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Involvement of tumor necrosis factor (TNF)-α in phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice

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ABSTRACT

Topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA) to mouse ear induced a prolonged skin inflammation. Histological analysis revealed that the early stage (~3 h) and later stage (6–24 h) of the skin reaction are characterized by dermal edema and cell accumulation, respectively. Topical application with TPA also induced increase in the level of TNF-α and prostagrandin E_2 (PGE_2) at the application site. The increase of TNF-α was transient with a peak at ~5 h, followed by a gradual elevation of PGE_2 level in the skin. An in vitro study with human keratinocytes as well as immunohistochemical analysis suggested that TNF-α induction in the skin might be produced by epidermis treated with TPA. Administration of a cyclooxygenase inhibitor indomethacin inhibited the later stage of the TPA-induced edema. In contrast, TNF-α antagonist etanercept inhibited exclusively the early stage of the reaction. Taken together, these data demonstrate that the prolongation of the skin inflammation induced by TPA may be due to the sequential production of proinflammatory mediators such as eicosanoids and cytokines, and show for the first time the importance of TNF-α in the TPA-induced dermatitis especially at the stage where dermal edema is significant.

1. Introduction

Mouse ear edema induced by phorbol ester 12-O-tetradecanoylphorbol-13 acetate (TPA) has been used as an animal model for testing anti-inflammatory activity. In this model, the skin edema is known to be associated with an increase in the content of eicosanoids such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) [1,2]. Moreover, not only inhibitors for cyclooxygenase or 5-lipoxygenase, but also antagonists for LTB4 inhibit the TPA-induced skin edema [1–4]. These findings suggest that eicosanoids play an important role in the TPA-induced skin inflammation. The mechanism of the TPA-induced increase of eicosanoids is not completely known but thought to include activation of protein kinase C [5] as well as phospholipase A2 [6], induction of cyclooxygenase [7,8] and translocation/activation of lipoxygenase [9].

In addition to the induction of eicosanoid production, TPA has been shown to induce expression of proinflammatory cytokines in keratinocytes [10,11], which are also known to play some role in skin inflammation. For example, an antibody to TNF-α abrogates the skin swelling in contact dermatitis induced by trinitrochlorobenzene in mice [12]. In IgE-mediated biphasic skin reaction in mice, moreover, antigen challenge increases mRNA level of TNF-α, and treatment with anti-TNF-α antibody inhibits both early-phase and late-phase skin reactions of the dermatitis [13].

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In the present study, we examined possible role of TNF-α in the skin edema induced by topical application with TPA in mice. The data presented here demonstrate that TPA treatment increases the TNF-α level in the application site of TPA, and that TNF-α antagonist etanercept significantly inhibits in this model the skin edema but not cell infiltration, both of which are typical aspects of dermal inflammation. It is thus possible that TNF-α, in conjunction with eicosanoids, may contribute to the TPA-induced edema formation.

2. Materials and methods

2.1. Mice

BALB/c mice were purchased from Charles River Japan Inc. (Yokohama, Japan). All animal experiments were approved by Institutional Review Board for animal studies of Daiichi Asubio Pharma Co. Limited, Biomedical Research Laboratories.

2.2. Materials

TPA was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Indomethacin was obtained from Sigma–Aldrich Japan. Etanercept and human IgG were purchased from Amgen (Thousand Oaks, CA) and ICN Pharmaceuticals Inc. (Costa Mesa, CA), respectively. Both etanercept and human IgG were reconstituted according to the manufacturer's instructions, and resuspended in PBS using PD-10 desalting column (Amersham Biosciences KK, Tokyo, Japan).

2.3. TPA-induced skin edema in mice

Twenty microlitres of TPA (100 μg/ml, i.e. 162 μM, in acetone) was topically applied to each ear of BALB/c mice, and ear thickness was measured using a thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo, Japan) at various time points. Increase in ear thickness (edema) was determined by subtracting ear thickness before the first painting from that of each time point. Indomethacin was suspended in 0.5% of hydroxy propyl cellulose (Nippon Soda, Tokyo, Japan) and administered p.o. to the mice 30 min before the TPA application. Etanercept (in PBS, 1 mg/head) was injected i.v. twice (1 day and 1.5 h prior to the TPA application), and human IgG was injected similarly to the control mice instead of etanercept. For histological analysis, ear samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 μm, and then stained with hematoxylin and eosin. The number of dermal infiltrated cells was determined by counting the stained cells in a high-power field (×200) for each section.

2.4. Measurement of TNF-α and PGE_2 in the skin of TPA-induced edema model

For measurement of TNF-α and PGE_2 in the ear samples in TPA-induced skin edema model, ear biopsy samples were taken using a punch (a diameter of 8 mm, Kai Industries Co. Ltd., Gifu, Japan). For TNF-α, the biopsies were homogenized vigorously in 50 mM Tris–HCl buffer (pH 7.5) with 1 mM EDTA and protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan) (70 μl/biopsy), and their homogenate was incubated on ice for 15 min in the presence of 0.1% Triton X-100. In case of PGE_2, the biopsies were homogenized in PBS containing indomethacin (10 μM), AA-861 (10 μM) and protease inhibitor cocktail (200 μl/biopsy). Their homogenate was centrifuged at 10,000 × *g* for 10 min. After the centrifugation, TNF-α and PGE_2 in the supernatants were measured using ELISA for TNF-α (Biosource International, Camarillo, CA) and PGE_2 (Amersham Biosciences KK), respectively.

2.5. TNF-α production by human keratinocytes in vitro

Human keratinocytes (Cambrex Bioscience Walkersville Inc., Walkersville, ML) were cultured in the presence of TPA for 5 h, and TNF-α in the supernatants was measured by ELISA (BD Biosciences, San Jose, CA).

2.6. Immunostaining

Immunohistochemical staining was performed on paraformaldehyde-fixed, paraffin-embedded sections using skin samples taken as described above. The sections were initially deparaffinized with xylene and rehydrated through graded alcohols. Antigen unmasking in the sections was then performed by pretreatment with proteinase K for 30 s. After quenching the endogenous peroxidase activity with incubation in 3% H_2O_2, the sections were incubated overnight with anti-TNF-α antibody (R&D systems, AF-410-NA) at 4 °C. After washing, the sections were incubated with biotin-conjugated anti-goat IgG (DAKO, E0466) at room temperature for 30 min, and then incubated with HRP-conjugated avidin (DAKO, K1016) at room temperature for 30 min. To visualize the immune complex, 3,3′-diaminobenzidine was used, and the sections were counterstained with hematoxylin.

2.7. Statistical analysis

The statistical analysis was performed with Dunnett's multiple comparison test or Student's *t*-test using SuperANOVA (Abacus Concepts, Berkeley, CA) or Statview (SAS Institute Inc., Cary, NC), respectively. The *p*-value less than 0.05 was considered significant.

3. Results

3.1. TPA-induced skin edema and histological changes

Possible role of TNF-α in skin inflammation was examined using the acute skin edema model induced by topical application with TPA in mice. As shown in Fig. 1A, skin thickness started to increase at 3 h following TPA application, reached to the plateau by 9 h and persisted for at least 24 h after the application. Histological analysis revealed that the TPA-induced skin inflammation divided into at least two stages. That is, at earlier stage (around 3 h following the TPA painting), thickening of dermis was evident (Fig. 1C) as compared with the sections of normal skin (Fig. 1B), but cell infiltration was little, if any (Fig. 1B and C). In contrast, at the later stage (6 h or thereafter), cell infiltration became

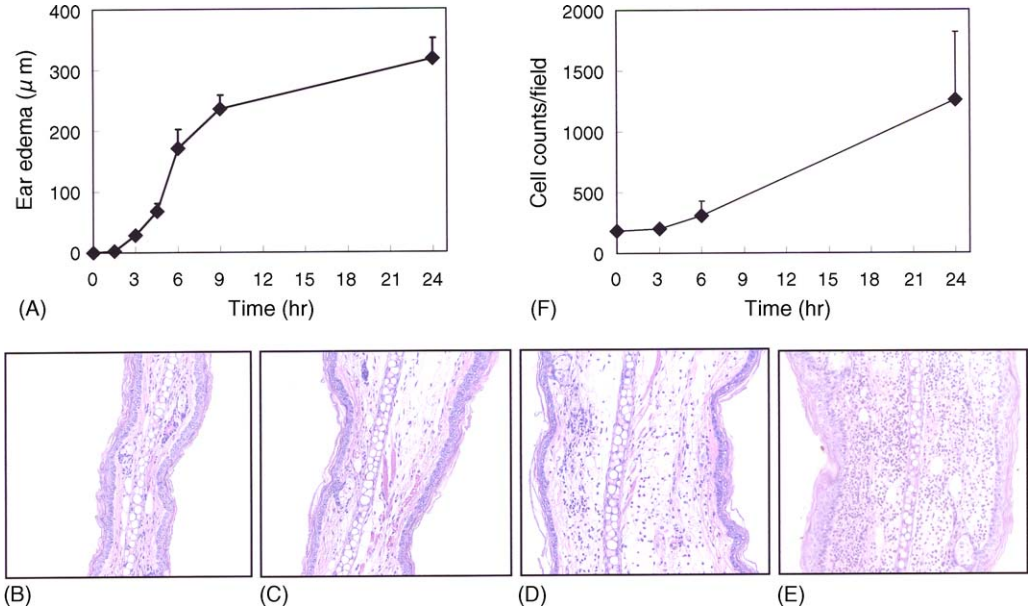

Fig. 1 – Change in skin thickness and infiltrated cells in dermis following topical application of TPA. Twenty microlitres of TPA (100 μg/ml in acetone) was applied to each ear of BALB/c mice, and the increase of ear thickness (A) was determined as described in Section 2. Data are mean ± S.D. ($N = 7$). For histological analysis (B–E), ear skin sections were prepared as described in Section 2 and stained with hematoxylin and eosin. (B) Normal skin; (C) 3 h; (D) 6 h; (E) 24 h after TPA treatment. The number of infiltrating cells in dermis (F) was counted in high-power fields (×200) (mean ± S.D., $N = 3$).

remarkable in addition to dermal edema (Fig. 1D and E). Cell accumulation still remained notable at 24 h (Fig. 1E). Counting the cell number in dermis showed that the cell number in the TPA-treated skin at 24 h was approximately six-fold that at 3 h (Fig. 1F).

3.2. *Production of TNF-α and eicosanoids in the skin by topical application with TPA*

The TPA application also increased the level of eicosanoid PGE_2 as well as that of TNF-α in the skin samples (Fig. 2). However, their kinetics were quite different each other. The level of TNF-α in the skin lesion began to increase at around 1.5 h, showed a maximal level at 4.5 h and then returned to the basal level by 24 h after the application. By contrast, the increase in the PGE_2 level occurred in a biphasic manner, with a slight transient reaction peaking at 3 h, followed by a gradual and significant rise persisting at least for 24 h. The first peak was trivial but likely important, as the PGE_2 level at 3 h was significantly higher than that at 1.5 h as well as that at 4.5 h ($p < 0.01$, Student's *t*-test). These findings suggest that TNF-α and PGE_2 may participate in distinct stages in the TPA-induced skin inflammation.

3.3. *Production of TNF-α in keratinocytes by TPA treatment*

Next, we examined further the ability of TPA to induce TNF-α production in vitro and in vivo. As shown in Fig. 3A, an addition of TPA to the culture of human keratinocytes resulted in increase of TNF-α content in the supernatant, with a maximal effect at 100 ng/ml (162 nM). This result suggests that the induction of TNF-α in the skin by the topical application with TPA is, at least in part, derived from epidermal keratinocytes. Consistently, immunohistochimical analysis revealed that TNF-α exists principally around epidermal keratinocytes in the TPA-treated skin (Fig. 3B), while little TNF-α protein was detected in the normal skin sample (data not shown). The localization of TNF-α induced by TPA is compatible with the previous data reported by Moore et al. [14].

3.4. *Effect of cyclooxygenase inhibitor on TPA-induced skin edema, cell infiltration and PGE_2 production*

The effect of inhibitors for cyclooxygenase pathways on TPA-induced skin inflammation has been extensively studied [1–4]. However, there is little report showing the detailed relationship between the kinetics of the production of metabolites of the pathways and the effect of such inhibitors. As shown in Fig. 4A, indomethacin (cyclooxygenase inhibitor) inhibited the TPA-induced edema in a dose-dependent manner. The effect of indomethacin was significant at later stage (6–24 h) but not at early stage (3 h). In contrast, indomethacin did not affect the TPA-induced cell infiltration at the late-phase (24 h) of the reaction (Fig. 4B), though it strongly blocked the production of PGE_2 in the skin (Fig. 4C). These results imply that PGE_2 does not contribute to cell infiltration, while the effect of indomethacin on skin edema is evident at late-phase where cell infiltration was significant.

3.5. *Effect of etanercept on TPA-induced skin edema, cell infiltration and PGE_2 production*

Etanercept is a TNF-α antagonist that is a soluble fusion protein composed of the extracellular domains of the human TNF-α receptor (p75) fused to the Fc region of human IgG1. This antagonist has been shown to exert inhibitory activity to

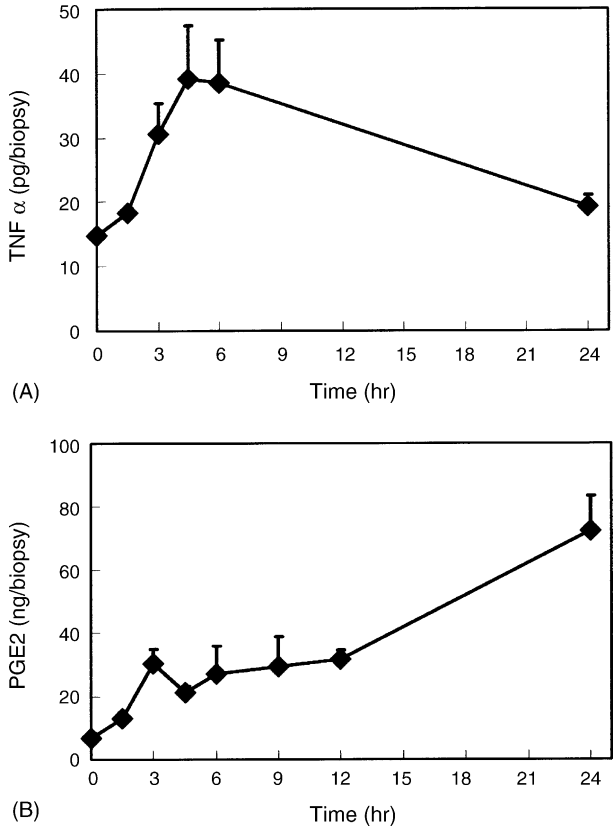

Fig. 2 – Effect of topical application of TPA on content of TNF-α and PGE_2 in the application site. Twenty microlitres of TPA (100 μg/ml in acetone) was applied to each ear of BALB/c mice, and ear biopsy samples were taken at the indicated time. The levels of TNF-α (A) and PGE_2 (B) in the skin were quantitated by ELISA as described in Section 2. Data are mean ± S.D. (*N* = 4–5).

mouse TNF-α in mouse models [15]. Thus, the effect of etanercept was examined on TPA-induced skin inflammation in mice so as to elucidate possible importance of TNF-α in the reaction. As shown in Fig. 5A, i.v. injection of etanercept reduced the TPA-induced skin swelling at early stage (3–6 h) of the TPA application, while little effect was detected by the treatment with PBS or human IgG. Etanercept injection, however, showed no effect on ear edema (Fig. 5A), cell accumulation (Fig. 5B) and PGE_2 production (Fig. 5C) at the later stage (24 h) of the skin reaction.

4. Discussion

The data in the present study showed that the skin edema induced by TPA is accompanied by increase in the level of TNF-α and PGE_2 at the inflamed site. The increase of TNF-α peaked at ~5 h following the application (Fig. 2), whereas the increase of PGE_2 was biphasic, composed of a slight transient response peaking at 3 h and a gradual but substantial increase lasting for 24 h. The effects of the TNF-α antagonist and the inhibitor of PGE_2 production on the TPA-induced edema seem to correlate with the increase of TNF-α and PGE_2 in the skin, respectively. Namely, indomethacin showed inhibitory activity at later stage of the skin reaction (Fig. 4A), while the effect of etanercept was significant at early stage (Fig. 5A). Our findings suggest that continuation of the TPA-induced skin edema reflects the sequential production of proinflammatory mediators, and that TNF-α also takes part in this reaction. The involvement of TNF-α in the TPA-induced skin inflammation may be in agreement with the data of Moore et al. who have reported that TNF-α-deficient mice fail to develop skin inflammation following the treatment with TPA [14].

Our data also showed that TPA induced the production of TNF-α by human keratinocytes in vitro (Fig. 3A), suggesting that TNF-α produced in vivo might be released from epidermis treated with TPA. Supporting this notion is that the TPA-induced TNF-α is mainly localized in or close to the epidermis (Fig. 3B). The mechanism by which TPA induces TNF-α production in keratinocytes is not fully explored to date. However, it has been shown that TPA regulates TNF-α production at both transcriptional and posttranscriptional levels in human monocytes [16]. The induction of TNF-α mRNA by TPA in human HL-60 cells is blocked by an inhibitor of protein kinase C or phospholipase A2 inhibitor [17],

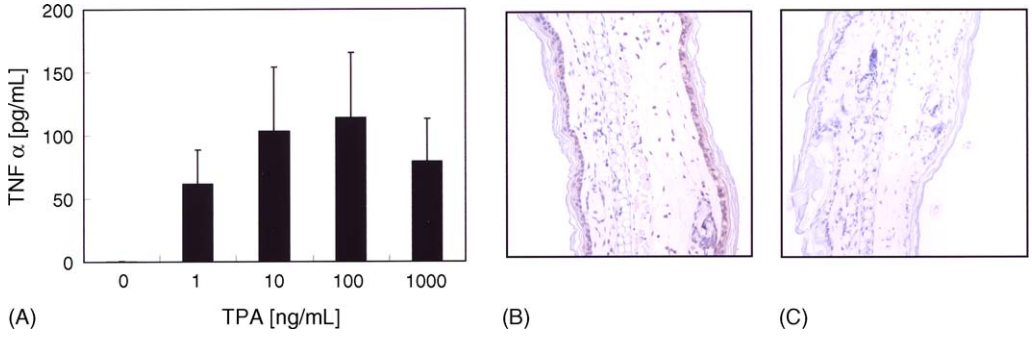

Fig. 3 – Effect of TPA on TNF-α production by keratinocytes in vitro and in vivo. (A) Human keratinocytes were seeded at 1.5×10^4 cells/well and cultured for 18 h, and then TPA was added to the culture. The cells were incubated for additional 5 h and the concentration of TNF-α in the supernatant was determined by ELISA. Data are mean ± S.D. (*N* = 3). (B, C) 20 μl of TPA (100 μg/ml in acetone) was applied to each ear of BALB/c mice, and ear biopsy samples were taken at 6 h and subjected to immunostaining with anti-mouse TNF-α antibody as described in Section 2. (B) TPA-treated (with anti-TNF-α antibody); (C) TPA-treated (with second antibody only).

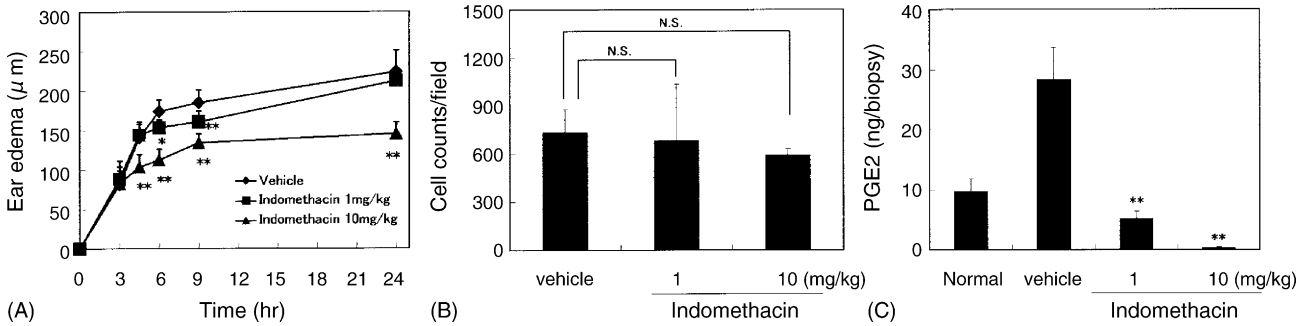

Fig. 4 – Effect of indomethacin on TPA-induced skin edema, cell infiltration and PGE_2 production. Twenty microlitres of TPA (100 μg/ml in acetone) was applied to each ear of BALB/c mice, and the increase of ear thickness (A), cell number in dermis (B) and PGE_2 content (C) were determined as described in Section 2. Indomethacin was suspended in 0.5% of hydroxy propyl cellulose and administered p.o. to the mice 30 min before the TPA application. The cell number and PGE_2 content were examined at 24 h after TPA treatment. Data are mean ± S.D. ($N = 7$ for A, $N = 5$ for B and C). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle control; N.S., not significant (Dunnet's test).

suggesting their role in the TPA-induced TNF-α production. Moreover, it is likely that TNF-α converting enzyme (TACE) and reactive oxygen species also mediate the TPA-induced TNF-α production [18].

The association of TNF-α with skin edema is likely explained by the ability of TNF-α to increase vascular permeability. For instance, TNF-α has been shown to increase the permeability of endothelial cell monolayers to macromolecules and lower molecular weight solutes by a mechanism involving a pertussis toxin-sensitive regulatory G protein [19]. The effect of TNF-α on vascular permeability is probably mediated by its specific receptors [20], leading to cytoskeletal changes and intercellular gap formation [21]. The idea that contribution of TNF-α in TPA-induced skin inflammation is mediated by its ability to increase vascular permeability is consistent with the finding that etanercept inhibit the early stage of the skin reaction, at which the major histological change is dermal edema.

It has been shown that anti-TNF-α antibody inhibited both early- and late-phase edematous responses in IgE-mediated biphasic cutanous reaction in mice [13], which is in marked contrast to our finding that the later response is not inhibited by etanercept. The difference of the effect of TNF-α inhibition could be accounted for by the mechanism of the model, e.g. TNF-α is likely released from mast cells upon IgE-mediated stimulation of the cells in the IgE-mediated biphasic dermatitis [13], whilst the major source of TNF-α is probably keratinocytes in our model. The release of TNF-α from mast cells might be more continuous as compared with that from keratinocytes. Alternatively, the difference between anti-TNF-α antibody and etanercept might explain the different effect in the two studies.

Another interesting point in the present study is that TPA induces PGE_2 production in a biphasic manner (Fig. 2). It has been reported that sialidase treatment, which affects the structure of selectins and inhibits leukocyte influx, significantly reduced the tissue level of PGE_2 and cyclooxygenase in the TPA-induced skin inflammation in mice [22]. It is thus possible that the PGE_2 production at the late-phase depends on the infiltrated leukocytes. The failure of etanercept to inhibit cell infiltration and PGE_2 production at the late-phase (Fig. 5) is consistent with this idea, and also suggests that TNF-α may not be related to cell infiltration in this model. The mechanism of early-phase PGE_2 production is not clear at present, but TPA is known to induce PGE_2 generation in mouse and human keratinocytes in vitro [23], suggesting direct action of TPA on keratinocytes to produce PGE_2 at the early stage of the reaction.

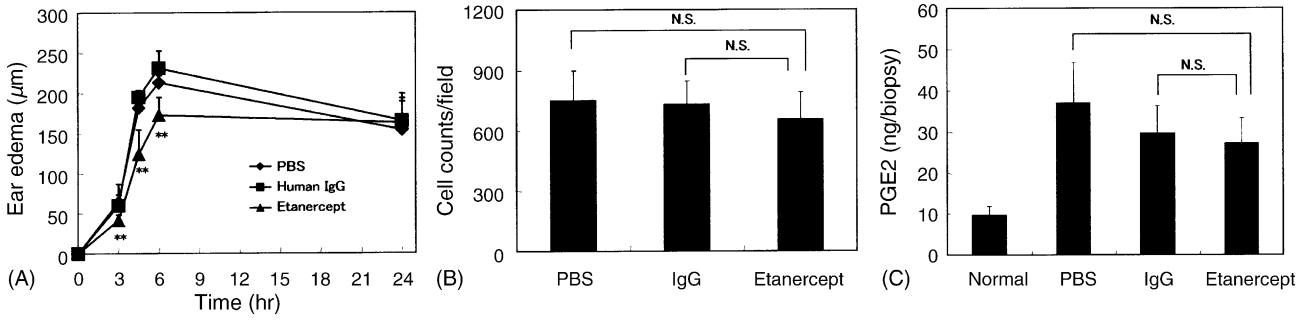

Fig. 5 – Effect of etanercept on TPA-induced skin edema, cell infiltration and PGE_2 production. Twenty microlitres of TPA (100 μg/ml in acetone) was applied to each ear of BALB/c mice, and the increase of ear thickness (A), cell number in dermis (B) and PGE_2 content (C) were determined as described in Section 2. Etanercept in PBS was injected i.v. to the mice twice (1 day and 1.5 h prior to the TPA application). Human IgG was injected similarly to the control mice instead of etanercept. The cell number and PGE_2 content were examined at 24 h after TPA treatment. Data are mean ± S.D. ($N = 7$ for A, $N = 5$ for B and C). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control; N.S., not significant (Student's t-test).

TPA is also known as a potent tumor promoter. Recently, it has been reported that TNF-α-deficient mice were resistant to papilloma development after treatment of skin with 9,10-dimethyl-1,2-benzanthracene followed by promotion with TPA for 15 weeks, compared with corresponding wild-type mice [24]. Treatment with monoclonal antibody to TNF-α in this papilloma development model during the early stages of tumor promotion (0–6 weeks) significantly decreased skin tumor development, suggesting that TNF-α is also critical for skin tumor promotion induced by TPA. In contrast to TNF-α, however, TPA-induced tumor promotion is enhanced by indomethacin [25]. Thus, the mechanisms underlying the inflammation and tumor promotion induced by TPA may not necessarily be the same.

In conclusion, our data indicate that TPA induces TNF-α production and release in the skin, and produces a biphasic response; one is TNF-α-dependent, and the other is not. It is not clear whether or not our findings relate to what occurs clinically, as the changes induced by TPA in the skin may be different from those occurring in human dermatitis such as eczema and psoriasis. While some cursory evidence implicating PKC in the early-phase of dermatitis do exist, further molecular characterization of clinical dermatitis and TPA-induced dermatitis would solidify this model and the context with which our findings might apply.

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