

# Suppression of Transglutaminase-2 is Involved in Anti-Inflammatory Actions of Glucosamine in 12-O-Tetradecanoylphorbol-13-Acetate-Induced Skin Inflammation

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## Abstract

Glucosamine (GS) is well known for the treatment of inflammation. However, the mechanism and efficacy of GS for skin inflammation are unclear. The aim of this study was to evaluate the effects and mechanism of GS in the mouse 12-O-tetradecanoyl 13-acetate (TPA)-induced ear edema model. TPA-induced ear edema was evoked in ICR or transglutaminase 2 (Tgase-2) (-/-) mice. GS was administered orally (10-100 mg/kg) or topically (0.5-2.0 w/v %) prior to TPA treatment. Orally administered GS at 10 mg/kg showed a 76 or 57% reduction in ear weight or myeloperoxidase, respectively, and a decreased expression of cyclooxygenase-2 (COX-2), NF- $\kappa$ B and Tgase-2 in TPA-induced ear edema by western blot and immunohistochemistry. Role of Tgase-2 in TPA ear edema is examined using Tgase-2 (-/-) mice and TPA did not induce COX-2 expression in ear of Tgase-2 (-/-) mice. These observations suggested that Tgase-2 is involved in TPA-induced COX-2 expression in the inflamed ear of mice and anti-inflammatory effects of glucosamine is mediated through suppression of Tgase-2 in TPA ear edema.

**Key Words:** Glucosamine, TPA-induced ear edema, Transglutaminase-2, Cyclooxygenase-2, NF- $\kappa$ B, Tgase-2 (-/-) mice

## INTRODUCTION

Glucosamine (GS), 2-amino-2-deoxy-D-glucose, is an amino monosaccharide that is one of the essential components of mucopolysaccharides and chitin. Glycosaminoglycans are components of connective tissue, skin, tendons, ligaments and cartilage. GS is readily synthesized in the body from glucose. Given the high concentration in joint tissues, the hypothesis that GS supplements would relieve the symptoms of osteoarthritis (OA) was developed more than 30 years ago (D'Ambrosio *et al.*, 1981). These effects of GS were shown in carrageenan- and cotton pellet-induced acute and subacute inflammation in rats at 25 mg/kg dose (Kim *et al.*, 2005). GS at 250 mg/kg showed a mild effect in carrageenan-induced edema and moderate inhibition of paw swelling against developing arthritis (Singh *et al.*, 2007).

Recently, glucosamine showed positive effects in atopic dermatitis-like skin lesions in NC/Nga mice via inhibition of

Th2 cell development (Kim *et al.*, 2011a). Combination treatments of glucosamine with FK-506 also produces a beneficial effects in atopic dermatitis-like skin lesions in NC/Nga mice (Kim *et al.*, 2012).

GS was also tested as a constituent of a new anti-inflammatory formulation (SAG) in a 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema model. SAG dose-dependently inhibited the edematous responses of arachidonic acid (AA)- and TPA-induced ear edema in mice (Choi *et al.*, 2005). But, in this case, efficacy of glucosamine by itself was not shown in TPA-induced ear edema and detailed histological studies on the effects of glucosamine were not done.

TPA promotes skin carcinogenesis via inflammatory responses and TPA-induced inflammatory responses are related with induction of pro-inflammatory cytokines, cyclooxygenase-2, reactive oxygen species and NF- $\kappa$ B (Chung *et al.*, 2007; Song *et al.*, 2008).

Recently, glucosamine was reported to act as a chemo-

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sensitizer via inhibition of transglutaminase-2 (Tgase-2) in doxorubicin-resistant MCF7 cells (Kim *et al.*, 2009; Jeong *et al.*, 2010).

Tgase-2 is a multifunctional protein with both intracellular and extracellular functions. In addition to catalyzing Ca<sup>2+</sup>-dependent transamidation reactions (Lorand and Graham, 2003; Mehta, 2005; Lee and Kim, 2009), it can bind and hydrolyze GTP/GDP with a similar affinity and catalytic rate to the  $\alpha$  subunit of large heterotrimeric G proteins and small Ras-type G proteins (Mhaouty-Kodja, 2004). Tgase-2 can activate NF- $\kappa$ B via polymerization of I- $\kappa$ B (Lee *et al.*, 2004). But role of Tgase-2 is not known in TPA-induced skin inflammation.

Therefore, we were interested in the effect and related mechanism of glucosamine on Tgase-2 in TPA-induced ear edema. To our knowledge, the role of Tgase-2 in TPA ear edema has not been reported yet.

In this report, we evaluated the efficacy of glucosamine in a TPA-induced dermatitis model and found that Tgase-2 expression suppressed by glucosamine is involved in the anti-inflammatory action of glucosamine by TPA-induced inflammation.

## MATERIALS AND METHODS

### Materials

Primary antibodies purchased: (1) rabbit polyclonal murine COX-2 antibody (Cayman Chemical, Ann Arbor, MI, USA), (2) rabbit polyclonal anti-NF- $\kappa$ B p65 antibody (Novus biological, Littleton, CO, USA), (3) polyclonal anti-actin antibody (Santa Cruz, CA, USA).

### Animals

Male ICR mice (Orientbio, Seoul, Korea), 7 weeks old were used in this experiment. They were acclimatized in the animal room at least 1 week prior to use. Throughout the experimental period, animals had free access to water and a commercial diet. The mice were randomly assigned into groups consisting of five animals per group, and were fasted overnight prior to experimentation. Tgase-2 knockout mice (C57BL/6) used were established by Dr. Ho Lee (Kim *et al.*, 2010). The experiments were conducted under the guidelines for the care and use of experimental animals of the Korea Association for Laboratory Animal Science.

### 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced mouse ear edema

Edema was induced on the right ear by topical application of 20  $\mu$ l of 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma, St. Louis, USA) in acetone (2.5  $\mu$ g/ear) with a micropipette (De Young *et al.*, 1989; Kim *et al.*, 2011b). To evaluate the inflammatory effects of TPA, ear lobe samples were collected using a 6 mm biopsy punch, weighed and measured for MPO (myeloperoxidase) levels 24 h after TPA application. The percentage of inhibition (% I) was calculated according to the following formula:

$$\% I = [1 - \text{Rt. Ear (Drug- No. treated)} / \text{Rt. Ear (TPA only- No. treated)}] \times 100.$$

### Myeloperoxidase (MPO) assay

The ear samples were placed in 0.5 ml of 80 mM sodium phosphate buffer, pH 5.4, containing 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma) (Bradley *et al.*, 1982)

and homogenized for 45 sec at 0°C, after which they were sonicated in an ice bath for 10 sec (Heat Systems-Ultrasonics, Plainview, NY). The sonicated samples were centrifugated at 12,000X g at 4°C for 15 min. Fifty  $\mu$ l of supernatant was added to a 96-well plate, and then 200  $\mu$ l of a mixture containing 0.167 mg/ml o-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxidase (Sigma) were added to each of the wells. The change of absorbance at 460 nm was measured with a SPECTRAMax<sup>®</sup> 190 microplate spectrophotometer (Molecular Device Corporation, Calif., USA).

### Evaluation of anti-inflammatory effects of glucosamine

Animals were orally administered 0.5 ml of D (+)-GS hydrochloride (Sigma) both immediately and 6 h after TPA application at doses of 5 mg/kg, 10 mg/kg or 20 mg/kg, respectively. The vehicle control animals were administered sterilized saline, and the positive control animals, with 10 mg/kg of dexamethasone. Also, the mice were topically administered 20  $\mu$ l of D(+)-GS hydrochloride (Sigma) in vehicle, both immediately and 6 h after TPA application at concentrations of 0.5% (w/v), 1% (w/v) and 2% (w/v) for the three groups, respectively. GS hydrochloride (Sigma) was dissolved in a mixture of distilled water (one part) and acetone (nine parts) for topical application. The vehicle control animals were topically administered a DW:acetone solution (1:9) and the positive control animals, 0.5% hydrocortisone.

### COX-2 and Tgase-2 western blot

Proteins from ear tissues were extracted in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.6), 1 mmol/L EDTA, 140 mmol/L NaCl, 1% NP40, 1% protease inhibitor, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium vanadate. Equivalent amounts of proteins from each treatment group were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotted with primary antibodies.

### Histological analysis

For histological analysis, ear tissue was fixed in 10% neutral buffered formaldehyde and embedded in paraffin wax according to standard methods. Sections were stained with hematoxylin and eosin.

Immunohistochemistry was performed by standard ABC technique. Tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. Antigenic retrieval was performed by pressure cooking in 10 mM citric acid buffer (pH 6.0). Hydrogen peroxide (3%) was used to quench endogenous peroxidase activity for 10 min. For blocking buffer, 10% normal goat serum was used for 30 min. Sections were then incubated with primary antibody for 1 hour at room temperature. Biotinylated goat anti-rabbit IgG antibody and ABC solution (Vector Laboratories, Burlingame, CA, USA) were applied sequentially. Diaminobenzidine (DAB) was used to visualize a positive signal. Immunostained sections were lightly counterstained in hematoxylin according to the manufacturer's instructions, dehydrated in graded ethanol, cleared in xylene and mounted with a coverslip using Canada balsam (Junsei Chemical, Tokyo, Japan).

### Statistics

All data are presented as means and S.D. Statistical significance was analyzed using a student's t-test;  $p < 0.05$  was

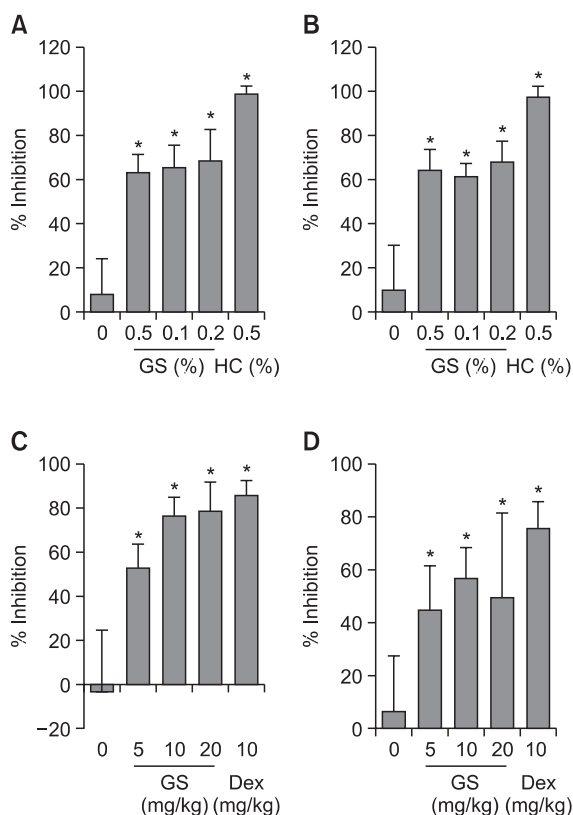
considered significant when compared with the TPA-only control group.

## RESULTS

### Effects of GS in TPA ear edema model

Twenty  $\mu$ l of D (+)-GS hydrochloride in vehicle were topically administered to the mice both immediately and 6 h after TPA application at concentrations of 0.5% (w/v), 1% (w/v) and 2% (w/v), respectively. GS hydrochloride was dissolved in a mixture of distilled water (one part) and acetone (nine parts) for topical application. We measured the weights of punched ear as well as MPO activity. After topical administration, GS hydrochloride showed significant anti-inflammatory effects in the TPA-induced ear edema mouse model evaluated by ear weight or MPO activity (Fig. 1A, 1B). Hydrocortisone (0.5%) was used as positive control and showed 99% and 97% inhibition in ear weight and MPO analysis, respectively.

Then, the animals were orally administered 0.5 ml of D(+)-GS hydrochloride both immediately and 6 h after TPA application at doses of 5 mg/kg, 10 mg/kg and 20 mg/kg, respectively. Oral administration of the GS hydrochloride showed significant anti-inflammatory effects in the TPA-induced skin inflam-



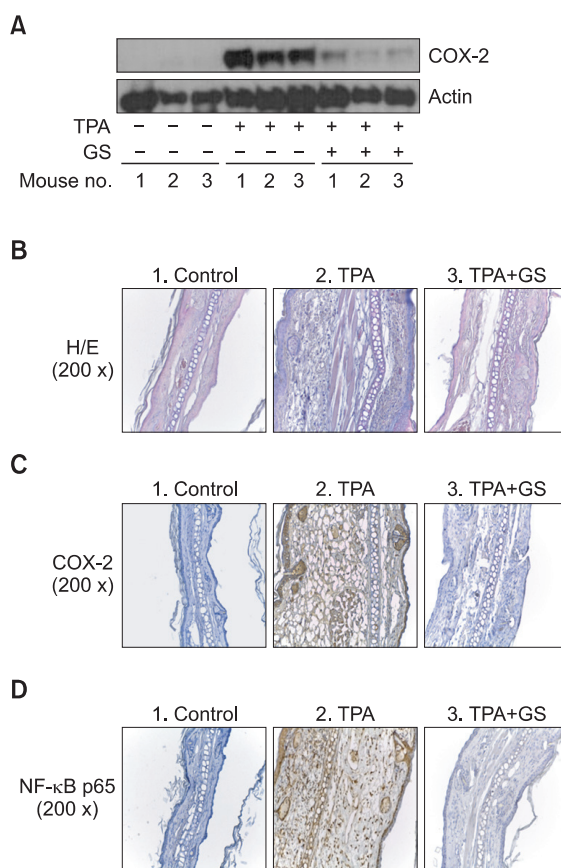
**Fig. 1.** Effects of GS on TPA-induced ear edema. GS (0.5-2% (w/v)) was applied topically to the ear of mice at the same time as a TPA application (2.5  $\mu$ g/ear; A, B), or orally administered (5-20 mg/kg; C, D). Ear edema was measured 4 h after application of TPA. Data are the means  $\pm$  SD of 10 animals. \* $p$ <0.05, relative to the control group, was considered to be significant. Dexamethasone or hydrocortisone was used as a positive control.

mation model (Fig. 1C, 1D). Specifically, GS at the doses of 5 and 10 mg/kg showed the strongest anti-inflammatory effects, that is, about 76-78% inhibition according to ear weights; further, there was a 50-57% inhibition in the MPO analysis (Fig. 1C, 1D). Dexamethasone (10 mg/kg) was used as positive control and showed 86% and 75% inhibitions in ear weight and MPO activity, respectively.

### Effects of orally administered GS on COX-2 expression and NF- $\kappa$ B activation in the TPA ear edema model

We examined the effects of GS on NF- $\kappa$ B target genes such as COX-2 in the TPA-ear edema model to confirm the anti-inflammatory effects of GS in an *in vivo* model as others have reported *in vitro* (Largo *et al.*, 2003).

In Fig. 2, it was confirmed by western blot that COX-2 was highly expressed in TPA-treated ears of mice compared with



**Fig. 2.** Effects of GS on COX-2 expression and NF- $\kappa$ B activation in the TPA-induced ear edema model. Three experimental groups were included three ICR mice/group. Group 1 received acetone, group 2 received TPA, group 3 received TPA and GS (10 mg/kg body weight). Edema was induced in both ears of the each mouse by topical application of 2.5  $\mu$ g TPA dissolved in 25  $\mu$ l of acetone to the inner ear surfaces. (A) Effect of GS on-TPA-induced changes of COX-2 protein in mouse ear skin. (B) For histological analysis, ear skin was prepared and stained with hematoxylin and eosin. Control: Normal skin, TPA: TPA-treated skin, TPA+GS: TPA plus orally administered GS skin. (C) 25  $\mu$ l of TPA (100  $\mu$ g/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 COX-2 antibody (1:10 dilution ratio). (D) Immunostaining with anti-mouse p65 of NF- $\kappa$ B antibody (1:50 dilution ratio).

the vehicle treated ear group. Orally GS administration at 10 mg/kg showed suppressed expression of COX-2 (Fig. 2A).

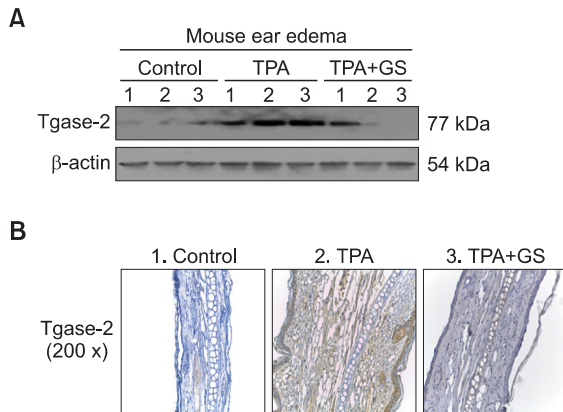
In histological studies, TPA treatment produced a marked increase in ear thickness and an abundant infiltration of inflammatory cells in the epidermis and dermis whereas the acetone-treated vehicle control group did not show histological and ear thickness changes. In contrast, the GS-treated group showed a remarkable reduction in ear thickness and inflammatory response in the epidermis and dermis (Fig. 2B).

Immunohistochemical analysis of COX-2 was performed. Strong positive signals for COX-2 were mostly found in epidermis. In the TPA-treated group, epidermal hyperplasia and high levels of COX-2 expression were found. On the contrary, the TPA and GS-treated group showed mild to moderate epidermal hyperplasia and dermal edema. The expression of COX-2 in the epidermis was remarkably suppressed in the TPA and GS-treated group; the acetone treated group was provided for comparison (Fig. 2C).

Immunolabelling of NF-κB p65 was found in epidermal keratinocytes and inflammatory cells in the epidermis and dermis. Cytoplasmic staining of NF-κB p65 was mostly found in epidermal keratinocytes, while nuclear staining of NF-κB p65 was found in inflammatory cells. Inflammatory cells within the lesion consisted of lymphocytes and a small number of neutrophils. NF-κB p65 was strongly expressed in lymphocytes and inflammatory cells which were present in dermis and epidermis of the TPA-treated group as well as the TPA and GS co-treated group. However, the number of inflammatory cells in the TPA and GS co-treated group was much smaller than in TPA-treated group (Fig. 2D).

**Effects of orally administered GS on Tgase-2 expression in the TPA ear edema model**

Considering the observation that GS suppressed COX-2 expression and inhibited Tgase-2 (Kim *et al.*, 2009), we exam-



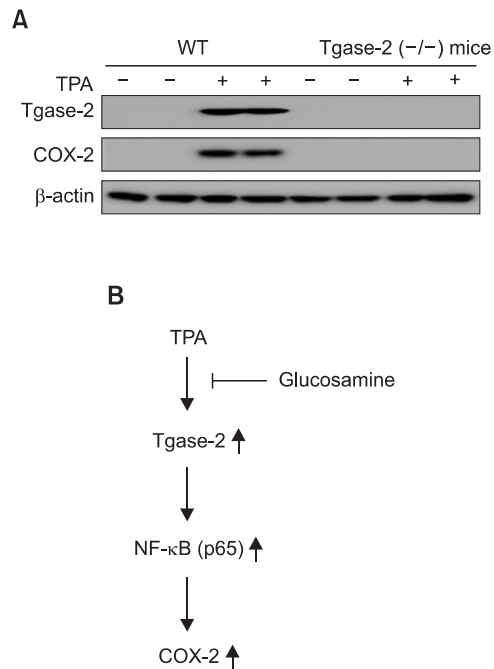
**Fig. 3.** Effects of orally administered GS on Tgase-2 expression in the TPA ear edema model. Three experimental groups included three ICR mice/group. Group 1 received acetone, group 2 received TPA, group 3 received TPA and GS (10 mg/kg body weight). Edema was induced in both ears of each mouse by topical application of 2.5 μg TPA dissolved in 25 μl of acetone to the inner ear surfaces. (A) Effect of GS on TPA-induced changes in Tgase-2 protein in mouse ear skin. (B) 25 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 Tgase-2 antibody (1:50 dilution ratio).

ined whether GS suppressed the expression of Tgase-2 in the TPA-ear edema model. At first, we examined the expression of Tgase-2 in TPA-treated ears of mice. As a result, the expression of Tgase-2 was increased in the TPA-treated group compared with vehicle control groups (Fig. 3A). We tested whether GS influenced the expression of Tgase-2 since Tgase-2 is one of target genes in the activation of NF-κB. As a result, the 10 mg/kg GS treated mice group showed suppression of Tgase-2 expression compared with the TPA only treated group determined by western blot (Fig. 3A).

Using immunohistochemical analysis, the expression of Tgase-2 was found in epidermal keratinocytes and inflammatory cells (Fig. 3B). Immunostaining pattern for Tgase-2 was nuclear and cytoplasmic. In the TPA-treated group, a high level of Tgase-2 expression was found in hyperplastic epidermal keratinocytes and lymphocytes in addition to inflammatory cells within the dermis and blood vessels. The expression of Tgase-2 in the TPA and GS co-treated group was less than that of TPA-treated group. On the contrary, expression of Tgase-2 was not found in vehicle control group (Fig. 3B).

**Tgase-2 involvement in COX-2 expression by TPA-induced ear edema**

To examine the effect of Tgase-2 on COX-2 expression in TPA ear edema, we topically applied TPA to ears of wild-type and Tgase-2 (-/-) mice in the same manner described in the Methods and ear tissue of TPA-treated Tgase-2 (-/-) were examined for COX-2 and Tgase-2 expression by western blot



**Fig. 4.** Tgase-2 involvement in COX-2 expression after TPA-induced ear edema. Two experimental groups included three or two wild type mice or Tgase-2 (-/-) mice/group. Group 1 received acetone, group 2 received TPA. Edema was induced in both ears of each mouse by topical application of 2.5 μg TPA dissolved in 25 μl of acetone to the inner ear surfaces. (A) Effects of TPA on Tgase-2 and COX-2 expression on ear skin of wild type mice or Tgase-2 (-/-) mice. (B) Scheme of involvement of Tgase-2 and effects of glucosamine on TPA-induced COX-2 expression.

analysis (Fig. 4A). Ears of TPA-applied mice showed induction of COX-2 and Tgase-2 but the increased expression of COX-2 was not observed in Tgase-2 (-/-) mice (Fig. 4A).

## DISCUSSION

In this study, we found glucosamine has anti-inflammatory activity on TPA-induced skin inflammation. Glucosamine suppressed Tgase-2 expression in inflamed ear tissue by TPA. The notion that COX-2 expression induced by TPA is related to expression of Tgase-2 is confirmed using Tgase-2 (-/-) mice.

GS has long been used by patients with arthritis. Glucosamine sulfate with potassium salt (GS-K) is effective in pain relief and is safe as glucosamine sulfate with sodium salt (GS-Na) for treatment of mild and moderate degrees of knee osteoarthritis (Wangroongsub *et al.*, 2010). Recently, efficacy of glucosamine was reported in a model of chronic skin inflammation including NC/Nga mice with or without FK-506 treatment (Kim *et al.*, 2012). However, the detailed role of GS as related to Tgase-2 has not been reported in these dermal inflammation models.

GS can decrease nitric oxide production and even control neutrophil functions (i.e. expression of adhesion molecules, p38 phosphorylation and chemotaxis) through the inhibition of NF- $\kappa$ B-dependent gene expression (Shikhman *et al.*, 2001; Hua *et al.*, 2002). But, how GS affects the NF- $\kappa$ B pathway is not clear. Recently, our colleagues reported that GS can act as a Tgase-2 inhibitor modulating the actions of NF- $\kappa$ B (Kim *et al.*, 2009).

As shown in Fig 1A and 1B, dose-dependency was difficult to achieve with topical application of GS in suppressing TPA-induced ear edema in mice. This reflects some of the hydrophilic characteristics of GS and the vehicle used (acetone-water). In Fig. 1C and 1D, GS demonstrated very good anti-inflammatory activity in the TPA ear edema model (Fig. 1C, 1D). These results suggested that GS might be also useful in controlling acute inflammation such as TPA-induced dermatitis.

GS suppressed COX-2 expression in the TPA-ear edema model (Fig. 2A). These results are consistent with the effect of GS in several cell lines such as macrophages and human chondrocytes (Largo *et al.*, 2003). GS inhibited significantly Tgase-2 at high concentrations (Kim *et al.*, 2009; Jeong *et al.*, 2010) and Tgase-2 activates NF- $\kappa$ B which is involved in TPA-induced ear edema models (Lee *et al.*, 2004; Rafi *et al.*, 2007). But, the role of Tgase-2 has not been studied in TPA-induced inflammation.

TPA-treated ears of mice showed increased expression of Tgase-2. TPA-induced Tgase-2 expression was also observed in HT-1080 cells (Park and Lee, 2011). The expression of Tgase-2 was suppressed in the GS-treated group after TPA-induced inflammation (Fig. 3). These results showed the possibility that Tgase-2 is involved in TPA-induced ear edema and suggested that the beneficial effect of GS might be related with suppression of Tgase-2. The role of Tgase-2 was examined in TPA-induced ear edema using Tgase-2 (-/-) and wild-type mice. We examined the expression of COX-2 which is a well-known target gene of NF- $\kappa$ B and one of the main targets for skin inflammation. The expression of COX-2 was not observed in TPA-induced ear edema of the TPA-treated Tgase-2 (-/-) mice (Fig. 4A). These results suggested that Tgase-2 is involved in the expression of COX-2 in TPA-induced inflam-

mation. The mechanism of involvement of Tgase-2 in COX-2 expression through NF- $\kappa$ B activation was suggested as polymerization of I $\kappa$ B in BV-1 microglia cells (Lee *et al.*, 2004; Mehta *et al.*, 2010).

Generally, several Tgases such as Tgase 1, 3, and 5, but not Tgase-2, are expressed in the epidermis and involved in terminal differentiation of keratinocytes (Eckert *et al.*, 2005). Tgase-2 is induced in TPA-treated skin and suggests a special role in skin inflammation. Similarly, in other inflammatory conditions such as renal ischemic injury, Tgase-2 induction is also observed (Kim *et al.*, 2010). But a more detailed role of Tgase-2 in skin requires further study.

Considering our results, Tgase-2 might be one of the key players of inflammation. The modulation of Tgase-2 expression by GS and the suppression of COX-2 by Tgase-2 inhibition suggests that Tgase-2 might be a new target for explaining the action of GS and Tgase-2 may be a new target for modulating skin inflammation (Fig. 4B). GS can also be used for skin inflammation if proved in clinical trials.

## ACKNOWLEDGMENTS

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