Protein Kinase Cε Is Linked to 12-O-tetradecanoylphorbol-13-acetate-induced Tumor Necrosis Factor-α Ectodomain Shedding and the Development of Metastatic Squamous Cell Carcinoma in Protein Kinase Cε Transgenic Mice


Departments of Human Oncology [D. L. W., K. J. N., A. K. V.] and Pathology and Laboratory Medicine [T. D. O.], Medical School, University of Wisconsin, Madison, Wisconsin 53792

ABSTRACT

Protein kinase Cε (PKCε), a Ca2+-independent, phospholipid-dependent serine/threonine kinase, is among the PKC isoforms expressed in mouse epidermis. We reported that FVB/N transgenic mice that overexpress PKCε, human epidermal cells and cells of the hair follicle develop papilloma-independent metastatic squamous cell carcinoma (mSCC) elicited by 7,12-dimethylbenz(a)anthracene-initiation and 12-O-tetradecanoylphorbol-13-acetate (TPA)-promotion protocol. We now present that PKCε transgenic mice elicit elevated serum tumor necrosis factor (TNFα) levels during skin tumor promotion by TPA, and this increase may be linked to the development of mSCC. A single topical application of TPA (5 nmol) to the skin, as early as 2.5 h after treatment, resulted in a significant (P < 0.01) increase (2-fold) in epidermal TNFα and more than a 6-fold increase in ectodomain shedding of TNFα into the serum of PKCε transgenic mice relative to their wild-type littermates. Furthermore, this TPA-stimulated TNFα shedding was proportional to the level of expression of PKCε in the epidermis. Using the TNFα-convertase enzyme (TACE) inhibitor, TAPI-1, TPA-stimulated TNFα shedding could be completely prevented in PKCε transgenic mice and isolated keratinocytes. These results indicate that PKCε protein signal transduction pathways to TPA-stimulated TNFα ectodomain shedding are mediated by TACE, a transmembrane metalloprotease. Using the superoxide dismutase mimetic CuDIPs and the glutathione reductase mimetic eb-Derivatives from epidermal keratinocytes; (c) TPA-induced TNFα shedding may be mediated through reactive oxygen species; and (d) TPA-induced TNFα shedding may play a role in the development of mSCC in PKCε transgenic mice.

INTRODUCTION

The multistep model of mouse skin carcinogenesis has been on the forefront of the identification of molecular and biochemical events unique to initiation, promotion, and progression of cancer (1, 2). A major breakthrough in understanding the mechanism of skin tumor promotion by TPA has been the identification of its major receptor, PKC ε (3–6). PKC ε represents a large family of PS-dependent serine/threonine kinases (7–9). On the basis of structural similarities and cofactor dependence, 11 PKC isoforms have been classified into three subfamilies: (a) the classical (cPKC); (b) the novel (nPKC); and (c) the atypical (aPKC). The cPKCs (α, βI, β II, and γ) are dependent on PS, DAG, and calcium for their activation. The nPKCs (δ, ε, η, and θ) retain responsiveness to DAG and PS but do not require calcium for full activation. The aPKCs (λ and σ) only require PS for their activation. The members of the PKC family exhibit functional diversity in their roles in the regulation of gene expression, cell growth, differentiation, and apoptosis (10–16). PKCε has been well documented as an oncogene (17, 18).

To determine the in vivo functional specificity of PKCε in TPA-activated PKC signals to skin tumor multiplicity, we generated transgenic mice that express T7-epitope-tagged PKCε in their epidermis. The expression of PKCε was directed to the basal cells of the epidermis and cells of the hair follicle using a human cytokeratin14 (K14) promoter (19, 20). This overexpression of PKCε in the mouse epidermis resulted in the rapid development of papilloma-independent metastatic squamous cell carcinomas (19, 20). However, the mechanism by which PKCε overexpression leads to the development of mSCC remains to be determined. Evidence indicates that the proinflammatory cytokine TNFα is linked to skin tumor promotion by TPA (21, 22) and UV light (23). Experiments using tumor promoters of the okadaic acid class have provided strong evidence that TNFα is the central mediator of tumor promotion in the mouse skin. Using either the two-stage model of carcinogenesis or UV light, mice deficient for TNFα or either of its receptors render the mice resistant to skin tumor formation (22–24).

TNFα is a potent proinflammatory cytokine that is produced by a multitude of cell types, including macrophages, lymphocytes, monocytes, fibroblasts, and keratinocytes. This molecule was originally discovered as a cytotoxic cytokine for tumor cells and its ability to cause necrosis of transplanted tumors (24). Mature murine TNFα consists of 156 amino acids (157 in humans); however, the molecule is translated with a 79 amino acid (76 in humans) long precursor sequence. For TNFα to exert its pleiotropic inflammatory responses at distant sites from its synthesis, it must be cleaved from the membrane in a process called ectodomain shedding. A specific enzyme called TACE cleaves pro-TNFα in response to extracellular stimuli (25, 26). The cloning of TACE (human and porcine; Refs. 25 and 26) revealed it to be a member of the “A disintegrin and metalloprotease” or ADAM family of proteins. The TACE protein is a multidomain, type I transmembrane protein that includes a zinc-dependent catalytic domain. The protein is broken into six domains: (a) prodomain; (b) catalytic domain; (c) disintegrin domain; (d) cysteine-rich domain; (e) transmembrane domain; and (f) the cytoplasmic domain. The prodo-

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2 To whom requests for reprints should be addressed, at Department of Human Oncology, Medical School, University of Wisconsin, Madison, WI 53792.

3 The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; PS, phosphatidylserine; cAMP, cyclic AMP; TNF, tumor necrosis factor; ROS, reactive oxygen species; TACE, tumor necrosis factor α converting enzyme; TAPI-1, tumor necrosis factor α processing inhibitor-1; TGF, transforming growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; PC, palmitoylcarnitine; KO, knockout; CuDIPs, Copper (II); 3,5-diisopropyl-salicylate; DMBA, 7,12-dimethylbenz[a]anthracene; OA, okadaic acid.

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main contains a cysteine residue that interacts with a zinc molecule in the catalytic domain. This interaction must be displaced for TACE activity and is believed to be mediated by ROS (27). On its release, TNFα exerts its biological effects by trimerizing and binding to two distinct receptors, TNFR1 and TNFR2. Binding of TNFα induces trimerization of each of these receptors, which then recruit several signaling proteins to the cytoplasmic membrane (28). With the ability to activate two distinct receptors and recruit different receptor signaling complexes, TNFα can regulate a vast array of cellular responses, including cellular inflammation, immunity, cell proliferation, differentiation, and apoptosis.

Here, we present evidence using FVB/N transgenic mice which overexpress PKCe in their basal epidermis and cells of the hair follicle: (a) PKCe mediates TPA-induced TNFα shedding, through the metalloprotease TACE; (b) generation of ROS is perhaps a PKCe downstream event in TPA-induced event in TPA-stimulated TNFα shedding; and (c) TPA-stimulated ectodomain shedding of TNFα may be linked to the development of mSCC.

MATERIALS AND METHODS

Materials. TPA was purchased from Alexis Corp. (San Diego, CA). DMBA was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).

PKCe Transgenic Mice. PKCe transgenic mice were generated as described (19, 20). Transgenic mice were maintained by mating hemizygous transgenic mice with wild-type FVB/N mice. The mice were housed in groups of two to three in plastic bottom cages in light-, humidity-, and temperature-controlled rooms; food and water were available ad libitum. The animals were kept in a normal rhythm of 12-h light and 12-h dark periods. The transgene was detected by PCR analysis using genomic DNA isolated from 1-cm tail clips.

Generation of PKCe-null Mice. The PKCe KO mice were generated and provided by Dr. Michael Leitges. Briefly, the ES cell line used for targeting was E14 from the mouse strain 129/Ola. The embryonic stem cells from 129/Ola were introduced into the blastocyst of C57BL/6. The germ-line chimeras were identified by the presence of agouti coat color in the F1 progeny. These chimeric mice (C57BL/6/129/Ola) were bred for eight generations for mutant transmission to FVB/N mice for a unified genetic background.

Tumor Induction Experiments. Mouse skin tumors were induced by the initiation-promotion regimen (19, 20). For mouse skin tumor initiation, a single 100-nmol dose of DMBA in 0.2 ml of acetone was applied topically to the shaved backs of mice. Two weeks after initiation, TPA in 0.2 ml of acetone was applied twice weekly to the skin for the duration of the experiment. Tumor multiplicity was observed every other week. Carcinomas were recorded in their basal epidermis and cells of the hair follicle, including cellular inflammation, immunity, cell proliferation, differentiation, and apoptosis.

Results

Overexpression of PKCe in the Mouse Epidermis Leads to Increased TPA-induced TNFα Ectodomain Shedding. TNFα has been proposed to be an endogenous tumor promoter of the mouse skin (21, 22). Two independent laboratories have shown that mice null for TNFα and their receptors are resistant to mouse skin tumor formation elicited by chemical carcinogenesis (DMBA-TPA, DMBA-OA protocols; Refs. 21 and 22) or by photocarcinogenesis (29). This prompted us to determine the role of TNFα in the development of mSCC in PKCe transgenic mice. First, we determined if PKCe transgenic mice have increased TNFα in their serum during skin tumor promotion. In this experiment, the dorsal skins of PKCe transgenic mice (n = 3) and their wild-type littermates were shaved and initiated with 100 nmol of DMBA and followed by twice weekly treatments of TPA (5 nmol) for 7 weeks (Fig. 1). The serum was collected at 3, 6, 12, 18, 24, 36, and 96 h after the 15th TPA treatment and analyzed by ELISA for TNFα. Chronic TPA treatment of PKCe transgenic mice led to sustained levels of TNFα in the serum at least until 36-h post-TPA, whereas the wild-type littermates had a peak of TNFα shed to the serum at 3 h and returned to undetectable levels by 6 h. We further determined the effects of a single application of TPA on TNFα shed into the serum in PKCe transgenic mice. In this experiment, TPA (5 nmol) in 0.2 ml of acetone or acetone alone was applied to the shaved backs of PKCe transgenic mice or their wild-type littermates. Mice were sacrificed at the indicated times, and serum was collected and analyzed by ELISA for TNFα. Serum TNFα was not detectable in either the acetone-treated PKCe transgenic mice or their wild-type littermates (Fig. 2A). However, the effect of a single TPA application on serum TNFα levels was especially dramatic in PKCe transgenic mice. TPA treatment resulted in an elevated level of serum TNFα in their basal epidermis and cells of the hair follicle, including cellular inflammation, immunity, cell proliferation, differentiation, and apoptosis.

The relative sensitivity of PKCe transgenic mice and their wild-type littermates to TPA-stimulated TNFα levels in the epidermis is illustrated in Figs. 2, B–D. TPA-stimulated epidermal TNFα levels in wild-type mice at 1, 2.5, 4, 6, 12, and 24 h after TPA treatment was 1.6, 2.6, 1.4, 1.8, 1.3, and 1.5-fold, respectively, above aceton control (Fig. 2B). TPA-stimulated epidermal TNFα levels in PKCe transgenic mice at 1, 2.5, 4, 6, 12, and 24 h after TPA treatment was 1.8, 3.8, 1.6, 1.6, 1.2, and 1-fold, respectively, above aceton control (Fig. 2C).
TPA treatment appears to elevate epidermal TNFα levels in PKCε transgenic mice at 2.5 h after treatment relative to their wild-type littermates (Fig. 2D). Epidermal TNFα levels in acetone treated wild-type and PKCε transgenic mice were not significantly different (P > 0.1) at 1, 2.5, 4, 6, and 12 h after treatment (Fig. 2, B and C).

TPA-induced TNFα Ectodomain Shedding Is Linked to the Level of Expression of PKCε in the Mouse Skin. We performed a series of experiments (Figs. 3 and 4) to determine whether PKCε expression level and activity in the mouse epidermis correlate with TNFα shed into the serum in PKCε transgenic mice. First, we compared two different PKCε transgenic mouse lines 224 and 215, which express PKCε 8- and 18-fold over wild type, respectively. As shown in Fig. 3, TPA-induced epidermal shedding of TNFα into the serum was directly proportional to the level of PKCε in the mouse epidermis (Fig. 3, inset).

The effects of general PKC inhibitors PC and Bis I on TNFα shedding are shown in Fig. 4. In these experiments (Fig. 4), the dorsal skins of PKCε transgenic and their wild-type littermates (n = 4) were shaved, and then 30 min before TPA (5 nmol) treatment, 3 μmol PC or 0.6 μmol of Bis I in acetone or acetone alone was applied to the shaved backs of PKCε transgenic mice and their wild-type littermates. The mice were sacrificed 2.5 h after application of TPA on epidermal and serum TNFα expression levels using the optEIA mouse TNFα ELISA kit (PharMingen). TNFα was not detectable in the serum of the acetone-treated mice. Each value is an average of determinations of pooled serum samples from four separate mice. Inset, Western blot analysis indicating the level of expression of PKCε in wild-type and PKCε transgenic mouse lines (224 and 215). Actin was used for equal protein loading control, which was expressed equally in all three samples. ND, not detected.
TPA treatment, and serum TNFα levels were measured. PC treatment completely blocked the shedding of TNFα into the serum in PKCe transgenic mice (Fig. 4A). Bis I also completely blocked shedding of TNFα into the serum of PKCe transgenic mice (Fig. 4B). The detection of serum TNFα in the wild-type was below the minimum level of detection by ELISA.

The role of PKCe in TPA-induced TNFα in mouse primary keratinocytes was further evaluated using PKCe KO mice. PKCe KO mice were generated with the LacZNeo cassette (Fig. 5A) by interrupting the initiating codon. The genetic background used was 129/Ola and C57Bl/6 strains. These mice (C57Bl/6/129/Ola) were bred for eight generations for mutant transmission to FVB/N for a unified genetic background. Western blot analysis of epidermal extract from the dorsal skin indicated a lack of PKCe protein in the PKCe KO FVB/N mouse epidermis, whereas the heterozygote mice contained less protein than wild-type mice (Fig. 5A, inset and B). The possibility was explored that loss of PKCe may result in compensatory alterations in the level of expression of other PKC isoforms (Fig. 5B). The levels of expression of various PKC isoforms in epidermal extracts from untreated wild-type mice and mice heterozygous and homozygous null at the PKCe allele were determined by immunoblot analysis. Using actin as an equal loading control, it appears that none of the other PKC isoforms was either lost or dramatically elevated. To determine the role of PKCe in TPA-induced TNFα shedding, keratinocytes from PKCe KO FVB/N mice and their wild-type littermates were prepared. The keratinocytes were treated with either the vehicle ethanol or 100 nm TPA for 24 h, and TNFα levels in the media were determined. As shown in Fig. 5C, both ethanol and TPA-induced TNFα release in the media at 24-h post-treatment was significantly (P < 0.01)
reduced in PKCe-null primary keratinocytes relative to the wild-type keratinocytes.

Keratinocytes Are the Source of TPA-induced TNFα Ectodomain Shedding in PKCe Transgenic Mice. Although treatment of PKCe transgenic mice with either chronic or single application of 5 nmol TPA led to increased levels of TNFα into the serum, these experiments did not provide clues about the source of TNFα. We determined whether mouse keratinocytes are the primary source of TNFα release into the serum. In these experiments (Fig. 6), we isolated primary keratinocytes from 1-day-old PKCe transgenic pups (line 215) and their wild-type littermates. The cells were plated and then treated with 100 nmol TPA in ethanol or ethanol alone. The media were collected and analyzed for TNFα. TNFα levels were normalized to the number of cells plated and expressed as picograms of TNFα per million cells. Fig. 6A illustrates that PKCe transgenic mice had a consistent significant increase ($P < 0.001$) in TNFα in the media relative to the wild-type keratinocytes after TPA stimulation. To link PKCe-induced TNFα shedding in PKCe primary keratinocytes to TACE activity, we used a TACE inhibitor called TAPI-1. TACE is a transmembrane metalloprotease that cleaves the membrane bound precursor of TNFα to its soluble form (25, 26). The specificity of TAPI-1 toward TACE inhibition as well as dosage required to inhibit TACE both in vivo and in vitro are reported by Mohler et al. (30). Primary keratinocytes isolated from PKCe transgenic mice (line 215) when treated with TAPI-1 (133 μM) in conjunction with 100 nmol TPA for 24 h significantly ($P < 0.001$) prevented shedding of TNFα in the media (Fig. 6B). It is noteworthy that although TAPI-1 treatment caused the prevention of TPA-induced release of TNFα in the media, this prevention was accompanied by increased accumulation of TNFα in the primary keratinocytes (Fig. 6C). To determine whether TAPI-1 could prevent release of TNFα in PKCe transgenic mice in vivo, the dorsal skin of PKCe transgenic mice and their wild-type littermates were shaved and then injected s.c. with 1.5 mg of TAPI-1 before treatment with TPA (5 nmol). The mice were sacrificed 2.5 h after
TPA treatment, and serum was collected for analysis of TNFα level. As shown in Fig. 6D, TPA-induced TNFα shedding from the intact mouse skin in vivo was completely prevented when treated with TAPI-1.

The Inhibitors of ROS Inhibit TPA-induced TNFα Ectodomain Shedding in PKCε Transgenic Mice. The regulation of TACE activity remains poorly understood. However, it is well known that cells exposed to phorbol esters result in an increase in shedding events. This TPA-induced shedding occurs within minutes of treatment with TPA followed by a rapid down-regulation of TACE (31). It has been shown that, in part, TACE can be regulated by ROS generated by the addition of TPA (27). To determine whether ROS may be linked to TPA-induced TNFα shedding in PKCε transgenic mice, we used inhibitors of the generation of ROS (CuDIPS and ebselen). CuDIPS is a superoxide dismutase biomimetic (32) and depletes superoxide anions, whereas ebselen is a Glutathione peroxidase (33). CuDIPS and ebselen may be linked to TPA-induced TNFα shedding in PKCε transgenic mice relative to their wild-type littermates (n = 4) 2.5 h after the last TPA treatment and assayed for serum TNFα in the serum. Fig. 7 shows that both antioxidants completely prevented TNFα ectodomain shedding in PKCε transgenic mice.

Inhibition of TNFα Synthesis Using Pentoxifylline Completely Prevents the Development of mSCC. To determine the link of TPA-induced TNFα shedding in the development of mSCC in PKCε transgenic mice, we performed tumor promotion experiments using pentoxifylline, the TNFα synthesis inhibitor. In this experiment, PKCε transgenic mice and their wild-type littermates (n = 25) were shaved and initiated with 100 nmol of DMBA in 0.2 ml of acetone. One week later, mice were injected i.p. with either 0.2 ml of sterile PBS or pentoxifylline (50 μg/gram body weight) in 0.2 ml of sterile PBS 30 min before the application of 5 nmol TPA to the dorsal skin twice weekly during the entire duration of the experiment. To ascertain that pentoxifylline inhibited the shedding of TNFα into the serum during the skin tumor promotion protocol, mice were collected (n = 4) 2.5 h after the last TPA treatment and assayed for serum TNFα (Fig. 8A). After 18 weeks of tumor promotion, wild-type mice had an average papilloma burden of 8.92 ± 0.913, whereas wild-type mice treated with pentoxifylline had a significant reduction in their average papilloma burden to 5.32 ± 0.579 (P < 0.005; Fig. 8B). A portion (40%; 10 of 25) of the PKCε transgenic mice receiving PBS injections had at least one carcinoma after 18 weeks of tumor promotion. However, none (0 of 25) of the PKCε transgenic mice receiving pentoxifylline injections before TPA treatment developed mSCC (Fig. 8C). Representative groups of mice are depicted in Fig. 8D. Mice in this tumor induction experiment were checked daily for any toxic side effects associated with pentoxifylline. Pentoxifylline treatment did not affect the body weight gains, and survival in each treatment group varied from 90 to 100%.

DISCUSSION

PKCe is a calcium-independent, TPA/DAG-activated serine/threonine kinase. PKCe participates in the regulation of diverse cellular functions, including gene expression (34–36), neoplastic transformation (17, 18), cell adhesion (37), mitogenicity (38, 39), and cellular motility (40). There is now direct evidence that PKCe may mediate its oncogenic properties by directly activating the classic mitogenic signaling pathway involving Ras and Raf-1 kinase (38, 39, 41–43). Alternatively, TGFβ family members have been proposed to be, in part, responsible for the downstream effects of PKCe (36). Rat-6 fibroblasts which overexpress PKCe have been shown to secrete active forms of TGFβ 2 and TGFβ 3 in conjunction with a yet unidentified mitogen, indicating that growth-stimulating autocrine/paracrine loops may be involved in the oncogenic activity of PKCe (36). In addition to being able to transform rodent cells, gene transfer experiments have shown that PKCe regulates tumor invasion in Matrigel assays and nude mice (44). Evidence indicates that the actin binding site in the C1 domain of PKCe may be critical for invasion but not necessary for tumorigenicity, implying that PKCe must interact with the actin cytoskeleton to enhance metastatic potential (44). This interaction was shown to stimulate polymerization and extensions of actin protrusions used for penetration of the surrounding tissue (44). We found PKCe mediates the development of mSCC when overexpressed in the mouse skin elicited by the initiation (DMBA), promotion (TPA) protocol (19, 20). We now present that TNFα may be a downstream messenger in PKCe signaling network to the development of mSCC.

TNFα levels are chronically elevated in the serum during mouse skin tumor promotion (Fig. 1). TNFα is a highly regulated molecule, and the source of increased circulating levels during TPA promotion in PKCε transgenic mice can be explained. Experiments using radio-labelled TNFα revealed that TNFα is cleared from the serum with a half-life of 6–7 min (45). This suggests that PKCe transgenic mice maintain the serum levels by chronic release of this molecule. In this context, the effects of TPA on TNFα level in mouse epidermis are noteworthy. TPA treatment resulted in a ~2-fold increase in epidermal TNFα, whereas a ~6-fold increase in serum TNFα in PKCε transgenic mice as compared with their wild-type littermates. TPA treatment resulted in ~2-fold increase in epidermal TNFα mRNA levels in PKCε transgenic mice relative to their wild-type littermates (data not shown). This may account for the TPA-induced increase (2-fold) in epidermal TNFα levels. An alternative interpretation for the TPA-induced epidermal rise in TNFα may be attributable to increased shedding of TNFα from the epidermal keratinocytes to the serum, its clearance from the blood, and subsequent concentration in the skin. Experiments reported previously using radiolabelled TNFα indicated that 30% of serum TNFα concentrated to the skin. Addi-
tional data to support that PKCε affects ectodomain shedding are supported by the results illustrated in Figs. 3 and 4. The release of TNFα to the blood was directly proportional to the level of the transgene expressed in the skin of PKCε transgenic mice. Furthermore, primary keratinocytes from PKCε transgenic mice were more sensitive than keratinocytes from wild-type littermates to TPA-induced TNFα release. However, TPA-induced TNFα release was not completely prevented in PKCε-null keratinocytes, indicating that there may be functional overlap in the regulation of TNFα release (Fig. 5).

TACE is the key metalloprotease that catalyzes the shedding of the proform of TNFα into its mature soluble form (25, 26). To determine whether PKCε mediates the shedding of TNFα from epidermal keratinocytes via TACE, we inhibited TACE activity using the TACE inhibitor TAPI-1 (25). This blockade both in intact mouse skin in vivo and cultured primary keratinocytes in vitro (Fig. 6) indicated that TPA-mediated shedding of TNFα in PKCε transgenic mice is regulated through increased TACE activity. The TACE molecule is a member of the ADAM family (a disintegrin and metalloprotease domain). This enzyme is synthesized initially in a latent form which contains a highly conserved inhibitory prodomain (25, 26). It has been shown that this prodomain inhibits TACE catalytic activity by interaction between the thiol group from a cysteine residue in the prodomain and a zinc molecule in the catalytic domain (46, 47). Disruption of this cysteine-zinc bond results in conformational changes, resulting in an active molecule. Furthermore, it has been shown that ROS and nitrogen radicals can oxidize this zinc thiol bond and thus create an active enzyme (48–51). This activation mediated through ROS has been shown for TACE (27). To explore the role of ROS in PKCε-mediated TNFα shedding, we used the antioxidants CuDIPs and ebselen, a superoxide dismutase and glutathione peroxidase mimetic, respectively (Fig. 7). Both of these ROS scavengers completely prevented the release of TNFα in PKCε transgenic mice. Taken together, these results indicate that overexpression of PKCε may result in generation of ROS leading to an activation of TACE by oxidation of the zinc thiol cysteine bond.

The finding that the inhibition of TPA-induced TNFα shedding completely prevents the development of mSCC indicates that TNFα is perhaps the key downstream component of PKCε signaling pathway to the development of mSCC (Fig. 8). In this experiment (Fig. 8), we used a pharmacological inhibitor pentoxifylline to inhibit the synthesis of TNFα in vivo. Pentoxifylline is a methylxanthine derivative that has been used for >20 years to treat patients with peripheral vascular disease (21, 22). A generally accepted mechanism of action for

![Graph A: Serum TNF alpha (pg/ml)]

![Graph B: Papillomas/Mouse (Weeks of Promotion)]

![Graph C: % Carcinoma (Weeks of Promotion)]

![Graph D: Photographs of representative mice at 16 weeks of skin tumor promotion. WT-PBS, wild-type mice receiving the PBS vehicle; WT-PTX, wild-type mice receiving pentoxifylline; PKCε-PBS, PKCε transgenic mice receiving the PBS vehicle; PKCε-PTX, PKCε transgenic mice receiving pentoxifylline. Each photograph depicts littermates. ND, not detected.

Fig. 8. PKCε-mediated mSCC is completely prevented in PKCε transgenic mice (line 215) treated with the TNFα synthesis inhibitor pentoxifylline. PKCε transgenic mice (line 215) and their wild-type littermates were initiated with a single 100 nmol dose of DMBA and promoted twice weekly with 5 nmol of TPA. There were 25 mice per group. A, after 16 weeks of skin tumor promotion, serum was collected 2.5 h the last treatment regimen and analyzed for TNFα release in mice treated with pentoxifylline. Serum TNFα value is an average of duplicate determinations from serum samples pooled from four of the same mice. B, papilloma multiplicity. The error bars indicate the SE of the papilloma multiplicity for each papilloma count. At all time points, wild-type mice treated with pentoxifylline had a significant reduction in their average papilloma burden (P < 0.005). C, carcinoma incidence refers to the percentage of mice with at least one carcinoma. D, the photographs of representative mice at 16 weeks of skin tumor promotion. WT-PBS, wild-type mice receiving the PBS vehicle; WT-PTX, wild-type mice receiving pentoxifylline; PKCε-PBS, PKCε transgenic mice receiving the PBS vehicle; PKCε-PTX, PKCε transgenic mice receiving pentoxifylline. Each photograph depicts littermates. ND, not detected.
pentoxifylline is the inhibition of phosphodiesterases, leading to increased intracellular levels of cAMP which negatively regulate the synthesis of TNFα (52). Inhibiting the synthesis and thus release of TNFα from epidermal keratinocytes in PKCe transgenic mice completely prevented the development of mSCC (Fig. 8). A previous study using pentoxifylline has shown that it decreases cutaneous inflammation and has decreased the DMBTA/TPA-induced papilloma formation (53). This suggests a role of inflammation in the development of papillomas in mice, and increased release of TNFα in PKCe transgenic mice may have dramatic effects on s.c. inflammation and thus lead to the rapid development of mSCC with interactions of inflammatory cells in the epidermis. Because pentoxifylline is not a specific inhibitor of TNFα, the results illustrated in Fig. 8, B–D should be interpreted with caution. Pentoxifylline caused inhibition of the development of mSCC in PKCe transgenic mice may be attributable to the effect of pentoxifylline on epidermal cAMP levels. Increased epidermal cAMP levels have been shown to inhibit carcinoma formation in the mouse skin (54). To prove the link of TNFα to the PKCe signaling to the development of mSCC, additional experiments using TNFα KO mice crossed with PKCe transgenic mice are warranted. Furthermore, PKCe signaling to the development of carcinomas via TNFα may involve the role of the AP-1 family member of transcription factors (e.g., c-fos) and p53 (55–57).

In summary, the results indicate that: (a) PKCe is an initial signal in TPA-induced TNFα ectodomain shedding; (b) keratinocytes appear to be the primary source of TPA-stimulated TNFα shedding in PKCe transgenic mice; (c) PKCe may regulate TNFα processing in epidermal keratinocytes through ROS and the membrane bound metalloprotease TACE; and (d) blocking of TNFα shedding in PKCe transgenic mice by inhibiting its synthesis can completely prevent the development of mSCC in the mouse skin. We conclude that TNFα may be a useful biomarker for the prognosis of squamous cell carcinoma of the skin and that intervention by neutralizing TNFα may be considered for therapy of mSCC.

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