Apoptosis in Proliferating, Senescent, and Immortalized Keratinocytes*

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Skin provides an attractive organ system for exploring coordinated regulation of keratinocyte (KC) proliferation, differentiation, senescence, and apoptosis. Our main objective was to determine whether various types of cell cycle arrest confer resistance to apoptosis. We postulated that KC cell cycle and cell death programs are tightly regulated to ensure epidermal homeostasis. In this report, simultaneous expression of cyclin-dependent kinase inhibitors (p15, p16, p21, and p27), a marker of early differentiation (keratin 1), mediators of apoptosis (caspases 3 and 8), and NF-κB were analyzed in three types of KCs. By comparing the response of proliferating, senescent, and immortalized KCs (HaCaT cells) to antiproliferative agents followed by UV exposure, we observed: 1) Normal KCs follow different pathways to abrupt cell cycle arrest; 2) KCs undergoing spontaneous replicative senescence or confluence predominantly express p16; 3) Abruptly induced growth arrest, confluence, and senescent pathways are associated with resistance to apoptosis; 4) The death-defying phenotype of KCs does not require early differentiation; 5) NF-κB is one regulator of resistance to apoptosis; and 6) HaCaT cells have undetectable p16 protein (hypermethylation of the promoter), dysfunctional NF-κB, and diminished capacity to respond to antiproliferative treatments, and they remain highly sensitive to apoptosis with cleavage of caspases 3 and 8. These data indicate that KCs (but not HaCaT cells) undergoing abruptly induced cell cycle arrest or senescence become resistant to apoptosis requiring properly regulated activation of NF-κB but not early differentiation.

Normal human skin is covered by a multi-layered epidermis in which keratinocytes (KCs) undergo a continuous process of proliferation, differentiation, senescence, and apoptosis. We postulated that there are multiple pathways leading to senescence and protection from apoptosis in other organ systems (4–9), and that KCs can also follow different biochemical pathways leading to cell cycle arrest. Furthermore, we postulated that these pathways will have distinctive characteristics as regards subsequent cell fate decisions such as differentiation, senescence, and susceptibility/resistance to apoptosis. The goal of this project was to begin to elucidate the phenotype and biochemical pathways regulating KC growth arrest, differentiation, and apoptosis. We postulated that there would be biochemical links between KC replication, senescence, and apoptosis and designed experiments to address how various types of KCs would respond to rapidly induced growth arrest or spontaneous replicative senescence, followed by exposure to high levels of ultraviolet radiation (UV light) that acutely triggers apoptosis.

Primary KC cultures proliferate for several passages in a low calcium, serum-free medium (11). Freshly isolated, proliferating, and relatively undifferentiated KCs can become growth-arrested and subsequently follow at least five different pathways. First, if growth supplements are removed, KCs maintained in basal medium will become quiescent and remain viable for at least several days, but retain the capacity to re-enter the cell cycle (12). Another distinct method for inducing reversible growth inhibition of KCs is exposure to anti-proliferative agents such as transforming growth factor β (TGF-β) (13). These initial two growth-arresting pathways can be reversed if quiescent cells are subsequently stimulated to re-enter the cell cycle and proliferate by addition of competence and progression factors following withdrawal of TGF-β (14). A third pathway involves growth arrest such that no further
proliferation is possible, and growth arrest does not induce early markers of differentiation (i.e., keratin 1), such as after exposure to phorbol ester and/or interferon γ (IFN-γ) (15, 16). A fourth pathway involves irreversible growth arrest and early differentiation, which occurs when extracellular calcium ion concentration is increased (17). A fifth pathway for KCs is to undergo replicative senescence, in which case they remain viable and metabolically active but not capable of any further replicative expansion (18).

The purpose of this investigation was to delineate the response of KCs to various stimuli that can influence all five potential pathways and to examine the interactive behavior of both cell cycle regulatory proteins that predominantly localize to the nucleus, with members of the caspase family that are present in the cytoplasm and regulate apoptosis (19). Because NF-κB is a key transcription regulator in KCs and plays a critically important role in both regulation of the cell cycle as well as influencing cell death pathways, particular focus was directed at this transcription factor (20–22). In addition to the aforementioned cell cycle arresting agents, UV light was also used because it can induce apoptosis in human skin and is regarded as an important etiological factor in development of skin cancer (23–25). The death-defying behavior of normal skin-derived KCs was compared with senescent KCs and immortalized HaCaT cells (26, 27).

Because the orderly process of KC proliferation, differentiation, and apoptosis occurs with a high degree of fidelity in skin, we postulated that the regulatory mechanisms involved in maintaining homeostasis would reveal a remarkable degree of coordination among the pathways that regulate the cell cycle (i.e., proliferation), and several key molecular participants involved in caspase cascades (i.e., cell death). Interestingly, just as KC differentiation is not required for cells to undergo apoptosis (19), we observed that early stages of differentiation were not required for growth-arrested or senescent KCs to acquire an apoptotic-resistant phenotype. Because the c-myc proto-oncogene is a central regulator of cell proliferation, differentiation, and apoptosis, expression of c-Myc protein levels were also examined (28, 29).

By comparing the behavior and response of normal KCs, senescent KCs, and immortalized KCs to antiproliferative agents followed by UV light, new insights were gained into the complex interactions of molecular mediators that regulate KC proliferation, growth arrest, differentiation, senescence, and apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary KCs were isolated from freshly excised neonatal foreskins as described previously (11) HaCaT cells, an immortalized KC cell line, were obtained from Professor N. Fusenig (Heidelberg, Germany) (20). Both normal KCs and HaCaT cells were maintained in a low calcium (0.15 mM) KC growth medium (KGM, Clonetics, San Diego, CA). Cells were treated with either KGM alone or KGM containing the manufacturer’s instruction. Also, 0.1–1 μM MG132 (0.1–1 mM BAP) (CLONTECH) was added to the cultures as indicated. KCs were pre-exposed for 2 h to the proteasome inhibitor MG132 (0.1–1 μM) and 1 μM BAP before the addition of trypsin/EDTA to detach the cells. The caspase inhibitor Z-VAD-FMK and the anti-apoptotic drug Bcl-x were added to the cultures as indicated.

Flow Cytometric Analysis—Flow cytometry was performed on single cell suspensions obtained using trypsin/EDTA as described previously (19). Briefly, for cell cycle analysis propidium iodide staining (50 μg/ml, 30 min) was performed following the manufacturer’s instruction. Cells were then washed with phosphate-buffered saline and were incubated in ice for ~80 °C. After washing, the cell cycle analysis was performed using an automated DNA sequencer as described previously (19).

UV Treatment and Suspension Culture Conditions to Induce Apoptosis—Apoptosis was induced by irradiating KCs with a Panelite Unit (Ultralite Enterprises, Inc., Lawrenceville, GA) equipped with four UV bulbs (FS6T12/UVB-VH0) that have the majority of their output in the UVB range (65%), with minor output in the UVA and UVC range (34 and 1%, respectively). KCs were irradiated with dose values 50 μmol/m2. The UV dose was monitored with an International Light Inc. (Newburyport, MA) radiometer fitted with a UVB detector. In selected experiments, cells were pretreated with caspase inhibitors for 30 min prior to irradiation as described previously (25). Another method for inducing apoptosis was to place a single cell suspension of KCs in KGM medium containing 1.68% methylcellulose (4000 centipoises, Sigma) for 48 h as described previously (19).

Western Blot Analysis—Nuclear cell lysate and whole cell lysate were prepared to detect different proteins. In brief, for nuclear lysates cells were washed with phosphate-buffered saline, pelleted in buffer A (20 mM HEPES, pH 7.8, 15 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol with 0.1% Nonidet P-40), incubated in ice for 15 min, and microcentrifuged, and the supernatant was discarded. The pellet was resuspended in buffer B (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 15 min on ice. Cells were vortexed and microcentrifuged, and the supernatant was saved and frozen at −80 °C. For the whole cell lysate, KCs were washed with phosphate-buffered saline and were incubated in ice for 15 min in CHAPS buffer (31). Cells were microcentrifuged, and supernatants were saved and frozen at −80 °C. Protein concentration of each sample was determined by Lowry assay.

30 μg of protein were run on 8–12.5% SDS-polyacrylamide gel, transferred to Immobilon-P (Millipore) membrane, and blocked in 5% nonfat powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20). The membrane was incubated with the primary antibody in 2.5% powdered milk in TBST and was washed extensively with TBST and then incubated with 1:1500 diluted anti-rabbit or mouse horseradish peroxidase (Amersham Pharmacia Biotech). Proteins were visualized with ECL reagents (Amersham Pharmacia Biotech) according to manufacturer’s instruction. Loading of proteins to verify equivalent distribution of proteins in each well was confirmed by Ponceau S staining.

RNase Protection Assay—Total cellular RNA was extracted using Trizol Reagent (Life Technologies, Inc.). The RNase protection assay was performed according to the supplier’s instructions (PharMingen, San Diego, CA). Briefly, human apoptosis template set hAP0–5 was labeled with α-32P]uridine triphosphate. RNA (10 μg) and 8 × 105 cpm of labeled probes were used for hybridization, and after RNase treatment, the protected probes were resolved on a 5% sequencing.

Electrophoretic Mobility Shift Assays and Supershift Assays—Electrophoretic mobility shift assays were performed as described previously (24). In brief, 1 μg of poly(dI-dC) (Amersham Pharmacia Biotech) and 200 ng of poly(dI-dC) (Amersham Pharmacia Biotech) and poly(dI-dC) was incubated with 5 μg of nuclear protein on ice for 30 min. The reaction mixture was separated on 4% native polyacrylamide gel, dried, and autoradiographed. The NF-κB oligonucleotide had the following sequence: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′. Competition analysis was performed by adding excess unlabeled oligonucleotides.

Analysis of p16 Gene in HaCaT Cells—Analysis of p16 gene in HaCaT cells was performed by nested PCR using specific primers for exons 1 and 2 using an automated DNA sequencer as described previously (32).

In addition, detection of p16 promoter methylation was performed by methylation-specific PCR. Amplification was performed using 1 μg of genomic DNA as template with the following sets of primers: forward, 5′-ACCTTACTGATACAAAGCGG-3′; reverse, 5′-TGGTTAGGAAGGAGGGTTC-3′. Slab-gel electrophoresis was performed using an automated DNA sequencer as described previously (31).
Southern blot analysis as described previously (33). Briefly, 10 μg of total genomic DNA was isolated from HaCaT cells, normal KCs, and a cell line known to have p16 DNA hypermethylation (SW13). DNA was subjected to restriction endonuclease digestion with EcoRI alone or EcoRI and SacII or EcoRI and EagI (Life Technologies, Inc.). After running on 1% agarose gel, DNA was transferred to nitrocellulose membrane. This membrane was analyzed with a genomic DNA fragment (1.1-kilobase probe) containing the promoter and exon 1 of human p16 gene as described previously (33).

RESULTS

Differential UV-induced Apoptotic Response of Normal versus Immortalized versus Senescent KCs—When neonatal foreskin-derived KCs are maintained at subconfluent density in a low calcium serum-free medium, they proliferate and are highly sensitive to induction of apoptosis by UV irradiation. A representative cell cycle profile for proliferating normal KCs is presented in Fig. 1A in which less than 1% of the cells have a sub-G0 DNA content and 5% are TUNEL-positive. Typically, proliferating KCs have 45–55% of cells in G1, 30–40% of cells in S, and 5–15% of cells in G2M. 18 h after UV irradiation (25 mJ/cm2), subconfluent KCs undergo apoptosis with over 55% of the KCs having sub-G0 DNA, and 39% becoming TUNEL-positive (Fig. 1B). However, if KCs become confluent and then exposed to UV (Fig. 1C), substantially less apoptosis (17% sub-G0 DNA, 22% TUNEL-positive) is induced. If confluent KCs cultures have their growth supplements removed (i.e. washed and maintained in KBM for 24 h), the resistance to apoptosis observed for the confluent cultures is reduced, and over 70% of KCs have sub-G0 DNA and greater than 40% of the cells are TUNEL-positive after UV exposure (Fig. 1D).

To further assess the consequences of growth-arresting treatments, normal KCs at 50–60% confluence were treated for 24 h with 2 mM Ca2+, TPA, IFN-γ, or TGF-β and then washed and maintained in KGM for an additional 24 h. In this scenario, there is irreversible growth arrest for all of the treatments except TGF-β. KC cultures exposed to elevated Ca2+, TPA, IFN-γ, or TGF-β had a reduction in proliferation assessed by manual cell counting of viable cells revealing substantial reductions compared with untreated cells in KGM of 79±8, 92±6, 93±6, and 83±6%, respectively. After the pulse/wash treatments, the dishes were then exposed to UV light (25 mJ/cm2), and the KCs were examined as before. KCs pulsed/washed with Ca2+ (Fig. 1E) were highly resistant to UV-induced apoptosis (10% sub-G0; 14% TUNEL-positive), as were KCs pulsed/washed after exposure to TPA (Fig. 1F) or IFN-γ (Fig. 1G). However, TGF-β-treated KCs were not as consistently protected as revealed by 45% sub-G0 DNA content and over 32% TUNEL-positive cells (Fig. 1H). Resistance to apoptosis for these treatments was not unique to UV light treatment, because similar results were observed when different KC cultures treated as above with identical pulse/wash protocols were trypsinized followed by 48 h of suspension in methylcellulose (data not shown). When the results using 24 h pulse/wash were compared with 48 h of continuous exposure similar levels of protection from UV-induced apoptosis were observed,
with the exception that continuous growth arrest produced by 48 h of treatment with TGF-β enhanced the resistance to apoptosis (29% sub G₀; 23% TUNEL-positive) compared with the pulse/wash protocol.

Interestingly, in cultures of normal KCs that underwent spontaneous replicative senescence (i.e. passages 3-5), the exposure to UV did not induce apoptosis in these subconfluent cultured cells (Fig. 1F). Pretreatment of senescent KCs with the aforementioned growth-arresting agents did not alter this resistance to UV-induced apoptosis (data not shown).

To determine whether a similar phenotypic response would occur in immortalized KCs, HaCaT cells were treated following the same protocol as described above for early passage normal human KCs. HaCaT cells were also growth-arrested by exposure to elevated Ca²⁺, TPA, or IFN-γ as determined by manual cell counting, although with an overall diminished antiproliferative response. Compared with untreated HaCaT cells, cultures (n=3) exposed for 72 h to elevated Ca²⁺, TPA, IFN-γ, or TGF-β had reductions in cell proliferation of 38 ± 5, 31 ± 85, 68 ± 8, and 54 ± 11%, respectively. In marked contrast to growth-arrested normal KCs, none of the treatments (i.e. either pulse/wash or continuous) reduced the extent of apoptosis present in HaCaT cells after UV exposure (Fig. 2). To better view the HaCaT cultures, only cell cycle DNA profiles are presented (although similar trends in TUNEL assays were also identified; data not shown). Briefly, subconfluent HaCaT cells had only approximately 3% of the cells undergoing spontaneous apoptosis (sub-G₀ DNA: Fig. 2A), whereas after UV exposure subconfluent (Fig. 2B) or confluent cultures (Fig. 2C) or cells placed in KBM (Fig. 2D) had markedly increased numbers of cells with sub-G₀ DNA (48-58%). Pulse/wash treatments using Ca²⁺, TPA, IFN-γ, or TGF-β prior to UV exposure provided no protective effects (Fig. 2, E–H, respectively). Also, 48 h of continuous exposure to these antiproliferative treatments did not change the ability of UV light to induce apoptosis in the HaCaT cells (data not shown).

Characterization of Caspase Cascade Involved in UV-induced Apoptosis—To determine the molecular mediators involved in the UV-induced apoptosis, Western blot analysis was performed on whole cell extracts before and after UV exposure with a focus on the caspase 8, caspase 3, and poly(ADP)ribose polymerase. UV-induced apoptosis in KCs is caspase-dependent, because it can be blocked by caspase inhibitors (25). Because caspase 3 is a primary executioner intermediate in this apoptotic pathway, the change in caspase 3 will be highlighted (Fig. 3), although similar changes were also observed for caspase 8 and poly(ADP)ribose polymerase (data not shown). Prior to UV irradiation, proliferating normal KCs and HaCaT cells had intact caspase 3, but after UV exposure both types of cells undergoing apoptosis had proteolysis (i.e. activation) of caspase 3.

Normal KCs in either a confluent state or after pretreatment/wash with Ca²⁺, TPA, IFN-γ, or TGF-β had less evidence of caspase 3 cleavage upon subsequent UV exposure, which correlated with diminished induction of apoptosis. HaCaT cells treated in a similar fashion had no or barely detectable intact caspase 3 levels, consistent with the induction of apoptosis under these conditions induced by UV.

To determine whether HaCaT cells were capable of acquiring an apoptotic resistant phenotype, several different caspase inhibitors such as z-VAD, DEVD, and IETD, were utilized (25). Pretreatment of HaCaT cells with these compounds prior to UV exposure decreased the apoptotic response (data not shown), with concomitant prevention of the cleavage of caspase 3 (Fig. 3). Overall, the degree of protection from UV-B-induced apoptosis and extent of caspase 3 degradation using these inhibitors was similar between KCs and HaCaT cells (data not shown).

Early Differentiation Marker Expression by Normal and Immortalized KCs—Two differentiation-related proteins were measured before and after the pulse/wash or continuous treatments including keratin-1 (present in suprabasilar KCs undergoing early differentiation) and keratin-14 (detectable in basal layer KCs in vivo and relatively undifferentiated cells in culture). Over 90% of cultured normal KCs were keratin 14-positive (expect for small foci of stratified clusters of KCs), and HaCaT cells were diffusely and uniformly expressed keratin-14 (Fig. 4, upper panel). In contrast, only rare focal clusters of keratin-1-positive normal KCs maintained in KGM were present in proliferating KCs (Fig. 4, middle panels). After exposure to elevated calcium, approximately 20% of KCs became kera-
antiproliferative agents. Only the inhibitors (DEVD, ZVAD, and IETD) blocked caspase 3 cleavage in HaCaT cells.

UV-induced caspase 3 cleavage. In contrast to KCs, HaCaT cells had all caspase 3 cleaved after UV exposure; even with pretreatment with inhibitors. Therefore, apoptosis was characterized by cleavage of caspase 3. KCs prior to UV exposure have detectable caspase 3.

KCs undergoing spontaneous replicative senescence were characterized by marked elevation in p16 with a slight increase in p27 but without changes in p15 or p21 levels. By contrast to normal KCs, when proliferating HaCaT cells were examined, only scant levels of intranuclear p21 was detected, and no p16 protein was identifiable, whereas low levels of p15 and p27 were present. Confluence did not produce complete growth arrest, and only a slight increase in p27 levels was detected. Addition of antiproliferative agents including elevated Ca²⁺, TPA, IFN-γ, or TGF-β did not produce enhanced nuclear levels of p21 or detection of p16. Some of these antiproliferative treatments did induce higher levels of either p15 or p27.

Given the complete absence of p16 in the HaCaT cells, the gene was examined for mutation, but no mutations in either exon 1 or 2 were identified. Next, the methylation status of the DNA was investigated, and there was DNA hypermethylation in the promoter sequence and first exon of p16. Some of these antiproliferative treatments did induce higher levels of either p15 or p27.

Constitutive and Inducible Levels of NF-κB in Normal KCs and HaCaT Cells—Because transcriptional activation by NF-κB can induce several cell survival genes (21, 22), the levels of the two subunits (i.e. p50 and p65), as well as DNA binding capacity were examined in this system. By Western analysis, proliferating KCs had relatively low but consistently detectable intranuclear p50 and p65 components of NF-κB. After exposure to TPA or IFN-γ, there was rapid induction (within 30–60 min) and enhancement of both transcriptional activation. This suggested that NF-κB might be involved in the resistance of proliferating KCs to UV-induced apoptosis.

KCs either before or after exposure to the treatments failed to express any keratin-1 except for rare isoforms. HaCaT cells either before or after exposure to the treatments failed to express any keratin-1 except for rare isoforms.
intranuclear levels. The functional activity of the nuclear p50/p65 was confirmed by DNA binding assays (Fig. 7, lower panel, left side) and supershift gel analysis in which p50 and p65 subunits were identified but not RelB or c-Rel (data not shown). However, addition of 2 mM calcium did not produce detectable increase in either p50 or p65 by Western blot or gel shift assays (data not shown).

In contrast to these results by Western blot analysis, HaCaT cells had relatively high constitutive intranuclear levels of p50 and p65 (Fig. 7, upper panel, right side), which was also confirmed by functional DNA binding (Fig. 7, lower panel, right side) and supershift gel assays (data not shown). Another difference noted was that after exposure to either TPA or IFN-γ, unlike normal KCs in which this treatment triggered nuclear translocation of p50/p65, no change in the relative levels of either of these subunits was observed in HaCaT cells by Western blot analysis (Fig. 7, upper panel, right side). The lack of further enhancement for p50 and p65 levels in stimulated HaCaT cells was confirmed by DNA binding (Fig. 7, lower panel, right side) and supershift gel assays (data not shown).

Because there were significant differences in the constitutive and inducible levels of NF-κB in normal KCs and HaCaT cells, we sought to determine whether there would be differences in the expression of anti-apoptotic transcripts triggered via NF-κB activation (34–36). Because HaCaT cells did not resist apoptosis and did not activate NF-κB, our hypothesis was that HaCaT cells would differ from normal KCs by not up-regulating apoptotic-resistant transcripts, as one mechanism to explain the response to UV light. To explore the transcriptional patterns of normal KCs and HaCaT cells with respect to cell survival pathways, RNase protection assays were performed. Fig. 8 reveals a panel of mRNA transcripts for numerous apoptosis-regulating genes. In proliferating normal KCs, several anti-apoptotic transcripts were present including XIAP, TRAF-2, and cIAP-1 mRNAs (34–36). However, after normal KCs are exposed to elevated calcium ion levels, TPA, IFN-γ, or TGF-β, several of these transcripts become more abundant including TRAF-1 and cIAP-2 mRNAs. By scanning laser densitometry, normal KCs had a 18-fold induction of TRAF-1 mRNA by TPA, a 1.8-fold increase by Ca²⁺, a 1.7-fold increase by IFN-γ, and a 2.0-fold increase by TGF-β. There was a 3.1-fold increase in cIAP-2 mRNA by Ca²⁺, as well as a 23.7-fold increase by TPA, a 2.6-fold increase by IFN-γ, and a 1.8-fold increase by TGF-β. The constitutive presence of L32 and glyceraldehyde-3-phosphate dehydrogenase served as loading controls. The other abbreviations represent human testes- one-repressed prostate message-2 (TRPM2), CRAF for TRAF3, and CART for TRAF4 as described previously (36).

The pattern of transcripts was very different in HaCaT cells. Consistent with the constitutive levels of intranuclear NF-κB, many of the transcripts were also constitutively present such as XIAP, TRAF-2, cIAP-1, and cIAP-2 mRNAs. However, even after exposure to elevated calcium ion concentrations or TPA,
UV-induced apoptosis was not due to any decrease or absence of Bcl-xL in both types of cells, but the susceptibility of HaCaT cells to UVB-induced apoptosis correlated with the lack of induction of NF-κB, the highly sensitive phenotype of both proliferating and growth-arrested HaCaT cells to UVB-induced apoptosis.

Expression of Cell Survival-related Proteins and c-Myc in Normal and Immortalized KCs—To explore expression beyond the transcriptional level for various cell survival genes, Western blot analysis was also performed. For normal KCs the relative levels of TRAF-1 and TRAF-2 at the protein level were similar to the mRNA levels for each of the different conditions portrayed in Fig. 8 (data not shown). Similarly, for HaCaT cells, there was no TRAF-1 detected at the protein level, and TRAF-2 levels were present constitutively with no significant change following exposure to the antiproliferative agents (data not shown). Because we had previously observed that in KCs derived from psoriatic plaques there was overexpression of Bcl-xL, correlated with enhanced survival (37, 38), the relative levels of Bcl-xL were examined. Western blot analysis of Bcl-xL in normal KCs and HaCaT cells revealed detectable Bcl-xL in both types of cells, but the susceptibility of HaCaT cells to UV-induced apoptosis was not due to any decrease or absence of Bcl-xL relative to the proliferating cells (Fig. 9). Furthermore, the enhanced survival of normal KCs that had become confluent or exposed to Ca2+ ion, TPA, IFN-γ, or TGF-β could not be correlated to the relative levels of Bcl-xL (Fig. 10).

Barely detectable levels of c-Myc are present in proliferating KCs, that are slightly increased after exposure to IFN-γ (Fig. 9). By contrast, HaCaT cells have constitutively high levels of c-Myc, which were only minimally changed in response to IFN-γ (Fig. 9).

Influence of Pharmacological Inhibition of NF-κB Activation on UV-induced Apoptosis—To determine whether the death-defying phenotype of IFN-γ treated KCs was related to NF-κB activation, KCs were pretreated with the proteasome inhibitor MG132, and the subsequent phenotype of the KCs was examined before and after IFN-γ treatment followed by UV irradiation. Fig. 10A reveals that at 1 μM (but not 0.1 μM), MG132 preincubated with KCs for 2 h blocked induction of NF-κB activation by IFN-γ. IFN-γ itself, MG132 at either 0.1 or 1 μM, and the combination of IFN-γ plus 1 μM MG132 did not induce significant apoptosis (<5%) over a 48-h incubation period (Fig. 10, B and C). Next, MG132 was added 2 h before IFN-γ (10³ units/ml) and after 24 h the KCs were irradiated with UV light. By blocking IFN-γ-induced NF-κB activation with 1 μM MG132, the anti-apoptotic phenotype of the KCs was reduced. Thus, although IFN-γ pretreatment could reduce the extent of apoptosis (sub-G₀ DNA) by 24 ± 8%, preincubation with MG132 (1 μM) prior to IFN-γ, led to an increase in the extent of apoptosis in these treated KCs to 52 ± 8% when subsequently analyzed 24 h hours after exposure to UV light.

DISCUSSION

Given the constant thickness of the epidermis throughout life, we postulated that there would be a tight link between the regulation of cell proliferation (i.e. cell cycle programs) and cell death (i.e. apoptosis). The results of these studies confirm our hypothesis for KCs by providing evidence that regulation of proliferation is critical for determination of susceptibility to apoptosis. We demonstrate, despite multiple experimental conditions that impact the cell cycle such as spontaneous replicative senescence, confluence, or exposure to several different antiproliferative agents, that all of these growth-arresting pathways lead to apoptosis resistance in normal KCs. By contrast, the immortalized KCs (i.e. HaCaT cells) that have abnormally antiproliferative responses remain susceptible to apoptosis. Although detailed molecular exploration for each of these responses is beyond the scope of this study, initial focus on the role for NF-κB in regulating KC apoptosis was pursued.

Two sets of results point to an important role for NF-κB activation in regulating KC apoptosis. First, although normal KCs responded to several antiproliferative agents such as IFN-γ or TPA by activating NF-κB (and several key anti-apoptotic transcripts), no such induction was observed in HaCaT cells, which were unable to acquire a resistance to apoptosis compared with normal KCs. Secondly, when this activation of NF-κB was blocked by MG132, the resistance to apoptosis by normal KCs was reduced. As discussed later, it is clear that there might be other pathways beyond that regulated by NF-κB, and we are currently investigating several other mechanistic leads to more fully explain the basis for resistance to apoptosis induced by other conditions.

There are several other notable observations in this report that link regulators of cell cycle with resistance to apoptosis. When normal human foreskin-derived KCs are proliferating, they express low nuclear levels of p15, p16, p21, and p27 as well as p50 and p65 subunits of NF-κB, and these replicating cells are highly susceptible to apoptosis, with rapid activation of the caspase cascade involving caspases 8 and 9 after UV light exposure. When neonatal KCs, which have tremendous replicative potential in vivo, are growth-arrested in vitro by exposure to anti-proliferative agents such as elevated calcium ion levels, IFN-γ, TPA or TGF-β or by allowing the cells to become confluent or following spontaneous replicative senescence, the normal KCs acquire a resistance to apoptosis. In contrast, immortalized HaCaT cells responded quite differently to the antiproliferative agents with less complete growth arrest and at the same time remained highly susceptible to UV-induced
apoptosis. Besides the well documented inactivation of both p53 alleles in HaCaTs (39), this apoptosis-related investigation uncovered several other previously unknown abnormalities in HaCaT cells, such as DNA hypermethylation involving the p16 promoter region of the gene resulting in no detectable protein, constitutively elevated levels of p50/p65 subunits of NF-κB, a failure to undergo further elevation of NF-κB levels, lack of induction of several transcripts associated with cell survival proteins, constitutively elevated c-Myc, and no resistance to apoptosis under the conditions examined.

There were also several differences observed in the pattern of cell cycle regulatory proteins present in the nucleus of normal KCs cultured under various conditions. Confluency-induced growth arrest produced the most striking enhancement of all four CDKIs measured with accompanying resistance to apoptosis. This result may be of particular relevance to in vivo conditions because KCs in skin are tightly aggregated with close cell-cell contact as simulated by the confluence experiments. The elevation in extracellular calcium ion concentration, while inducing growth arrest, only partially induced early markers of differentiation (i.e. keratin-1) in a relatively small percentage of KCs, compared with a marked increase in p21. Recently, p21 was found to inhibit differentiation of murine KCs (40). A previous investigation noted that TPA or elevated calcium ion levels could up-regulate p21 in KCs (41), but the current work indicates that induction of p21 can also be triggered by several other growth-arresting agents including IFN-γ as well as TGF-β. Moreover, because the other antiproliferative treatments also strongly induced p21, it is possible that the failure of TPA, IFN-γ, or TGF-β to enhance keratin-1 expression was related in part to the induction of p21. Our results are in agreement with an earlier report that up-regulation of these CDKIs was not sufficient by itself to induce differentiation of KCs (42). The reversible growth arrest induced by TGF-β led to enhanced levels of p15 (43), whereas spontaneous replicative senescence was associated most prominently with an increase in p16 (18). These differences in induction of specific CDKIs in response to various antiproliferative agents point to multiple pathways by which normal KCs undergo growth arrest.

The immortalized HaCaT cells had a substantially different profile of CDKIs compared with the normal KCs. HaCaT cells had barely detectable nuclear levels of p15 or p21, and there was complete absence of p16. The only consistently detectable CDKI that was constitutively expressed was p27, which was further enhanced after exposure to the antiproliferative agents: TPA and TGF-β (44). Given the relatively high constitutive levels of c-Myc in HaCaT cells, we postulate that more significant growth arrest was not induced by these antiproliferative agents in HaCaT cells, relative to normal KCs with lower levels of c-Myc, because of the previous reported ability of c-Myc to abrogate growth arrest mediated by p27 (28, 29). Given the complex role c-Myc plays in regulating apoptosis (45–47), more work is required to determine the significance of c-Myc overexpression in KCs.

In this report, the first evidence documenting the silencing of the p16 gene in HaCaT cells is presented. Although there is evidence for p16 alterations in several skin-derived cancers and cell lines (48, 49), the HaCaT cells have not been previously shown to harbor such an alteration in which the DNA encoding sequence within exons 1 and 2 lacks a mutation, but the p16 promoter region is hypermethylated.

The most dramatic phenotypic difference between normal KCs and HaCaT cells was revealed by their respective responses to UV light-mediated induction of apoptosis. Previous reports using fibroblasts and myocytes documented that CDKIs play a role in protecting cells from apoptosis (4–9, 50), and our results using KCs also clearly demonstrate a correlation between growth arrest, induction of CDKIs, and resistance to apoptosis. Indirect evidence to support a link between CDKIs and apoptosis was revealed using the HaCaT cells in that these cells fail to induce either p16 or p21 and remain highly susceptible to apoptotic stimuli. To explore the molecular mechanism of this anti-apoptotic phenotype, the expression of NF-κB and several cell survival genes and proteins were studied. In many cell types including KCs, NF-κB transcriptional activity mediates enhancement of cell survival gene products (20, 21). Cell survival genes of interest in this study included Bel-xL because transgenic mice overexpressing Bel-xL yield epidermal KCs with resistance to apoptosis (51), as well as IAP genes (34, 35), and TRAF1/TRAF2 (36). As regards NF-κB, dramatic differences were noted between normal KCs and HaCaT cells. Using several different assays, it was clearly established that HaCaT cells have a dysregulated NF-κB pathway in which there is extremely high constitutive intranuclear levels of p50 and p65. Although such abnormalities have been seen in other cell lines, this is the first report documenting such a dysregulated state in HaCaT cells (52, 53). RNase protection assays confirmed the electrophoretic mobility shift assay results, in that HaCaT cells did not respond to either TPA or IFN-γ, as did normal KCs, by enhanced NF-κB nuclear translocation and up-regulation of several cell survival mRNA transcripts and proteins. We pos-
tulate that proliferating normal KCs were susceptible to apoptosis in part because they did not produce either TRAF-1 or c-IAP-1. It was recently demonstrated that expression of TRAF-1 with c-IAP-1 substantially increased the anti-apoptotic response in a different human cell system (36). The precise mechanism by which the regulatory events described herein mediate the resistance to apoptosis remains largely unknown. Because elevated Ca\(^{2+}\) ion levels do not trigger NF-\(\kappa\)B activation, it is possible that the Ca\(^{2+}\) mediated anti-apoptotic mechanism is significantly different from the mechanism involving exposure to either IFN-\(\gamma\) or TPA, which do activate NF-\(\kappa\)B. Furthermore, the exact contribution of the various NF-\(\kappa\)B-inducible gene products such as XIAP, c-IAP-1, c-IAP-2, TRAF-1, and TRAF-2 are currently under investigation.

However, a role for NF-\(\kappa\)B in the anti-apoptotic phenotype of IFN-\(\gamma\) treated KCs was demonstrated using a pharmacological approach with the proteasome inhibitor MG132. By inhibiting the activation of NF-\(\kappa\)B, IFN-\(\gamma\) treated KCs become more susceptible to UV-induced apoptosis in agreement with an earlier report focusing on IL-1 and KC survival (30). Possible mechanisms involving cell cycle regulators such as p21 and the caspase cascade as seen in other cell types (54, 55) were found not to be operative in this system involving KCs. There was also no modulation of heat shock protein 27 to suggest a role for this protein in the anti-apoptotic phenotype of KCs (56).

It is conceivable that the exposure of neonatal KCs to these growth-arresting agents converts these cells to a phenotypic state that resembles premature senescence (57). We postulate that on one hand HaCaT cells have become immortalized by several genetic mutations that disrupt cell cycle regulation and NF-\(\kappa\)B activation in a way that also prevents them from undergoing replicative senescence. This resistance to induction of senescence by the HaCaT cells is accompanied by an inability of these cells to acquire an anti-apoptotic phenotype. The resistance to apoptosis in this system could not be attributed solely to levels of Bcl-X\(_{L}\), because HaCaT cells contained similar levels of Bcl-X\(_{L}\) compared with normal KCs but remained highly sensitive to UV-induced apoptosis.

In conclusion, these results highlight potential cross-talk between pathways that regulate cell proliferation and cell survival (58–62). Given the qualitative and quantitative differences in CDKI profiles induced by various antiproliferative treatments, it appears there are multiple pathways leading to G\(_1\) arrest in KCs. Clearly susceptibility to apoptosis is also linked to the cell cycle activity of KCs, and the anti-apoptotic mechanism in KCs requires properly regulated NF-\(\kappa\)B activity. Once a KC becomes growth-arrested, it may or may not begin to express early differentiation markers, but resistance to apoptosis does not require induction of differentiation. Future studies are required to better understand the molecular complexities associated with regulation of KC proliferation, differentiation, senescence, and apoptosis.

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23367