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RESEARCH PAPER

Anti-inflammatory effects of the R2 peptide, an inhibitor of transglutaminase 2, in a mouse model of allergic asthma, induced by ovalbumin

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BACKGROUND AND PURPOSE
Transglutaminase 2 (TGase 2) expression is increased in inflammatory diseases, and TGase 2 inhibitors block these increases. We examined whether the R2 peptide inhibited the expression of TGase 2 in a mouse model of inflammatory allergic asthma.

EXPERIMENTAL APPROACH
C57BL/6 mice were sensitized and challenged by ovalbumin (OVA) to induce asthma. OVA-specific serum IgE and leukotrienes (LTs) levels were measured by enzyme-linked immunosorbent assay. Recruitment of inflammatory cells into bronchoalveolar lavage (BAL) fluid or lung tissues and goblet cell hyperplasia were assessed histologically. Airway hyperresponsiveness was determined in a barometric plethysmographic chamber. Expression of TGase 2, eosinophil major basic protein (EMBP), the adhesion molecule vascular cell adhesion molecule-1, Muc5ac and phospholipase A_2 (PLA_2) protein were determined by Western blot. Expression of mRNAs of Muc5ac, cytokines, matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) were measured by reverse transcriptase-polymerase chain reaction and nuclear factor-κB (NF-κB) by electrophoretic mobility shift assay.

KEY RESULTS
R2 peptide reduced OVA-specific IgE levels; the number of total inflammatory cells, macrophages, neutrophils, lymphocytes and eosinophils in BAL fluid and the number of goblet cells. Airway hyperresponsiveness, TGase 2 and EMBP levels, mRNA levels of interleukin (IL)-4, IL-5, IL-6, IL-8, IL-13, RANTES, tumour necrosis factor-α, and MMP2/9, Muc5ac, NF-κB activity, PLA_2 activity and expressions, and LT levels in BAL cells and lung tissues were all reduced by R2 peptide. R2 peptide also restored expression of TIMP1/2.

CONCLUSION AND IMPLICATIONS
R2 peptide reduced allergic responses by regulating NF-κB/TGase 2 activity in a mouse model of allergic asthma. This peptide may be useful in the treatment of allergic asthma.

Abbreviations
BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; EMBP, eosinophil major basic protein; EMSA, electrophoretic mobility shift assay; MMP, matrix metalloproteinase; NF-κB, nuclear factor-kappa B; OVA, ovalbumin; PAS, periodic acid-Schiff; PBS, phosphate buffered saline; PLA_2, phospholipase A_2; RT-PCR, reverse transcriptase-polymerase chain reaction; TGase 2, transglutaminase 2; TIMP, tissue inhibitor of matrix metalloproteinase

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Introduction

Transglutaminase 2 (TGase 2) is a cross-linking enzyme that stabilizes tissues and is an immediate defense against injury or infection. TGase 2 is aberrantly activated in tissues and cells and contributes to neurodegenerative diseases, such as Alzheimer's disease (Citron *et al*., 2001), Parkinson's disease (Junn *et al*., 2003; Andrings *et al*., 2004), neuro-AIDS (Roberts *et al*., 2003), Huntington's diseases (Kim *et al*., 2005), hippocampal CA 1 ischemia (Hwang *et al*., 2009), celiac disease (Naiyer *et al*., 2008), lupus (Sanchez *et al*., 2000), autoimmune diseases like rheumatic arthritis (Picarelli *et al*., 2003), human atherosclerotic coronary arteries (Cho *et al*., 2008), breast cancers (Park *et al*., 2009a) and drug resistance in variety of cancer cells (Kim *et al*., 2006; Park *et al*., 2009b, 2010). TGase 2 expression is increased in inflammatory diseases such as activation of microglia, a hallmark of brain inflammation (Park *et al*., 2004), idiopathic inflammatory myopathies (Kim, 2006) and allergic asthma (Hallstrand *et al*., 2010). TGase 2 also regulates the transcription factor, nuclear factor-κB (NF-κB) which itself plays a pivotal role in inflammatory processes. Thus, TGase 2 polymerizes the inhibitor of κBα (IκBα) and depletes free IκBα during lipopolysaccharide-induced microglia activation. It also activates NF-κB via two different pathways, a IκB kinase (IKK)-independent and IKK-dependent pathways (Lee *et al*., 2004; Kim, 2006), and is responsible for constitutive NF-κB activation in breast cancer cells without canonical activation (Lee *et al*., 2004; Kim *et al*., 2008; Park *et al*., 2009a). Therefore, TGase 2 inhibition may provide a novel therapeutic approach for the treatment of inflammatory disease.

The recombinant R2 peptide (KVLDGQDP) combines antiflamins (Miele *et al*., 1988) and pro-elafin sequences (Molhuizen *et al*., 1993) and has anti-TGase 2 and anti-phospholipase A_2 (PLA_2) activities. This peptide is known to regulate eicosanoid production in various cell types (Mehrotra and Henderson, 2009; Hallstrand *et al*., 2010). It decreases inflammation in an allergic conjunctivitis model in guinea pigs (Sohn *et al*., 2003), and decreases tumour necrosis factor-α (TNF-α) immunoreactive protein in the serum of mice injected with lipopolysaccharide (Suh *et al*., 2006).

Allergic asthma is a chronic, complex inflammatory disease caused by inappropriate responses to inhaled allergens, and is characterized by reversible obstruction of airway hyperresponsiveness, infiltration of inflammatory cells into lung tissues, mucus overproduction, allergen-specific IgE, the overexpression of Th2-mediated or Th1-mediated cytokines, including interleukin (IL)-4, IL-5 and IL-13, and IL-8 and TNF-α (Corry, 2002), respectively, and chemokines such as eotaxin (CCL11) and RANTES (CCL5) (Berkman *et al*., 1996; Zimmermann *et al*., 2003). Despite the increasing prevalence of this disease, its pathophysiological mechanism is still unclear, and current treatments are not satisfactory.

R2 peptide can inhibit TGase 2, but the pathophysiological mechanisms of R2 peptide in allergic asthma are not known. Therefore, we tested whether TGase 2 was expressed in mice sensitized to and challenged with, ovalbumin (OVA) and whether treatment with the R2 peptide reduced the symptoms of OVA challenge in our model of allergic asthma.

Methods

Animals

All animal care and experimental procedures were in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care and were approved by the institutional review board and conducted in the Laboratory Animal Research Center of Sungkyunkwan University. Female C57BL/6 mice, 6–8 weeks old, were obtained from ORIENT BIO (Seongnam, Korea) and maintained in specific pathogen-free conditions before the experiments.

Sensitization, challenge and experimental protocol

C57BL/6 mice were divided into eight groups (eight mice/group): NC (negative control), mice sensitized and nebulized with phosphate buffered saline (PBS); GL (OVA-mice), mice sensitized and nebulized with OVA; R2 peptide (5, 10 or 20 $mg \cdot kg^{-1}$), OVA-mice pretreated with each dose of R2 peptide; Scrambled (S2) peptide (5, 10 or 20 $mg \cdot kg^{-1}$), OVA-mice pretreated with each dose of S2 peptide. Mice were sensitized with 20 $\mu g \cdot 200\ \mu L^{-1}$ OVA adsorbed in 1.0 $mg \cdot 50\ \mu L^{-1}$ aluminum hydroxide gel adjuvant by i.p. injection on days 0 and 14. Mice were then challenged with nebulised 5% OVA in PBS for 30 min once a day for 3 days, from day 20 until day 22. The nebulizer was from Mega Medical (Seoul, Korea). Control mice (Con) were sensitized and challenged with PBS. All mice were killed on day 25.

Preparation and treatment with R2 or scrambled (S2) peptide

R2 or S2 peptide (5, 10, or 20 $mg \cdot kg^{-1}$) was dissolved in PBS and given by i.p. injection once a day 10 min before OVA challenge for 3 days (from day 20 to day 22). All the data for pretreatment with R2 or S2 peptide alone in non-sensitized and non-challenged

mice are not shown, as these treatments were without effect. In Figures and Tables, we show only the data for the highest dose (20 mg·kg^{-1}) of S2 peptide (control mice pretreated with S2 peptide alone and OVA-mice pretreated with S2 peptide) because the lower doses of S2 peptide did not show any effect on control and OVA-mice.

Bronchoalveolar lavage (BAL) fluid

Immediately after blood collection, thoracic cavities were carefully opened. Tracheas were exposed, and BAL fluid was collected by cannulating the upper part of the trachea and lavaging twice with 1 mL and then 0.8 mL PBS (85–90% of the lavage volume was recovered). Lavaged samples from each mouse were kept on ice, and were centrifuged at 400× *g* for 5 min at 4°C. After centrifugation, lavage supernatants were removed, pellets were resuspended in 100 µL PBS, and total viable cell numbers were counted by Trypan blue exclusion using a haemacytometer. BAL cells were adjusted to a concentration of 5×10^4 cells·mL^{-1} in PBS. For cytospin preparations, cells were centrifuged at 400× *g* for 3 min using a Cytospin III (Shandon, Pittsburg, PA), and were stained with Diff-Quik (International Reagents Corp., Japan) for inflammatory cells. Differential cell counting was performed using standard morphological criteria (Kim *et al*., 2007).

Lung tissue histology

Lungs were perfused with 5 mL PBS via the right ventricle to wash out blood. Left lung tissues were removed from the chest cavity, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 µm. Tissue sections were subjected to haematoxylin and eosin staining to examine changes in general histology and periodic acid-Schiff (PAS) staining to examine goblet cell hyperplasia in the bronchial epithelium (Kim *et al*., 2007). Histological changes were scored in terms of the extent of infiltration of inflammatory cells into lung tissues: (i) none; (ii) slight; (iii) mild; (iv) moderate; and (v) severe. Goblet cell hyperplasia was quantified as percentage of PAS-positive cells/total cells in 10 sites of 100 µm × 100 µm areas after microscopy (Kim *et al*., 2007). The grading data are presented in histograms. The magnification used was 200 times. Right lung tissues were removed and stored at –70°C for assays of activities and amounts of various proteins.

Measurement of airway hyperresponsiveness (AHR)

AHR was assessed as described previously (Choi *et al*., 2006) by measuring changes in the airway resistance (cmH_2O·mL^{-1}·s^{-1}) and lung compliance (mL·cmH_2O^{-1}) using the Flexivent system (SCIREQ, Montreal, Quebec, Canada) after mice were treated with methacholine (MCh). Anaesthetized (pentobarbital sodium 70–90 mg·kg^{-1}, i.p.) and tracheostomized (18 G cannula) mice were mechanically ventilated (160 breaths·min^{-1}, tidal volume of 10 mL·kg^{-1}, positive end-expiratory pressure of 3 cmH_2O). Aerosols of increasing concentrations of MCh (3–25 mg·mL^{-1}) were administered by nebulization. Airway resistance (R_L) and lung compliance (C_L) were measured at 60 and 120 s after administration of MCh. Result of compliance was expressed as % change from the baseline values [(baseline value – value at each Mch concentration)/baseline value × 100].

Measurement of OVA-specific serum IgE

Blood samples immediately after anaesthesia were collected by cardiac puncture. Blood was allowed to clot at room temperature, and then centrifuged at 550× *g* for 30 min. Aliquots of serum were stored at –70°C until analysis for OVA-specific serum IgE by enzyme-linked immunosorbent assay (ELISA) (Kim *et al*., 2007). For OVA-specific IgE, microplates were coated with purified rat anti-mouse IgE, and then treated with mouse sera followed by biotinylated OVA which is generated with a peroxidase-conjugated goat anti-biotin antibody, which was used as secondary antibody. Reactions were read using an ELISA plate reader at 415 nm. Mouse IgE was used for calibrating the assay.

Western blot analysis

Immunoblotting were performed as described previously (Kim *et al*., 2007). BAL cells (1×10^6 cells) or lung tissues (50 mg·50 µL^{-1}) were suspended in a low-salt lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 µg·mL^{-1} aprotinin, 2.0 µg·mL^{-1} leupeptin] and allowed to swell on ice for 30 min. The cells or tissues were then homogenized using a Polytron (Kinematica, Littau, Switzerland). After centrifugation, supernatants obtained from cells or tissue extracts (20 µg) were analysed by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were washed with PBS containing 0.1% Tween 20 (PBST), and then blocked for 1 h in 5% skim milk in PBST. After the membranes were washed with PBST, they were incubated for 60 min at room temperature with primary antibodies against actin, TGase 2, eosinophil major basic protein (EMBP), Muc5ac, vascular cell adhesion molecule (VCAM)-1 or PLA_2 diluted with PBST

(1:1000). Membranes were washed with PBST and treated with horseradish peroxidise (HRP)-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG (diluted to 1:5000–1:10 000) in PBST for 60 min. After washing, the protein bands were visualized by enhanced chemiluminescence using a chemiluminometer (Amersham Biosciences, Buckinghamshire, UK).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from BAL cells (1×10^6 cells) or lung tissues (50 mg·50 μL^{-1}) using Trizol reagent. RT-PCR was performed in a final volume of 50 µL using a amfiRivert one-step RT-PCR kit (GenDEPOT, Barker, TX, USA) in an automated thermal cycler (BIOER Technology, Hangzhou, China). PCR assays were performed for 35 cycles. Each cycle consisted of the following steps: denaturation at 94°C for 30 s, annealling at 56°C for 45 s, and extension at 72°C for 1 min. PCR products were analysed using 1.0% agarose gel containing ethidium bromide (Kim *et al*., 2007).

The primer sequences used were as follows: Muc5ac sense, 5-AAA GAC ACC AGT AGT CAC TCA GCA A-3; Muc5ac anti-sense, 5-CTG GGA AGT CAG TGT CAA ACC A-3; IL-4 sense, 5-TCG GCA TTT TGA ACG AGG TC-3; IL-4 anti-sense, 5-GAA AAG CCC GAA AGA GTC TC-3; IL-5 sense, 5-ATG GAG ATT CCC ATG AGC AC-3; IL-5 anti-sense, 5-GTC TCT CCT CGC CAC ACT TC-3; IL-6 sense, 5-TGG AGT CAC AGA AGG AGT GGC TAA G-3-3; IL-6 anti-sense, 5-TCT GAC CAC AGT GAG GAA TGT CCA C-3; IL-8 sense, 5-CAA ACC TTT CCA CCC CAA AT-3; IL-8 anti-sense, 5-ATT GCA TCT GGC AAC CCT AC-3; IL-13 sense, 5-CAG CTC CCT GGT TCT CTC AC-3; IL-13 anti-sense, 5-CCA CAC TCC ATA CCA TGC TG-3; TNF-α sense, 5-TTA TCT CTC AGC TCC ACG CC-3; TNF-α anti-sense, 5-TGC GCA CTG AAA GCA TGA TC-3; RANTES sense, 5-GAT GGA CAT AGA GGA CAC AAC T-3; RANTES anti-sense, 5-TGG GAC GGC AGA TCT GAG GG-3; matrix metalloproteinase2 (MMP2) sense, CAC ACC AGG TGA ACC ATG TG; MMP2 anti-sense, AGG GCT GCA TTG CAA ATA TC; MMP9 sense, GTA TGG TCG TGG CTC TAA GC; MMP9 anti-sense, AAA ACC CTC TTG GTC TGC GG; tissue inhibitor of MMP1 (TIMP1) sense, CAC CAC CTT ATA CCA GCG TT; TIMP1 anti-sense, GTC ACT CTC CAG TTT GCA AG; TIMP2 sense, CCA GGT CCT TTT CAT CCT GA; TIMP2 anti-sense, TCC ATT CGC TGA AGT CTG TG; GAPDH sense, 5-GAT GCA GGG ATG ATG TTC TG-3; GAPDH anti-sense, 5-GTG AAG GTC GGT AAC GG-3.

Electrophoretic mobility shift assay (EMSA)

BAL cells (1×10^6 cells) or lung tissues (50 mg·50 μL^{-1}) were washed twice with ice-cold PBS, resuspended in 1 mL of an ice-cold cell lysis buffer [10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 1 $\mu g \cdot mL^{-1}$ leupeptin and 1 $\mu g \cdot mL^{-1}$ aprotinin] on ice for 15 min, and mixed with Nonidet P-40 (a final concentration of 0.625%, v/v) by vortexing immediately for 10 s. Nuclei were harvested by centrifugation at 20 000× *g* for 10 min and resuspended in 40 µL of an ice-cold nuclear lysis buffer [20 mM HEPES/KOH (pH 7.9), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.2 mM PMSF, 1 $\mu g \cdot mL^{-1}$ leupeptin and 1 $\mu g \cdot mL^{-1}$ aprotinin] at 4°C for 20 min on a shaking platform. After centrifugation at 15 000× *g* for 10 min, the supernatants containing the nuclear extracts were stored at –70°C. Using these nuclear extracts and NF-κB oligonucleotides (5′-AGT TGA GGG GAC TTT CCC AGG C-3′, 3′-TCA ACT CCC CTG AAA GGG TCC G-5′), EMSA for NF-κB was performed as described previously (Kim *et al*., 2007). Briefly, 10 µL of a mixture of NF-κB oligonucleotide (1.75 pmol·μL^{-1}), T4 polynucleotide kinase 10X buffer, [α-^{32}P]ATP (10 µCi; 3000 Ci·$mmol^{-1}$), nuclease-free water and T4 polynucleotide kinase (5–10 U·μL^{-1}) were incubated at 37°C for 30 min. The reaction was stopped by adding 1 µL EDTA (0.5 M), mixed with 89 µL of a buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] and eluting through a G-25 chromatographic spin column to separate unlabeled nucleotides. The ^{32}P-labeled nucleotides obtained (20–30 fmol) were incubated at room temperature for 20 min with the nuclear extracts that was already incubated with a gel shift binding 5X buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5 and 0.25 mg·mL^{-1} poly (dI-dC)] for 10 min. Each reaction was mixed with 10× gel loading buffer (1 µL) and separated by electrophoresis on 6% polyacrylamide gels. The radioactive bands on the gels were analysed using Phosphor Imager FLA-2000 (Fujifilm, Japan). The experiments were repeated four times and a representative result of the four experiments is presented.

Determination of levels of cytokines or MMPs

Amounts of cytokines (IL-4, IL-5, IL-13, TNF-α) or MMP2/9 in the supernatants obtained from lung tissues were determined using an ELISA kit. Lung tissues were homogenized in PBS (50 mg lung tissues·mL^{-1} PBS) using a Polytron (Kinematica, Littau, Switzerland) and centrifuged (770× *g*, 10 min) and then supernatants were collected. Standard curves were generated using different

concentrations of the recombinant cytokines. The limit of detection of this method was better than 7.8 pg·mL^{-1}.

Measurement of PLA_2 activity

PLA_2 activity was measured in 96-ELISA well plates by the technique described by Holzer and Mackessy (1996). The standard assay mixture contained 200 µL of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 µL of substrate (4-nitro-3-octanoyloxy-benzoic acid; 4N3OBA), 20 µL of water and 20 µL of sample (supernatant of lung tissue homogenates) in a final volume of 260 µL. After addition of sample, the mixture was incubated for up to 40 min at 37°C, and read at absorbance (425 nm) in 10 min intervals using a Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Enzyme activity, expressed as initial velocity of the reaction (V_o), was calculated based on the increase in absorbance after 20 min. All assays were in triplicate.

Leukotrienes (LTs) immunoassay

The LTs contents in BAL fluid and supernatants obtained from lung tissue homogenates were determined using an enzyme immunoassay kit according to the manufacturer's instructions. Briefly, 50 µL samples were incubated with anti-LT antiserum (diluted 1:120) and acetylcholinesterase-linked LTs (diluted 1:120) in wells that had been coated with mouse monoclonal antibodies for 18 h at room temperature. After rinsing with washing buffer, colour was developed using Ellman's reagent, and the plates were read at 412 nm with a spectrophotometer. The concentrations of LTs were then calculated using standard curves generated with specific LTs standards. The results are expressed in ng·mL^{-1} (1×10^6 cells). The LTs concentration was calculated using analysis tools on the Cayman Chemical website (Ann Arbor, MI, USA; http://www.caymanchem.com/app/template/analysis%2CEIA.vm/a/z).

Data analysis

Unless otherwise noted, experimental data are presented as mean ± SEM, $n = 8$. In Tables 1 and 2, the data are presented as geometric means with 95% confidence interval. Multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by Scheffe's *post hoc* test, using SPSS (SPSS Inc., Chicago, IL, USA). P-values < 0.05 were regarded as significant, but significant symbols among R2 peptide-treated groups were not shown in all Tables and Figures. The densitometry analysis of immunoblots, PCR and EMSA was performed with Quantity One version 4.6.3 (BIO-RAD, Hercules, CA, USA). Summary data from densitometry analysis are shown as mean ± SEM obtained from four independent experiments.

Materials

Ovalbumin (Grade V) and PAS stain were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aluminum hydroxide gel adjuvant (2% Alhydrogel) was purchased from Superfos Biosector (Vedbaek, Denmark). Diff-Quik from International Reagents Corp. (Kove, Japan). Antibody against mouse IgE was purchased from Bethyl Laboratories (Montgomery, TX). Antibodies against TGase 2, EMBP, Muc5ac and VCAM-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and the LT assay kit from Cayman Chemical. Antibody against HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Trizol reagent was from Molecular Research Center, Inc. (Cincinnati, OH, USA). 4-nitro-3-octanoyloxy-benzoic acid (4N3OBA) was from Life Sciences (Farmingdale, NY, USA), ELISA kit for each cytokines and MMPs from BD Bioscience (San Jose, CA, USA). All primers were from Bionics, Korea. NF-κB oligonucleotide was from Promega (Madison, WI, USA). R2 peptide and scrambled (S2) peptide (GVLDGGDP) were provided by Dr Soo Youl Kim (National Cancer Center, Seoul, Korea).

Results

Effect of R2 peptide on expression of TGase 2

TGase 2 expression was remarkably increased in BAL cells (Figure 1A left panel) and lung tissues (Figure 1A right panel) from mice sensitised to and challenged with OVA (OVA-mice), compared with levels in BAL cells and lung tissue from control mice. Pretreatment with R2 peptide, a TGase 2 inhibitor, dose-dependently reduced TGase 2 expression in OVA-induced BAL cells and lung tissues (Figure 1A). However, pretreatment with a range of doses of S2 peptide (5, 10 or 20 mg·kg^{-1}) did not affect TGase 2 expression. Therefore, we have shown only the data for the highest dose (20 mg·kg^{-1}) of S2 peptide in the all experiments.

Effect of R2 peptide on inflammatory cell recruitment and histopathological changes

Next, we examined the effects of R2 peptide on the infiltration of inflammatory cells into BAL fluid in OVA-mice. Total inflammatory cell levels (i.e. eosinophils, neutrophils, lymphocytes and macrophages) in BAL fluid were significantly elevated in

Table 1

Effect of R2 peptide on cytokine or MMP2/9 in the lung tissues from mice sensitised to and challenged with ovalbumin (OVA-mice)

		IL-4 (pg·mL^{-1})	IL-13 (pg·mL^{-1})	IL-5 (pg·mL^{-1})
	NC	106 (102–110)	0653 (626–680)	380 (364–397)
	GL	397 (385–409)***	1597 (1586–1608)***	694 (665–723)***
R2 peptide (mg·kg^{-1})	5	368 (359–376)	1487 (1473–1502)	601 (585–618)
	10	309 (301–316)$^{+}$	0920 (854–985)$^{++}$	540 (493–587)$^{++}$
	20	238 (226–250)$^{+}$	0713 (651–774)$^{++}$	423 (408–438)$^{++}$
S2 peptide (mg·kg^{-1})	Con	109 (103–115)	0657 (628–685)	386 (376–397)
	20	386 (367–404)***	1584 (1565–1603)***	685 (638–733)***
		TNF-α (pg·mL^{-1})	**MMP2 (ng·mL^{-1})**	**MMP9 (ng·mL^{-})**
	NC	0838 (796–881)	07.2 (6.1–8.2)	1.6 (0.7–2.5)
	GL	1750 (1672–1828)***	54.2 (47.6–60.8)***	28.5 (24.4–32.6)***
R2 peptide (mg·kg^{-1})	5	1636 (1581–1691)	55.4 (49.5–61.3)	26.6 (24.3–28.9)
	10	1404 (1214–1595)$^{++}$	37.8 (35.7–39.8)$^{++}$	17.8 (17.1–18.4)$^{+++}$
	20	0902 (862–942)$^{+++}$	24.1 (19.6–28.7)$^{+++}$	10.3 (7.9–12.7)$^{+++}$
S2 peptide (mg·kg^{-1})	Con	0842 (797–888)	07.5 (6.9–8.1)	02.0 (1.3–2.7)
	20	1784 (1656–1912)***	57.1 (53.0–61.1)***	29.9 (25.5–34.2)***

C57BL/6 mice were sensitized and challenged with OVA as described in Methods. R2 or scrambled (S2) peptide (5, 10 or 20 mg·kg^{-1}) was given i.p. once a day 10 min before every OVA challenge for 3 days. Individual cytokine and MMP2/9 levels in lung tissues were determined by ELISA. NC (negative control), mice sensitized and challenged with PBS; GL (OVA-mice), mice sensitized and challenged with OVA; R2 peptide, OVA-mice pretreated with different doses of R2 peptide (5, 10, 20 mg·kg^{-1}); Con (control), control mice pretreated with 20 mg·kg^{-1} S2 peptide (20), OVA-mice pretreated with S2 peptide (the data for only the highest dose (20 mg·kg^{-1}) is shown). Data are expressed as geometric mean with 95% confidence interval ($n = 8$). ***$P < 0.001$ versus the control (NC or Con) mice; $^{+}P < 0.05$; $^{++}P < 0.01$, $^{+++}P < 0.001$ versus GL mice.
ELISA, enzyme-linked immunosorbent assay; MMP, matrix metalloproteinase; TNF-α, tumour necrosis factor-α.

Table 2

Effect of R2 peptide on the leukotrienes (LTs) in bronchoalveolar lavage (BAL) fluid or lung tissues from mice sensitized to and challenged with ovalbumin (OVA-mice)

Groups	Leukotrienes (pg·mL^{-1}) BAL fluid	Lung tissues
NC	010.8 (8.4–13.1)	026.4 (24.2–28.7)
GL	296.7 (267.7–325.7)***	614.3 (577.9–650.7)***
R2 peptide (mg·kg^{-1})		
5	267.5 (242.8–292.1) [9.9]	569.1 (540.7–597.6) [7.4]
10	234.1 (197.3–270.9) [21.1]$^{+}$	467.2 (380.7–553.6) [24.0]$^{+}$
20	133.7 (109.3–158.0) [54.9]$^{+++}$	269.3 (211.2–327.3) [56.2]$^{+++}$
S2 peptide (mg·kg^{-1})		
Con	011.8 (10.1–13.6)	027.6 (26.4–28.8)
20	299.8 (265.8–333.7)***	626.9 (569.9–683.8)***

C57BL/6 mice were sensitized and challenged with OVA as described in Methods. R2 or scrambled (S2) peptide (5, 10 or 20 mg·kg^{-1}) was given i.p. once a day 10 min before every OVA challenge for 3 days. Treatment groups are as defined in Table 1. LTs released in BAL fluid or in supernatants obtained from lung tissue homogenates were determined using enzyme-linked immunosorbent assay. Data are expressed as geometric mean with 95% confidence intervals ($n = 8$). ***$P < 0.001$ versus the control (NC or Con) mice; $^{+}P < 0.05$; $^{++}P < 0.01$, $^{+++}P < 0.001$ versus GL mice.
Con, control for S2 pretreatment alone; GL, OVA-mice; NC, negative control.

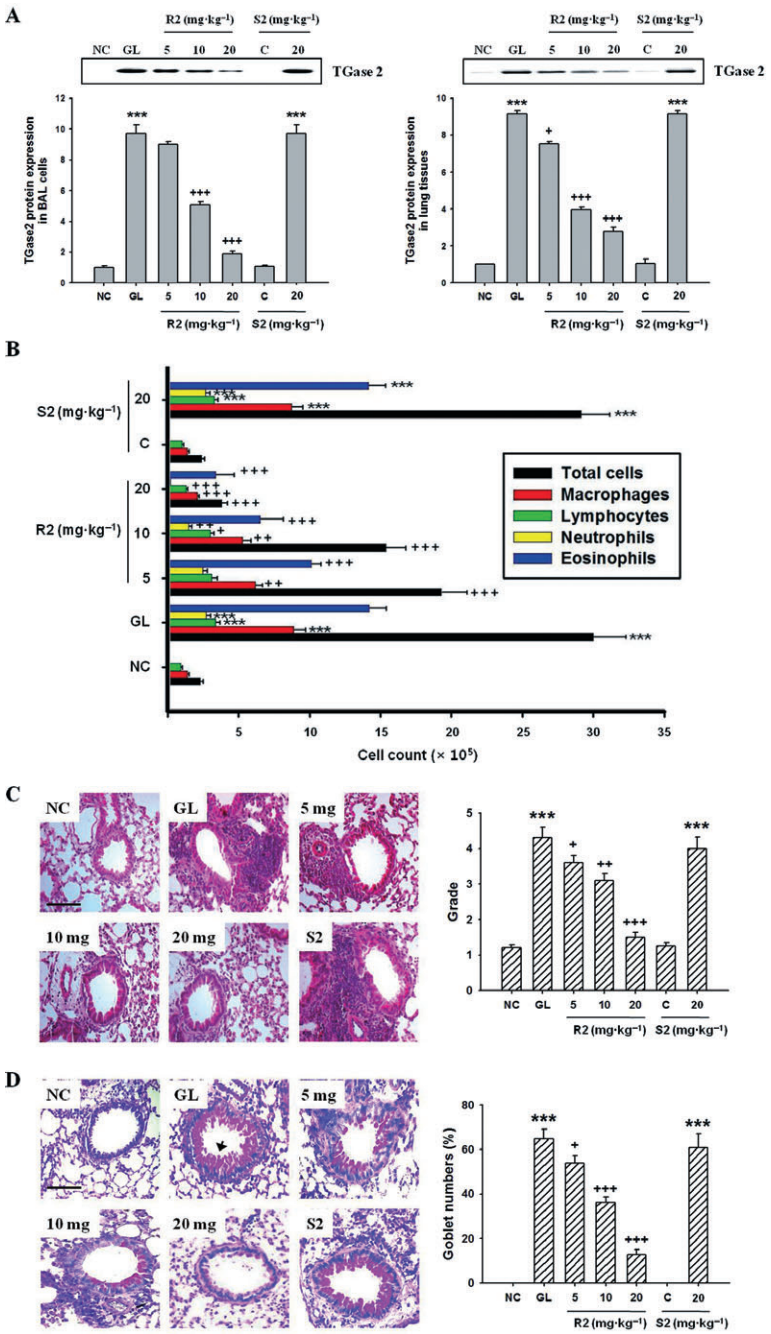

Figure 1

Effect of R2 peptide on the expressions of Transglutaminase 2 (TGase 2), recruitment of inflammatory cells, and histopathological changes in bronchoalveolar lavage (BAL) cells or lung tissues from mice sensitised to and challenged with ovalbumin (OVA) (OVA-mice). Experimental details are given in Methods. R2 or scrambled (S2) peptide (5, 10 or 20 mg·kg^{-1}) was given i.p. once a day 10 min before every OVA challenge for 3 days. NC (negative control), mice sensitized and nebulized with phosphate buffered saline (PBS); GL (OVA-mice), mice sensitized and challenged with OVA; R2 peptide, OVA-mice pre-treated with R2 peptide (5, 10 or 20 mg·kg^{-1}); C (control), control mice pretreated with 20 mg·kg^{-1} S2 peptide; S2 peptide (5, 10 or 20 mg·kg^{-1}), OVA-mice pre-treated with S2 peptide only data for highest dose,(20 mg·kg^{-1}) is shown. A, band images and summary data for TGase 2 expressions in BAL cells (left panel) or lung tissues (right panel) using Western blotting. Data shown are mean ± SEM (n = 4) for values from densitometry analysis. B, numbers of a variety of BAL cells counted by standard morphological criteria. C, lung tissues stained with haematoxylin and eosin (magnification: ×200). Histological appearances were scored for the presence of peribronchial and perivascular inflammation, and these scores were added together to give a total lung inflammation score; summary data is shown in the histogram. D, lung tissues stained with periodic acid-Schiff (PAS) (magnification: ×200). Numbers of goblet cell were quantified as the percentage of PAS-positive cells/total cells in 10 sites of 100 μm × 100 μm areas. Arrow in Figure 1D (GL) indicates goblet cells. ***P < 0.001 versus the control. ^{+}P < 0.05; ^{++}P < 0.01; ^{+++}P < 0.001 versus the OVA-mice. Bar in control group (NC) indicates 100 μm. Data for Con (control for S2 peptide) is indicated only in the histogram, for clarity. Data in B, C and D panels are expressed as mean ± SEM (n = 8).

OVA-mice relative to those in the NC mice (Figure 1B). Differential cell counts disclosed that eosinophils were most, and the lymphocytes least, increased. Pretreatment with the R2 peptide (5, 10 or 20 mg·kg^{-1}) dose-dependently prevented these increases in BAL cells in OVA-mice (Figure 1B).

Histological examination of lung tissue from OVA-mice also showed inflammatory cell infiltration in the peribronchial and perivascular areas (Figure 1C GL) and pretreatment with R2 peptide reduced inflammatory cell infiltration in these areas (Figure 1C). OVA challenge increased Goblet cells (pink dots) containing mucus were also increased in lungs from OVA-mice (Figure 1D GL) and the R2 peptide (5, 10, 20 mg·kg^{-1}) decreased goblet cell numbers, dose-dependently (Figure 1D).

Pretreatment with S2 peptide at doses up to 20 mg·kg^{-1}, did not affect inflammatory cell recruitment (Figure 1B and C) or goblet cell hyperplasia (Figure 1D).

Effect of R2 peptide on expressions of EMBP, Muc5ac or VCAM-1

As R2 peptide particularly reduced infiltration of eosinophils (Figure 1B), we examined the effect of R2 peptide on EMBP, a constituent of eosinophil secondary granules that is elevated in biological fluids from patients with asthma and other eosinophil-associated diseases (Srelts *et al.*, 1998; Plager *et al.*, 2006). R2 peptide pretreatment reduced the increased EMBP expression in BAL cells (Figure 2A left panel) or lung tissues (Figure 2A right panel) of OVA-mice. We observed that the ratios of EMBP expressions in each group mice are similar to the ratios of eosinophil numbers in OVA-induced mice and R2 peptide pretreatment mice

Airway mucous hypersecretion originates from hyperplastic goblet cells which typically express the protein Muc5ac (Ordonez *et al.*, 2001). Expression of Muc5ac mRNA (Figure 2B) and protein (Figure 2C) was increased in BAL cells (left panel) or lung tissues (right panel) of OVA-mice and this exspression was clearly reduced by pretreatment with R2 peptide.

OVA-mice increases the expression of the adhesion molecule, VCAM-1 (Kim *et al.*, 2007), which directs the extravasation of inflammatory cells into inflamed sites (Sriramarao *et al.*, 2000) was increased in BAL cells (Figure 2D left panel) or lung tissues (Figure 2D right panel) from OVA-mice and these increases were dose-dependently reduced by pretreatment with R2 peptide.

S2 peptide (20 mg·kg^{-1}) did not affect expressions of EMBP, Muc5ac mRNA and protein, or VCAM-1 in BAL cells or lung tissues of OVA-mice.

Effect of R2 peptide on serum levels of OVA-specific IgE

Antigen-specific Th2 responses induce antigen-specific IgE antibody production. We therefore tested whether the R2 peptide decreased OVA-specific IgE production in our allergic asthma mouse model. Serum OVA-specific IgE levels were elevated in OVA-mice (250 ± 8.4 ng·mL^{-1}) compared with control mice (0.8 ± 0.16 ng·mL^{-1}). R2 peptide (5, 10 or 20 mg·kg^{-1}) dose-dependently decreased OVA-specific IgE antibody levels (196 ± 13 ng·mL^{-1}for 5 mg·kg^{-1}; 140 ± 16 ng·mL^{-1} for 10 mg·kg^{-1}; 91.2 ± 10.3 ng·mL^{-1} for 20 mg·kg^{-1}). However, levels of OVA-specific IgE after pretreatment with S2 peptide (249 ± 6.9 ng·mL^{-1}, after 20 mg·kg^{-1}) were similar to those in OVA-mice (see above).

Effect of R2 peptide on AHR to MCh

Data presented in Figure 1B, C and D suggest that R2 peptide has anti-allergic activity. Therefore, we examined the effect of R2 peptide on AHR, a typical symptom in asthmatic disorders. AHR was assessed by the changes in R_L and C_L in response to increasing doses of MCh administration. The R_L values of OVA-mice were significantly increased by MCh administration, compared to those of the NC mice (Figure 3A). However, this MCh-induced increase of R_L in OVA-mice was effectively reduced by R2 peptide (5, 10 or 20 mg·kg^{-1}) pretreatment. The maximum effect seemed to be attained at 20 mg·kg^{-1} R2 peptide. The OVA-mice required a significantly decreased concentration of MCh to induce a 100% increase in R_L (the PC_{100}). R2 peptide pretreatment dose-dependently restored PC_{100} values, but pretreatment with S2 peptide did not affect this variable (Figure 3B). The lung compliance (C_L) was dramatically decreased in OVA-mice, compared with that in NC mice. This decrease was also significantly prevented by pretreatment with R2 peptide (Figure 3C) but not by the S2 peptide.

Effect of R2 peptide on mRNA and protein expressions of cytokines or MMPs

Allergic asthmatic inflammation and airway remodelling are associated with the secretion of a series of inflammatory cytokines, particularly IL-4, IL-5, IL-6, IL-8, IL-13, CCL5 and TNF-α (Kim *et al.*, 2007; Zhu and Gilmour, 2009). Expression of cytokines and chemokines in BAL cells (Figure 4A) and lung tissues (Figure 4B) from OVA-mice were increased, and pretreatment with the R2 peptide dose-dependently inhibited these increases (Figure 4A, B).

MMPs and TIMPs modulate the nature and outcome of allergic lung inflammation in murine asthma models (Sands *et al.*, 2009). OVA challenge

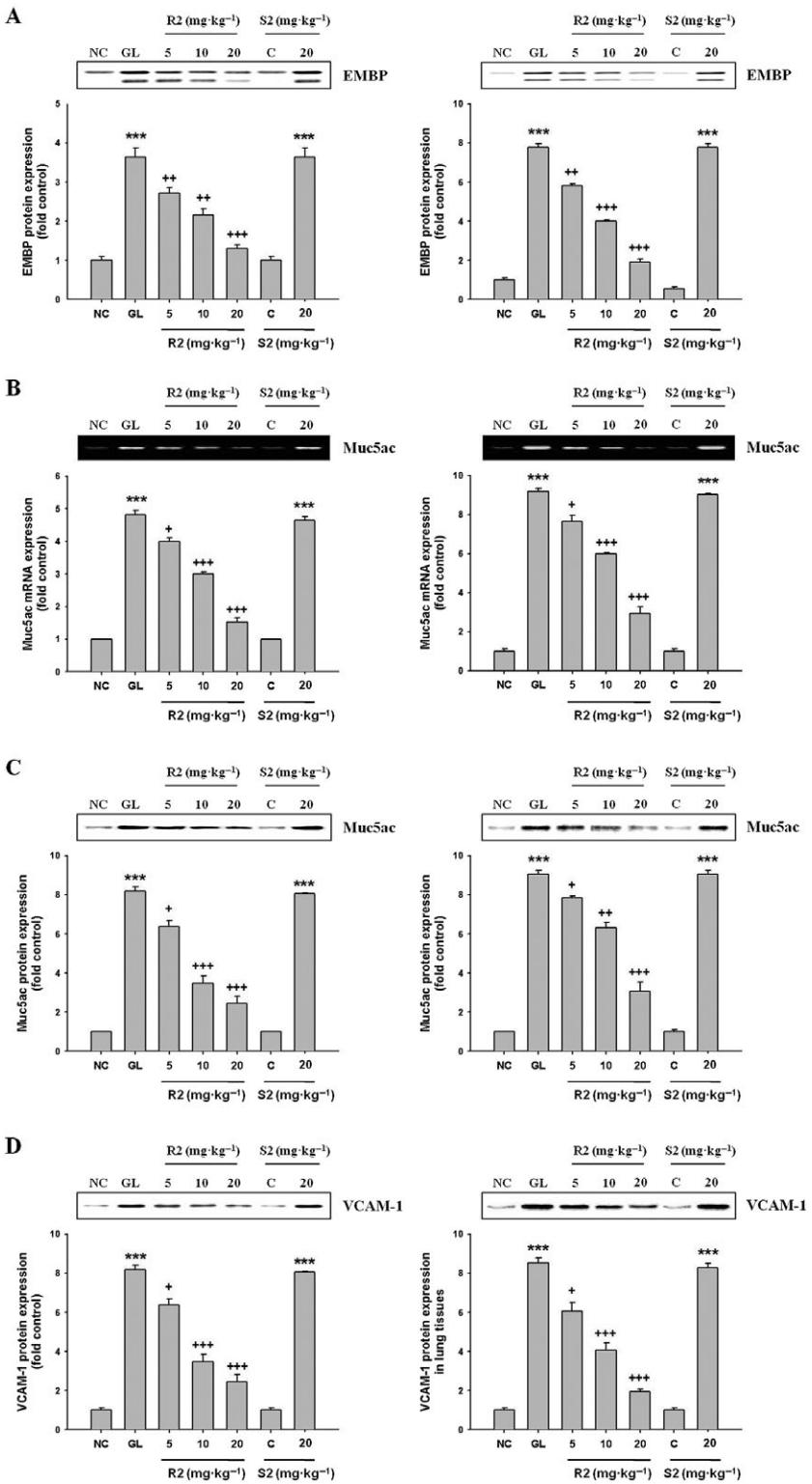

Figure 2

Effects of R2 peptide on the expressions of eosinophil major basic protein (EMBP), Muc5ac or vascular cell adhesion molecule (VCAM)-1 in bronchoalveolar lavage (BAL) cells and lung tissues from mice sensitised to and challenged with ovalbumin (OVA) (OVA-mice). A, band images and histogram for EMBP expression. B, C, band images and histogram for expressions of Muc5ac mRNA and protein respectively. D, band images and histogram for VCAM-1 protein expression. Data in histogram are means ± SEM (n = 4) for values from densitometry analysis. S2 peptide pretreatment showed only the data for high dose, 20 mg·kg^{-1}. Left and right panel, BAL cells and lung tissues respectively. ***P < 0.001 versus the control. ^{+}P < 0.05; ^{++}P < 0.01; ^{+++}P < 0.001 versus the OVA-mice group.

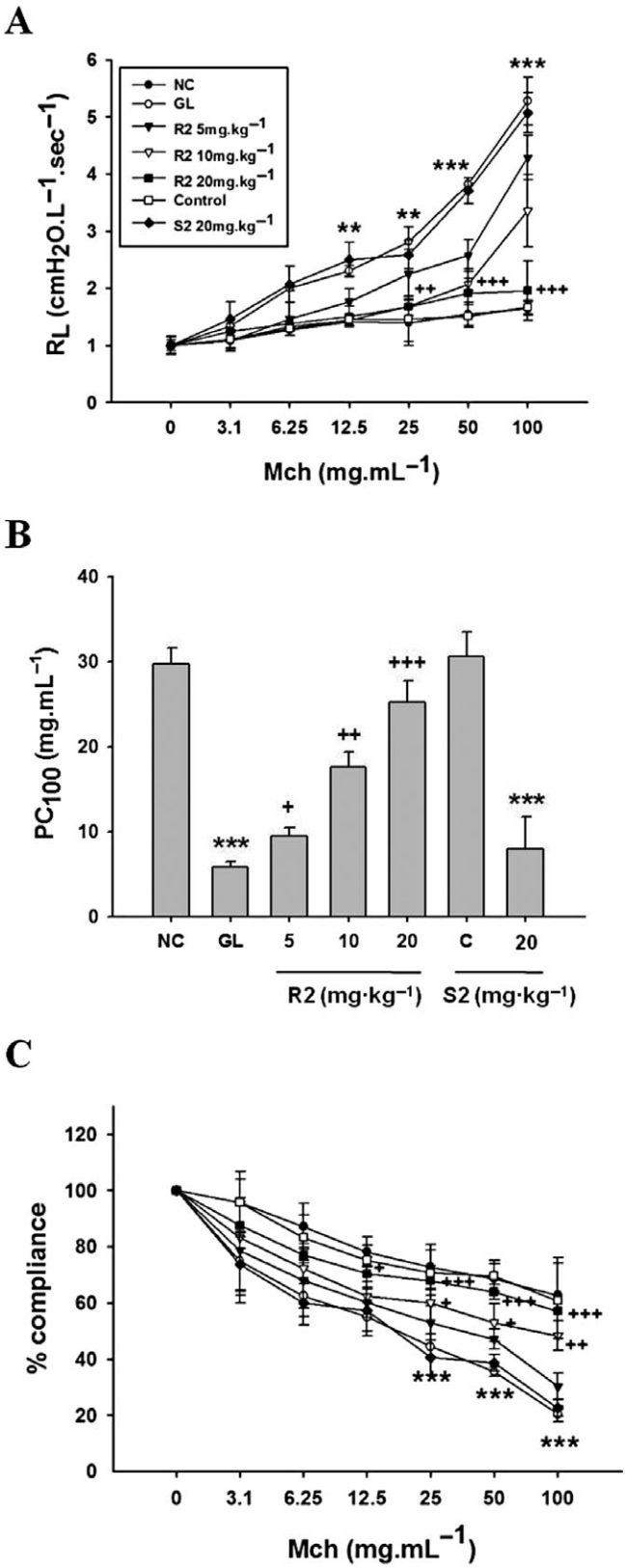

Figure 3

Effect of R2 peptide on airway hyperresponsiveness in mice sensitised to and challenged with ovalbumin (OVA) (OVA-mice). AHR at 48 h after last challenge were assessed by measuring changes in airway resistance (R_L) in 3A and compliance (C_L) in 3C, using the Flexivant system. The compliance values are expressed relative to the corresponding values in the control group (% control). The PC_{100} (in 3B) represents the dose of methacholine (MCh) which induces 100% increase in R_L. Negative control (NC), mice sensitized and nebulized with phosphate buffered saline (PBS); GL (OVA), mice sensitized and nebulized with OVA; R2 5 mg·kg^{-1}, 10 mg·kg^{-1} or 20 mg·kg^{-1}, OVA-mice received an i.p. injection of each dose of R2 peptide; Control, control mice received an i.p. injection of 20 mg·kg^{-1} S2 peptide; S2 20 mg·kg^{-1}, OVA-mice received an i.p. injection of S2 peptide. $^{**}P < 0.01$; $^{***}P < 0.001$ versus NC mice. $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$ versus OVA-mice. Data are expressed as mean ± SEM ($n = 8$).

of sensitized mice increased the levels of constitutive MMP-2 and inducible MMP-9, and decreased the levels of TIMP1/2. R2 peptide dose-dependently reduced expressions of MMP-2 and MMP-9 mRNA, but restored TIMP1/2 mRNA levels in BAL cells and lung tissues from OVA-mice (Figure 4C).

As expression of the mRNA for cytokines had been inhibited by the R2 peptide (Figure 4A B), we examined the effect of the R2 peptide on the protein levels of some cytokines (IL-4, IL-5, IL-13 and TNF-α) and MMP2/9. As shown in Table 1, R2 peptide inhibited the production of these cytokines and MMP2/9 in OVA-mice (Table 1).

However, S2 peptide (20 mg·kg^{-1}) had no effect on the expression of mRNA for cytokines (data not shown), MMP2/9 or TIMP1/2, or expressions of the corresponding proteins (Table 1).

Effect of R2 peptide on activity of NF-κB

NF-κB is a major transcriptional regulator of inflammation and the pathophysiology of asthma. OVA-mice exhibited increased NF-κB DNA binding activity in nuclear extracts from BAL cells (Figure 5A left panel) and lung tissues (Figure 5A right panel) which were inhibited by pretreatment with R2 peptide (Figure 5). Also, IκB protein expression was decreased and phosphorylation of IκB was increased in BAL cells and lung tissues of OVA-mice and pretreatment with the R2 peptide reversed these changes in IκB expression and phosphorylation (Figure 5B). S2 peptide (20 mg·kg^{-1}) did not affect NF-κB activation, IκB expression or phosphorylation of IκB in OVA-mice (Figure 5A, B).

Effect of R2 peptide on PLA_2 activity and protein expression

Transglutaminase 2 has anti-PLA_2 activity (Sohn *et al.*, 2003; Lee *et al.*, 2004) and R2 peptide inhibited TGase 2 expression in our experiments (Figure 1A).

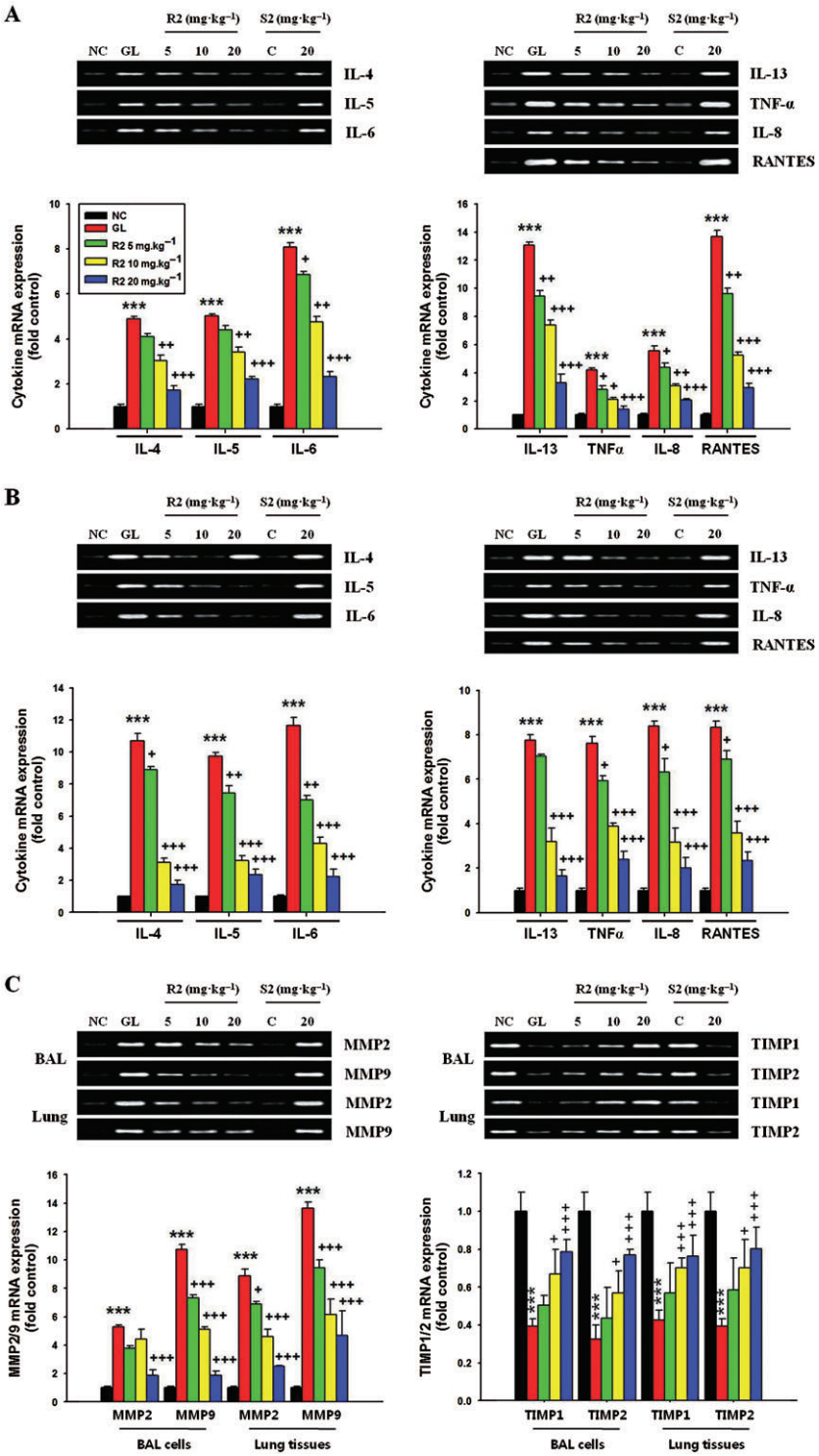

Figure 4

Effect of R2 peptide on gene expression in bronchoalveolar lavage (BAL) cells or lung tissues from mice sensitised to and challenged with ovalbumin (OVA) (OVA-mice). mRNA expression of cytokines or chemokines from homogenized BAL cells (1×10^6 cells) or lung tissues (50 mg·50 μL^{-1}) were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). A, B, band images and histogram for expression of cytokines or chemokines in bronchoalveolar lavage (BAL) cells (A) and lung tissues (B) respectively. C, band images and histogram for expression of matrix metalloproteinase (MMP)2/9 or tissue inhibitor of matrix metalloproteinase (TIMP)1/2. Data in histogram are means ± SEM ($n = 4$) for values from densitometry analysis. Data for S2 peptide are not shown as none of the doses of S2 peptide (5, 10, 20 mg·kg^{-1}) had any effect. MMP2/9, matrix metalloproteinase2, 9; TIMP1/2, tissue inhibitor of metalloproteinase1, 2. ***$P < 0.001$ versus control mice. $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$ versus OVA-mice.

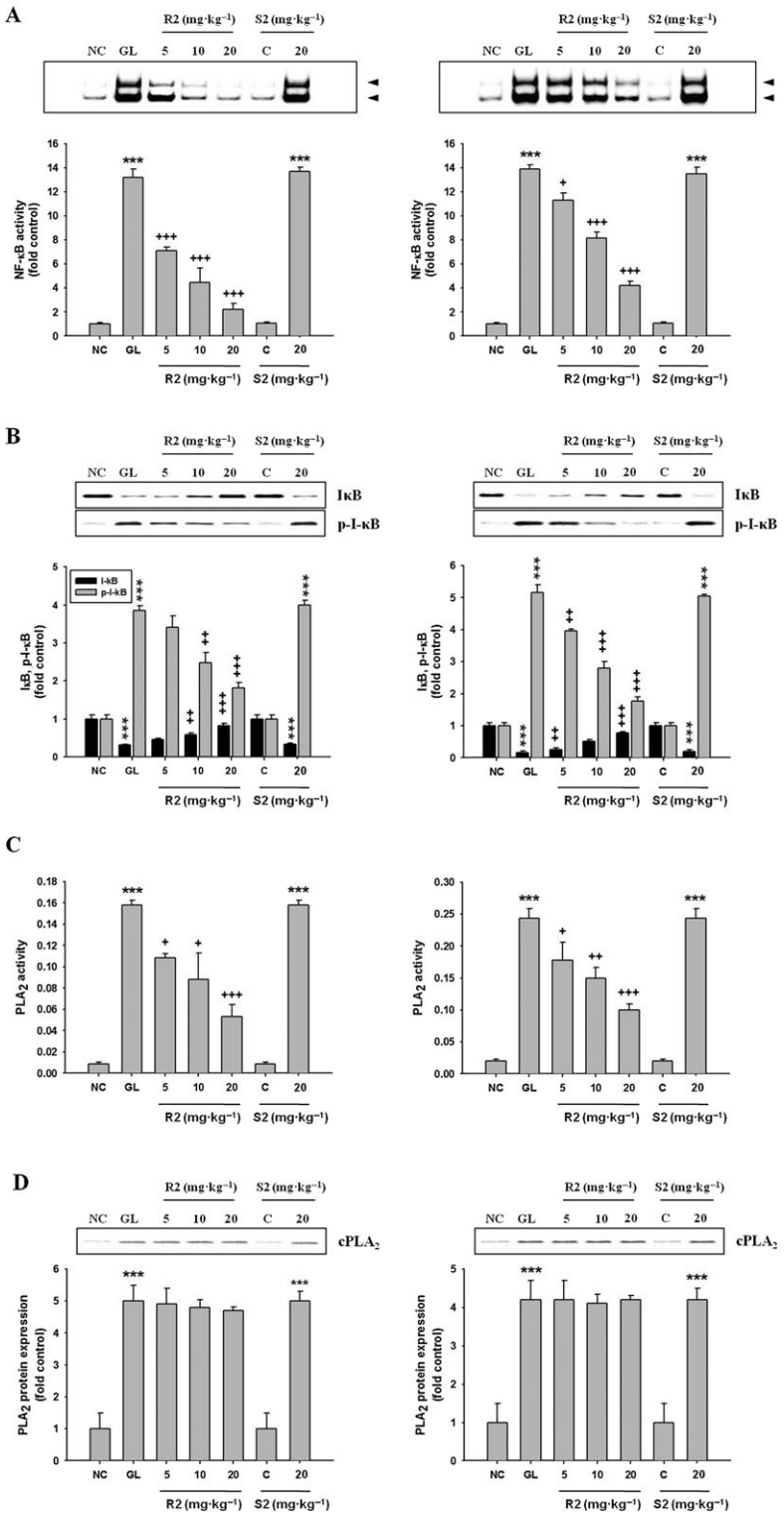

Figure 5

Effect of R2 peptide on nuclear factor-kappa B (NF-κB) and inhibitor of kappa B (IκB) activities in bronchoalveolar lavage (BAL) cells or lung tissues from mice sensitised to and challenged with ovalbumin (OVA) (OVA-mice). NF-κB or IκB activity from homogenized BAL cells (1×10^6 cells) or lung tissues (50 mg·50 μL^{-1}) was determined in nuclear extracts and protein using EMSA and Western blot respectively. A, band images and histogram for NF-κB activity. B, histogram for phospho-IκB activity and IκB protein expression. C, histogram for PLA_2 activity. D, band images and histogram for PLA_2 protein. Data in histogram are means ± SEM ($n = 4$) for values from densitometry analysis. ***$P < 0.001$ versus control mice. $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$ versus OVA-mice group.

Therefore, we examined the effects of R2 peptide on PLA_2 enzyme activity and protein expression in OVA-mice. Pretreatment with R2 peptide inhibited PLA_2 enzyme activity (Figure 5C), which was increased in OVA-mice, without inhibiting PLA_2 protein expression (Figure 5D) in BAL cells (left panel) or lung tissues (right panel). S2 peptide had no effect on PLA_2 activity or protein expression.

Effect of R2 peptide on the release of LTs

As PLA_2 activity was inhibited by R2 peptide pretreatment, we examined whether R2 peptide inhibited LTs release in OVA-mice. R2 peptide dose-dependently inhibited release of LTs, which was increased in the BAL fluid or lung tissues (Table 2). However, S2 peptide had no effect on the LTs release in the group of OVA-mice.

Discussion and conclusions

The prevalence of allergic asthma, one of the most common diseases encountered in the clinic, has increased worldwide over the last two decades. However, current allergic asthma therapy is not satisfactory. TGase 2 is associated with a variety of diseases (Sanchez *et al*., 2000; Citron *et al*., 2001; Junn *et al*., 2003; Picarelli *et al*., 2003; Roberts *et al*., 2003; Andrings *et al*., 2004; Kim *et al*., 2005; Cho *et al*., 2008; Naiyer *et al*., 2008; Hwang *et al*., 2009; Park *et al*., 2009a) including human allergic asthma (Hallstrand *et al*., 2010). R2 peptide inhibits TGase 2 to block inflammation in an allergic conjunctivitis model in guinea pigs (Sohn *et al*., 2003). However, there have been no reports on the effects of the R2 peptide in allergic asthma models in mice. Here, we have demonstrated that R2 peptide had anti-allergic activity by showing an inhibition of OVA-induced changes in AHR to MCh, and a marked reduction of increased eosinophil infiltration in BAL fluid and of increased inflammatory cells in the lung tissues. The anti-allergic activity of R2 peptide was further supported by the immunological data, showing an inhibition of increased OVA-specific IgE levels and an inhibition of expressions of cytokines and VCAM-1. R2 peptide also prevented airway tissue remodelling by reducing the increased goblet cell hyperplasia, collagen deposition and imbalance of MMPs and TIMPs.

The migration of inflammatory cells from blood into the airway occurs through binding to specific adhesion molecules such as VCAM-1 and chemokines such as CCL5 and IL-8 (Berkman *et al*., 1996; Zietkowski *et al*., 2009). VCAM-1 expression is enhanced by Th2-mediated cytokines such as IL-4, IL-13 and TNF-α (Sriramarao *et al*., 2000; Wills-Karp and Karp, 2004). The predominant inflammatory cells recruited into asthmatic lung tissues are eosinophils (Srelts *et al*., 1998; Kim *et al*., 2007). Eosinophilia in asthmatic BAL fluid is also associated with the production of IL-5, which plays a critical role in the differentiation, infiltration, activation of pulmonary eosinophils (Kim *et al*., 2007; Zhu and Gilmour, 2009) and VCAM-1 expression. Therefore, our data suggest that R2 peptide may reduce the numbers and infiltration of eosinophils by inhibiting IL-5 expression, and may reduce the trafficking of other inflammatory cells including eosinophils by inhibiting the adhesion molecule VCAM-1 expressed via Th2-mediated cytokines, and by inhibiting the chemokines such as IL-8 and CCL5, produced via NF-κB activation. This is further supported by similar ratios for inhibition by R2 peptide of eosinophil numbers (Figure 1B) and granular EMBP expression (Figure 2A), which is a constituent of eosinophil secondary granules and has been found to be elevated in asthmatic patients.

Neutrophils, lymphocytes (Th1 and Th2), and macrophages are also elevated in BAL fluid and lung tissues (Mckay *et al*., 2004), and neutrophils are particularly recruited by IL-8 (Gibson *et al*., 2003). It can be inferred from our data that R2 peptide may reduce migration of these cells (Figure 1B) via inhibiting the expressions of VCAM-1 and chemokines such as IL-8 and CCL5.

B cells require IL-4 and IL-13 and the CD40 ligand to switch to IgE production (Kim *et al*., 2007; Poulsen and Hummelshoj, 2007). R2 peptide blocked OVA-induced increases in IL-4 and IL-13 expression levels (Figure 4A, B; Table 1) and OVA-specific IgE levels. Therefore, our data suggest that R2 peptide inhibited the production of IgE via IL-4 and IL-13, although we did not evaluate the effects of the R2 peptide on CD40.

Tissue remodelling is characterized by goblet cell hyperplasia, the deposition of collagen, airway smooth muscle hypertrophy (Wills-Karp and Karp, 2004; Kim *et al*., 2007), and MMPs produced by IL-8, TNF-α and IL-1β (Gibson *et al*., 2003; Johnson *et al*., 2004; Sands *et al*., 2009). MMPs are proteolytic enzymes that can cleave proteins that contribute to the extracellular matrix. Alveolar macrophage-derived metalloproteinases in sputum lead to tissue remodelling in asthma (Ko *et al*., 2005). TIMP1/2 specifically modulates the activities of MMP2 and MMP9 (Atkinson *et al*., 2003; Sands *et al*., 2009). Goblet cell hyperplasia in asthma is also stimulated by IL-4 and IL-13, and their inhibition may provide an important therapeutic strategy for mucus hypersecretion (Hoshino *et al*., 2005). Our data suggested that R2 peptide may reduce tissue remodelling by balancing MMP2/9 and TIMP1/2 (Figure 4C;

Table 1) via regulation of the expression of IL-8 and TNF-α and by reducing goblet cell hyperplasia and Muc5ac expression (Figures 1D and 2B) caused by IL-4, 5 and 13.

NF-κB is released from its complex with IκB by phosphorylation of IκB and is subsequently translocated to act in the nucleus. NF-κB increases the expression of many genes related to airway inflammation in asthma (Bureau *et al.*, 2000). Thus, inflammatory cytokines (Zimmermann *et al.*, 2003; Sheller *et al.*, 2009), adhesion molecules (Sriramarao *et al.*, 2000), and MMPs (Ko *et al.*, 2005; Kim *et al.*, 2007; Sands *et al.*, 2009) are up-regulated in asthmatic airways downstream of NF-κB activation. NF-κB activation in airway epithelium also enhances the AHR and mucus secretion in mouse allergic inflammation (Sheller *et al.*, 2009). Recently, TGase 2, which is over-expressed in asthmatic airways, was found to be a novel mediator in the pathogenesis of asthma (Hallstrand *et al.*, 2010). TGase 2 induced by NF-κB may also activate NF-κB without kinase activation (Lee *et al.*, 2004; Kim, 2006). Therefore, our findings suggest that R2 peptide may reduce allergic inflammation and tissue remodelling by suppressing NF-κB activation (Figure 5A) via regulation of the phosphorylation of IκB (Figure 5B). This is supported by reduction of AHR and the migration of inflammatory cells caused with up-regulation of cytokines/chemokines and adhesion molecules, and by reduction of tissue remodelling via regulation of MMPs and goblet cell hyperplasia, in OVA-specific allergic responses shown after R2 peptide pretreatment.

TGase 2 is expressed after NF-κB activation via a IKK-dependent pathway and the expressed TGase 2 activates NF-κB via an IKK-independent pathway during immune cell activation (Lee *et al.*, 2004), because the TGase 2 promoter has a NF-κB binding motif (Mirza *et al.*, 1997). Therefore, it can be inferred from our data that TGase 2 expression may be induced by NF-κB activation via a IKK-dependent pathway in airway epithelial cells or in the infiltrated cells in lungs from mice sensitized to and challenged with OVA, as demonstrated by Hallstrand *et al.*, (2010). Alternatively, the NF-κB activation could be via inflammatory cytokines which are produced by activation of BAL cells such as eosinophils and neutrophils, as supported by the report that inflammatory cytokines can increase the expression of TGase 2 (Kim *et al.*, 2002). However, our present data cannot differentiate between these two possibilities and further experiments are required to clarify this situation.

Moreover, it has been known that TGase 2 increases the catalytic activity of PLA_2 by forming intramolecular linkage facilitating dimerization of PLA_2 (Cordella-Miele *et al.*, 1990). TGase 2 is expressed in the asthmatic airway epithelium (Hallstrand *et al.*, 2010), and induces airway inflammation and structural remodelling in asthma by acting on the PLA_2-cysteinyl-LTs axis (Bowton *et al.*, 1997; Henderson *et al.*, 2007; Ram *et al.*, 2008; Mehrotra and Henderson, 2009; Hallstrand *et al.*, 2010). Our data also suggest that R2 peptide reduced PLA_2 activity by suppressing TGase 2 expression via regulating NF-κB activation in BAL cells and lung epithelial cells of OVA-challenged mice. However, TGase 2 expression was not directly measured in lung epithelium, when LT production was assayed (Table 2). Hence further studies are needed to better understand the order of events.

In conclusion, the data suggest that the R2 peptide reduced the symptoms of this allergic asthma model through regulation of a variety of molecules associated with allergic inflammation and lung tissue remodelling, by inhibiting NF-κB activation through IKK-dependent pathway. In addition the R peptide inhibited PLA_2, activated by TGase 2 expressed with NF-κB activation in our model. We suggest that the R2 peptide should be evaluated as a potential therapeutic agent for airway inflammation and tissue remodelling in allergic asthma.

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Conflicts of interest

None

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