Genetic deletion of TNFα inhibits ultraviolet radiation-induced development of cutaneous squamous cell carcinomas in PKCε transgenic mice via inhibition of cell survival signals


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Abstract

Protein kinase C epsilon (PKCε), a Ca2+-independent phospholipid-dependent serine/threonine kinase, is among the six PKC isoforms (α, δ, ε, η, μ, ζ) expressed in both mouse and human skin. Epidermal PKCε level dictates the susceptibility of PKCε transgenic (TG) mice to the development of cutaneous squamous cell carcinomas (SCC) elicited either by repeated exposure to ultraviolet radiation (UVR) or by using the DMBA-TPA (12-O-tetradecanoylphorbol-13-acetate) tumor promotion protocol (Wheeler, D.L. et al. (2004) Protein kinase C epsilon is an endogenous photosensitizer that enhances ultraviolet radiation-induced cutaneous damage and development of squamous cell carcinomas. Cancer Res., 64, 7756–7765). Histologically, SCC in TG mice, like human SCC, is poorly differentiated and metastatic. Our earlier studies to elucidate mechanisms of PKCε-mediated development of SCC, using either DMBA-TPA or UVR, indicated elevated release of cytokine TNFα. To determine whether TNFα is essential for the development of SCC in TG mice, we generated PKCε transgenic mice/TNFα-knockout (TG/TNFαKO) by crossbreeding TNFαKO with TG mice. We now present that deletion of TNFα in TG mice inhibited the development of SCC either by repeated UVR exposures or by the DMBA-TPA protocol. TG mice deficient in TNFα elicited both increase in SCC latency and decrease in SCC incidence. Inhibition of UVR-induced SCC development in TG/TNFαKO was accompanied by inhibition of (i) the expression levels of TNFα receptors TNFRI and TNFRII and cell proliferation marker ornithine decarboxylase and metastatic markers MMP7 and MMP9, (ii) the activation of transcription factors Stat3 and NF-κB and (iii) proliferation of hair follicle stem cells and epidermal hyperplasia. The results presented here provide the first genetic evidence that TNFα is linked to PKCε-mediated sensitivity to DMBA-TPA or UVR-induced development of cutaneous SCC.

Introduction

Chronic ultraviolet radiation (UVR) exposure is the most common etiologic factor linked to the development of cutaneous squamous cell carcinomas (SCC), a non-melanoma form of skin cancer that can metastasize (1). Protein kinase C epsilon (PKC), a family of phospholipid-dependent serine/threonine kinases, is the major intracellular receptor for the mouse skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (2-5) and is activated by a variety of stress factors including UVR (6). PKCε is among six isoforms (α, δ, ε, η, μ, ζ) of PKC expressed in both human and mouse skin (2-8). To determine the in vivo functional
role of PKCε in mouse skin carcinogenesis, we generated PKCε transgenic (TG) mouse lines (224 and 215) on FVB/N background that overexpress PKCε approximately 8- and 18-fold, respectively over endogenous levels in basal epidermal keratinocytes and cells of the hair follicle. We observed that epidermal PKCε level dictates the susceptibility of transgenic mice to the development of papilloma-independent SCC elicited by either repeated exposure to UVR or using the DMBA initiation-TPA tumor promotion protocol (9-16). Histologically, SCC in PKCε transgenic mice, like human SCC, is poorly differentiated and metastatic (11).

During studies to find clues about how PKCε overexpression mediates development of metastatic SCC, we found that PKCε transgenic mice have dramatically elevated TNFα serum levels relative to their wild-type littersmates following UVR exposure or TPA treatment (13,16). Several reports have implicated TNFα in the development of papillomas, but not in SCC (17-19). To conclusively determine whether TNFα is essential for the development of SCC in PKCε transgenic mice, we generated PKCε transgenic mice/TNFα-knockout (TG/TNFαKO) transgenic mice by crossbreeding PKCε transgenic mice line 224 (TG) with TNFα knockout mice. We now present that depletion of TNFα in PKCε transgenic mice inhibited the development of SCC either by repeated UVR exposures or by the DMBA initiation-TPA promotion protocol via inhibition of survival signals to epidermal cell proliferation including hair follicle stem cells (HSCs).

Materials and Methods

Chemicals, antibodies and kits

TPA was purchased from Alexis Corp (San Diego, CA) and 7,12-Dimethylbenzanthracene (DMBA) was from Aldrich Chemical Company (Milwaukee, WI). Surface staining conjugated antibodies for FACs (α–integrin PE, CD34 FITC), isotype controls and 7-aminoactinomycin D (6-ACT) dye were purchased from BD Biosciences (San Jose, CA). The anti-β-actin for western blotting were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA); pStat3Ser727, pStat3Tyr705 and CD34 antibodies to PKCε (7AAD) dye were purchased from BD Biosciences (San Jose, CA). The anti-B-p65, proliferating cell nuclear antigen (PCNA) and α-actin for western blotting were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA); pStat3Ser727, pStat3Tyr705 and CD34 were from BD Biosciences (San Jose, CA). Secondary antibodies anti-rat IgG (H + L) (Invitrogen; 1:1000 dilution) for 1 h light fixation, and then embedded in paraffin. Four to five μm sections were cut for H&E staining and immunostaining of skin stem cells. Briefly, the extra paraffin was removed using three xylene gradient washes followed by alcohol gradient for 10 min each. The slides were washed with Milli-Q water and then 1XPBS. The antigen retrieval was done using antigen unmasking solution as per protocol (vector laboratories). The blocking process was done in normal goat and normal horse serum for 1 h at room temperature (RT). After blocking, primary antibody to CD34 (dilution 1:200) was incubated to tissue section on the slides overnight. Tissue sections were incubated with their secondary antibodies (Alexa-Fluor 594-Donkey anti-rat IgG (H + L) (Invitrogen; 1:1000 dilution) for 1 h at room temperature. After incubation with secondary antibody, slides were washed three times with 1XPBS, mounted with DAPI (Vector Laboratories) and observed under the fluorescent microscope (Vectra). The fluorescent pictures of CD34 staining were captured through TRITC channel (red color) in a Fluence Fluorescent microscope.

Karainocyte isolation and flow cytometric analysis

For the skin stem cell estimation, the dorsal skin of mice was harvested for keratinocyte isolation 24 h post UVR (2 kJ/m²) exposure (22). In each experiment an equal size of skin was excised from the indicated four groups of mice. Viable cell counts were determined using Trypan Blue (0.4%). Keratinocytes were incubated for 1 h in the dark at 4°C with PE-conjugated Rat Anti-Human α6-integrin antibody at 10 μl per 10⁵ cells and FITC-conjugated rat anti-mouse CD34 antibody at 2 μg per 10⁵ cells. Keratinocyte preparations of single cell suspension were sorted based on α6-integrin and CD34 status using a FACs Aria cell sorter (BD Biosciences). A 488 nm laser was used to detect FITC with a 530/30 filter and a 532 nm laser for PE with a 575/25 filter. The nozzle size was 130 nm and the pressure used was 14 p.s.i. The live cell population gate was estimated using forward and side scatter positioning and confirmed with 7AAD staining.

Phenotyping and estimation of the frequency of CD34+/α6-integrin+ stem cells (HSCs)

The phenotyping assays were acquired on a BD FACScalibur (BD Biosciences). The BD FACScalibur instrument was calibrated daily by the University of Wisconsin Carbone Cancer Center Flow Cytometry.
Laboratory staff using the manufacturer's Cytometer Settings and Tracking calibration software. Data were analyzed using FlowJo software version 9.4.3 (Treestar, Ashland, OR). Positive staining and gating strategy were determined by comparison to isotype controls. Dead cells were excluded using 7AAD staining on FACSCalibur assays. Data demonstrate frequency of cells in a parent population (of live intact cells for α-6-integrin and CD34 expression).

The frequency of CD34/α-6-integrin* stem cells (Hair follicle stem cells or HSCs) represent the percent of CD34/α-6-integrin*7AAD* cells (‘cells’ determined by FSC/SSC morphologic gate) in the total 7AAD* population. The absolute number of HSCs in individual samples was calculated by multiplying percentage frequency of HSCs by the total number of Trypan-Blue excluding cells in the single cell keratinocyte preparation. The data represent absolute number of HSCs from the equal size of dorsal skin from all four indicated mice groups used in the study.

Analysis of PCNA-positive cells
At the end of the tumor induction experiment, all four groups of mice (WT, TNFαKO, TG and TG/TNFαKO) (n = 3) were used for evaluation of UVR-induced levels of PCNA and epidermal hyperplasia. Mice were killed at 24 h post-last UVR treatment. Skin specimens were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Five-μM-thick sections were cut for PCNA staining. Briefly, the slides were incubated overnight at 4°C with PCNA primary antibody. Antigen retrieval pretreatment was done by using Tris-urea solution in a digital decloaking chamber. The process of blocking and secondary antibody incubation was done using R.T.U Vectastain Kit. A detailed process of PCNA staining and quantitation is described elsewhere (12). The visualization was performed using diaminobenzidine as a substrate for the peroxidase reaction. Pictures were taken using a Nuance Bright field Microscope (Vectra). An average percentage was calculated based on the total number of cells and the number of PCNA positive epidermal keratinocyte cells from each set of 10 fields count. Results are expressed as mean of percentages ± SEM.

Western blot analysis
Mice were shaved and depilated before experimentation. The mouse skin was excised and scraped to remove the subcutaneous fat. The epidermis was scraped off on an ice-cold glass plate and homogenized in lysis buffer [50 mmol/l HEPES, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/l MgCl₂, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 200 μmol/l Na₃VO₄, 200 μmol/l NaF and 1 mmol/l EDTA (final pH 7.5)]. The homogenate was centrifuged at 14,000g for 30 min at 4°C. Protease and phosphatase inhibitors were protected from Sigma-Aldrich (St. Louis). Epidermal cell lysate proteins (50–100μg) were fractionated on 10% Criterion precast SDS–polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA). The protein was transferred to a 0.45 μm Hybond-P polyvinylidene difluoride (PVDF) transfer membrane (Amersham Life Sciences, Piscataway, NJ). The membrane was then incubated with the indicated antibody followed by a horseradish peroxidase secondary antibody (Thermo Scientific), and the detection signal was developed with Amersham’s enhanced chemiluminescence reagent and using FOTO/Analyst Luminary Work Station (Fotodyne). The Western blots were quantitated (normalized to β-actin) by densitometric analysis using TotalLab Nonlinear Dynamic Image analysis software (Nonlinear USA, Durham, NC).

Statistical analysis
Number of SCCs and papillomas (where applicable) were recorded weekly starting at week 10 for the DMBA challenged arms and week 14 for the UVR challenged arms. Time to first SCC was plotted using Kaplan–Meier curves, and differences in risk between treatment arms for time to first SCC were tested using Cox proportional hazard regression models. Differences between the two treatment arms in mean numbers of papillomas/mouse were tested using Negative Binomial regression models. Differences in quantitated staining levels and stem cell analysis were examined using Student’s t-test. Computations were performed with SAS and R software; figures were created with R software (23,24).

Figure 1. Deletion of TNFα in PKCε transgenic mice inhibited the development of SCC elicited by repeated UVR exposures. Four groups of 6–8-week-old FVB/N mice [WT (n = 15), TNFαKO (n = 16), TG transgenic (n = 12) and TG/TNFαKO (n = 13)] were shaved and depilated 2–3 days before the start of UVR exposure. The UVR source was Kodacel-filtered FS-40 sun lamps (approximately 60% UVB and 40% UVA). Mice were exposed to UVR (2 kJ/m²) three times weekly (Monday, Wednesday and Friday) for 45 weeks. Carcinomas were recorded grossly as downward-invading lesions, which were confirmed histologically. Shown are the Kaplan–Meier survival curves (A), SCC multiplicity data (SCC/mouse) (B) and representative photographs of indicated mice at the end of the experiment (C).
Results

TNFα deletion in PKCε transgenic mice and their wild-type littermates inhibits development of SCC elicited either by repeated UVR exposures or by the DMBA-TPA protocol

UVR-induced SCC

In this experiment (Figure 1), four groups of mice (WT, TNFα KO, TG, TG/TNFα KO) were exposed to UVR (2 kJ/m²) thrice weekly for 45 weeks. The results are shown in Figure 1A-C. As compared to wild-type littermates, PKCε over-expressing transgenic mice exhibit decrease in tumor latency and increases in SCC incidence, the results are in accord with our previous finding (12,25). PKCε overexpressing TNFα-deficient (TG/TNFα KO) mice exhibited a reduction in risk of SCC compared to their WT littermates (HR: 0.14; 95% CI: 0.05–0.37, P = 0.001). Also, TG/TNFα KO mice exhibited both an increase in SCC latency (21 weeks) and a decrease in SCC incidence (55%). Similarly, wild-type mice deficient in TNFα exhibited a non-significant reduction in risk of SCC compared to WT mice (HR: 0.69; CI: 0.14–2.81, P = 0.54) (Figure 1A and B). It is notable that papilloma multiplicity is shown only in WT mice and not in TG mice (Figure 1C). TG mice develop only SCC and no papilloma (12).

DMBA-TPA-induced SCC

We have previously reported that epidermal PKCε transgenic mice are sensitive to the development of SCC elicited not only by repeated exposure to UVR but also by the DMBA-TPA tumor promotion protocol (9,12,16,21). Also, PKCε transgenic mice are more sensitive than their wild-type littermates to both UVR- and TPA-induced increase in TNFα levels in serum (13). These findings prompted us to determine the role of TNFα in the development of SCC elicited by the DMBA-TPA protocol in PKCε transgenic mice. In this experiment (Figure 2), FVB/N mice (WT, TNFα KO, TG, TG/TNFα KO) were topically treated with a single dose of DMBA (100 nmol) followed a week later by repeated treatment of TPA (10 nmol) twice a week for 37 weeks. Carcinoma and papilloma were recorded weekly. PKCε overexpressing mice deficient in TNFα (TG/TNFα KO) exhibited a reduction in risk of SCC compared to TG mice (HR: 0.07; 95% CI: 0.01–0.30, P < 0.001) (Figure 2A and B). Moreover, TG/TNFα KO mice exhibited both an increase in SCC latency (14 weeks) and a decrease in SCC incidence (8%) (Figure 2A and B). Similarly the wild-type mice deficient in TNFα (TNFα KO) exhibited a non-significant reduction in risk of SCC compared to WT mice (HR: 0.69; CI: 0.14–2.81, P = 0.54) (Figure 2). Also, TNFα KO exhibited both an increase in SCC latency (8 weeks) and a decrease in SCC incidence (45%) (Figure 2A and B). TNFα deletion significantly (P = 0.0014) inhibited papilloma multiplicity elicited by the DMBA-TPA protocol in wild-type mice (Figure 2B and C). It is notable that papilloma multiplicity is shown only in WT mice and not in TG mice (Figure 2C). TG mice develop only SCC and no papilloma (12).

TNFα deletion in PKCε transgenic mice and their wild-type littermates inhibits UVR-induced epidermal hyperplasia and epidermal proliferative cell nuclear antigen

We explored the possibility whether TNFα is linked to UVR-induced epidermal cell proliferation and hyperplasia. In this experiment (Figure 3), FVB/N Mice (WT, TNFα KO, TG, TG/TNFα KO) were exposed to UVR (2.0 kJ/m²) three times weekly (Monday, Wednesday and Friday) for 45 weeks, and were killed...
at 24 h after the last UVR exposure. For histochemistry, uninvolved skin specimens (n = 3 each) were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Five μm skin sections were used for hematoxylin and eosin and PCNA staining. Uninvolved skin of TNFα deficient mice, as compared to TG and WT mice, elicited inhibition in UVR-induced hyperplasia (Figure 3A). As reported before (12), PKCe overexpression in WT mice resulted in UVR-induced increase in number of PCNA stained epidermal keratinocyte cells (P = 0.04). TNFα deletion significantly inhibited UVR-induced increase in number of PCNA stained epidermal keratinocyte cells in both WT (P = 0.002) and TG (P = 0.001) mice (Figure 3B). Similarly, we observed the significant (P = 0.002) increase in PCNA positive epidermal keratinocytes in TG/TNFα KO mice compared to TNFα KO (Figure 3B).

**TNFα deletion in PKCe transgenic mice and their wild-type littermates inhibits UVR-stimulated putative HSCs proliferation**

The epidermal keratinocytes positive for both cell surface marker CD34+ and α6-integrin mark mouse hair follicle bulge stem cell populations (Figure 4A) which have stem cell properties such as quiescence and multipotency. We have previously reported that both single and chronic UVR treatments (1.8 kJ/m², Monday, Wednesday and Friday) result in an increase in the frequency of double positive HSCs in TG mice as compared to their WT littermates (22). We explored the possibility that the mechanism of resistance of TNFα-deficient mice to UVR-induced development of SCC may involve the role of HSCs. In this experiment (Figure 4), we compared TNFα-deficient mice and their wild-type littermates for the effects of single UVR exposure on frequencies and absolute number of HSCs. The percentages of double-positive HSCs (CD34+ and α6-integrin+) in the isolated keratinocytes were determined by flow cytometric analysis after staining with α6-integrin and CD34 antibodies conjugated with fluorochrome, the cell surface markers of mouse hair follicle bulge cells. For quantitative analysis of HSCs among all four groups we have calculated the frequencies (Figure 4B) and total absolute number of CD34+/α6-integrin+ cells (Figure 4C) from dorsal skin. We observed that UVR-induced HSCs proliferation is less in TNFα KO and TG/TNFα KO mice compared to their respective wild-type littermates (Figure 4B and C). UVR exposure resulted in significant

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![Figure 3](image-url)  
**Figure 3.** Deletion of TNFα in PKCe transgenic mice inhibited UVR-induced proliferative marker PCNA and epidermal hyperplasia. At the end of the tumor induction experiment (Figure 1), the same groups of mice (WT, TNFαKO, TG and TG/TNFαKO) (n = 3) were used for evaluation of UVR-induced levels of PCNA and epidermal hyperplasia. The mice were killed at 24 h post last UVR exposure. For histochrometry, uninvolved tumor free mouse skin specimens (n = 3) were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin for sectioning (~5 μM), and stained with H&E. PCNA staining for epidermal proliferation was done as described in Materials and Methods. Pictures were taken in a Nuance bright field microscope at 20X magnification for HE and PCNA staining. Upper panel and lower panel are showing the representative pictures of H&E stained sections and nuclear PCNA staining, respectively (A). (B) Showing the quantitation data of PCNA count from all four indicated mouse groups (n = 3) in the uninvolved epidermis. Each bar value is the percent mean ±SEM of PCNA positive nuclei counted from 10 random areas from each mouse. Black arrows are showing PCNA positive proliferative nuclei (Abbreviation: HF, hair follicle; SG, sebaceous gland). (Q) Two and three star denotes P < 0.05 and ≤0.001, respectively.)
HSCs increase in both WT ($P = 0.01$) and TG ($P = 0.001$) mice. We also observed that UVR-induced HSCs proliferation is significantly less ($P = 0.001$) in TG/TNF-α KO mice compared to TG (Figure 4C).

Genetic deletion of TNF-α in mice inhibits UVR-induced expression of TNF-α receptors and associated signals to epidermal cell survival

The TNF-α signal transduction pathway, which may be linked to UVR-induced development of SCC, was explored. The biological effects of pro-inflammatory cytokine TNF-α are mediated through their receptors, namely TNFR1 and TNFR2. Although TNF-α can signal through these two receptors, the majority of TNF-mediated biological events are mediated through TNFRII signaling (26–30). Both TNF-α and TNFR1 have been linked to UVR carcinogenesis (17–19). TNF-α mediates the activation of two transcriptional factors, AP-1 and NF-κB, linked to the expression of TNF-α-induced genes involved in immunity and inflammatory responses and control of cellular proliferation, differentiation and apoptosis (31,32). As shown in Figure 5, deletion of TNFα in mice suppressed the expression levels of TNFα receptors TNFR1 and TNFR2, inhibited the activation of transcription factors AP-1, Stat3 and NF-κB-p65, and decreased expression levels of cell proliferation marker ornithine decarboxylase (ODC) and metastatic markers MMP7 and MMP9 (Figure 5).

**Discussion**

Skin cancer is the most common malignancy encountered in the United States with an expected diagnosis of 1.3 million new cases of non-melanoma skin cancer each year (1). SCC and basal cell carcinoma (BCC) are the most common non-melanoma forms of human skin cancer (33,34). BCC is rarely life threatening because it is slow-growing and is mostly localized. SCC, unlike BCC, invades the nearby tissues (1). The most important risk factor for non-melanoma skin cancer is chronic exposure...
to UVR in sunlight (1). We found that PKCε is an important component of UVR-induced signal transduction pathways to the development of SCC (9–16). Our FVB/N transgenic mice, which overexpress PKCε in the epidermis, develop papilloma-independent SCC elicited either by UVR (12,16) or by the DMBA-TPA protocol (9–11,21). PKCε transgenic mice provide a unique model to investigate SCC (10). We have previously reported that epidermal PKCε level in PKCε transgenic mice dictates UVR- or TPA-induced TNFα release (13,16). We now present genetic evidence that TNFα is linked to UVR- or TPA-induced development of SCC.

Both PKCε transgenic mice and their wild-type littermates develop mostly carcinomas in response to repeated UVR exposures (Figure 1). Both PKCε transgenic mice and their wild-type littermates deficient in TNFα elicited both an increase in SCC latency and a decrease in SCC incidence (Figure 1). Skin PKCε level dictates the levels of TNFα release and the susceptibility of mice to UVR-induced development of SCC (13,16). A significant inhibition of SCC incidence in TG/TNFα KO mice clearly indicates that TNFα is the downstream component of PKCε signal transduction pathways to UVR-induced development of SCC (Figures 1 and 2).

The mechanism by which PKCε-induced TNFα mediates UVR-induced cutaneous SCC is not clearly understood. TNFα has the ability to regulate a vast array of cellular responses including proapoptotic, anti-apoptotic, proliferation and inflammation (35). We have previously presented evidence that UVR-induced increased expression of TNFα is proapoptotic or a proliferating signal in UVR carcinogenesis. In this context, it is noteworthy that FADD is the key component of both Fas- and TNFR-mediated apoptosis (36–39). The UVR-induced loss of FADD expression in PKCε transgenic mice lends support to the conclusion that UVR-induced level of TNFα is not proapoptotic, but rather may contribute to an increased cell proliferation of preneoplastic cells (39). We also reported that UVR-induced severe cutaneous damage (ulceration, hyperplasia and infiltration of inflammatory cells) in PKCε transgenic mice was partially prevented in bigenic PKCε transgenic TNFα knockout mice (12).

Moreover, TNFα-deletion resulted decrease in both the frequencies and total number of HSCs (CD34+/6-integrin+) in WT and TG transgenic mice (Figure 4). These results imply that TNFα is linked to UVR-induced HSCs proliferation. These skin HSCs are known to be the putative precursor cells for SCC (40–43). These HSCs reside in the bulge region of hair follicles and are characterized by their attributes such as slow cycling, clonogenic, label retaining and role in induction of skin papillomas and carcinomas (44,45). It has been observed that the mice deficient in TNFα converting enzyme (TACE) show abnormal hair follicles and decreased number of HSCs (CD34+/6-integrin+) (46). We have previously reported an association of PKCε with HSCs (22). PKCε overexpression in mice increased the clonogenicity of isolated keratinocytes, a property commonly ascribed to stem cells. Both single and chronic UVR treatments resulted in an increase in the frequency of double positive HSCs in PKCε TG mice as compared to their WT littermates. In TG mice HSCs cycle at a faster rate as compared to wild-type mice. A comparison of gene expression profiles of FACS sorted double positive keratinocytes isolated from UVR treated WT and TG mice indicated increased expression in TG mice of genes (Pes1, Rad21, Tfdp1 and Cks1b) linked to cell transformation, invasion and metastasis of cancer cells (22).

The current results (Figure 4) lend support to the conclusion that PKCε-induced TNFα mediates induction of SCC through direct effects on stem cells in the mouse hair follicle.

UVR-induced TNFα in PKCε transgenic mice mediates proliferative signals is further evidenced by the findings that TNFα-deficiency in PKCε transgenic mice resulted in suppression of UVR-induced activation of transcription factors Stat3, c-Jun/AP-1 and NF-κB and expression of cell proliferative markers such as PCNA and ODC (Figure 5). The constitutive expression of phosphorylated Stat3 at both pSTAT3Tyr705 and Stat3Ser727 residues are linked with UVR-induced skin carcinogenesis (47,48). The

Figure 5. Genetic deletion of TNFα in mice inhibits UVR-induced expression of TNFα receptors and associated signals to epidermal cell survival. Groups of WT, TNFαKO, TG and TG/TNFαKO mice (n = 2) were exposed once to UVR (2.0 kJ/m²) and were killed at 24-h post UVR. Dorsal skins of the mice were removed for the preparation of epidermal cell lysates. Shown is the western blot analysis from indicated mice for expression of PKCε, MMP7, MMP9, TNFRI, TNFRII, ODC (A) and total Stat3, pStat3Tyr705, Stat3Ser727, NF-κB-p65 and c-Jun/AP-1 (B). AN, arbitrary number of the quantitation of the western blots. Experiments were repeated three times with similar results.
phosphorylation of both of these residues is also crucial for maximum activation of Stat3, a downstream signaling component of PKCε. Inhibition of the phosphorylation of these Stat3 residues and other transcriptional factors also reveals the role of these signaling molecules in the inhibition of UVR-induced SCC in TG/TNFα KO mice. We also found inhibition of UVR-induced increased expression of TNFα receptors (TNFRI, TNFRII) in TG/TNFα KO mice (Figure 5). Notably, it is known that the biological effects of TNFα are mediated through its receptors, namely TNFRI and TNFRII. These receptors are also required for the optimal development of tumor in mice (49). The inhibition of these TNFα receptors may be one of the mechanisms of abrogation of TNFα signaling in TG/TNFα KO mice. Also, protein level of MMP7 and MMP9 are also inhibited in TG/TNFα KO mice (Figure 5), which are linked to the development, progression and metastasis of skin cancer (49–53). WT mice hardly elicited detectable changes in the indicated proteins (Figure 5). Consequently, it is difficult to conclude whether TNFα deletion has any effect on the expression of these proteins.

In summary, UVR has been shown to induce the release of pro-inflammatory (IL-1, TNFα), chemotactic (IL-6, IL-7, IL-15, GM-CSF, TNFα) and immune-regulatory cytokines (IL-10, IL-12, IL-18) in epidermal keratinocytes (54–57). Additionally, UVR-induced mRNA and protein level of epidermal TNFα correlates with the expression level of PKCε in PKCε overexpressing mouse lines (13). Our finding indicates that the inhibition of UVR-induced SCC development in TG/TNFα KO mice is accompanied by inhibition of (1) TNFα receptors (TNFRI and TNFRII), cell proliferation marker ODC, metastatic markers MMP7 and MMP9 (2), the activation of transcription factors Stat3, c-Jun/AP-1 and NF-κB, and (3) proliferation of HSCs and epidermal hyperplasia. We conclude that the genetic deletion of TNFα in PKCε transgenic mice (TG/TNFα KO) and their littermates inhibit UVR-induced SCC via abrogation of TNFα signaling and activation of cell survival pathway as evidenced by hyperplasia, HSCs proliferation and western blot analysis.

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