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TNF Receptor p55 Plays a Pivotal Role in Murine Keratinocyte Apoptosis Induced by Ultraviolet B Irradiation

Lihua Zhuang,* Binghe Wang,* Gayle A. Shinder,* Gulnar M. Shivji,* Tak W. Mak,† and Daniel N. Sauder2,*

Excess exposure of skin to ultraviolet B (UVB) results in the appearance of so-called sunburn cells. Although it has been demonstrated that sunburn cells represent apoptotic keratinocytes, the molecular mechanisms for UVB-induced apoptosis in keratinocytes have not been fully elucidated. The cytokine, TNF-α, has been shown to induce apoptosis in a variety of cell types. Since UVB induces keratinocytes to release TNF-α, we hypothesized that TNF-α is involved in UVB-induced apoptosis in keratinocytes. In order to confirm this hypothesis and to further delineate which type of TNF receptor signaling mediates the apoptosis pathway, we performed both in vivo and in vitro experiments using gene-targeted knockout mice lacking either the TNF p55 receptor or the TNF p75 receptor. In the in vivo study, wild-type and mutant mice were exposed to UVB, and apoptotic keratinocytes were detected by examining DNA fragmentation using in situ nick-end labeling. For the in vitro experiments, keratinocytes derived from the wild-type and mutant mice were irradiated with UVB, and the degree of apoptosis was determined by flow cytometry, nick-end labeling of DNA, and a DNA ladder assay. Both in vivo and in vitro studies demonstrated that the deletion of TNF receptor p55 could suppress UVB-induced apoptosis in keratinocytes. Our observations support the notion that TNF-α is involved in UVB-induced keratinocyte apoptosis, and demonstrate that p55 receptor signaling plays a pivotal role in this event. The Journal of Immunology, 1999, 162: 1440–1447.

A poptosis, or programmed cell death, has been recognized as an important control mechanism in both the maintenance of tissue homeostasis and the elimination of cells with damaged DNA (1). Morphological effects of apoptosis include changes in the plasma membrane such as loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation, chromosomal DNA fragmentation, disruption of the nucleus, cytoplasmic blebbing (zeiosis), and the formation of membrane-bound apoptotic bodies containing intact cytoplasmic organelles and nuclear fragments (1). Apoptosis is essential in most physiological processes including maturation of the immune system; embryonic development of tissues, organs and limbs; development of the nervous system; and hormone-dependent tissue remodeling (2). Apoptosis is also part of the normal process of epithelial renewal in human epidermis, and is an important mechanism for controlling the size of its major cell population, the keratinocytes (3). Dysregulation of apoptosis is observed in various disease states including skin cancer (4).

Environmental stimuli such as UV radiation, may trigger apoptosis (5). Excess exposure of the skin of humans and laboratory animals to UV light causes sunburn, photoaging, actinic keratosis, and skin cancers (6). Ultraviolet B (UVB)3 (290–320 nm) accounts for most of the documented harmful biologic effects of sunlight. Epidermal cells are considered to be a major target of UVB radiation since the vast majority of UVB is absorbed within the epidermis, causing molecular damage to nucleic acids (7). It is now clear that sunburn cells caused by exposure of the skin to UVB, are keratinocytes that have undergone apoptosis (5, 8). However, the molecular mechanisms inducing keratinocyte apoptosis have not yet been fully elucidated. Since TNF-α can be induced by UVB (9) and TNF-α has been reported to induce apoptosis in various cell types (10, 11), this cytokine has been thought to be involved in UVB-induced keratinocyte apoptosis (8).

TNF-α is a multifunctional cytokine produced by a variety of cell types, including macrophages, T cells, mast cells, and keratinocytes (12). Two distinct membrane receptors for TNF-α have been identified (13). The TNF-α receptor, with a molecular mass of 55 kDa, is referred to as the p55 receptor (Rp55 or RII). The p75 receptor (Rp75 or RII) has a molecular mass of 75 kDa. The two receptors are encoded by distinct genes; however, they are ~30% homologous in their extracellular, cysteine-rich, and ligand-binding regions. The genes for the p55 receptor and p75 receptor map to human chromosomes 12 and 1, respectively, and in the mouse, to conserved syntenic regions on chromosomes 6 and 4, respectively (14, 15). TNF-Rp55 is ubiquitously expressed, whereas TNF-Rp75 is found predominantly on hematopoietic and endothelial cells (16). TNF-Rp55 and TNF-Rp75 mediate distinct biological activities. TNF-Rp55 signaling is thought to be involved in mediating cytotoxicity, antiviral activity, fibroblast proliferation, and induction of superoxide dismutase, while TNF-Rp75 signaling is

3 Abbreviations used in this paper: UVB, ultraviolet B; KO, gene knockout; hpf, high power fields; TNF-Rp55, TNF receptor p55; KO, knockout; TNF-Rp75, TNF receptor p75; WT, wild type; TUNEL, terminal deoxynucleotidyltransferase-mediated deoxyuridine 5′-triphosphate nick-end labeling; PI, propidium iodide; TdT, terminal deoxynucleotidyltransferase; TRADD, TNF-Rp55-associated death domain protein; FADD, Fas-associated protein with death domain.
involved in the proliferation of thymocytes and cytotoxic T cells (17). Although it is known that TNF-α induces apoptosis in a variety of cell types, the role of the two receptors in triggering cell death is disputed. Some studies suggest that receptor p55 is the main mediator of TNF-α-induced apoptosis (18), whereas others contend that receptor p75 may be equally effective (11, 19).

In this study, we have used gene-targeted knockout (KO) mice deficient in either TNF-Rp55 (20) or TNF-Rp75 (21) in order to confirm the role of TNF-α in UVB-induced keratinocyte apoptosis, and to identify which TNF receptor mediates this apoptotic pathway. The results of this study demonstrated that TNF p55 receptor signaling plays a significant role in UVB-induced keratinocyte apoptosis.

Materials and Methods

Animals

The generation of TNF-Rp55 KO mice has been described previously (20) and this mutation was interbred 10 times into the C57BL/6 background. TNF-Rp75 KO mice on a C57BL/6 background were initially constructed by Dr. Moore (Genetech, San Francisco, CA) (21). These mutant mice were maintained under a specific pathogen-free environment at the animal facility of Sunnybrook Health Science Centre, University of Toronto (Toronto, Canada). C57BL/6 mice were obtained from the Charles River Breeding Laboratories (Quebec, Canada) and used as a wild-type (WT) control. All mice were used at 8–12 wk of age. For keratinocyte cultures, newborn mice were used. Each experimental group consisted of five mice. All procedures were approved by the Animal Care Committee of Sunnybrook Health Science Centre.

In vivo UVB irradiation of mice

WT, TNF-Rp55, and TNF-Rp75 KO mice were shaved on their abdomens and exposed to UVB irradiation as described previously (22). Briefly, UVB irradiation was delivered with polychromatic light from a four-tube fluorescent sun lamp (FS20T12-UVB, National Biological, Twinsburg, OH). These lamps emit wavelengths mainly between 280 and 320 nm, peaking at 313 nm. The irradiation intensity was 0.45 mW/cm² at a target distance of 15 cm, as measured by an IL-1400 A radiometer equipped with a SEL240/UVB 1/1 DT UVB detector with spectral sensitivity in the range of 280–320 nm (International Light, Montreal, Quebec, Canada). Following anesthesia by i.p. injection of pentobarbital (50 mg/kg), mice were given a single exposure of 200 mJ/cm² on their abdomens. Negative control mice were treated in an identical way, but the UVB lamp was not switched on.

Nick-end labeling of DNA from apoptotic mouse skin cells

Twenty-four hours following UVB irradiation, skin biopsies were taken from the irradiated and control areas, fixed in buffered formaldehyde, and then embedded in paraffin. Five-micron paraffin sections were placed on slides pretreated with 0.01% aqueous solution of poly-L-lysine (300,000 m.w.; Sigma, St. Louis, MO). Slides were deparaffinized by heating overnight at 37°C and then rehydrated. The sections were analyzed by the terminal deoxynucleotidyltransferase (TdT)-mediated deoxyuridine 5' triphosphate (dTUTP) nick-end labeling (TUNEL) technique using an in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) (23). The TUNEL reaction preferentially labels DNA strand breaks generating a fluorescent signal (24). Labeling was performed according to the manufacturer’s instructions with minor modifications. The sections were preincubated with 20% normal bovine serum for 30 min at room temperature, and then 3'-OH termini of internucleosomal DNA strand breaks were labeled with fluorescein-dUTP, TdT, and alkaline phosphatase-labeled anti-fluorescein Ab. After the fast red (Boehringer Mannheim) color reaction, counterstaining was performed with hematoxylin stain (Fisher Scientific, Fair Lawn, NJ). Sections were visualized by light microscopy. TUNEL results were quantified by counting 10 high power fields (hpf) of epidermis (magnification, X400) for each stained section. The number of apoptotic cells in the epidermis was expressed as a mean of 10 hpf.

Keratinocyte culture and in vitro UVB irradiation

Primary keratinocyte cultures were prepared from newborn WT and TNF receptor KO mice as described previously, but with some modifications (25). The skin samples were treated with 1% dispase II (Boehringer Mannheim) solution overnight at 4°C. Epidermal sheets were separated from the dermis and stirred in a trypsin-EDTA solution (0.05% trypsin and 0.53 mM EDTA) for 20 min at room temperature. The cell suspension was filtered through nylon mesh and centrifuged at 300 × g for 10 min. Cell pellets were resuspended in Eagle’s MEM with 10% heat-inactivated FBS (Life Technologies, Grand Island, NY) and plated at 2 × 10⁶ cells/10-cm dish. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The medium was renewed and replaced with fresh medium every 2 days. For all experiments, trypsin-passage cells were used at subconfluence.

For UVB irradiation, the medium was removed and keratinocytes were washed three times with prewarmed PBS and irradiated with UVB (0–20 mJ/cm²) in the presence of 0.5 ml PBS. Immediately after UVB treatment, the PBS was removed, replaced with medium, and the cells were cultured in Eagle’s MEM supplemented with 10% FBS for a further 24 h. The cells and supernatants were collected. Control cells were subjected to the identical procedure but were only sham irradiated.

Immunolabeling of TNF-Rp55 and TNF-Rp75

WT keratinocytes were labeled with anti-TNF-Rp55 or TNF-Rp75 Ab by a three-step immunostaining procedure (26). Briefly, 10⁶ cells were incubated with rat anti-mouse TNF-Rp55 (100 μg/ml; Cedarlane, Hornby, Ontario, Canada), rat anti-mouse TNF-Rp75 (100 μg/ml; Cedarlane), or the equivalent amount of a rat IgG2a isotype control (Cedarlane) for 30 min at 4°C, washed, and then incubated with biotin-conjugated goat anti-rat IgG2a (Cedarlane) for 30 min on ice. After washing, cells were reacted with Streptavidin-phycocerythrin (Cedarlane) for 30 min on ice. Cells were then washed and analyzed using flow cytometry (FACScan, Becton Dickenson, San Jose, CA).

Apoptotic analysis by flow cytometry

Flow cytometric analysis was performed for quantification of cell death by apoptosis according to the method described previously (27). Due to DNA degradation and subsequent leakage from cells, apoptotic cells can be detected via their diminished staining with DNA-specific fluorochromes such as propidium iodide (PI) (Sigma). Keratinocytes (2 × 10⁶ cells) from WT, TNF-Rp55 KO, and TNF-Rp75 KO mice were harvested and then fixed using 70% ethanol at 4°C for 1 h. The fixed cells were washed with PBS and incubated with 1 ml of hypotonic fluorochrome solution (50 μg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100), and RNase-A (50 μg/ml) (Boehringer Mannheim) for 15 min at room temperature in the dark, and then kept on ice. DNA fluorescence was analyzed by quantitative flow cytometry using CellQuest Software. The percentage of apoptotic cells was identified by analyzing hypoploid areas.

Nick-end labeling of DNA from apoptotic keratinocytes

Keratinocytes derived from WT, TNF-Rp55 KO, and TNF-Rp75 KO mice were exposed to UVB at a dose of 20 mJ/cm², and 24 h later nick-end labeling of DNA in apoptotic cells was performed by using the APO-DIRECT kit (PharMingen, Mississauga, Ontario, Canada). A total of 2 × 10⁶ cells were fixed with 1% paraformaldehyde solution, resuspended in 70% cold ethanol, and stored at −20°C. After rehydrating in PBS, the fixed cells were incubated in 50 μl of solution containing FITC-labeled dUTP and TdT for 1 h at 37°C, and then resuspended in PI/RNase solution for 30 min at room temperature in the dark. Following incubation in staining solution, the cells were rinsed in PBS. Individual cells were analyzed by fluorescence microscopy using conjugated FITC (green) and PI (red) markers. The two negative control cells were treated in an identical way, however, one control was sham irradiated and the other control was incubated without FITC. FITC-labeled cells (apoptotic cells) were counted from about 100 randomly selected cells and expressed as a percentage of the total cells.

DNA fragmentation analysis

Detection of DNA fragmentation was performed after extraction of DNA, as previously reported, with some modifications (28). Untreated or irradiated keratinocytes (2 × 10⁶) were collected, washed once with PBS, resuspended in 1 ml of lysis solution (10 mM Tris, pH 7.5, 1 nM EDTA, 0.2% Triton X-100) containing 5% SDS and proteinase K (0.5 mg/ml) (Sigma), and incubated overnight at 37°C. After centrifugation at 1000 × g for 10 min, the supernatants were extracted with phenol-chloroform. The genomic DNA was precipitated with ice-cold 100% ethanol. After centrifuging for 20 min at 1000 × g, the DNA pellet was resuspended in 15 μl of TE (10 mM Tris, pH 8.0, 1.0 mM EDTA), and then treated with 100 μg/ml RNase-A for 1 h at 37°C. Loading buffer (2 μl) was added to each sample. Samples were then electrophoresed on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and visualized under UV light. The 100-bp ladder, which was used as a marker, was purchased from Pharmacia Biotech (Piscataway, NJ).
Reverse transcription and PCR
Total RNA was extracted from either mouse skin that had been exposed to UVB or cultured keratinocytes, by the acid guanidinium thiocyanate-phenol-chloroform method and RT-PCR was performed as previously described (13, 29). Primer sets and positive cDNA template controls for mouse TNF-α, and β-actin were obtained from Clontech Laboratories (Palo Alto, CA). RNA from both TNF-Rp55 and TNF-Rp75 expressing BW5147.3 cells (mouse T cell lymphoma-derived cell line) was used as positive control. The sequences for each primer were as follows: TNF-Rp55, 5′-GGGA TAC ATG CAG GGA GTG-3′ and 5′-TCC ACC GGG GAT ATC GGC ACA T-3′; TNF-Rp75, 5′-GAC AGG AAG GCT CAG ATG TGC T-3′ and 5′-GCA TTT CCG GGA GAA AGC ATG-3′; TNF-α, 5′-ATG AGC ACA GAA AGC ATG ATG ATC CGC-3′ and 5′-CCA AAC TAG ACC TGC CCG GAC TC-3′; and β-actin, 5′-GTG GGC CGC TCT AGG CAC CAA-3′ and 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′. Specific cDNA obtained from reverse transcription was amplified in a total volume of 10 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 200 μM each of all four dNTPs, 10 μM each of all primers, and 0.5 U of Taq DNA polymerase (Pharmacia). The mixture was overlaid with 15 μl of mineral oil and PCR cycles were performed in a Perkin-Elmer Cetus Thermal Cycler 480 (Perkin-Elmer/Cetus, Norwalk, CT) with denaturation at 94°C for 15 s, annealing at 55–60°C for 45 s, and extension at 72°C for 30 s. PCR signals for β-actin, TNF-Rp55, TNF-Rp75, and TNF-α were obtained after 26, 32, 32, and 38 cycles, respectively. An aliquot (4 μl) of the PCR product was electrophoresed on a 1.6% agarose gel and visualized by ethidium bromide staining and UV illumination. After photographing the gel, relative amounts of PCR products were determined by scanning the negative films using a laser densitometer (LKB 2222-020, Ultroscan XL, Pharmacia). For relative quantity, the densitometric value of each sample was normalized to that of β-actin.

Quantitation of TNF-α protein
Keratinocytes were allowed to grow to subconfluence in Eagle’s MEM containing 10% FBS. The medium was replaced with PBS and the cells were exposed to UVB (0–20 mJ/cm2). Immediately after UVB treatment, the PBS was removed and replaced with the medium. After another 24 h of incubation, the concentration of TNF-α in culture supernatants was measured using a commercially available ELISA kit (Genzyme, Cambridge, MA) according to the manufacturer’s procedures. TNF-α concentration was determined from the linear portion of a standard curve that was obtained using rTNF-α (Genzyme). The detection limit of the assay is 15 pg/ml. Each supernatant was analyzed in triplicate.

Neutralization assay
Subconfluent keratinocytes from WT, TNF-Rp55 KO, and TNF-Rp75 KO mice were exposed to UVB at a dose of 20 mJ/cm2 as described above. Immediately after UVB irradiation, cells were incubated with fresh medium containing various dilutions of polyclonal rabbit anti-mouse TNF-α (Genzyme) (0.5–10 μl/ml). We had previously determined that 10 μl of anti-TNF-α Ab can neutralize up to 1000 U of rTNF-α bioactivity. As a control, cultures were incubated with medium containing identical concentrations of rabbit IgG. After 24 h, the cells were collected and the percentages of apoptotic keratinocytes were determined by flow cytometry.

Statistical analysis
A minimum of three experiments was performed for each assay. All the data are expressed as mean values, with the SEM. The statistical significance was determined by either Student’s t test or ANOVA (when more than two groups were compared). A difference was considered to be statistically significant with p < 0.05.

Results

Murine keratinocytes express both TNF-Rp55 and TNF-Rp75
In order to determine whether TNF-α-mediated keratinocyte apoptosis involved signaling through TNF-Rp55 or TNF-Rp75, it was necessary to determine that these receptors were present on murine keratinocytes. Flow cytometric analysis demonstrated that both TNF-Rp55 and TNF-Rp75 were consistently detectable in WT murine keratinocytes (Fig. 1). Using primers specific for mouse TNF-Rp55 or TNF-Rp75, PCR-amplified TNF-Rp55, and TNF-Rp75 cDNA fragments were detected in WT murine keratinocytes, however, the latter fragment was present at a lower level (Fig. 2).

Fewer apoptotic keratinocytes are induced by in vivo UVB irradiation in TNF-Rp55 KO mice
In order to determine if TNF-α signaling involving either one of the TNF receptors plays an important role in UVB-induced keratinocyte apoptosis, we performed in vivo experiments using C57BL/6 mice that are deficient in either TNF-Rp55 or TNF-Rp75. Mouse skin was irradiated with UVB at a dose of 200 mJ/cm2 and, 24 h later, biopsies were obtained, mounted on slides, and analyzed using the TUNEL technique. As shown in Fig. 3, TUNEL-labeled keratinocytes appeared in the epidermis of WT (Fig. 3a), TNF-Rp55 KO (Fig. 3b), and TNF-Rp75 KO (Fig. 3c) mice after UVB irradiation. Light microscopy revealed shrunken cells, with irregularly-shaped and condensed nuclei, which differentiated them from their normal neighbors. Apoptotic keratinocytes were absent from the sham-irradiated epidermis of WT mice (Fig. 3d). As an additional negative control, we used UVB-exposed samples in which TdT was omitted. No TUNEL-positive staining cells were seen in this control (data not shown). The number of keratinocytes/hpf within epidermal sections from skin irradiated with UVB (200 mJ/cm2) was 50.1 ± 3.1 (mean ± SEM). The numbers of TUNEL-positive cells/hpf from WT mice, TNF-Rp55 KO mice, and TNF-Rp75 KO mice were 15.0 ± 1.2, 9.0 ± 0.6, and 13.6 ± 1.4, respectively. These results are reported in Fig.

FIGURE 1. Flow cytometric analysis of anti-TNF-Rp55 or anti-TNF-Rp75 activity from WT murine keratinocytes. WT keratinocytes were immunolabeled with anti-TNF-Rp55 or anti-TNF-Rp75 Ab as described in Materials and Methods. IgG2a isotype control Ab did not exceed the background fluorescence (top panel), whereas anti-TNF-Rp55 (middle panel) and anti-TNF-Rp75 (lower panel) treatment demonstrated specific reactivities. Data are given as histograms of fluorescence intensity vs cell number and represent one of three representative experiments.
4 as the absolute number of positive keratinocytes/hpf. No statistical difference was observed between WT and TNF-Rp55 KO mice; however, the number of apoptotic keratinocytes was significantly lower in TNF-Rp55 KO mice, compared with WT mice or TNF-Rp75 KO mice.

In vitro UVB irradiation induces less apoptosis in TNF-Rp55-deficient keratinocytes

In the in vitro experiments, keratinocytes isolated from the WT, TNF-Rp55 KO, and TNF-Rp75 KO mice were grown to subconfluence and then irradiated with UVB. After 24 h, direct microscopic examination of the culture dishes revealed a dose-dependent increase in the number of detached cells possessing condensed nuclei. Apoptosis was quantitated by flow cytometry. To test the sensitivity of this method, keratinocytes from WT mice were exposed to UVB (0–20 mJ/cm²), and showed a dose-dependent increase of subdiploid (apoptotic) cells with increasing UVB (Fig. 5). Although UVB irradiation induced keratinocyte apoptosis in WT, TNF-Rp55-deficient, and TNF-Rp75-deficient keratinocytes, a significantly lower number of apoptotic cells were found in TNF-Rp55-deficient keratinocytes at all UVB doses tested (Fig. 5).

Apoptotic keratinocytes were further examined by the TUNEL technique. Apoptotic keratinocytes were clearly labeled with FITC and exhibited a yellowish green fluorescence as well as condensation and fragmentation of the nucleus, whereas nonapoptotic keratinocytes stained with PI (red fluorescence). Fig. 6 shows the results of an experiment in which cultured keratinocytes from WT (Fig. 6a), TNF-Rp55 KO (Fig. 6b), and TNF-Rp75 KO (Fig. 6c) mice were exposed to UVB (20 mJ/cm²), and apoptosis was analyzed 24 h later. No apoptotic keratinocytes were observed in the sham-irradiated WT keratinocytes (Fig. 6d) or in the absence of TdT (data not shown). The FITC-labeled keratinocytes was counted under a fluorescent microscope and the number of apoptotic cells/100 cells was evaluated in three independent experiments. The number of FITC-labeled cells in WT, TNF-Rp55-deficient, and TNF-Rp75-deficient keratinocytes was 28.9 ± 3.4, 15.2 ± 2.1, and 28.3 ± 3.2, respectively (Fig. 7). The degree of apoptosis in TNF-Rp55-deficient keratinocytes was significantly lower than that in WT keratinocytes or TNF-Rp75-deficient keratinocytes (p < 0.05).

DNA fragmentation is reduced in UVB-exposed TNF-Rp55-deficient keratinocytes

To detect DNA fragmentation in UVB-irradiated keratinocytes, DNA was extracted and analyzed electrophoretically. Within 24 h of UVB irradiation, both WT keratinocytes and TNF-Rp75-deficient keratinocytes demonstrated the discontinuous electrophoretic pattern of DNA degradation into nucleosomal fragments, while the internucleosomal DNA fragmentation was not evident in TNF-Rp55-deficient keratinocytes (data not shown). DNA ladders were completely absent in WT keratinocytes that had not been irradiated (data not shown).

UVB induces TNF-α mRNA expression in skin and TNF-α protein release from keratinocytes

To examine whether the in vivo dose of UVB used in these studies was sufficient to up-regulate endogenous TNF-α production in the skin, RNA was extracted from WT mouse skin exposed to 200 mJ/cm² UVB. RT-PCR for TNF-α and β-actin was performed at 0 and 24 h after UVB irradiation, and PCR products were resolved on an agarose gel. Although low levels of TNF-α mRNA were detectable before exposure to UVB, TNF-α mRNA markedly increased 24 h after UVB irradiation (Fig. 8A). Densitometric analysis revealed that this increase was ~6.3-fold (Fig. 8B).

In order to confirm that keratinocytes are a source of TNF-α in the skin, cultured keratinocytes from WT, TNF-Rp55 KO, and
TNF-Rp75 KO mice were irradiated with the same doses of UVB used for the in vitro apoptosis assays, namely 0 to 20 mJ/cm². After 24 h, TNF-α levels were measured from the culture supernatant by ELISA. As shown in Fig. 8C, in WT keratinocytes, TNF-α protein was below the detection level in the supernatant from the sham-irradiated controls. As the UVB dose increased to 5 mJ/cm², 10 mJ/cm², and 20 mJ/cm², the TNF-α concentration also increased (80.3 ± 9.5 pg/ml, 103.1 ± 11.2 pg/ml, and 180.2 ± 13.8 pg/ml, respectively). Similar dose-dependent induction of TNF-α was observed for both TNF-Rp55 KO and TNF-Rp75 KO mice (data not shown).

Anti-TNF-α Ab inhibits UVB-induced apoptosis
To further confirm the role of TNF-α signaling in UVB-induced apoptosis, we treated the keratinocytes with a neutralizing Ab against murine TNF-α immediately after UVB irradiation (20 mJ/cm²). Apoptosis was assessed using flow cytometric analysis 24 h later. Neutralizing TNF-α Ab caused a concentration-dependent suppression of apoptosis in WT- and TNF-Rp75-deficient keratinocytes (data not shown). As illustrated in Fig. 9, treatment with anti-TNF-α Ab (1 μl/ml) significantly decreased the frequency of UVB-induced apoptosis in WT and TNF-Rp75 KO mice (apoptotic cell count of 25.1 ± 2.3% and 24.7 ± 2.5%, respectively), as compared with keratinocytes treated with control rabbit IgG (39.1 ± 2.2% and 37.2 ± 1.9% of apoptotic cells, respectively). A decrease of approximately 33–36% in apoptosis occurred in both UVB-irradiated WT and TNF-Rp75-deficient keratinocytes treated with anti-TNF-α Ab (compared with the control IgG-treated cells).

FIGURE 4. Fewer apoptotic keratinocytes seen in TNF-Rp55-deficient mice. Mice were irradiated with 200 mJ/cm² of UVB and skin biopsies were taken 24 h later. Apoptotic cells were examined by TUNEL staining as in Figure 3. The number of apoptotic keratinocytes per hpf was quantitated. Data are reported as the mean ± SEM (n = 3). A significant reduction in apoptosis was found for TNF-Rp55 KO mice. p < 0.01 vs WT mice; p < 0.05 vs TNF-Rp75 KO mice.

FIGURE 5. Flow cytometry analysis of WT and TNF-R-deficient keratinocytes. After UVB irradiation of keratinocytes from WT, TNF-Rp55 KO, and TNF-Rp75 KO mice, apoptosis was analyzed by flow cytometry. The percentages of apoptotic cells in each sample are derived from the mean of three experiments ± SEM. *p < 0.05, between TNF-Rp55-deficient keratinocytes and either WT- or TNF-Rp75-deficient keratinocytes over a range of UVB doses.

FIGURE 6. TUNEL analysis of keratinocytes exposed to UVB in culture. Keratinocytes were irradiated with UVB (20 mJ/cm²), and 24 h later were nick-end labeled with FITC-dUTP as described in Materials and Methods. Photomicrograph of keratinocytes (magnification, ×400) from WT (a); TNF-Rp55 KO (b); TNF-Rp75 KO (c); and Sham-irradiated WT keratinocytes (d). Chromatin in fragmented nuclei of apoptotic keratinocytes are labeled with FITC (green), while nonapoptotic keratinocytes are labeled with PI (red).

FIGURE 7. Fewer apoptotic keratinocytes seen after in vitro UVB irradiation in TNF-Rp55 deficient keratinocytes. The percentage of FITC-labeled apoptotic cells was quantitated under a fluorescent microscope. The data represent the mean ± SEM of three independent experiments. A significantly lower number of apoptotic keratinocytes were found in TNF-Rp55-deficient keratinocytes, vs either WT keratinocytes or TNF-Rp75-deficient keratinocytes (*, p < 0.05).
TNF-α Ab treatment of both WT- and TNF-Rp75-deficient keratinocytes reduced apoptosis to approximately the same level as that seen in TNF-Rp55-deficient keratinocytes, suggesting that all TNF-α signaling for UVB-induced apoptosis was via the TNF-Rp55 receptor. In contrast, neutralizing TNF-α Abs had no effect on apoptosis in TNF-Rp55-deficient keratinocytes.

Discussion

UVB radiation accounts for most of the harmful biologic effects of sunlight on the skin. Acute exposure of suberythemal doses of UVB impairs the function of epidermal APCs (Langerhans cells) and up-regulates the expression of immunomodulatory cytokines, leading to impaired induction of delayed-type hypersensitivity and contact hypersensitivity (30, 31). Chronic exposure of the skin to UVB radiation has been implicated as a cause of certain skin cancers (32, 33). UVB-induced apoptosis may play an important role in preventing irreversibly damaged keratinocytes from remaining in the skin and therefore being a risk factor for malignant transformation (34). Thus, UVB-induced apoptosis has become a subject of interest, due to the potential link between this process and UVB-induced skin carcinogenesis.

Cytokines are associated with the regulation of apoptosis (10–11, 35–39). Some cytokines such as IL-6 and IL-10 protect cells from apoptosis (35, 36). However, certain cytokines such as IFN-α and IL-2 induce apoptosis (38, 39). TNF-α is involved in apoptosis of a variety of cell types such as T cells, fibroblasts, epithelial cells, macrophages, and carcinoma cells (11, 40–42).

Keratinocytes are the major source of a variety of cytokines, including IL-1, IL-6, IL-7, IL-10, IL-12, IL-15, IL-18, and TNF-α, as well as chemokines (9, 43, 44). The constitutive production of most of these cytokines by keratinocytes is very low, but can increase in the presence of various stimuli, including UVB. UVB irradiation of keratinocytes induces almost all tested cytokines except for IL-7 (45). To determine whether the dose of UVB irradiation used in our experiments up-regulates TNF-α production in mouse skin, we examined the levels of TNF-α mRNA and protein.
after UVB irradiation. Our results confirm that UVB significantly induces keratinocytes to synthesize and release TNF-α.

TNF-α has been thought to be involved in UVB-induced keratinocyte apoptosis. Studies from Schwarz et al. (8) have provided evidence that TNF-α plays a role in UVB-induced apoptosis of murine keratinocytes. The present study demonstrates that anti-TNF-α Abs reduced UVB-induced apoptosis. These findings support the notion that TNF-α plays an important role in UVB-induced keratinocyte apoptosis (8).

Of the two TNF receptors, p55 and p75, the former has been implicated as the main mediator of apoptosis in nonlymphoid cells such as 3T3 cells, neurons, murine hepatocytes, and human hepatoma cells (41, 46, 47). TNF-Rp55 and another member of the TNF receptor superfamily, Fas (APO-1/CD95), are homologous in a 70-amino acid region known as the “death domain.” This domain is required for transducing the death signal through binding such mediators as TNF-Rp55-associated death domain protein (TRADD), MORT1/Fas-associating protein with death domain (FADD), and receptor-interacting protein (RIP), which may act as adapter proteins in both the TNF-Rp55 and Fas-activated signaling cascades (18). FADD and TRADD were found to bind differentially to Fas and TNF-Rp55, as well as to each other, and both bound effectively to RIP. The death domain of TRADD recruits FADD, which is responsible for downstream signal transduction by recruiting cytosolic aspartate-specific cysteine proteases 8 (caspase-8) (48). The C-terminal region of caspase-8 is related to the caspase family, specifically to caspase-3. The activated caspase members can cleave various substrates that lead to morphological changes known to accompany apoptosis (49). Recently, it has been demonstrated that TNF-Rp75 may also be involved in apoptosis of activated human synovial T cells, activated murine T cells, and TA1 cells (11, 40). The induction of apoptosis by TNF-Rp75 is strongly associated with the down-regulation of Bcl-XL mRNA and protein expression in activated T cells (50).

The present study demonstrates both TNF-Rp55 and TNF-Rp75 expression in murine keratinocytes. We utilized gene-targeted mutant mice lacking either TNF-Rp55 or TNF-Rp75, to elucidate the role of TNF-α in UVB-induced keratinocyte apoptosis as well as to further delineate which type of TNF receptor signaling mediates the apoptotic pathway. Both in vivo and in vitro studies demonstrated that there was a significant reduction of UVB-induced keratinocyte apoptosis in TNF-Rp55 KO mice, compared with WT mice or TNF-Rp75 KO mice. These results suggest that TNF-Rp55, but not TNF-Rp75, plays an important role in UVB-induced keratinocyte apoptosis.

Although significantly down-regulated, UVB-induced apoptosis of keratinocytes in TNF-Rp55 KO mice was not completely abolished. Neutralizing TNF-α Ab did not completely inhibit UVB-induced apoptosis of keratinocytes, even at a concentration 100-fold greater than that necessary for complete neutralization of autocrine TNF-α bioactivity in the supernatants. Moreover, we were not able to induce apoptotic cell formation in WT keratinocytes by the addition of increasing concentrations of rTNF-α (data not shown). This result confirms previous reports, which demonstrated that TNF-α alone is insufficient to induce keratinocyte apoptosis (8). RNA or protein synthesis inhibitors are necessary in order to trigger TNF-Rp55-mediated apoptosis (41, 51). Two major TNF-induced responses have been described: apoptosis and activation of (NF-κB). Activation of NF-κB can inhibit TNF-Rp55-mediated apoptosis (52). Blockage of the protective pathway encourages the induction of cytotoxicity by triggering the apoptotic process (51). These studies suggest that, although TNF-α is clearly involved in UVB-induced keratinocyte apoptosis via TNF-Rp55, other molecules likely play a role as well. In fact, it has been demonstrated that the cytotoxicity of TNF-α can actually be enhanced by other cytokines, such as IL-1 and IFN-γ (53).

It has been shown that the suppressor gene, p53, has a direct effect on apoptosis by down-regulating Bcl-2 expression and up-regulating Bax expression (54). It is also involved in UVB-induced apoptosis in keratinocytes and, in fact, levels of p53 protein in human epidermis are significantly increased following UV irradiation (34, 55). In addition, Fas/Fas ligand, which was shown to be expressed on keratinocytes, may also play an important role (56). However, UV light is known to directly stimulate Fas receptor itself, thereby activating the Fas-dependent death pathway independently of Fas ligand (57). Clearly, the exact role of these multiple components of the keratinocyte survival machinery requires further studies.

Nevertheless, our observations support the notion that TNF-α is involved in UVB-induced keratinocyte apoptosis and demonstrate that p55 receptor signaling plays a pivotal role in this complex event. Understanding the mechanisms that regulate apoptosis and identifying individual control points of the apoptotic pathway in keratinocytes will likely have an impact on future strategies for therapeutic intervention in UVB-related skin diseases.

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References


