et al*, 1994). The mechanism underlying p53 protein upregulation may be a post-translational stabilization of the protein that has been shown by Maltzman *et al* (1984) in UV-

irradiated mouse fibroblasts. In the present study, levels of p53 protein were even found to be slightly enhanced by the addition of a protein synthesis inhibitor. Candidate proteins whose inhibition could lead to an accumulation of p53 protein are, for example, the enzymes of the ubiquitin protein degradation cascade that are responsible for a rapid degradation of p53 (Scheffner *et al*, 1990).

Regarding apoptosis, there are conflicting results about its dependence on protein synthesis (Duke *et al*, 1983; Sellins and Cohen, 1987). In the present study, induction of apoptosis by UVB irradiation in HaCaT keratinocytes was not found to be inhibited and was even found to be enhanced by cycloheximide. In studies with HL-60 cells, it has been speculated that the inhibition of continuously synthesized suppressors of apoptosis might be responsible for this phenomenon (Martin *et al*, 1990; Gong *et al*, 1993)

In agreement with our findings, others studying p53-dependent induction of apoptosis by UV light in GHFT1 cells also noted that the process of apoptosis cannot be inhibited by protein or RNA synthesis inhibitor (Caelles *et al*, 1994). Therefore, these authors speculated that p53 activity, through participation in DNA repair or DNA cleavage rather than activation of other genes, is responsible for the induction of apoptosis.

In order to provide definitive proof that p53 also plays a key role in UVB-induced apoptosis of HaCaT keratinocytes, we transfected HaCaT cells with WT p53 cDNA in sense (pC53-SN3) and antisense (pC53-ASN) orientation. Transfection with pC53-SN3 yielded only very few colonies as already observed in WT p53 transfected colorectal carcinoma cells (Baker *et al*, 1990) and in a rapidly dying murine cell line normally lacking p53 (Yonish-Rouch *et al*, 1991). In the latter cells, the mechanisms of cell death have been shown to be apoptotic (Yonish-Rouch *et al*, 1991). In our study, a proof for apoptosis was, however, not possible due to the small cell numbers that might, on the other hand, indicate that overexpression of p53 leads inevitably to apoptosis. Those few colonies that had formed after transfection with pC53-SN3 did not express the transfected cDNA construct, indicating a deletion or rearrangement of the inserted sequence, as described for the p53 transfected colorectal carcinoma cells (Baker *et al*, 1990).

In contrast, all cell clones derived after transfection with pC53-ASN expressed this cDNA construct that was able to clearly reduce p53 protein expression, although not in all cell clones examined. UVB irradiation of two clones with reduced p53 protein showed a marked reduction of internucleosomal DNA fragmentation and morphologic changes typical for apoptotic cell death.

Our *in vitro* results thus confirm and extend previous findings of an *in vivo* study demonstrating a reduced number of sunburn cells (apoptotic keratinocytes) in murine skin after inactivating the p53 gene (Ziegler *et al*, 1994). The data altogether prove the crucial role for p53 in UV-induced apoptosis in keratinocytes. Moreover, our results clearly demonstrate that p53 must at least in part be functional in spite of its point mutations in HaCaT keratinocytes. This may indicate that different p53 mutations found in skin tumors may have different phenotypes that may be mechanistically based on their different profiles of promotor specifity (Hall *et al*, 1996). In addition, our data may also be indicative of additional apoptotic pathways operative in HaCaT cells due to UVB irradiation.

The exact means by which p53 induces apoptosis in HaCaT keratinocytes is, however, unclear and may be different from cells bearing WT p53. Involvement of p21 as mediator of the p53 response seems to be unlikely in this case, because the mutated p53 of the HaCaT cells fails to transcriptionally activate the p21 promotor as does WT p53 (Datto *et al*, 1995). In line with this result, we detected no induction of p21 protein due to UVB irradiation of the HaCaT keratinocytes (not shown). Overall, there is now a growing number of reports about transcription independent p53 activities (Hall *et al*, 1996), including the induction of apoptosis (Haupt *et al*, 1995).

Furthermore, it has been demonstrated that the mutated form of p53 in HaCaT cells does not promote G1 arrest in contrast to WT p53, but a prolonged G2 arrest due to UVB irradiation (Herzinger *et al*, 1995). The G2 arrest has also been considered to enable DNA repair, and it has recently been reported that cells with radiation-induced damage may be eliminated by apoptosis subsequent to a G2/M block (Palayoor *et al*, 1995).

Because this G2/M checkpoint seems to be an additional important control mechanism in preventing proliferation of cells with damaged DNA, HaCaT keratinocytes and particularly our p53-anti-sense transfected HaCaT cell clones thus represent a useful model to further study this alternative way of inducing apoptotic cell death.

The authors thank Prof. N. Fusenig (Deutsches Krebsforschungszentrum Heidelberg, Germany) for providing the HaCaT cell line, Dr. B. Vogelstein for providing the p53 cDNA constructs, G. Schielke and F. Glowacki for excellent technical assistance, and Prof. B.M. Henz for helpful discussions and critical reading of the manuscript. Parts of this study were supported by DFG grant He 2524/1–1.

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