Down-Regulation of p63 Is Required for Epidermal UV-B-induced Apoptosis

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Abstract
In the epidermis, p53 plays an important role in UV-B protection that led us to examine the role, if any, that p63, a p53 homologue highly expressed in the basal layer of the epidermis, might play in the epidermal UV-B response. One p63 isoform, ΔNp63α, decreased dramatically in normal keratinocytes or newborn epidermis at both the protein and RNA levels after UV-B irradiation. In an attempt to further investigate the significance of the UV-B-induced decrease of this p63 isoform as well as further delineate the function of p63 in the epidermis, we generated transgenic mice that constitutively express ΔNp63α in the mouse epidermis using the loricrin promoter (ML.ΔNp63α). The ML.ΔNp63α mouse epidermis developed normally, with no overt phenotype and an unaltered proliferation rate. When challenged by UV-B exposure, the ML.ΔNp63α mice exhibited a 40–45% decrease in the number of apoptotic cells in the epidermis as compared with nontransgenic littermates. These results suggest that aberrant expression of ΔNp63α altered the UV-B-induced apoptotic pathway in the transgenic epidermis, proving that down-regulation of ΔNp63α in response to UV-B is important to epidermal apoptosis. The forced overexpression of ΔNp63α may act via a dominant negative effect on the endogenous p53 transcriptional activity required for UV-B-induced apoptosis.

Introduction
For many years, p53 has been considered the prototypical tumor suppressor and remains the subject of intense research. The protein product of the p53 gene responds to cellular stresses such as DNA damage and hypoxia and plays important roles in regulating cell cycle progression, genomic stability, and apoptosis (1, 2). Mutations in the p53 gene have been detected in >50% of all human cancers (3), demonstrating the universality of this tumor suppressor.

DNA damage can be caused by a number of genotoxic agents, and UV-B radiation is one of the most biologically relevant inducers of DNA damage. UV-B radiation in sunlight is the carcinogen responsible for most human skin cancers (4, 5). In response to the DNA damage induced by UV-B, the p53 protein is stabilized and translocated to the nucleus, where it triggers an arrest of the cell cycle or induces apoptosis (1, 4, 6). In the epidermis, the response to UV-B irradiation is frequently characterized by the induction of apoptosis, primarily mediated by p53 (7). In the absence of p53, fewer apoptotic cells are observed in the epidermis in response to UV-B (7). Also, mice null for p53 are more susceptible to UV-B-induced tumorigenesis than wild-type mice, implying a critical function for p53 in combating the detrimental effects on the epidermis of this carcinogen (8, 9).

Recently, two homologues of p53, p63 and p73, have been discovered and cloned, generating a new family of p53-like genes. The p63 gene shares extensive homology to p53 and produces multiple transcripts with varying functions (10–13). Unlike the fairly ubiquitous nature of the p53 protein, p63 exhibits a rather tissue-specific distribution in that it is most detectable in the basal layer of stratified epithelia, including the epidermis (13). It is reported that the most highly expressed p63 isoform in the epidermis is ΔNp63α (13). This isoform lacks the 5’ region that exhibits extensive homology to the transactivation domain of p53. In contrast to p53, ΔNp63α fails to induce apoptosis when overexpressed in cultured cells. ΔNp63α has also been shown to inhibit p53 transcriptional activity (13). The p63 gene was disrupted by homologous recombination, resulting in severe limb, craniofacial, and epithelial defects and leading to death shortly after birth (14, 15). The newborns lacking functional p63 did not have a recognizable epidermis or hair follicles but possessed a thin, single cell layer covering the body (14, 15). This cell layer did not exhibit epidermal characteristics such as keratin expression, but in one model, this cell layer showed a few isolated patches of highly differentiated clusters of epidermal-like cells (14, 15).

Because p63 is essential to normal epidermal development and is a homologue of the p53 tumor suppressor, we investigated the role of p63 in the epidermis in response to UV-B irradiation. We found that in contrast to p53, ΔNp63α is down-regulated in response to UV-B. To further characterize the importance of this down-regulation as well as the functional importance of p63 in the epidermis, we generated transgenic mice that overexpress ΔNp63α in the epidermis. The epidermis of these transgenic mice developed normally but exhibited a reduction in UV-B-induced apoptosis, indicating that the down-regulation of this p63 isoform is critical for normal epidermal UV-B-induced apoptosis.

Materials and Methods
Primary Keratinocyte Culture. Primary keratinocytes were obtained from newborn ICR mouse epidermis as described previously (16). Cells (1.5 × 105) were plated on 60-mm dishes and cultured in 50% fibroblast-conditioned medium supplemented with 0.05 mM calcium and 4 ng/ml epidermal growth factor. Media were refreshed 1 day after plating, and cells were exposed to UV-B 2 days after plating.

UV-B Treatment of Keratinocytes and Mice. Before exposure to UV radiation, the media were removed, and cells were rinsed twice with Dulbecco’s PBS (Life Technologies, Inc., Gaithersburg, MD) and covered with 1.5 ml of PBS during the UV treatment. Cells were exposed to 50 mJ/cm2 UV-B from FS40T12 bulbs (National Biological/ETA Systems, Twinsburg, OH) as monitored by a radiometer/photometer (International Light, Inc., Newburyport, MA). PBS was removed, and media were added to cells as soon as UV treatment was complete. Samples were taken at 0 (no UV radiation), 6, 12, and 24 h after UV treatment. Culture dishes were rinsed three times with PBS, and PBS was removed before freezing the dish on dry ice and storing it at ~80°C until protein or RNA extraction was performed. Newborn pups were immobilized, and back skin was exposed to 100 mJ/cm2 UV-B from FS40T12 lamps (National Biological/ETA Systems) as monitored by a photometer/radiometer (International Light, Inc.). Samples of back skin were taken at 0 (no UV radiation), 6, 12, and 24 h after UV exposure for normal ICR pups or at 24 h after UV exposure for transgenic litters. Portions of the skin were frozen in OCT, fixed in 10% neutral buffered formalin, and embedded in paraffin or frozen in liquid nitrogen for storage. Adult mice were exposed to 100 mJ/cm2 UV-B, monitored at the height of the ears. Ear samples were processed as described here for newborn back skin.

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Protein Extraction and Western Blotting. Protein was extracted from cultured keratinocytes and neonatal mouse epidermis and subjected to Western blotting as described previously (17). Membranes were blocked with TBST/NFDM [50 mM Tris (pH 7.5), 150 mM NaCl, 0.075% Tween 20 (Sigma, St. Louis, MO), and 5% nonfat dried milk] for 30 min at room temperature. Membranes were probed for p53 (Ab-7, sheep anti-p53; Oncogene Research Products, Cambridge, MA), stripped using ImmunoPure IgG Elution Buffer (Pierce Chemical Company, Rockford, IL) according to the manufacturer’s instructions, probed for p63 (mAb4A4, mouse monoclonal anti-p63; McKeon Laboratory), stripped, and probed for K14 [sheep anti-K14 (18)] as a loading control. Ab-7 and mAb4A4 were diluted 1:2000 or 1:100, respectively, in 1:3 TSBT/NFDM:TBS and incubated overnight at 4°C. Probing for K14 was performed at room temperature for 1 h using a 1:500 dilution in 1% NFDM in TBS. Blots were rinsed with TBS and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (Sigma) diluted in 1:3 TSBT/NFDM:TBS at 1:2-500 (against p53 and p63) or 1:20,000 (against K14). After rinsing with TBS, blots were exposed to PicoWest SuperSignal ECL Substrate (Pierce Chemical Company) and exposed to Biomax MR film (Kodak, Rochester, NY). A quantitative comparison of protein expression was determined by densitometric scanning of the films and normalizing each sample to the K14 signal.

RNA Extraction and RPAs. RNA was extracted from cultured keratinocytes and neonatal mouse epidermis using RNezol B (TeTest, Friendswood, TX) according to the manufacturer’s instructions. A 544-bp fragment from the 3’-most end of p63 (Fig. 2) was cloned by RT-PCR from an epidermal RNA sample and ligated to pGEM-T-Easy (Promega, Madison, WI) and sequenced. This clone was linearized with SpeI and transcribed in the presence of [32P]CTP with T7 RNA polymerase. RPAs were performed using the RPA II Kit (Ambion, Austin, TX). A 32P-labeled cyclophilin riboprobe was included in each reaction as a loading control. The intensity of protected product was determined by densitometric scanning of the films, and samples were normalized to the cyclophilin signal.

Generation of ML.Np63a Mice. The cDNA encoding the mouse Np63a transcript was modified by PCR to incorporate amenable restriction sites for cloning into the ML promoter to generate the ML.Np63a transgene (Fig. 2). The sequence was confirmed, the transgene was liberated from plasmid backbone by restriction digestion with BamH I and gel-purified, and transgenic mice were generated by standard techniques. Founder mice were identified by PCR of tail tip DNA using primers ML-1f (5’-GCTCTTCTGCTCTTCTTCC-3’) and 63-2r (5’-GACATCGTITTCACACCTCG-3’; Fig. 2).

Detection of ML.Np63a Transgene Expression. Reverse transcription was performed on 2 μg of epidermal RNA using random hexamers (Boehringer Mannheim, Indianapolis, IN) and Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. PCR reactions were performed using primers 63-5f (5’-GTGCTCTTACCTGGCTAGGTTG-3’) and mxeX6 (5’-AACAACAGCTGGAAACGGG-3’) for 35 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C. RT-PCR results were verified by immunohistochemistry on newborn epidermis using mAb4A4. Formalin-fixed, paraffin-embedded sections were deparaffinized in HemoDe: Xylene (3:1), rehydrated, and treated with HistoMouse Kit reagents 1A (30 min) and 1B (10 min; Zymed Laboratories, San Francisco, CA). Slides were incubated with mAb4A4 (1:250 dilution) for 2 h at room temperature followed by biotinylated horse antirabbit (Vector Laboratories, Burlingame, CA) and ABC Elite reagent (Vector Laboratories). Slides were incubated with SigmaFast 3,3’-diaminobenzidine (Sigma) and counterstained with hematoxylin (Gill’s #3; Fisher Biotech, Fair Lawn, NJ). Coverslips were mounted with GVA mount (Zymed Laboratories).

In Vivo BrdUrd Incorporation and Analysis. Newborn mice were injected i.p. with 250 μg of BrdUrd (Sigma) in 0.9% sterile saline solution. One h later, mice were sacrificed, and skin samples were fixed in Carnoy’s fixative or frozen in OCT (Sakuro Finetek, Torrance, CA). BrdUrd staining was performed as described previously (19).

TUNEL Analysis. Formalin-fixed, paraffin-embedded sections were sectioned at 6 μm. The sections were deparaffinized and rehydrated, and TUNEL analysis was performed using the Apoptosis Detection System, Fluorescein (Promega) according to the manufacturer’s instructions. Sections were then stained with propidium iodide (Sigma). The number of apoptotic cells per millimeter of epidermis was examined.

Results

Down-Regulation of p63 in Response to UV-B. After exposure to UV-B, the level of p53 protein in keratinocytes is increased, whereas the level of p53 transcript is unaltered (6). Because p63 is a new p53 family member and is highly expressed in the basal cells of the epidermis, the levels of p63 protein and transcript were analyzed after exposing primary keratinocytes to UV-B. At 6 h after UV-B treatment, the protein level of one p63 isoform, Np63α, increased slightly over basal levels (Fig. 1a). This is the p63 isoform that is the most highly expressed isoform in the normal epidermis. However, a dramatic decrease at the protein level of Np63α occurred at 12 h after UV-B treatment (Fig. 1a). Another p63 protein product, Np63γ, was expressed at low levels before UV-B treatment and was undetectable at 12 and 24 h after UV-B treatment (data not shown). To determine whether the decrease in p63 protein was a posttranscriptional event or whether it reflected a decreased level of transcription, RPA was performed. The p63α transcript was readily detected before UV-B treatment and decreased sharply after UV-B exposure (Fig. 1b). This is in contrast to p53 transcripts, which are detected at a constant level before and after UV-B exposure (6). Similar results were obtained after exposing normal newborn pups to 100 ml/cm2 UV-B and analyzing epidermal protein and RNA (data not shown).

Expression of the ML.Np63a Transgene in the Epidermis. Because the p63-null mouse does not survive, we were interested in developing a mouse model that would allow us to further investigate the significance of this decrease in p63 in response to UV-B and to better understand the function of p63 in the epidermis. The Np63α cDNA was placed under the control of the ML promoter (ML.Np63α), and transgenic mice were generated (Fig. 2a). This transgenic promoter construct was generated in our laboratory and has been shown to successfully drive expression to the epidermis in the superbasal layers as well as a portion of the basal cells (20). Three

Fig. 1. The levels of p63 protein and transcript from normal keratinocytes were examined after UV-B exposure. a, levels of Np63α protein transiently increase at 6 h after UV-B treatment and sharply decrease at 12 h after UV-B treatment, p63 was probed to verify that the UV-B dose was sufficient to elicit stabilization of p53, a typical UV-B response of keratinocytes. K14 was probed as a loading control. b, RPA showing that the levels of p63α transcript decreased corresponding to the decreased levels of protein. Cyclophilin (cyc.) is shown to confirm equal loading.

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founder lines were identified by PCR of tail tip DNA: (a) D1588; (b) D1593; and (c) D1595. To determine whether the transgene was being expressed, RT-PCR was performed. These three lines were all positive for the transgene transcript (Fig. 2b). To confirm transgenic protein expression, immunohistochemistry was performed on newborn transgenic and nontransgenic epidermis using mAb4A4. Because this ML promoter expresses transgenes throughout the epidermis, and endogenous p63 protein is detected strictly in the basal layer, any p63 detected suprabasally is a result of transgene expression. The immunohistochemistry confirmed the RT-PCR results, and it appeared that line D1588 had the highest level of ML.AΔNp63α transgene expression (Fig. 2c). In this line, most suprabasal cells showed a strong p63 signal, whereas the control epidermis failed to exhibit any suprabasal p63 staining (Fig. 2c). Lines D1593 and D1595 also showed p63 staining in suprabasal nuclei (data not shown).

Effects of ML.AΔNp63α Transgene Expression in the Epidermis. The ML.AΔNp63α transgenic epidermis appeared to develop normally, with no macroscopically detectable difference between transgenic and nontransgenic littermates (data not shown). Immunofluorescence analysis of epidermal differentiation markers, keratin 1 and loricrin, revealed no overt alteration in epidermal differentiation (data not shown). To determine whether there was an alteration of the epidermal proliferation rate, incorporation of BrdUrd was examined. There was no statistically significant difference between the transgenic and nontransgenic newborn epidermal proliferation rates (Fig. 3).

Forced Expression of AΔNp63α Altered UV-B-induced Apoptosis. Because the levels of ΔNp63α decreased in normal skin after UV-B exposure, we examined the effect of forced expression of this isoform on the UV-B response of the epidermis. After exposure to 100 mJ/cm² UV-B, apoptotic keratinocytes were detected using TUNEL analysis. We found that the ML.AΔNp63α transgenic epidermis was less susceptible to UV-B-induced apoptosis than the nontransgenic epidermis. As shown in Fig. 4a, there are fewer TUNEL-positive nuclei in the transgenic epidermis than in the nontransgenic littermate. All three ML.AΔNp63α lines exhibited a statistically significant decrease in TUNEL-positive nuclei in the transgenic epidermis than in the nontransgenic littermate.

Fig. 2. Detection of ML.AΔNp63α expression in transgenic epidermis. a, schematic diagram of the transgene construct and primers used for PCR and RT-PCR analysis. Details of the ML promoter and 3’ noncoding sequence used for transgenic analysis have been described previously (20). The ~1.7-kb ΔNp63α cDNA was inserted at the Cldn sites, and BamHI was used to liberate the entire transgene. Primers used for PCR genotyping of mice are ML.1F and 63.2R. Primers used for RT-PCR analysis are 63.5F and mceX46. The riboprobe used for RPA is indicated by the thick black line. b, RT-PCR analysis shows three transgenic lines positive for the ML.AΔNp63α transcript. Lanes 1 and 7, DNA HyperLadder (BioLine, Kaysville, UT); Lane 2, D1588; Lane 3, D1593; Lane 4, D1595; Lane 5, nontransgenic littermate; Lane 6, DNA positive control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected to verify the presence of cDNA in each sample. Mock RT-PCR analysis excluding Moloney murine leukemia virus reverse transcriptase was negative for all samples, indicating a lack of DNA contamination (data not shown). c, immunohistochemical detection of p63 in control and ML.AΔNp63α transgenic newborn epidermis. Control epidermis shows p63 expression restricted to the basal layer. Each transgenic line in which transgenic transcript was detected exhibits aberrant suprabasal expression of p63, consistent with the expected expression pattern from this ML promoter. In line D1588, nearly all suprabasal cells are strongly positive for p63 expression (arrows), whereas lines D1593 and D1595 exhibited fewer p63-positive suprabasal nuclei (data not shown).

Fig. 3. Proliferation rate analysis of ML.AΔNp63α transgenic epidermis. BrdUrd (green) was detected by immunofluorescence in the transgenic (a) and wild-type (b) newborn epidermis. Tissues were counterstained with K14 (red) to highlight the epidermis. c, quantitative analysis of three transgenic lines compared with control littermates revealed no significant difference in the proliferation rate of the ML.AΔNp63α transgenic epidermis and the wild-type epidermis.
crease in the number of apoptotic keratinocytes after UV-B exposure (Fig. 4b).

Discussion

In the present study, we investigated the role of p63 in the epidermal UV-B response. First, we found that the p63 isoform that is predominantly expressed in the epidermis, ΔNp63α, is dramatically reduced at both the transcript and protein levels in response to UV-B. This was intriguing because the p53 protein is stabilized posttranscriptionally in response to UV-B. We generated a transgenic mouse model to assess the effect of forced overexpression of ΔNp63α in the epidermis, particularly in response to UV-B. Although the MLΔNp63α epidermis appeared to develop normally, the ability of epidermal keratinocytes to undergo apoptosis in response to UV-B exposure was compromised, highlighting the importance of down-regulating this p63 isoform in response to UV-B.

The observation that the MLΔNp63α transgenic epidermis appeared to develop normally with an unaltered proliferation rate was not completely surprising because it has been shown that overexpression of ΔNp63α alone in cultured cells that were not stressed by DNA-damaging agents did not result in apoptosis (13). The need to down-regulate ΔNp63α after UV-B exposure raises questions regarding p63 function in the normal versus stressed epidermis. The ΔNp63α isoform fails to transactivate a p53-responsive reporter construct; however, other p63 isoforms that contain the 5′ region homologous to the p53 transactivation domain have been shown to activate p53-responsive promoters such as the p21/WAF1, BAX, and MDM2 promoters (13, 21). Additionally, at least one p63 isoform, ΔNp63γ, has been shown to interact directly with p53-binding sites in an electrophoretic mobility shift assay (13). Because this p63 isoform has the same DNA binding domain as the ΔNp63α isoform, it is not unreasonable to think that the ΔNp63α isoform may also interact directly with p53-binding sites. Furthermore, the ΔNp63α isoform has been shown to suppress p53 transcriptional activity (13). Whether this suppression occurs via direct protein-protein interaction or via promoter binding competition remains unclear. However, it has been shown that the various p63 isoforms can form oligomeric complexes among themselves (13, 22) but fail to interact directly with p53 or a p53 mutant in in vitro assays. These results imply that p63 is affecting the ability of p53 to transactivate target genes via an indirect mechanism such as competition for DNA binding sites.

This report is the first to assess the functional consequences of deregulated p63 in the epidermis of transgenic mice. It was proposed that p63 plays an essential role in maintaining the proliferative capacity of the basal cells of stratified epithelia (13, 23), a scenario that the results presented here support nicely. If activation of p53 results in cell cycle arrest or cell suicide, then maintaining a situation in which p53 is not allowed to function, i.e., rapid p53 turnover or blocking target promoter binding sites, is important in the proliferative basal compartment. Perhaps in addition to altering the ability of p53 to transcribe its targets, p63 may control other genes that function in excluding p53 from the nucleus or contribute to the rapid turnover of p53 in the cytoplasm. When a cell incurs DNA damage, p63 is down-regulated, allowing p53 to step into action, protecting the cell from perpetuating damaged DNA. Deregressed p63 expression would interfere with this protective role of p53, possibly by competing with p53 for binding to DNA targets and/or exclusion from the nucleus and degradation via the actions of newly synthesized p63.

There are many questions that remain to be explored regarding the function of p63 in both the unperturbed state as well as in response to DNA-damaging events. Defining a role, if any, for p63 in epithelial tumorigenesis awaits further analysis. Interestingly, Osada et al. (10) report mutations in the p63 DNA binding domain in three epidermal carcinomas. Additionally, a syndrome known as EEC (ectodactyly, ectodermal dysplasia and facial clefts) has been linked to germ-line mutations in p63, primarily within the DNA-binding domain (24). Amplification of chromosome 3q, the chromosomal location of the p63 gene, has been observed in squamous cell carcinomas and advanced cervical carcinomas, supporting a role for deregulated p63 expression in epithelial tumorigenesis (25–27). This MLΔNp63α transgenic model will provide a useful in vivo system for further assessing the role of p63 in the epidermis and in epidermal tumorigenesis, both UV-B-induced and chemically induced tumorigenesis.

Note Added in Proof

While this paper was under review, two reports that support an oncogenic role for ΔNp63 isoforms in squamous cell carcinoma were published (28, 29).
References

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