Transglutaminase II interacts with rac1, regulates production of reactive oxygen species, expression of snail, secretion of Th2 cytokines and mediates in vitro and in vivo allergic inflammation

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\textbf{A B S T R A C T}

Transglutaminase II (TGase II) is a protein cross-linking enzyme with diverse biological functions. Here we report the role of TGase II in allergic inflammation. Antigen stimulation induced expression and activity of TGase II by activation of NF-κB in rat basophilic leukemia (RBL2H3) cells. This induction of TGase II was dependent on FcεRI and EGFR. Interaction between TGase II and rac1 was induced following antigen stimulation. TGase II was responsible for the increased production of reactive oxygen species, expression of prostaglandin E2 synthase (PGES) and was responsible for increased secretion of prostaglandin E2. ChIP assay showed that TGase II, through interaction with NF-κB, was responsible for the induction of histone deacetylase-3 (HDAC3) and snail by direct binding to promoter sequences. HDAC3 and snail induced by TGase II, exerted transcriptional repression on E-cadherin. Snail exerted negative effect on expression of MMP-2, and secretion of Th2 cytokines. Inhibition of matrix metalloproteinase-2 (MMP-2) inhibited secretion of Th2 cytokines.

\section{1. Introduction}

Transglutaminase II (TGase II) is a protein cross-linking enzyme with diverse functions. TGase II plays important role in liver injury via cross-linking of Sp1 (Tatsukawa et al., 2009). Cross-linking by transglutaminase II inhibits Rb binding of human papillomavirus E7 (Jeon et al., 2003). Cross-linking of omega-5 gliadin by TGase II enhances IgE reactivity in wheat-dependent, exercise-induced anaphylaxis. (Palosuo et al., 2003). Protein cross-linking activity of TGase II is dependent on GTP (Begg et al., 2006a,b; Jeon et al., 2002). Prolonged increase of calcium ion increases TGase II activity (Yoo et al., 2005). Protein cross-linking activity by TGase II confers resistance to anti-cancer drugs (Park et al., 2009a,b; Cao et al., 2008; Kim et al., 2006). Physiological roles of TGase II have been reported in wound healing (Stephens et al., 2004), fibrosis (Shweke et al., 2008), apoptosis (Yamaguchi and Wang, 2006) and matrix formation (Aeschlimann and Thomazy, 2000). Transglutaminase II is necessary for the stabilization of tissue inflammation (Quan et al., 2005). Increased TGase II expression has been reported to occur in many inflammatory diseases, such as celiac disease (Bruce et al., 1985), cystic fibrosis (Maiuri et al., 2008), idiopathic inflammatory myopathies (Choi et al., 2004), and sporadic inclusion-body myositis (Choi et al., 2000). Inhibition of transglutaminase reverses the inflammation of allergic conjunctivitis (Sohn et al., 2003). The lack of TGase II leads to abnormal inflammatory responses in macrophages (Falasca et al., 2005).

Rat basophilic leukemia cells (RBL2H3) have been used to understand mechanism of IgE-induced allergic inflammation. IgE-induced allergic inflammation in RBL2H3 cells involves FceRI cross-linking, which in turn leads to cascade of signaling events. FceRI interacts with various intracellular signaling molecules, including Lyn, Syk and PKCa/δ (Kim et al., 2009). Antigen

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stimulation is accompanied by degranulation, increases in calcium influx and leads to activation of PKC, rac1 and MAPK signaling (Kim et al., 2008a,b,c). Given the fact that TGase II activity is regulated by intracellular calcium (Yoo et al., 2005), it is possible that increased calcium influx in antigen-stimulated RBL2H3 cells may affect expression of TGase II. Antigen stimulation induces interaction between CD44 and PKCα in RBL2H3 cells (Kim et al., 2008a,b,c). Hyaluronic acid exerts anti-allergic effect by inhibiting interaction between CD44 and PKCα (Kim et al., 2008a,b,c). In our preliminary data, antigen stimulation leads to activation of EGFR. Although TGase II has been known to be involved in various inflammatory diseases, its role in allergic inflammation has not been studied. We wanted to examine potential value of TGase II as a target for the development of allergy therapeutics.

Here we report novel role of TGase II in allergic inflammation. TGase II was induced by activation of NF-κB in antigen-stimulated RBL2H3 cells. In response to antigen, TGase II interacted with rac1 to increase production of reactive oxygen species, which led to the induction of COX-2, PGES and decreased expression of PGDH. TGase II, through interaction with NF-κB, was shown to induce expression of HDAC3 and snail, which in turn exert transcriptional repression on E-cadherin. Induction of HDAC3 and snail occurred by direct binding of TGase II to promoter sequences of these genes. Snail and MMP-2, induced by TGase II was responsible for the increased secretion of Th2 cytokines. Expression of TGase II was induced in both phorbol myristate acetate (PMA)-induced Balb/c mouse model of atopic dermatitis and IgE-induced Balb/c mouse model of passive cutaneous anaphylaxis. Chemical inhibition of TGase II by cystamine exerted negative effect on both IgE and PMA-induced mouse model of allergic inflammation.

2. Materials and methods

2.1. Cell culture

RBL2H3 cells were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco’s modified Eagle’s medium containing heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, San Diego, CA, USA). Cultures were maintained in 5% CO₂ at 37 °C.

2.2. Chemicals and reagents

DNP-HSA and DNP-specific IgE antibody were purchased from Sigma Chemical Company. Chemicals used in this study were purchased from Sigma Chemical Company. Bioneer Company (Daejon, Korea) synthesized primers used in this study. Superscript reverse transcriptase was purchased from Life Technologies Inc. Trizol was purchased from Invitrogen. Anti-ERK, anti-phospho ERK, anti-Akt, anti-phospho Akt $^{5473}$, and anti-Rac1 antibodies were purchased from R&D Company (Minneapolis, MN). Anti-p47phox, anti-p67phox, anti-phospho rhoGDI, anti-EGFR, anti-pY, anti-MMP-2, anti-TGase II, anti-IκB, anti-phospho IκB, anti-phospho-IκBα, anti-phospho-IκBβ, anti-FceRIβ, anti-FceRIγ antibodies were purchased from Cell Signaling Company (Beverly, MA, USA). All other antibodies used in this study were purchased from Sigma Company (St. Louis, MO, USA). Anti mouse and anti rabbit IgG–horse radish peroxidase conjugate antibody was purchased from Pierce Company (Rockford, IL, USA). Lipofectamin and Plus™ reagent were purchased from Invitrogen (San Diego, CA, USA).

2.3. Western blot analysis

For Western blot analysis, cells were solubilized by lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue, 10 mM NaF, 1% (v/v) protease inhibitor cocktail, 1 mM sodium orthovanadate). The samples were boiled for 5 min, and equal amounts of protein (20 μg/well) were analyzed on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membrane and subjected to Western blot analysis. The membrane was incubated with primary antibody (diluted according to manufacturer’s instructions) for 1 h. After extensive washing, blot was further incubated with antimouse or anti-rabbit horseradish peroxidase-conjugated antibody at a 1:3000 for 1 h and developed using enhanced chemiluminescence kit (Amersham).

For immunoprecipitation, cell lysates were immunoprecipitated with respective antibody (each at 2 μg) on ice for 1 h. Protein G-sepharose was then added and the reaction was performed at 4 °C for 2 h on a rotary shaker. Immune complexes were washed three times with lysis buffer, and 2X sample buffer was added to the beads. Boiled samples were then loaded on gels. Western blot analyses were followed according to standard procedures.

2.4. TGase II activity assay

TGase II activity was determined by a confocal microscopic method Briefly, cells were incubated with 1 mM of 5-(biotinamido) pentylamine (a pseudosubstrate of TGase II) for 1 h at 37 °C and treated with AA, MTX, and LPA. The treated cells were fixed with 3.7% formaldehyde in PBS for 30 min and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. Then, the cells were incubated with the blocking solution and treated with FITC-conjugated streptavidin (1:200) in the blocking solution for 1 h and observed with the confocal microscope. Approximately, 20 cells were randomly selected from three separate experiments and their fluorescence intensity was determined by processing the FITC-images in a single cell level. TGase II activity was determined by comparing the fluorescence intensity of antigen stimulated cells with that of unstimulated cells (fold).

2.5. HDAC3 activity assay

HDAC3 activity was measured by using a fluorescence activity assay kit (Cayman Chemical, Ann Arbor, MI, USA). The activity was assayed according to the manufacturer’s instructions. For immunoprecipitation, cells were lysed with ice-cold buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 15 mM MgCl₂, 250 mM sucrose, 0.12 mM EDTA, 0.5% NP-40, and a cocktail of protease inhibitors). The lysates were suspended with nuclear extraction buffer (50 mM HEPES, pH 7.5, 420 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, and 10% glycerol), sonicated for 30 s, and then centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant containing nuclear extract were immunoprecipitated with anti-HDAC3 antibody (2 μg/ml) and incubated with 200 μM acetylated fluorometric substrate for 30 min at 37 °C and 40 μL of developer was added. After 15 min, the fluorescence was measured using an excitation wavelength of 340–360 nm and an emission wavelength of 440–460 nm.

2.6. Measurement of PGE2 level

The level of PGE2 in antigen-stimulated RBL2H3 cells, using culture medium, was quantified by using an ELISA kit (Cayman Chemical Company) according to the manufacturer’s instructions.

2.7. Gelatin zymography

Conditioned medium from RBL2H3 cells cultured in serum-free medium was mixed 3:1 with substrate gel sample buffer [40%,...
v/v, glycerol, 0.25 M Tris–HCl, pH 6.8, and 0.1%, w/v, bromophenol blue] and loaded onto a 7.5% SDS-PAGE containing type I gelatin (2 mg/ml). After electrophoresis, the gel was soaked in Triton X-100 with shaking for 30 min with one change of solution. The gel was rinsed and incubated for 24 h at 37°C in substrate buffer [50 mM Tris–HCl, pH 7.5, 5 mM CaCl2 and 0.02% NaN3]. After incubation, the gel was stained with 0.1% Coomassie brilliant blue G-250 and destained in 50% methanol 10% acetic acid and 40% water. To determine the effect of snail on the secretion and induction of MPP-2, cells were transiently transfected with control siRNA (10 nM) or snail siRNA (10 nM). Gelatin zymography was performed as described.

2.8. RT-PCR

Total RNA was isolated by trizol and converted into cDNA using Superscript reverse transcriptase. The following primer pairs were used for PCR. MPP-2, 5′-ATGACACGTGACACACTGAG-3′ and 5′-CTCCTGAATGCGCTTGATGT-3′; actin, 5′-TAA CCA ACT GGG ACG AGA TG-3′ and 5′-ATA CAG GGA CACAG AGC CT-3′. The PCR conditions were as follows: MPP-2: 95°C for 45 s, 58°C for 45 s, 72°C for 1 min and 32 cycles; actin: 94°C for 1 min, 58°C for 45 s, 72°C for 45 s and 20 cycles.

2.9. Preparation of siRNA duplexes

The siRNA duplexes were constructed with the following target sequences. TGaše II, sense [5′-AATGCTCTTATTGGGCCTACGTCTC-3′]; antisense [5′-AACGTGTAGACAGCATCTGCTCT-3′]; FceRIβ, sense [5′-AAGCCAGATGCTTCACGCTGCT-3′]; antisense [5′-AATATGGGTCAGATGCTGCT-3′]; TGase II, sense [5′-AACTGACAGCTGCACCACTGACCC-3′]; antisense [5′-AACGTGACACGTTC-3′]; Snail, sense [5′-AATGCACCAATGTTGAACGCTGCT-3′]; antisense [5′-AACTGGTGATTCAATATGAGCCACGT-3′]; control, sense [5′-AATTCCTCGAAGCTGTCACGCTTGCT-3′]; antisense [5′-AAGCCTGACACGCTTGACAGAATCCTGCTC-3′]. The construction of siRNA was carried out according to the instruction manual provided by the manufacturer (Ambion, Austin, TX, USA).

2.10. The measurements of reactive oxygen species

IgE-sensitized cells were incubated with 5 μM of 2,7-dichlorodihydrofluorescein diacetate for 30 min. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added. After incubation, cells were immediately observed by a laser scanning confocal microscope (LSM410, Carl Zeiss). The samples were excited by a 488 nm Ar laser and a 515 nm long-pass filter. About 20 cells were randomly selected from three separate experiments. The IgE-sensitized RBL2H3 cells were incubated with cystamine (10 μM) for 15 min in the presence of 5 μM of 2,7-dichlorodihydrofluorescein diacetate. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added and ROS measurement was performed. To measure level of peroxynitrite (ONOO−), 5 μM of dihydrothoradamine 123 (DHR123) was used.

2.11. Rac1 activity assays

Rac1 activity assays were performed according to the well established procedures (Kim et al., 2008a,b,c). Approximately 10 μg of total lysates were subjected to Western blot analysis.

2.12. Cytokine ELISA

Assays were performed according to manufacturer’s instruction (Koma Biotech, Korea). Briefly, anti-Mouse IL-13 Antibody was coated onto flat-bottomed microtitre plates. The plates were washed twice with PBS, 0.02% (v/v) Tween 20 (Sigma) and incubated for 1 h with PBS containing 1% BSA. After washing, culture media from antigen-stimulated RBL2H3 cells were added to the plates and incubated for 2 h. After washing, biotinylated purified anti-Mouse IL-13 antibody (0.25 μg/ml) was added and incubated for 2 h. The plates were washed three times and incubated with Avidin-HRP Conjugate (1:2000 dilutions) for 30 min. The reaction was stopped by adding a 2 M sulfuric acid to the wells. The plates were read at 450 nm using a micro-titer plate reader. OD readings were converted to pg/ml using a standard curve and the appropriate dilution factor. IL-5 ELISA assay was performed identically to the IL-13 ELISA.

2.13. Cellular fractionation

Nuclear and cytosolic extracts were prepared with a nuclear/cytosol Fractionation kit (Biovision, Mountain View, CA, USA). Cells were collected by centrifugation at 600 × g for 5 min at 4°C. Cell pellets were washed twice with ice-cold PBS, followed by the addition of 0.2 ml of Cytosol Extraction Buffer A and vigorous mixing for 5 s. Ice-cold Cytosol Extraction Buffer B (11 μl) was then added to the solution. After mixing, nuclei and cytosolic fractions were separated by centrifugation at 16,000 × g for 5 min (supernatants were cytosolic fraction). Nuclear extraction buffer was added to the nuclei. After vortexing for a total of 40 min, nuclei were centrifuged at 16,000 × g for 10 min. Supernatants thus obtained were the nuclear fraction. Protein concentration of each fraction was determined using the DC Protein Assay Kit (Bio-Rad). Equal amounts of nuclear/cytoplasmic extracts were loaded for SDS-PAGE and Western blot analysis was performed. Purity of the cytosolic and nuclear fraction was confirmed by GAPDH and histone H1, respectively. For fractionation of cytosol and membrane, Cells were resuspended in a buffer containing 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and were lysed by sonication. The lysates were then centrifuged at 100,000 × g for 1 h at 4°C. The supernatants constitute the cytosolic fraction. The pellet was resuspended in the above buffer, which also contained 0.1% Triton X-100, and the mixture was lysed by sonication and centrifuged again at 100,000 × g for 1 h at 4°C to obtain the membrane fraction (supernatant). Purity of the cytosol and membrane fraction was confirmed by GAPDH and rac1, respectively.

2.14. Chromatin immunoprecipitation assays (ChIP)

Assays were performed according to manufacturer’s instruction (Upstate). The anti-p65 (subunit of NF-κB) antibody immunoprecipitates were reverse cross-linked. PCR was done on the phenol-chloroform-extracted DNA with specific primers of NF-κB site in the TGaše II promoter [5′-CTCAAAAGTTACCCACTCTC-3′ (sense) and 5′-TGCGAGCTGATAGATCA-3′ (antisense)]. Specific primers of HDAC3 promoter [5′-AAATAGAGGGTGGGAGGAGG-3′ (sense) and 5′-CCGAGACTGAGCTGAGAG-3′ (antisense)] and snail promoter [5′-AACTGGTACACGCTGACAGAATCCTGCTC-3′ (sense) and 5′-GGTAAAGGCTGGGAGAGG-3′ (antisense)] were used in ChIP to determine binding of TGaše II. ChIP assays employing anti-HDAC3 antibody or anti-snail antibody were also done with specific primers of Prostaglandin dehydrogenase promoter [5′-GGGAACTGACAGCTGAGAG-3′ (sense) and 5′-ATGACACGTGACACACTGAG-3′ (antisense)] and E-cadherin promoter [5′-CGACAGGGGTTGGAGGAAT-3′ (sense) and 5′-TGCGAGCTGACAGCTGAGAG-3′ (antisense)].
2.15. Animal experiment

BALB/C mouse (4-6 weeks) were purchased from the Dae Han Experimental Animal Center (Eumsung, Korea). Animals were housed in the animal facilities at the College of Natural Sciences of Kangwon National University. This study was done in accordance with the institutional guidelines.

2.16. Induction of IgE-induced passive cutaneous anaphylaxis in mouse

Anti DNP-specific IgE (0.5 μg) was injected into a mouse ear. The next day, cystamine was injected via tail vein in a dose of 50 mg/kg. 1 h later, the mice were challenged with intravenous injection of a 250 μg of DNP-HSA in 250 μl PBS containing 4% Evansblue solution. The mice were killed an hour after antigen challenge, followed by the removal of an ear for measurement of the amount of dye extravasated by DNP-HSA. The dye was eluted from the ear in 700 μl of formamide at 63 °C. The absorbance was measured at 620 nm.

2.17. Induction of skin inflammation in BALB/c mouse

Phorbol myristate acetate (PMA) was used as an inducer of skin inflammation. The thickness of both ears of untreated mouse was measured using dial thickness gauge (Mitutoyo Corporation, Kanakawa, Japan) as a reference. 20 μl of cystamine (5%) was pretreated for 30 min by topical application, and 20 μl of PMA (0.015%) was painted to dorsal surface of both ears to induce skin inflammation. 12 h after, the topical application of inducer, ear thickness was measured again and changes in ear thickness were calculated.

2.18. Histological analyses

Ear samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 μm, and then stained with hematoxylin and eosin.

3. Results

3.1. Transglutaminase II is induced in antigen-stimulated RBL2H3 cells

In order to examine potential role of TGase II in allergic inflammation, we examined whether antigen stimulation would affect expression level of TGase II in rat basophilic leukemia (RBL2H3) cells. Antigen stimulation induces expression of TGase II in RBL2H3 cells, in a time and dose-dependent manner (Fig. 1A). Hyaluronic acid, which exerts anti-allergic effect by inhibiting interaction between CD44 and PKCζ (Kim et al., 2008a,b,c), prevents antigen from increasing expression of TGase II (Fig. 1B). Previously we reported activation of epidermal growth factor receptor (EGFR) in antigen-stimulated RBL2H3 cells (Kim et al., 2008a,b,c). Chem-

Fig. 1. Antigen stimulation induces expression of TGase II in RBL2H3 cells. (A) The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA (100 ng/ml) for various time intervals (upper panel). The IgE-sensitized RBL2H3 cells were treated with various concentrations of DNP-HSA for 30 min (lower panel). Cell lysates prepared at each time point were subjected to Western blot analysis. (B) The IgE-sensitized RBL2H3 cells were pretreated with or without hyaluronic acid (200 μg/ml) for 30 min, followed by antigen stimulation for 30 min. Cell lysates were subjected to Western blot analysis. (C) The IgE-sensitized RBL2H3 cells were pretreated with or without AG1478 (1 μM) or cystamine (100 μg/ml) for 30 min, followed by antigen stimulation. Cell lysates were subjected to Western blot analysis. (D) TGase II activity assay in antigen-stimulated RBL2H3 cells was performed by confocal microscopy as described in methods (upper panel). Each value represents average of three independent experiments (lower panel). P<0.05 compared with IgE-sensitized RBL2H3 cells unstimulated with DNP-HSA. **P<0.005 compared with IgE-sensitized RBL2H3 cells unstimulated with DNP-HSA. (E) The IgE-sensitized RBL2H3 cells were pretreated with or without AG1478 (1 μM) for 30 min, followed by stimulation with DNP-HSA for various time intervals. Cell lysates were immunoprecipitated with anti-EGFR antibody (2 μg/ml) or anti-FcεRIβ (2 μg/ml), followed by Western blot analysis. (F) RBL2H3 cells were transiently transfected with various siRNA (each at 10 nM) as indicated. At 48 h after transfection, cell lysates were prepared and subjected to Western blot analysis.
3.2. Induction of TGase II by activation of NF-κB

NF-κB activation regulates mast cell degranulation (Suzuki and Verma, 2008). NF-κB plays important role in inflammation in monocytes (Schmidt et al., 2008). We therefore checked the involvement of NF-κB in the induction of TGase II by antigen stimulation. Antigen stimulation decreases expression of IκB, increases phosphorylation of IκB, phosphorylation of IκKα (Fig. 2A). Antigen stimulation induces translocation of p65 subunit of NF-κB (Fig. 2B). IκKα shows interaction with Akt and ERK (Fig. 2C), suggesting that the increased phosphorylation of IκKα is due to its interaction with Akt and ERK. The inhibition of NF-κB by its super repressor construct prevents antigen from inducing expression of TGase II (Fig. 2D). The inactivation of ERK or Akt by respective dominant negative construct prevents antigen from inducing expression of TGase II (Fig. 2E). Downregulation of TGase II does not affect activation of Akt or ERK by antigen stimulation (data not shown), suggesting that Akt and ERK function upstream of TGase II. ChIP assay shows binding of p65 subunit of NF-κB to promoter sequences of TGase II (Fig. 2F). Taken together, these results suggest that NF-κB activation occurs by activation of Akt and ERK which interact with IκKα, and regulates expression of TGase II in antigen-stimulated RBL2H3 cells. We also observed induction of c-jun and the increased phosphorylation of JNK (data not shown). Therefore it is also possible that other transcription factor, such as AP-1, is involved in induction of TGase II in antigen-stimulated RBL2H3 cells.

3.3. TGase II interacts with and is necessary for activation of rac1 in antigen-stimulated RBL2H3 cells

Reactive oxygen species play important roles in allergic inflammation (Kim et al., 2008a,b,c). We therefore examined relationship between TGase II and production of reactive oxygen species in RBL2H3 cells. Antigen stimulation leads to the interaction between TGase II and rac1 (Fig. 3A). Induction of TGase II occurs in both cytoplasm and membrane (Fig. 3B). Cystamine, an inhibitor of TGase II, prevents translocation of rac1 into membrane by antigen stimulation (Fig. 3B). Downregulation of TGase II prevents antigen from inducing rac1 activity (Fig. 3C). Overexpression of TGase II leads to the interaction between TGase II and rac1 (Fig. 3D). Since TGase II interacts with rac1, it is probable that TGase II is necessary for production of reactive oxygen species in RBL2H3 cells.
3.4. TGase II increases production of reactive oxygen species, which in turn regulate expression of COX-2, PGES, and the level of prostaglandin E2

We examined whether TGase II would be necessary for the increased production of reactive oxygen species in RBL2H3 cells. Chemical inhibition of TGase II by cystamine prevents antigen from increasing production of reactive oxygen species in RBL2H3 cells (Fig. 4A), Overexpression of TGase II increases production of hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$) in RBL2H3 cells (Fig. 4B). Downregulation of TGase II prevents antigen from increasing expression of cyclooxygenase-2 (COX-2) and prostaglandin E2 synthase (PGES synthase) (Fig. 4C). Prostaglandin E2 is known to promote immune inflammation, such as skin disorders (Yao et al., 2009). Downregulation of prostaglandin dehydrogenase (PGDH) is accompanied by an increased expression of PGE2 synthase (Mann et al., 2006). TGase II is responsible for decreased expression of PGDH (Fig. 4C). TGase II is responsible for the increased secretion of PGE2 (Fig. 4D). Inactivation of rac1 by dominant negative construct (rac1N17) prevents antigen from inducing expression of COX-2, PGES and decreasing expression of PGDH (Fig. 4E). These results suggest that TGase II may mediate allergic inflammation by increasing production of reactive oxygen species, which in turn regulates expression of PGES and secretion of prostaglandin E2.

3.5. TGase II mediates effect of antigen on regulation of expression of epithelial mesenchymal transition (EMT)-related proteins

Antigen stimulation leads to aberrant expression of epithelial mesenchymal transition (EMT)-related proteins in RBL2H3 cells (Kim et al., 2009). Vimentin, an EMT-related protein, is a substrate of TGase II (Gupta et al., 2007). Reactive oxygen species induce EMT (Radisky et al., 2005). COX-2 enhances EMT through PGE2-dependent mechanism in breast cancers (Neil et al., 2008). Since TGase II is responsible for the increased production of reactive oxygen species in antigen-stimulated RBL2H3 cells (Fig. 4A and B), we examined whether TGase II would exert regulation on expression of EMT-related proteins. Downregulation of TGase II prevents antigen from increasing expression of snail, from decreasing expression of E-cadherin (Fig. 5A). Overexpression of TGase II increases expression of snail while decreasing expression of TGase II (Fig. 5B). Inactivation of rac1 by its dominant negative construct prevents antigen from regulating expression of EMT-related proteins (Fig. 5C), suggesting that rac1 is responsible for expression regulation of EMT-related proteins. Theses results suggest that TGase II regulates expression of EMT-related proteins by interaction with rac1. Since expression of snail is regulated by TGase II, it is probable that EMT-related proteins may be involved in allergic inflammation.

3.6. Interaction between TGase II and NF-κB is responsible for induction of HDAC3 and snail, which in turn exert transcriptional repression on E-cadherin

Antigen stimulation leads to decreased expression of PGDH expression in RBL2H3 cells (Fig. 4C). Interaction between histone deacetylase 2 (HDAC2) and snail is necessary for decreased expression of PGDH (Backlund et al., 2008). The involvement of HDAC(s) in allergic inflammation has not been reported. Therefore we investigated role of HDAC(s) in allergic inflammation. First, we examined whether expression levels of HDAC(s) would be affected in RBL2H3 cells following antigen stimulation. Antigen stimulation leads to
Fig. 4. TGase II is necessary for the increased production of reactive oxygen species, expression of PGE2 synthase and PGE2 level. (A) The IgE-sensitized RBL2H3 cells were pretreated with or without cystamine (1 μM) for 30 min, followed by stimulation with DNP-HSA for 30 min. Determination of production of reactive oxygen species was performed by using 5 μM of DCFH-DA. (B) RBL2H3 cells were transiently transfected with control vector (1 μg) or TGase II cDNA (1 μg). Measurement of level of reactive oxygen species was performed by using DCFH-DA for hydrogen peroxide or DHR123 (5 μM) for peroxynitrite. (C) RBL2H3 cells were transiently transfected with control siRNA (10 nM) or TGase II siRNA (10 nM). At 48 h after transfection, Western blot analysis was performed. (D) Same as (C) except that measurement of PGE2 level was performed. **P < 0.005 compared with IgE-sensitized RBL2H3 cells unstimulated with DNP-HSA; ++P < 0.005 compared with cells transfected with control siRNA. (E) RBL2H3 cells were transiently transfected with control vector (1 μg) or rac1 dominant negative construct (1 μg). The next day, cells were sensitized by DNP-specific anti-IgE antibody, followed by stimulation with DNP-HSA for 30 min. Western blot analysis was performed.

Fig. 5. TGase II regulates expression of epithelial mesenchymal transition (EMT)-related proteins. (A) RBL2H3 cells were transiently transfected with control siRNA (10 nM) or TGase II siRNA (10 nM). Next day, cells were sensitized with DNP-specific anti-IgE antibody for 16 h. The IgE-sensitized RBL2H3 cells were then stimulated with DNP-HSA for 1 h, followed by Western blot analysis. (B) RBL2H3 cells were transiently transfected with control vector (1 μg) or TGase II cDNA (1 μg). Cell lysates were subjected to Western blot analysis. (C) RBL2H3 cells were transiently transfected with control vector (1 μg) or dominant negative rac1 construct (1 μg). Next day, cells were sensitized with DNP-specific anti-IgE antibody for 16 h. The IgE-sensitized RBL2H3 cells were stimulated with DNP-HSA for 1 h, followed by Western blot analysis.
(Fig. 6C). Downregulation of TGase II also exerted negative effect on induction of HDAC3 and snail (data not shown). Cellular fractionation study shows that TGase II is induced in the nucleus, as well as in the cytosol (Fig. 6D, upper panel). Interaction between TGase II and NF-κB occurs in the nucleus (Fig. 6D, lower panel). ChIP assays show that TGase II binds to the promoter sequences of HDAC3 (Fig. 6E) and snail (Fig. 6F). This suggests that TGase II induces expression of HDAC3 and snail by direct binding to promoter sequences of these genes. ChIP assays show binding of snail to promoter sequences of E-cadherin (Fig. 6G). Snail, but not HDAC3, exerts transcriptional repression on E-cadherin to mediate allergic inflammation.

3.7. Downregulation of snail exerts negative effect on secretion of Th2 cytokines in antigen-stimulated RBL2H3 cells

Allergic inflammation is closely related with increased secretion of Th2 cytokines (Kim et al., 2008a,b,c). Snail increases expression of pro-inflammatory cytokines, such as interleukin-1β, interleukin-6 and interleukin-8, in head and neck squamous cell carcinomas (Lyons et al., 2008). We hypothesized that snail would affect secretion of Th2 cytokines in antigen-stimulated RBL2H3 cells. Downregulation of snail, a transcriptional repressor of E-cadherin, restores expression of E-cadherin and leads to downregulation of vimentin and N-cadherin (Fig. 7A). Downregulation of snail prevents antigen from increasing expression (Fig. 7A) and secretion (Fig. 7A) of MMP-2.

Downregulation of snail prevents antigen from increasing secretion of Th2 cytokines, such as IL-5 and IL-13 in RBL2H3 cells (Fig. 7B). These results point to the role of snail in allergic inflammation by regulating secretion of Th2 cytokines.

3.8. MMP-2 is necessary for enhanced secretion of Th2 cytokines in antigen-stimulated RBL2H3 cells

TGase II regulates expression and secretion of MMP-2 (Fig. 5A and B). Downregulation of snail leads to decreased expression and secretion of MMP-2 (Fig. 7A). Therefore we examined role of MMP-2 in secretion of Th2 cytokines in RBL2H3. Antigen stimulation, through activation of EGFR, leads to increases expression and secretion of MMP-2 in RBL2H3 cells (Fig. 8A). Chemical inhibition of MMP-2 exerts negative effect on activation of EGFR by antigen (Fig. 8B), suggesting feedback regulation by MMP-2 on EGFR signaling. Chemical inhibition of MMP-2 prevents antigen from increasing secretion of IL-5 and IL-5 in RBL2H3 cells (Fig. 8C). These results suggest that MMP-2, induced by TGase II, exerts feedback regulation to increase secretion of Th2 cytokines.

3.9. The role of TGase II in IgE-induced passive cutaneous anaphylaxis

Since TGase II mediates allergic inflammation, we examined role of TGase II in vivo allergic inflammation. We employed IgE-induced
Balb/c mouse model of systemic passive cutaneous anaphylaxis. Chemical inhibition of TGase II by cystamine exerts negative effect on IgE-induced Balb/c mouse model of passive cutaneous anaphylaxis (Fig. 9A and B). Western blot analysis shows an increased expression of TGase II, snail and other proteins involved in allergic inflammation in ear tissue of IgE-induced Balb/c mouse model of passive cutaneous anaphylaxis (Fig. 9C). Chemical inhibition of TGase II exerts negative effect on the induction of these proteins (Fig. 9C). Activation of epidermal growth factor EGFR and rac1 was also evident in this tissue (data not shown). These results suggest that TGase II mediates IgE-dependent in vivo allergic inflammation. It is necessary to examine whether TGase II would be necessary for mediating IgE-independent in vivo allergic inflammation.

3.10. TGase II is necessary for PMA-induced Balb/c mouse model of atopic dermatitis

Next, we examined the role of TGase II in phorbol myristate acetate (PMA)-induced Balb/c mouse model of atopic dermatitis.
Fig. 9. TGase II is necessary for IgE-induced passive cutaneous anaphylaxis. (A) IgE-induced Balb/c mouse model of passive cutaneous anaphylaxis was employed to determine effect of TGase II in allergic inflammation. The extent of inflammation was determined by measuring optical density as described. Each picture is representative of three independent experiments. (B) The means ± SEMs of values from three independent experiments. Each with five mice, are shown. **P < 0.005 compared with mice unstimulated with DNP-HSA; +++P < 0.005 compared with mice untreated with cystamine. (C) Lysates prepared from ear tissue of Balb/c mouse were subjected to Western blot analysis.

PMA induces expression of TGase II and HDAC3 in RBL2H3 cells (Fig. 10A). This suggests that induction of TGase II also occur independent of IgE-sensitization. Chemical inhibition of TGase II decreases ear thickness in PMA-induced Balb/c mouse model of atopic dermatitis (Fig. 10B), and also decreases lymphocyte infiltration (Fig. 10C). Western blot of ear tissue of Balb/c treated with PMA shows increased expression of TGase II, snail, MMP-2, and PGES (Fig. 10D) Chemical inhibition of TGase II exerts negative effect on induction of these hall marks of allergic inflammation (Fig. 10D). Activation of EGFR and rac1 was also detected [data not shown]. These results point to the role of TGase II in IgE-independent in vivo allergic inflammation.

Fig. 10. TGase II is necessary for PMA-induced atopic dermatitis. (A) RBL2H3 cells were treated with or without PMA (100 ng/ml) for various time intervals. Cell lysates were subjected to Western blot analysis. (B) Increase in ear thickness in PMA-induced Balb/c mouse was decreased by cystamine treatment. Means ± SEMs of values from three independent experiments are shown. Each with five mice is shown. **P < 0.005 compared with mice unstimulated with PMA; +++P < 0.005 compared with mice untreated with cystamine. (C) Histological analyses of skin lesions induced by PMA in Balb/c mouse. Cystamine reduced skin inflammation in Balb/c mouse treated with PMA. (D) Lysates prepared from ear tissue of Balb/c mouse treated with PMA in the absence or presence of cystamine, were subjected to Western blot analysis.
4. Discussion

In this study, we investigated role of TGase II in allergic inflammation. Induction of TGase II has been reported in various inflammatory diseases. Hyaluronic acid, which was shown to exert anti-allergic effect by inhibiting interaction between CD44 and PKCα in RBL2H3 cells (Kim et al., 2008a,b,c), decreased expression of TGase II (Fig. 1B), suggesting that TGase II may mediate allergic inflammation. Antigen stimulation was shown to activate EGFR and induce interaction between EGFR and CD44 (Kim et al., 2008a,b,c). The inhibition of EGFR by AG1478 prevented antigen from increasing expression of TGase II (Fig. 1C), suggesting role of EGFR in the induction of TGase II. FceRI is composed of three subunits. In response to antigen stimulation, FceRIIβ interacted with Akt and lyn while FceRIγ shows interaction with PKCθ (Fig. 1D). Chemical inhibition of EGFR prevented antigen from activating FceRI signaling (Fig. 1E), suggesting that induction of TGase II by antigen stimulation occurs through cross-talk between EGFR and FceRI.

Aggregation of the high-affinity IgE receptor FceRI on human monocytes and dendritic cells induces NF-κB activation (Kraft et al., 2002). The inhibition of NF-κB improves atopic dermatitis in NC/Nga mouse (Tanaka et al., 2007). We found an activation of NF-κB in antigen-stimulated RBL2H3 cells (Fig. 2A and B). Akt activates NF-κB by inducing interaction between Ikappa kinase and mTOR (Dan et al., 2008). Antigen stimulation led to interaction between IkBα and Akt and IkBα and ERK (Fig. 2C), suggesting that increased phosphorylation of IkBα by Akt and ERK may be responsible for the activation of NF-κB. NF-κB activation by Akt protects against stress-induced apoptosis (Shant et al., 2009). NF-κB was responsible for the induction of TGase II (Fig. 2D). NF-κB induces activation of TGase II activity (Chen et al., 2008). ChIP assay clearly shows that NF-κB regulates expression of TGase II (Fig. 2F). PKCα which interacts with EGFR was shown to be necessary for the induction of TGase II (data not shown), confirming role of EGFR signaling in the induction of TGase II by antigen in RBL2H3 cells.

Increased production of reactive oxygen species is a major feature of allergic inflammation (Kim et al., 2008a,b,c; Springer et al., 2007). Rac1 regulates NF-κB activation (Chen et al., 2009; Kim et al., 2008a,b,c). Rac1 regulates COX-2 expression in RAW264.7 macrophages (Chen et al., 2009). TGase II induces nitric oxide synthesis in BV-2 microglia (Park et al., 2004). The above reports suggest role of TGase II in the production of reactive oxygen species. Antioxidant down-regulates interleukin-18 expression in asthma (Lee et al., 2006), suggesting role of reactive oxygen species in allergic inflammation. Downregulation of TGase II prevented antigen from increasing rac1 activity (Fig. 3C). Overexpression of TGase II increased production of reactive oxygen species (Fig. 4B). The effect of TGase II on the production of reactive oxygen species may result from interaction between TGase II and rac1 (Fig. 3A). Prostaglandin E2 production is dependent with LPS-induced inflammation (Inada et al., 2006; Ikeda-Matsuou et al., 2005). Prostaglandin E2 production is dependent on COX-2 (Murakami et al., 2003). Our data showed that rac1 regulated expression of COX-2, PGE2 synthase (Fig. 4C) and the production of PGE2 (Fig. 4E). Rac1 is known to regulate COX-2 expression RAW264.7 macrophages by activating PI3 kinase/Akt pathway (Chen et al., 2009). In this study, we found that downregulation of PGDH by RNA interference led to the increased expression of PGES (data not shown). The inhibition of rac1 by dominant negative construct was shown to prevent antigen from inducing expression of PGES and COX-2 (Fig. 4D). This confirms role of reactive oxygen species in the regulation of PGE2 level in RBL2H3 cells. Inhibition of inducible nitric oxide synthase (iNOS) attenuates chronic allergic inflammation (Prado et al., 2006). In our data, increased expression of iNOS in response to antigen stimulation is dependent on rac1 (data not shown). Matrix metalloproteinases are necessary allergen-induced airway inflammation in a murine model of asthma (Kumagai et al., 1999). Transglutaminase regulates matrix metalloproteinase-2 in ovarian cancer by modulating CAMP-response element-binding protein activity. (Satpathy et al., 2009). Secretion of MMPs is regulated by E prostaglandins in gastric epithelial cells (Pillinger et al., 2005). MMP-2 exerts feedback regulation on EGFR to promote cellular invasion (Gong et al., 2008). Therefore we hypothesized that increased secretion of MMP-2 would affect EGFR signaling to induce secretion of Th2 cytokines.

Snail up-regulates proinflammatory mediators in oral keratinocytes (Lyons et al., 2008). Down-regulation of E-cadherin in human bronchial epithelial cells leads to increased secretion of Th2 cytokines (Heijink et al., 2007). These reports suggest that proteins involved in epithelial mesenchymal transition (EMT) maybe involved in allergic inflammation. In our data, TGase II and rac1 were shown to regulate expression of EMT-related proteins (Fig. 5A and B). Our data showed that rac1 mediated effect of antigen on downregulation of E-cadherin (Fig. 5C). Reactive oxygen species plays important role in the induction of snail, which in turn recruits HDAC1 and DNA methyl transferase to E-cadherin promoter (Lim et al., 2008). Epigenetic changes induced by reactive oxygen species exert transcriptional repression on E-cadherin (Lim et al., 2008). Prostaglandin dehydrogenase expression is repressed by snail and HDAC2 in colorectal cancer (Backlund et al., 2008). Repression of PGDH by snail increases PGE2 synthase and promotes cancer progression (Mann et al., 2006). HDACs 1, 2 and 3 are highly expressed in colon cancer and play important role in cellular proliferation (Weichert et al., 2008). Histone deacetylases (HDACs) exert transcriptional repression on a variety of genes (Evert et al., 2006). Snail represses E-cadherin expression by recruiting HDAC1/2 complex (Peinado et al., 2004). Snail recruits HDAC3 to exert transcriptional repression (Qi et al., 2008). Antigen stimulation decreases expression of HDAC2 (Fig. 6A). This decrease in expression of HDAC2 by antigen stimulation is accompanied by tyrosine nitration of HDAC2 and decreased HDAC2 activity (data not shown). Tyrosine nitration of HDAC2 by oxidative stress leads to inactivation of HDAC2 activity (Osoata et al., 2009). Tyrosine nitration of HDAC2 may occur by TGase II which increases production of reactive oxygen species, and this may lead to degradation of HDAC2. Since antigen stimulation decreases expression of HDAC2, it is possible that HDAC2 may exert transcriptional repression on TGase II in the absence of antigen. Inhibition of NF-κB prevented antigen from inducing expression of HDAC3 and snail (Fig. 6C). Chemical inhibition of TGase II and downregulation of TGase II also prevented antigen from inducing expression of HDAC3 and snail (data not shown). This led us to further examine relationship between TGase II and NF-κB in the induction of HDAC3. Our study showed that the induction of TGase II by antigen stimulation also occurred in the nucleus (Fig. 6D, upper panel). Interaction between TGase II and NF-κB occurred in the nucleus (Fig. 6D, lower panel). TGase II was shown to be induced and translocated into the nucleus during neutrophil granulocyte differentiation (Balajthy et al., 2006). It is probable that TGase II, through interaction with NF-κB, regulates expression of HDAC3 and snail. ChIP assays revealed that TGase II was shown to bind to promoter sequences of HDAC3 (Fig. 6E) and snail (Fig. 6F). This suggests that TGase II may directly regulate expression of HDAC3 and snail. We found that overexpression of TGase II led to the induction of HDAC3 in RBL2H3 cells (data not shown), HDAC3 and snail showed binding to promoter sequences of E-cadherin (Fig. 6G). It would be necessary to examine effect of overexpression of HDAC3 on expression of E-cadherin. Since HDAC3 and snail do not show interaction in the nucleus (data not shown), it is reasonable that HDAC3 and snail exert transcriptional repression on E-cadherin independent of each other. Downregulation of snail was shown to prevent antigen from decreasing expression of E-cadherin in RBL2H3 cells (data not shown). It would be necessary to carry out further studies to...
functional role of HDAC3 in allergic inflammation. This may prove potential value of HDAC3 as a target for the development of allergy therapeutics.

FcεRI receptor cross-linkage leads to the increased production of IL-13 in human mast cells (Kobayashi et al., 1998). Our data showed that snail (Fig. 7B) and MMP-2 (Fig. 8C) were necessary for the increased secretion of Th2 cytokines, such as IL-5 and IL-13, in antigen-stimulated RBL2H3 cells. Downregulation of snail prevented antigen from increasing secretion of PGE2, as well (data not shown). Downregulation of TGase II was shown to prevent antigen from increasing secretion of Th2 cytokines (data not shown). Chemical inhibition of TGase II prevented concanavalin A from stimulating secretion of Th2 cytokines, such as IL-5 and IL-13, in mouse splenocytes (data not shown).

TGase II was necessary for the development of systemic passive cutaneous anaphylaxis in IgE-induced Balb/c mouse model (Fig. 9A and B). Induction of TGase II was evident in ears of Balb/c mouse sensitized by DNP-specific IgE and challenged with DNP-HSA (Fig. 9C). Induction of TGase II also occurred in IgE-independent Balb/c mouse model of atop dermatitis (Fig. 10D). These results suggest that TGase II may be valuable as a target for the development of therapeutics against various forms of allergic diseases.

In this study, we found a novel role of TGase II in allergic inflammation. Induction of TGase II occurs in IgE-dependent and -independent manner. The fact that TGase II, through interaction with HDAC3 and snail, exerts transcriptional repression on PCDH and E-cadherin has not been previously reported. TGase II functions downstream of FcεRI signaling and mediates allergic inflammation. In vivo mouse allergy model also suggest role of TGase II in allergic inflammation. The development of allergy therapeutics targeting TGase II therefore would be reasonable. It would be necessary to define domain of TGase II that is necessary for interaction with NF-κB or rac1 to develop such therapeutics.

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