Role of NF-κB in the Apoptotic-resistant Phenotype of Keratinocytes*

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Several studies point to a role for NF-κB in modulating epidermal thickness and apoptotic susceptibility of keratinocytes. When phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) are topically applied, prominent epidermal thickening occurs, and exposure to interferon (IFN)-γ promotes increased epidermal thickness producing psoriatic lesions. While keratinocytes derived from psoriatic plaque resist apoptosis, and combination of TPA and IFN-γ activates NF-κB, the molecular mechanism linking NF-κB activation and keratinocyte apoptosis resistance was unknown. Therefore, we examined the ability of IFN-γ plus TPA to influence NF-κB activity, gene expression, and response to UV light-induced apoptosis. These responses in normal keratinocytes were compared with immortalized keratinocytes (HaCaT cells). Exposure of normal keratinocytes to IFN-γ plus TPA produced a synergistic activation of NF-κB, compared with when each reagent was used individually. Normal keratinocytes when exposed to IFN-γ plus TPA acquired a resistance to UV light-induced apoptosis, which was dependent on NF-κB because expression of a dominant negative form of IκBα overcame the resistance. Compared with normal keratinocytes, HaCaT cells have a dysfunctional constitutive NF-κB signaling pathway not induced by IFN-γ and TPA, rendering HaCaT cells highly susceptible to UV-induced apoptosis. Thus, immortalized HaCaT cells have an abnormal constitutive and dysfunctional NF-κB signaling system. These results provide evidence that activation and proper regulation of NF-κB is essential for acquisition of an apoptotic-resistant phenotype for epidermal-derived keratinocytes.

The thickness of human epidermis remains relatively constant throughout life. Yet the molecular basis regulating keratinocyte life and death is poorly understood. Several studies indicate a role for NF-κB function in the regulation of epidermopoiesis in vivo (1–4). The activity of NF-κB3 is primarily regulated by translocation from the cytoplasm into the nucleus (5), where it can alter expression of target genes (6). The inactive form of NF-κB is sequestered in the cytoplasm, being bound by IκBα and IκBβ (7–10). These inhibitors of NF-κB are regulated by IKK which can phosphorylate them, leading to their degradation in proteosomes (11–15). The ability of cytokines and pro-inflammatory signals to liberate NF-κB dimers that are translocated to the nucleus is mediated by the aforementioned IKK activity (2–4, 11–15).

In normal murine skin the p50 subunit of NF-κB is found in the cytoplasm of basal keratinocytes, but in the suprabasilar keratinocytes that have become mitotically quiescent and begun to differentiate, p50 is found in the nucleus (1). If p50 or p65 is overexpressed by being constitutively activated using a keratin 14 promoter, there is profound epidermal thinning and premature death of the transgenic mice associated with increased intranuclear levels of p50 and p65 (1). Conversely by blocking NF-κB activity using either a pharmacological inhibitor, or a dominant negative inhibiting protein of NF-κB (i.e. mutant IκBα), there is striking epidermal hyperplasia, which is also associated with failure to thrive and early death of transgenic mice (1). By targeting proximal regulators of NF-κB such as the IκB kinases (IKK), similar phenotypes have been produced in which blocking of NF-κB activation was associated with pathologically thickened murine epidermis (2–4). Taken together, these in vivo results clearly point to a key role for NF-κB modulating the phenotype of epidermal keratinocytes.

Currently it is not clear how NF-κB activity modulates epidermal thickness, because NF-κB signaling impacts a number of important intracellular cascades governing cell cycle, differentiation, apoptosis, cytokine release, and oncogenic pathways (16). Moreover, NF-κB activity has also been implicated in chronic inflammatory skin diseases including psoriasis (17, 18). We previously examined the role for γ-interferon (IFN-γ) and phorbol ester (i.e. TPA) in psoriasis with respect to their ability to modulate the immunophenotype of keratinocytes, and expression of cell survival proteins such as Bcl-xL (19, 20). Since keratinocytes derived from psoriatic plaques are resistant to apoptosis (21), we explored the impact of these combined stimuli on the apoptotic pathway of keratinocytes. While the genetic basis for psoriasis is unclear, T cell derived cytokines such as IFN-γ and signal transduction pathways involving protein kinase C have been implicated in the production of a thickened epidermis (22, 23). Moreover, topical application of protein kinase C agonist such as phorbol esters on rodent skin produces a rapid and marked thickening of the skin associated with inflammation and cytokine release (23). Earlier (24), it was determined that IFN-γ and TPA when used individually could trigger NF-κB activation and induced a relative resistance to apoptosis in normal keratinocytes, but not in an immortalized cell line (HaCaT cells). To extend these findings we have now combined IFN-γ with TPA, and observed a synergis-
tic enhancement in the activation of NF-κB as well as the anti-apoptotic phenotype of normal keratinocytes. We also detected significant abnormalities in the NF-κB signaling pathway in HaCaT cells which can explain the lack of protection from apoptosis by pretreatment with IFN-γ and TPA. These studies provide some of the molecular insights necessary to begin to unravel the mechanism by which NF-κB regulates epidermal thickness.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—Primary keratinocytes were isolated from freshly excised neonatal foreskins as described previously (24). An immortalized human keratinocyte cell line, HaCaT cells, was obtained from Professor N. Fusenig (Heidelberg, Germany). All cells were cultured in keratinocyte growth medium (Clonetics, San Diego, CA) with 0.15 mM calcium.

Both keratinocytes and HaCaT cells were treated with either keratinocyte growth medium alone or exposed to IFN-γ (10 μg/ml, Gentech, Inc., San Francisco, CA) plus TPA (100 nM, Sigma) for 24 h. Then the cells were washed with PBS for three times and fed with fresh medium for 12–24 h. To induce apoptosis, cells were irradiated by a Panelite Unit (Ultralite Enterprise, Inc., Lawrenceville GA) equipped with four UVB bulbs (FS36T/12/UVB-VHO), which have the majority of output in the UVB range (65%), with minor output in the UVA and UVC range (34 and 1%, respectively). The cells were irradiated with dish lids removed using a dosage of 25 mJ/cm² or 30 mJ/cm². The UVB treatment was performed in a Panelite Unit (Ultralite Enterprise, Inc., Lawrenceville GA) equipped with four UVB bulbs (FS36T/12/UVB-VHO), which have the majority of output in the UVB range (65%), with minor output in the UVA and UVC range (34 and 1%, respectively). The cells were irradiated with dish lids removed using a dosage of 25 mJ/cm² or 30 mJ/cm². The UV dose was measured with an International Light Inc. (Newburyport, MA) radiometer fitted with a UVB detector.

For induction of NF-κB, the cells at 70–80% confluency were treated with IFN-γ (10 μg/ml) and/or TPA (100 nM) for different times as indicated. To assess proliferation counting of cells was performed using a calibrated slide chamber hemacytometer as described previously (24).

Antibodies—The anti-caspase 3/CPP32 antibody (C31T20) was purchased from Transduction Labs (Lexington, KY). The anti-caspase 8/Flice (DO7) antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the anti-caspase 9 (9A7) antibody was from Transduction Labs (Lexington, KY).

Fluorescence-activated cell sorting (FACS) analysis was performed with a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) as described previously (26). Briefly, cells were detached, fixed with 70% ethanol, and stained with propidium iodide (50 μg/ml, Sigma) for 30 min. For analysis of cell cycle distribution, the stained cells were analyzed with a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) as described previously (26).

Isolation of Cytosplasmic and Nuclear Extracts—Nuclear and cytoplasmic extracts were prepared following the method of Gerber et al. (25). Monolayers of keratinocytes and HaCaT cells were washed with ice-cold PBS, harvested by scraping into 1 ml of PBS, and pelleted in a 1.5-ml microcentrifuge tube. The pellet was suspended in 400 μl of lysis buffer. After 20 min incubation on ice, 25 μl of 10% Nonidet P-40 was added and then centrifuged briefly. The supernatant was analyzed by SDS-PAGE.

Preparation of cell lysate and luciferase activity measurements were made with Dual Luciferase™ Reporter Assay System (Promega, Madison WI) according to the manufacturer’s instructions. The sample was placed in a TD-20/20 luminometer (CLONTECH Laboratories Inc., Palo Alto, CA) for detection of light intensity. Differences were analyzed for statistical significance using the Student’s t test.

Ribonuclease Protection Assay—Keratinocytes and HaCaT cells were treated with IFN-γ (10 μg/ml) and TPA (100 nM) for 6 h. Total cellular RNA was extracted using Trizol Reagent (Life Technologies, Inc., Grand Island, NY). The ribonuclease protection assay was performed according to the supplier’s instructions (Pharmingen, San Diego, CA). Briefly, human apoptosis template set hAPO-5 was labeled with [32P]uridine triphosphate. RNA (10–20 μg) and 8 × 10⁶ cpm of labeled probes were used for hybridization and after RNase treatment, the protected probes were resolved on 5% urea-polyacrylamide gels.

Infection of Cells with Expression Vector of IκB Dominant Negative Fusion Protein—IκBα C2-βCDN (IκBαΔCDN) was kindly provided by Dr. Tim Ellis (Loyola University Medical Center) and subcloned into the BamHI and NotI of LZRS and MGB-based retroviral expression vector. The LZRS vector (1) containing enhanced green fluorescent protein was kindly provided by Dr. Paul A. Khavari (Stanford University School of Medicine, Stanford CA). The Phoenix-Ampo retroviral packaging cells were obtained from American Type Culture Collection (Manassas, VA) with permission from Dr. Gary P. Nolan (Stanford University Medical Center, Stanford, CA). The packaging cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Grand Island, NY) and transfected with LZRS-IκBα CDN vector by using CaCl₂ and 2 × HBSS. After overnight incubation, the cells were fed with fresh medium and incubated at 32 °C for an additional 24–48 h. The supernatants were collected for cell infection.

The normal keratinocytes were seeded into 6-well plates and infected with 300 μl of viral supernatant in the presence of 4 μg/ml hexadimethrine bromide (Polybrene) (Sigma H-9268) for 1 h at 32 °C, then the supernatant was removed and replaced with fresh medium, incubated at 37 °C in 5% CO₂ overnight. After being washed with PBS, the infected cells were propagated and treated with IFN-γ plus TPA and/or UV light. The overexpression of the IκBα dominant negative protein was detected with Western blot and immunocytochemistry. Immunostaining was performed using a primary antibody against the IκBα peptide, followed by a secondary antibody conjugated to Cy3. The confocal images were obtained using a Leica TCS SP confocal microscope.

RESULTS

IFN-γ Plus TPA Renders Normal Human Keratinocytes but Not HaCaT Cells Resistant to UV-induced Apoptosis—Previously we demonstrated that the growth arrest treatment by IFN-γ or TPA alone renders normal human keratinocytes re-
sistant to subsequent UV-induced apoptosis (24). In a typical experiment, when normal human keratinocytes were exposed to UV light approximately 50–60% of the cells are induced to become apoptotic (24). However, if these keratinocytes are pre-treated with either TPA alone or IFN-γ alone, the subsequent exposure of UV light induced only approximately 15–30% of the keratinocytes to become apoptotic (24). In this study we investigated the role of combined treatment by IFN-γ and TPA on the keratinocyte apoptotic response to UV irradiation.

The neonatal foreskin-derived keratinocytes at 60–70% confluence were treated with IFN-γ (10^3 units/ml) and TPA (100 nM) for 24 h, washed and maintained in fresh medium for an additional 24 h in keratinocyte growth medium. Next the cultures were rinsed with PBS prior to UV (30 mJ/cm²) radiation. The cellular DNA was stained with propidium iodide and analyzed by flow cytometry 18 h after UV exposure. The displayed percentage of cells with sub-G_1 DNA content is the average from three independent experiments. B, the cells were also treated as above and subjected to TUNEL staining. The positive cells were detected by flow cytometry. Insets depicts percentage of positive cells of a representative experiment. C, equal amounts of proteins from the keratinocytes treated as above were separated by SDS-polyacrylamide gel electrophoresis and detected by immunoblotting using antibodies against caspase 8, caspase 3 (D), and PARP (E). The arrows indicate the intact or cleaved fragments of the corresponding proteins.

FIG. 1. Pretreatment of normal keratinocytes with IFN-γ plus TPA renders them resistant to UV-induced apoptosis. A, cells were treated with IFN-γ (10^3 units/ml) plus TPA (100 nM) for 24 h, washed and incubated for an additional 24 h in keratinocyte growth medium. Next the cultures were rinsed with PBS prior to UV (30 mJ/cm²) radiation. The cellular DNA was stained with propidium iodide and analyzed by flow cytometry 18 h after UV exposure. The displayed percentage of cells with sub-G_1 DNA content is the average from three independent experiments. B, the cells were also treated as above and subjected to TUNEL staining. The positive cells were detected by flow cytometry. Insets depicts percentage of positive cells of a representative experiment. C, equal amounts of proteins from the keratinocytes treated as above were separated by SDS-polyacrylamide gel electrophoresis and detected by immunoblotting using antibodies against caspase 8, caspase 3 (D), and PARP (E). The arrows indicate the intact or cleaved fragments of the corresponding proteins.

FIG. 2. HaCaT cells retain the sensitivity to UV induced apoptosis despite pretreatment with IFN-γ plus TPA. A, HaCaT cells treated with the same protocol as described in the legend to Fig. 1 and analyzed by flow cytometry. The inset represents percentage of cells with sub-G_1 DNA content from three independent experiments. Immunodetection of caspase 8 (B), caspase 3 (C), and PARP (D) in the HaCaT cells with or without the pretreatment as described in the legend to Fig. 1. Note that after UV exposure (25 mJ/cm²), no blockage of activation of both caspase 8 and caspase 3 and PARP cleavage was detected in the pretreated HaCaT cells.

prior to UV (25 mJ/cm²) irradiation. Thus, significantly greater protection of keratinocytes against UV-induced apoptosis was observed when IFN-γ and TPA were combined compared with when they were used individually.

Since caspase activation and PARP cleavage have been described to be one of the molecular mechanisms in UV-induced apoptotic process (24), and are hallmarks for apoptosis (26), we also examined levels of caspase 8 and caspase 3 (the two major caspases responsible for cell apoptosis) activation, as well as PARP (one of the substrates of caspase 3) cleavage. UV exposure led to obvious proteolysis of both caspase 8 and caspase 3, as well as PARP cleavage, in keratinocytes without pretreatment. However, pretreatment with IFN-γ plus TPA partially prevented caspase proteolysis and PARP cleavage caused by UV irradiation (Fig. 1, panels C, D, and E).

To determine if similar protection against UV-induced apoptosis would also occur in immortalized keratinocytes, HaCaT cells were treated following the same protocol as above for normal keratinocytes. In marked contrast to normal keratinocytes, after the same pretreatment with IFN-γ plus TPA, Ha-
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p65 and p50 proteins, respectively. Normal keratinocytes were stimulated with IFN-γ (10^3 units/ml) alone or TPA (100 nM) alone, or IFN-γ plus TPA for the indicated time. The nuclear extracts prepared were tested for NF-κB DNA binding activity. The NF-κB complex is indicated by arrows. Specificity of binding of NF-κB with its consensus DNA sequence was demonstrated by competition with 100 total excess of unlabeled oligonucleotide.

**FIG. 3.** The synergistic induction of DNA binding activity of NF-κB in keratinocytes by pretreatment with IFN-γ plus TPA. Normal keratinocytes were stimulated with IFN-γ (10^3 units/ml) alone or TPA (100 nM) alone, or IFN-γ plus TPA for the indicated time. The nuclear extracts prepared were tested for NF-κB DNA binding activity. The NF-κB complex is indicated by arrows. Specificity of binding of NF-κB with its consensus DNA sequence was demonstrated by competition with 100 total excess of unlabeled oligonucleotide.

**FIG. 4.** Degradation of IκBα is followed by the nuclear translocation of NF-κB and DNA binding induced by IFN-γ plus TPA in keratinocytes. A, keratinocytes were treated with IFN-γ (10^3 units/ml) plus TPA (100 nM) for the indicated times. Equal amounts (30 μg) of cytoplasmic extracts were analyzed with Western blot using IκBα specific antibody. Degradation of IκBα was revealed by the lower level of IκBα protein (see lanes 2 and 3). B, nuclear extracts prepared from the same cells as in A were also analyzed by Western blot with antibodies against p65 and p50. The arrows indicate the specific bands for p65 and p50 proteins, respectively. C, EMSA was performed using 5 μg of nuclear extracts from keratinocyte after the same treatment as in panel A. The two major NF-κB-DNA complexes are indicated with arrows.

**FIG. 5.** Increased constitutive DNA binding activity and low transcription activity of NF-κB in HaCaT cells. A, HaCaT cells were stimulated with IFN-γ (10^3 units/ml) and TPA (100 nM) for different times as indicated. Equal amounts (30 μg) of cytoplasmic extracts were used to detect the IκBα level by Western blot. B, equal amounts (30 μg) of nuclear extracts as above in A were also analyzed by Western blot with antibodies against p65 and p50. C, the same nuclear extracts as above were also used to detect the DNA binding activity of NF-κB by EMSA. D, HaCaT cells or normal keratinocytes were transiently transfected with a plasmid in which the expression of luciferase reporter gene was driven by NF-κB enhancer as described under “Experimental Procedures.” Luciferase activity was assayed after cells were untreated or treated with IFN-γ (10^3 units/ml) plus TPA (100 nM) for 6 h. The similar transfection and assay were also performed using vector control plasmid. Error bars represent standard deviations from three determinations.

CaT cells were still highly sensitive to UV-induced apoptosis as revealed by the cell cycle analysis portrayed in Fig. 2A. The fraction of HaCaT cells with sub-G0/G1 pretreated with IFN-γ plus TPA was actually slightly higher than that of untreated cells after UV exposure (65 versus 58.1%). This result is consistent with our previous observation that IFN-γ or TPA when used as single pretreatments provide no protection to HaCaT cells subsequently exposed to UV light. Consistent with the apoptosis assays, pretreatment of HaCaT cells with IFN-γ plus TPA did not prevent either caspase 8 or caspase 3 activation, or the PARP cleavage (Fig. 2, panels B, C, and D). It should be noted that the combination of IFN-γ plus TPA produced over 80% inhibition of the proliferation for both normal keratinocytes as well as HaCaT cells as determined by manual cell counts (data not shown).

**IFN-γ Plus TPA Synergistically Induce NF-κB DNA Binding Activity in Normal Keratinocytes—**Activation of transcription factor NF-κB has recently been found to prevent apoptosis in a diversity of cell types (5, 16, 27–30). Since both IFN-γ and TPA can individually induce NF-κB activity in keratinocytes and this induction is defective in HaCaT cells (24), we postulated that the inducible NF-κB activity in normal keratinocytes in some way contribute to the protection of apoptosis caused by UV irradiation of normal keratinocytes.

We first determined whether IFN-γ plus TPA could induce NF-κB in normal keratinocytes. The normal keratinocytes were stimulated with IFN-γ alone, TPA alone, or IFN-γ plus TPA for various lengths of time. Then nuclear extracts were prepared to detect the DNA binding activity of NF-κB. EMSA revealed that the proliferating keratinocytes contain low but detectable basal level of NF-κB activity. Exposure of keratinocytes to TPA alone led to a moderate and transient induction of the p50/p50 homodimers, whereas IFN-γ alone led to a relatively weak and more transient induction of the p65/p50 heterodimer. In contrast, when IFN-γ and TPA were combined, the keratinocyte...
response revealed a rapid and sustained induction of both the homodimers as well as heterodimers of NF-κB (Fig. 3). Supershift analysis of IFN-γ and TPA activated DNA-protein complexes confirmed that the major components of NF-κB were p65 and p50 proteins, with only minor to no RelB and c-Rel (data not shown).

The activation of NF-κB in response to a variety of stimuli involves the phosphorylation of IκBα on two N-terminal serines followed by degradation of the protein via the ubiquitin-proteasome pathway (11–15). We then checked if the activation of NF-κB induced by IFN-γ and TPA was also through this classical pathway. By Western blot analysis using the cytoplasmic extracts from treated cells, IκBα degradation was identified at 30 and 60 min after exposure to IFN-γ plus TPA (Fig. 4, panel A). The IκBα degradation was followed by NF-κB translocation as determined by the increased p65 and p50 protein in nuclear extracts by Western blot analysis (Fig. 4, panel B), which also was confirmed by EMSA (Fig. 4, panel C). No obvious IκBβ degradation was detected at any of the tested time points (data not shown). Thus, the activation of NF-κB stimulated by IFN-γ plus TPA was accompanied by rapid degradation of IκBα, followed by nuclear translocation of p50 and p65 subunits of NF-κB.

Lack of Protection from UV-induced Apoptosis in HaCaT Cells Was Correlated to Deficient Induction of NF-κB by IFN-γ and TPA—In normal keratinocytes, prevention of UV-induced apoptosis and the activation of NF-κB by IFN-γ plus TPA were rather striking. Therefore, we postulated that the lack of protection in HaCaT cells may be related to deficient induction of NF-κB. EMSA showed that proliferating HaCaT cells had a high constitutive NF-κB DNA binding activity which was not further increased at any of the tested time points in response of IFN-γ plus TPA (Fig. 5, panel C). The longer incubation with IFN-γ and TPA led to an decrease in the constitutive NF-κB DNA binding activity (data not shown). Consistent with the EMSA results, no obvious increased p65 and p50 protein level in the nuclear extracts could be detected by Western blot analysis (Fig. 5, panel B). Furthermore, no obvious IκBα degradation was found in the cytoplasmic extracts of HaCaT cells (Fig. 5, panel A), and the IκBβ was undetectable in HaCaT cells, although it was easy to detect in normal keratinocytes (data not shown).

Because the high constitutive NF-κB binding activity did not appear to be correlated with the prevention of apoptosis by IFN-γ plus TPA pretreatment, we investigated the transcriptional activity of intranuclear NF-κB proteins in HaCaT cells. To this end, HaCaT cells and normal keratinocytes that had been transiently transfected with an NF-κB-driven luciferase reporter were stimulated with IFN-γ plus TPA, and then luciferase activity was measured. Interestingly, despite the high constitutive DNA binding activity of NF-κB in unstimulated HaCaT cells, their luciferase activity was significantly lower than that of untreated normal keratinocytes (p < 0.05). Furthermore, after stimulation with IFN-γ plus TPA, the luciferase activity increased nearly 2-fold in normal keratinocytes (p < 0.05), however, nearly no enhancement was detected in the treated HaCaT cells (Fig. 5, panel D), suggesting a functional deficiency occurred in the ability of regulating gene transcription in HaCaT cells. These results demonstrate the importance of distinguishing between DNA binding and transcriptional activity of NF-κB in HaCaT cells.

IFN-γ Plus TPA Increase the Level of Transcripts of Several Apoptosis Inhibitors in Normal Keratinocytes but Not HaCaT Cells—IFN-γ plus TPA pretreatment renders normal keratinocytes resistant to subsequent apoptotic challenge with UV irradiation, thus suggesting induction of proteins that mediate protection against apoptosis. Recently, a group of inhibitors of apoptosis has been characterized and many of them can be up-regulated by NF-κB (31–35). We hypothesized that treatment of IFN-γ plus TPA could promote the expression of these proteins via NF-κB activation in normal keratinocytes, and
FIG. 8. Overexpression IκBαΔN in keratinocytes reverses the apoptosis resistance by pretreatment with IFN-γ plus TPA. A, mock infected (upper panel) and IκBαΔN infected (lower panel) keratinocytes were treated or not with IFN-γ (10^5 units/ml) plus TPA (100 nM) for 24 h, then washed and irradiated with UV (30 mJ/cm^2). Apoptotic cells were measured by DNA staining with propidium iodide and flow cytometer analysis. Insets depicts the percentage of cells with sub-G_0 DNA content from a representative experiment. B, equal amounts (30 μg) of whole cell lysates from both mock infected (left side) or IκBαΔN-infected keratinocytes treated with similar protocol as above were analyzed by Western blot to detect the activation of caspase 8 (upper panel) and caspase 3 (lower panel).
that HaCaT cells would differ from normal keratinocytes by not up-regulating apoptotic resistant transcripts because of their dysfunctional NF-κB signaling pathway.

To explore the transcriptional patterns of normal keratinocytes and HaCaT with respect to cell survival proteins, RNase protection assays were performed. In proliferating normal keratinocytes, several anti-apoptotic transcripts were present including x-IAP, TRAF-2, and cTAP-1 mRNAs. After treatment with IFN-γ plus TPA, some of these transcripts became more abundant, especially the TRAF-1 and cIAP-2 mRNAs (Fig. 6, panel A). The constitutive presence of L32 and glyceraldehyde-3-phosphate dehydrogenase served as the loading control. By Western blot, we also detected an increase in TRAF-1 protein in normal keratinocytes treated with IFN-γ plus TPA (data not shown). In HaCaT cells, consistent with dysfunctional intranuclear NF-κB, many of the transcripts were only detectable when used twice as much RNA in this assay (Fig. 6B). For example, x-IAP, TRAF-2, cIAP-1, and cIAP-2 mRNAs were visible in untreated HaCaT cells. In addition, no induction of any of these cell survival gene products was identified in this assay. The relatively low levels of these transcripts in proliferating HaCaT cells and absence of induction in response to IFN-γ plus TPA correlated with the lack of functional NF-κB activation and resistance to UV-induced apoptosis.

**Inhibition of NF-κB Translocation Induced by IFN-γ Plus TPA by Overexpression of Dominant Negative IκBα**—From the above results, it is clear that pretreatment of normal keratinocytes with IFN-γ plus TPA can simultaneously block UV-induced apoptosis and activate NF-κB. To convincingly establish a cause/effect relationship between these two events, a dominant negative retrovirus specific for NF-κB was used. As previously demonstrated, the NF-κB translocation induced by IFN-γ plus TPA occurred in conjunction with IκB degradation. To block the NF-κB activity we infected the normal keratinocytes with a retroviral expression vector encoding dominant negative IκBα protein. The phosphorylation-defective IκBα protein acts by sequestering the cytoplasmic NF-κB pool in a manner that is insensitive to extracellular stimuli and hence prevents nuclear translocation of p50/p65 subunits into the nucleus (1). Overexpression of the mutant IκBα was detected in the cytoplasm by immunocytochemistry (Fig. 7A) and Western blot analysis (Fig. 7B). The IκBα detected in mock infected cells represented the wild type level (Fig. 7, panels A and B). When keratinocytes were infected with the dominant negative IκBα retroviruses and then treated with IFN-γ plus TPA, no increased NF-κB translocation was detected in their nuclear extracts by Western blot. However, the mock infected cells still maintained their responsiveness to NF-κB activation revealed by increased p65 and p50 protein levels after stimulation (Fig. 7, panel C).

**Repression of NF-κB Activation Reverses the Resistance to UV-induced Apoptosis**—After the successful inhibition of NF-κB activity by this retroviral construct, we then analyzed the influence of blocking NF-κB on UV-induced apoptosis. This mutant IκBα protein produced after infection by the retrovirus reduced the constitutive level of NF-κB and rendered normal proliferating keratinocytes slightly more sensitive to UV-induced apoptosis. The normal keratinocytes which had mock infected or infected with IκBα dominant negative retroviruses were subsequently exposed to IFN-γ plus TPA for 24 h followed by a 6-h wash and then irradiated with UV light. The apoptotic process was determined by cell cycle analysis and caspase activation as shown in Fig. 8. A representative cell cycle analysis demonstrated that after blocking NF-κB, the sub-G1/G0 fraction in IFN-γ plus TPA-treated keratinocytes was as high as that of untreated cells (panel A). Furthermore, by Western blot, the blocking of NF-κB activity led to both caspase 3 and caspase 8 cleavage. However, in mock infected cells, the induction of NF-κB could still partially prevent this caspase cleavage (panels B and C). Thus, it was clearly demonstrated that the NF-κB activation is necessary to the resistance to UV-induced apoptosis.

**Blocking NF-κB Activity Decreases the Level for Transcripts of Several Inhibitors of Apoptosis**—As it has been demonstrated above that induction of NF-κB was correlated with increased levels of mRNA of several anti-apoptotic proteins, we next determined whether blocking of NF-κB activation would also inhibit the induction of anti-apoptotic genes. Again, by RNase protection assay, we found that in mock infected keratinocytes, IFN-γ plus TPA enhanced the mRNAs of TRAF-1, c-IAP-1, and c-IAP-2. However, after inhibition of NF-κB using the retrovirus, the same treatment led to a decreased induction of these transcripts compared with that of mock infected cells (Fig. 9).

**DISCUSSION**

These results clearly establish that combining IFN-γ plus TPA produces a synergistic activation of NF-κB as determined by Western blot, EMSA, supershift analysis, and by transactivation of a luciferase reporter construct in normal human keratinocytes. Conversely, immortalized HaCaT cells, despite having constitutive intracellular protein levels of NF-κB, have low to absent functional transcriptional activity, which is not further enhanced by treatment with IFN-γ plus TPA. These highly divergent biochemical characteristics between normal keratinocytes and HaCaT cells became phenotypically manifest by exposing the cells to UV light. While proliferating untreated normal keratinocytes and HaCaT cells were equally sensitive to the antiproliferative effects of IFN-γ plus TPA, and to UV-light-induced apoptosis, pretreatment with IFN-γ plus TPA produced a death-defying phenotype in normal keratinocytes, that was not acquired by HaCaT cells. Using an IκBα dominant negative retrovirus to block NF-κB activation, the ability of IFN-γ plus TPA to confer resistance to apoptosis in normal keratinocytes was drastically reduced demonstrating that this resistance is directly linked to NF-κB activation.

To examine the potential genes activated by IFN-γ plus TPA, focus was directed at several known transcripts that can produce anti-apoptotic proteins. Indeed, in normal keratinocytes,
but not HaCaT cells, IFN-γ plus TPA increased levels of TRAF1, cIAP-1, and cIAP-2. Previous investigators have determined that several of these anti-apoptotic transcripts are regulated by NF-κB (5). Their importance in conferring resistance to apoptosis was supported by the observation that the IKKα dominant negative retrovirus also blocked their expression, with abrogation of the apoptotic resistance phenotype for normal keratinocytes. Furthermore, in HaCaT cells the dysfunctional NF-κB transcription activity was reflected by the relatively low levels of several anti-apoptotic transcripts, and their complete lack of induction by IFN-γ plus TPA. Studies are in progress to determine the molecular basis for the dysfunctional NF-κB transcriptional apparatus in HaCaT cells responsible for these intracellular signaling defects.

Despite these clear-cut in vitro results demonstrating the striking apoptotic resistance associated with NF-κB activation in normal keratinocytes, the in vivo findings reported by several groups in which blocking keratinocyte NF-κB activity produced an increased epidermal thickness requires further discussion. Based on the current studies involving keratinocytes, and many other reports in different cell types (5, 16, 24, 27–30), NF-κB activation is associated with resistance to apoptosis. Thus, one would predict that blocking NF-κB activation would make keratinocytes more susceptible to apoptosis, with fewer rather than more viable cells in the epidermis. To resolve this paradox it is necessary to consider the context in which NF-κB activity is present, and the role for other molecular regulators of apoptosis in skin. One of the other major intracellular pathways regulated by NF-κB (besides apoptosis) is the cell cycle (1, 24). NF-κB activating stimuli such as IFN-γ plus TPA induce complete and irreversible growth arrest in vitro, as observed in this study for both normal keratinocytes and HaCaT cells. The transgenic mice phenotype in which activation of NF-κB in keratinocytes produced profound epidermal thinning may reflect this cytostatic activity, that cannot be compensated by an increased survival of these keratinocytes. Tumor biologists have also begun to appreciate that the overall mass of neoplastic cells represents a net sum of both rates of proliferation, as well as cell death (36). Extrapolating to the skin, it appears that the anti-proliferative effects mediated by NF-κB activation are dominant over the anti-apoptotic effects since the cutaneous phenotype of mice with activated NF-κB in the absence of inflammatory or apoptotic stimuli is epidermal thinning, whereas blockade of NF-κB produces epidermal thickening. Indeed under normal conditions transgenic mice that overexpress only proteins that enhance survival (but without significant cell cycle changes) such as Bel-X or Bel-2 have either no significant increase (37), or only focally thickened epidermis (38). It is only when the homeostasis of the skin is perturbed by various proinflammatory and/or tumorigenic stimuli does a distinctive abnormal phenotype become apparent in these transgenic mice (37–39).

While our results reported herein demonstrate that induction of NF-κB by IFN-γ plus TPA exposure is necessary for resistance to apoptosis, there may be other conditions that can also suffice for enhancing keratinocyte survival. For example, it was established that exposure of keratinocytes to elevated extracellular calcium ion, or during confluence, or after replicative senescence, there was also resistance to apoptosis, but this did not require NF-κB activation (24). Taken together, more work will be required to fully dissect individual contributions of NF-κB activation in the immunobiology of normal and diseased human skin, in regards to cell cycle control and regulation of apoptosis.

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