



Immunopharmacology and Inflammation

8-oxo-2'-deoxyguanosine suppresses allergy-induced lung tissue remodeling in mice

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ABSTRACT

We previously reported that 8-oxo-2'-deoxyguanosine (8-oxo-dG) suppressed airway hyperresponsiveness and allergy-associated immune responses in ovalbumin-induced allergic mice by inactivating Rac. In the present study, 8-oxo-dG was investigated for its suppression of inflammation and remodeling in lung tissues induced by allergic reaction in mice. Mice were sensitized and challenged with ovalbumin without or with oral administration of 8-oxo-dG. The mice without 8-oxo-dG administration showed the following inflammatory and airway remodeling signs: infiltration of inflammatory cells into peribronchial area, hyperplasia of mucus-secreting goblet cells in bronchial walls, increase of expressions of Muc5ac and vascular cell adhesion molecule (VCAM)-1, collagen deposition and protein expression, and matrix metalloproteinase (MMP)-2/-9 expressions. We also observed an increase of various inflammation-mediating proteins, namely IL-4, IL-5, IL-8, IL-13, TNF-α and IFN-γ, and activation of STAT1 and NF-κB. Production of reactive oxygen species and nitric oxide (NO) was increased as indicated by a dramatic increase in formation of nitro-tyrosine. Importantly, Rac1 and 2 were also markedly activated. However, 8-oxo-dG suppressed all these inflammatory and tissue remodeling signs as well as activation of Rac1 and 2. These results indicate that 8-oxo-dG can inhibit allergy-induced inflammation and remodeling in airway and lung tissues through Rac inactivation.

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1. Introduction

Thus far, 8-oxo-2'-deoxyguanosine triphosphate (8-oxo-dGTP) and 8-oxo-2'-deoxyguanosine triphosphate (8-oxo-GTP) were regarded simply as mutagenic wastes since they are incorporated into DNA and RNA, making 8-oxo-guanine-containing DNA and RNA, respectively, unless they are hydrolyzed by 8-oxo-dGTPase into 8-oxo-2'-deoxyguanosine monophosphate (8-oxo-dGMP) or 8-oxo-guanosine monophosphate (8-oxo-GMP) (Hayakawa et al., 1999; Taddei et al., 1997). However, we recently found that 8-oxo-GTP inactivates Rac while GTP activates it (Kim et al., 2007a,b). Interestingly, 8-oxo-2'-deoxyguanosine (8-oxo-dG) and 8-oxo-guanosine (8-oxo-G), both of which are phosphate-less 8-oxo-guanine-containing nucleosides, were also found to be able to inactivate Rac (Choi et al., 2007; Kim and Chung, 2006; Lee et al., 2009). Rac is known to be essentially linked to inflammation-provoking activities of phagocytes, which include phagocytosis and production of reactive oxygen species (Yamauchi et al., 2004), chemotaxis (Weiss-Haljiti et al., 2004), release of pro-

inflammatory cytokines (Abdel-Latif et al., 2004; Bokoch, 2005), and production of nitric oxide (NO) and prostaglandins (Kim et al., 2006a). Rac also serves important functions in cells of myeloid lineage which contribute to inflammation (Abreu et al., 2010; Benvenuti et al., 2004). We also observed that 8-oxo-dG, 8-oxo-G, the Rac-inactivating nucleosides inhibited these Rac-linked functions of phagocytes: production of reactive oxygen species, chemotaxis and release of pro-inflammatory cytokines (Lee et al., 2009), and production of NO and prostaglandins (Kim et al., 2006a).

Consistent with these observations, the nucleosides were shown to exhibit anti-inflammatory activity by inhibiting elevation of serum levels of the pro-inflammatory cytokines, infiltration of neutrophils into lung tissues and animal death in LPS-treated mice (Choi et al., 2007). Simultaneously, the suppression of Rac activity was observed in lung tissues of these mice (Choi et al., 2007), suggesting that the anti-inflammatory activity was mediated by inactivation of Rac. In the inhibition of Rac-linked functions and inflammatory reaction, 8-oxo-dG exhibited more potency than 8-oxo-G while deoxyguanosine (dG), guanosine (G) and adenosine (A) showed no effect (Choi et al., 2007; Kim et al., 2006a; Lee et al., 2009). Furthermore, 8-oxo-dG suppressed airway hyperresponsiveness and allergy-associated immune responses in bronchoalveolar lavage fluid and lung tissues of ovalbumin-induced allergic mice by inactivating Rac (Ro et al., 2009).

Airway obstruction is the most common and distressing symptom of allergic asthma and thus, the primary target of asthma management. However, airway inflammation is also an important target of

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treatment since it is the most common etiological factor associated with the initiation of asthma and is a key player in the modulation and perpetuation of allergic asthmatic responses (Levine, 2003) because the persistent inflammation in airway tissues may lead to structural changes known as airway remodeling (DiGiovanni et al., 2009; Tagaya and Tamaoki, 2007). Therefore, suppression of inflammation and structural changes in asthma is an important requisite for reduction of airway hyperresponsiveness and exacerbations of asthmatic symptoms, as well as the improvement of lung function (Levine, 2003; Tagaya and Tamaoki, 2007). Based on the above observations, it is strongly suggested that 8-oxo-dG is able to inhibit the tissue remodeling related to the inflammatory reaction induced by allergic/asthmatic condition. In the present study, this assumption was tested by observing whether 8-oxo-dG can suppress inflammatory responses and lung tissue remodeling manifested in mice with allergic condition induced by ovalbumin. We observed that 8-oxo-dG effectively inhibited the inflammatory responses and remodeling in lung tissues of mice, together with suppression of Rac activation.

2. Materials and methods

2.1. Materials

Ovalbumin (Grade V), aluminum hydroxide gel adjuvant (2% Alhydrogel), hematoxylin, eosin and periodic acid-Schiff (PAS) were purchased from Sigma-Aldrich (St. Louis, MO); Diff-Quik from international Reagents Corp. (Kove, Japan); Sircol collagen kit from Biocolor Ltd. (Carrickfergus, UK); aprotinin, leupeptin from Roche (Basel, Switzerland); antibodies against VCAM-1 and Muc5ac from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); antibody against STAT1 from cell signaling (Beverly, MA); antibody against HRP-conjugated goat anti-mouse, HRP-conjugated rabbit anti-goat IgG and fluorescence-conjugated anti-rabbit IgG secondary antibody from Zymed Laboratories Inc. (San Francisco, CA); Trizol reagent from Molecular Research Center, Inc. (Cincinnati, OH); nitrocellulose membranes, chemiluminescent, [γ - ^{32} P]ATP (specific activity, 3000 Ci/mmol) from Amersham Biosciences (Buckinghamshire, UK); amfiR-ivert one-step RT-PCR kit from GenDEPOT (Barker, TX); ELISA kit for each cytokines and MMPs from BD Bioscience (San Jose, CA); NF- κ B oligonucleotide from Promega (Madison, WI); anti-nitrotyrosine antibody from Upstate (Billerica, MA); EZ-Detect protein activation kits from Upstate Biotechnology (Lake Placid, NY). All primers were purchased from Bionics, Korea.

2.2. Animals

Female BALB/c mice, 6–8 weeks old, were obtained from ORIENT BIO (Seongnam Co, Korea) and maintained in specific pathogen-free conditions before sacrifice. All animals used in experiments were housed in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all protocols were approved by the institutional review board and conducted in the Laboratory Animal Research Center of Sungkyunkwan University.

2.3. Allergy induction with ovalbumin and treatment with 8-oxo-dG

The experimental mice were divided into six groups, each group containing 8 animals ($n=8$). Control (Con, negative control), mice sensitized with phosphate buffered saline (PBS) and nebulized with PBS; General sensitization (GS), mice sensitized with ovalbumin and nebulized with PBS; Ovalbumin (general and local challenge), mice sensitized and nebulized with ovalbumin; 6 mg, 30 mg or 60 mg, ovalbumin mice orally administered 6, 30 or 60 mg/kg of 8-oxo-dG, respectively, 6 h before every challenge with ovalbumin. The symbols

and abbreviated names for each group were used consistently to avoid confusion.

Allergy was induced in mice by sensitization and then challenge with ovalbumin as previously described (Kim et al., 2009). For purposes of the sensitization, 250 μ l PBS (pH 7.4) or 250 μ l PBS containing 10 μ g ovalbumin adsorbed in 12.5 μ l aluminum hydroxide gel adjuvant were injected into the mice intraperitoneally on days 0, 5, 14, 21 and 28. For purposes of the challenge, mice were subjected to whole-body mainstream nebulization in a Plexiglas chamber (23 $\times$ 23 $\times$ 19 cm) connected to a Stand nebulizer (Mega medical, Seoul, Korea). That is, mice sensitized with PBS or ovalbumin were nebulized with PBS or 2% ovalbumin in PBS for 10 min once a day for 7 days, beginning on day 35 (one week after the final injection of ovalbumin) until day 41, and nebulized on day 49 as the final challenge. All mice were sacrificed on day 50.

For 8-oxo-dG treatment, 8-oxo-dG (6, 30 or 60 mg/kg) was dissolved in PBS and administered orally once a day 6 h before every ovalbumin challenge for 7 days (from day 35 to day 41). The data from control (Con) or general sensitization mice treated with 8-oxo-dG (6, 30, or 60 mg/kg) were not presented because the compound did not have any significant effects in these groups.

2.4. Lung tissue histology

Mouse lungs were perfused with 5 ml PBS via the right ventricle. Left lung tissues were removed from the chest cavity, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 μ m. Tissue sections were subjected to hematoxylin and eosin (H & E) staining to examine changes in the general histology of lung tissues, and periodic acid-Schiff (PAS) staining to examine goblet cell hyperplasia in the bronchial epithelium (Kim et al., 2009), and van Gieson staining for collagen deposition (Masuda et al., 2003). Histological changes for H & E staining were scored in terms of the extent of infiltration of inflammatory cells including eosinophils and neutrophils into lung tissues, which were 1, none; 2, slight; 3, mild; 4, moderate; 5, severe. Goblet cell hyperplasia was quantified as the percentage of PAS-positive cells/total cells in 10 sites of 100 μ m $\times$ 100 μ m areas under microscopy. Histological changes for collagen deposition were scored into 8 grades using Scarford score (Masuda et al., 2003): which were 0, normal lung; 1 to 2, minimal fibrous thickening of alveolar or bronchiolar walls; 3 to 4, moderate thickening of walls without obvious damage to the lung architecture; 5 to 6, increased fibrous with definite damage to the lung architecture and formation of fibrous bands or small fibrous masses; 7, severe distortion of the architecture and a large fibrous area; and 8, total fibrous obliteration of the field using conventional and polarized light microscopy (Axiophot, Carl Zeiss, Oberkochen, Germany). The scoring results in each panel are presented as histograms. Right lung tissues were removed and stored at -70°C for assays of activities and amounts of various proteins.

2.5. Immunohistochemistry for VCAM (vascular cell adhesion molecule)-1 expression

Briefly, the paraffin-embedded sections of right lung lobes were deparaffinized with xylene and then rehydrated. Section slides were incubated at 4°C with 3% hydrogen peroxide for 5 min, 1% BSA in PBS (blocking solution) for 1 h, and then incubated with anti-VCAM-1 antibody (1:50 dilution) in blocking solution for 24 h. After washing with PBS, the slides were treated with biotinylated secondary antibody for 10 min, streptavidin-HRP (horse radish peroxidase) for 10 min and 3, 3'-diaminobenzidine (chromogen) for 5 to 10 min (DAKO, Carpinteria, CA). The intensity of dark brown color developed in each tissue section was scored into 5 grades, which were 0, normal (no color); 1, slight; 2, mild; 3, moderate; 4, dark. The grading data obtained is presented as a histogram.

2.6. Collagen content in lung tissues

Collagen amounts in lung tissues were assessed by the Sircol collagen assay, according to the manufacturers' instruction (Pilling et al., 2007). Briefly, the lung tissues (100 mg) were homogenized and mixed with 1 ml of Sircol Dye Reagent for 30 min at room temperature in a mechanical shaker. The precipitate of collagen-dye complex was obtained by centrifugation at $10,000\times g$ for 10 min. The unbound dye solution was then carefully removed. The precipitated complex was resolved in 1 ml of 0.5 M sodium hydroxide and the obtained solution was placed in a 96 well flat-bottomed plate and evaluated in a plate reader at 550 nm. Collagen content was calculated by comparing the sample values to a standard curve.

2.7. Immunoblot analysis

Lung tissues (50 mg/50 μ l) 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 μ g/ml aprotinin, 2.0 μ g/ml leupeptin], allowed to swell on ice for 30 min. The tissues were then homogenized using a Polytron (Kinematica, Littau, Switzerland). After centrifugation, supernatants obtained from tissue extracts (20 μ g) were subjected to 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. 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 washing with PBST, the membranes were incubated for 60 min at room temperature with primary antibodies against STAT (signal transduction and signal transducer and activator of transcription) 1, mucin 5ac (Muc5ac) and VCAM-1 or against phosphorylated STAT1 p-STAT1, which were diluted with PBST (1:1,000). Membranes were washed with TBST and treated with 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 (Kim et al., 2009).

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from lung tissues (50 mg/50 μ l) using Trizol reagent. RT-PCR was performed in a final volume of 50 μ l using a amfiRivert one-step RT-PCR kit 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 sec, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. PCR products were analyzed using 1.0% agarose gel containing ethidium bromide (Kim et al., 2009).

The primer sequences used were as follows: Muc5ac sense, 5'-AAA GAC ACC AGT CAC TCA GCA A-3', Muc5ac anti-sense, 5'-CTG GGA AGT CAG TGT CAA ACC A-3'; VCAM-1 sense, 5'-CCC AAC GAT CCA GAG ATT CA-3', VCAM-1 anti-sense, 5'-TAA GGT GAG GGT GGC ATT TC-3'; MMP2 sense, 5'-CAC ACC AGG TGA ACC ATG TG-3'; MMP2 anti-sense, 5'-AGG GCT GCA TTG CAA ATA TC-3'; MMP9 sense, 5'-GTA TGG TCG TGG CTC TAA GC-3'; MMP9 anti-sense, 5'-AAA ACC CTC TTG GTC TGC GG-3'; TIMP1 sense, 5'-CAC CAC CTT ATA CCA GCG TT-3'; TIMP1 anti-sense, 5'-GTC ACT CTC CAG TTT GCA AG-3'; TIMP2 sense, 5'-CCA GGT CCT TTT CAT CCT GA-3'; TIMP2 anti-sense, 5'-TCC ATT CGC TGA AGT CTG TG-3';

GAPDH sense, 5'-GAT GCA GGG ATG ATG TTC TG-3', GAPDH anti-sense, 5'-GTG AAG GTC GGT AAC GG-3'.

2.9. Measurement of levels of cytokines and matrix metalloproteinase (MMP)-2 and -9

Lung tissues (100 mg/100 μ l) were homogenized in PBS using a Polytron (Kinematica, Littau, Switzerland) and centrifuged ($10,000\times g$, 10 min). The supernatants obtained were used for assays of interleukin-4 (IL-4), IL-5, IL-13, tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ) and MMP-2 and -9 by ELISA according to the manufacturer's instructions. The amounts of cytokines and MMPs were calculated by comparing the sample values to standard curves. Standard curves were generated using different concentrations of the recombinant cytokines. The lowest detection limit for IL-4 and IL-8 was 7.8 pg/ml, IL-5 was 15.6 pg/ml, IL-13 was 6 pg/ml, TNF- α was 60 pg/ml, IFN- γ was 5 pg/ml, MMP-2 was 25 pg/ml and MMP-9 was 50 pg/ml.

2.10. Estimation of nitrotyrosine by immunofluorescence

Oxidative stress in lung tissues of allergic mice was measured in terms of peroxynitrite-mediated tyrosine nitration, which was estimated using an anti-nitrotyrosine antibody/immunofluorescence technique (Thiyagarajan and Shaarma, 2004). Briefly, left lungs were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 μ m. The tissue sections were then fixed with 0.1% glutaraldehyde in PBS for 15 min on ice, washed with PBS, and permeabilized with 0.1% Triton X-100 for 15 min. After blocking with 2% BSA (bovine serum albumin) for 1 h at room temperature, the sections were incubated with 10 μ g/ml anti-nitrotyrosine antibody overnight at room temperature. They were then washed with PBS, incubated with a 1:100 dilution of fluorescence-conjugated anti-rabbit IgG secondary antibody for 1 h in the dark. After mounting, the tissue sections were examined under a fluorescent microscope.

2.11. Rac assay

Rac activity was assayed as previously described (Yoon et al., 2005) using EZ-Detect protein activation kits. Mouse lung tissues (50 mg) were suspended in 50 μ l of a lysis buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 1 mM DTT, 5% glycerol, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin] for 30 min on ice and homogenized. Supernatants were obtained by centrifugation ($13,000\times g$ for 20 min). According to the manufacturer's protocol, the active form of Rac was obtained from the supernatants by affinity precipitation using a Pak-1 Rac-binding domain (an effector protein of Rac), which was fused to GST (glutathione-S-transferase) and visualized by immunoblotting with anti-rabbit Rac1 (1:1000; PIERCE). To measure the total level of Rac protein, supernatants were used directly for immunoblotting. The band densities were quantified using a LAS 2000 imaging analyzer (Fujifilm, Tokyo, Japan).

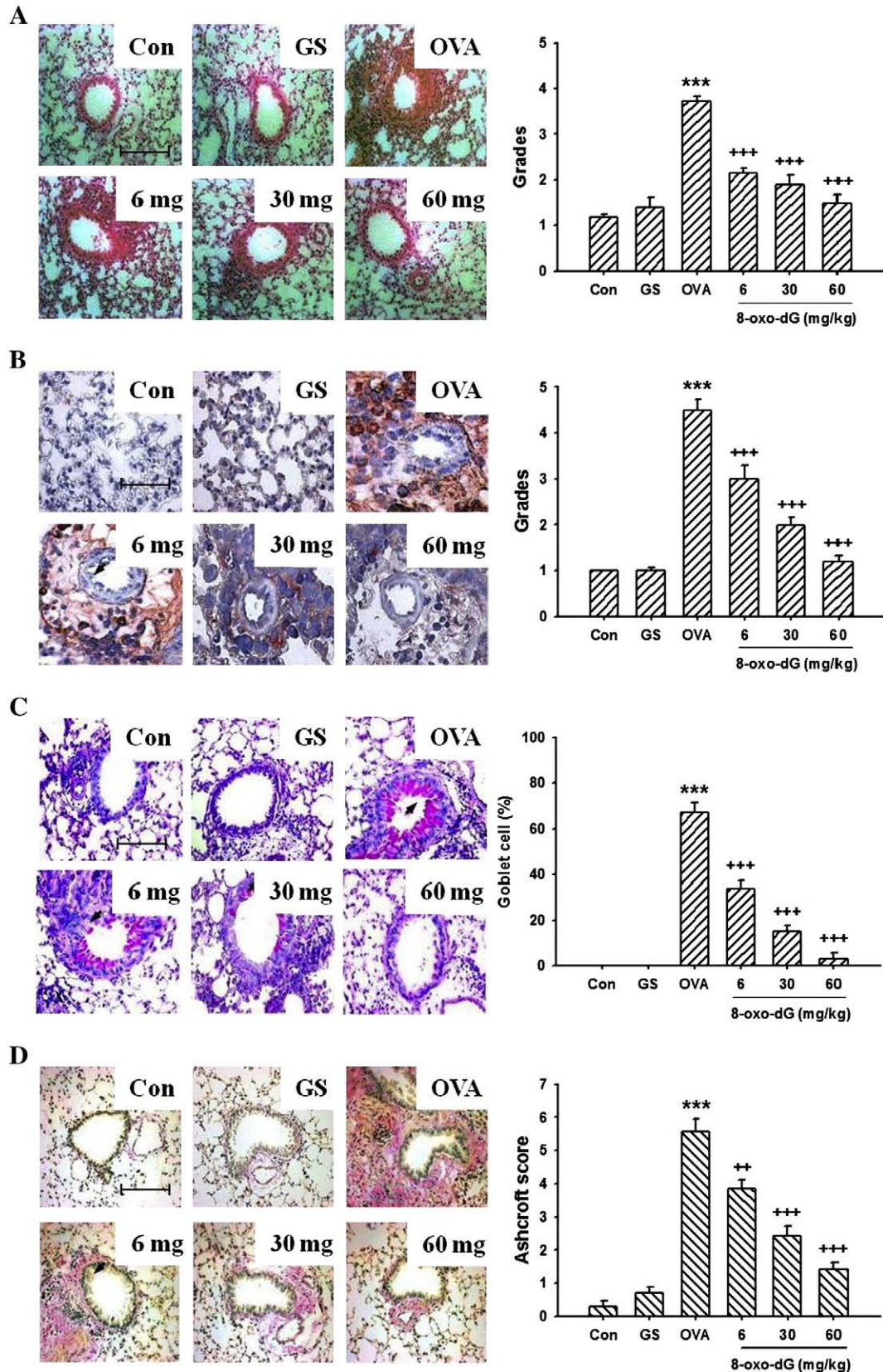
2.12. Electrophoretic mobility shift assay (EMSA) for NF- κ B

Lung tissues (50 mg/50 μ l) were washed twice with ice-cold PBS, resuspended in 1 ml of an ice-cold cell lysis buffer [10 mM HEPES/

Fig. 1. Effect of 8-oxo-dG on inflammatory cell infiltration, goblet cell hyperplasia, collagen deposition and VCAM-1 expression in lung tissues of ovalbumin-challenged allergic mice. Mice were sensitized/challenged with PBS or OVA and treated with 8-oxo-dG as described in Materials and Methods. Each group is described as follows. Control (Con), mice sensitized and challenged with PBS; General sensitization (GS), mice sensitized with OVA and challenged with PBS; Ovalbumin (OVA), mice sensitized and challenged with OVA; 6 mg, 30 mg, and 60 mg, OVA mice orally administered with 6, 30, or 60 mg/kg 8-oxo-dG, respectively, 6 h before every OVA challenge. A and B, H & E staining for inflammatory cell infiltration and Immunohistochemistry for VCAM-1 expression, respectively, which was scored into 5 grades as described in Materials and Methods. C, PAS staining for goblet cell hyperplasia, which was quantified as described in Materials and Methods. D, van Gieson staining for collagen deposition, which was scored into 8 grades as described in Materials and Methods. The scoring results in each panel are presented as histograms (n = 8). ***, P < 0.001 versus control or general sensitization mice; ++, P < 0.01; +, P < 0.05 versus ovalbumin mice. Bars in control group indicate 100 μ m.

KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 1 μ g/ml leupeptin, and 1 μ g/ml 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 $\times$ g for 10 min and resuspended in 40 μ l of an ice-cold nucleus 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 μ g/ml leupeptin, and 1 μ g/ml aprotinin] at 4 °C for 20 min on a shaking



platform. After centrifugation at $15,000 \times g$ for 10 min, the supernatants were stored as nuclear extracts at -70°C . Using these nuclear extracts and NF- κ B oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3', 3'-TCA ACT CCC CTG AAA GGG TCC G-5'), EMSA for NF- κ B was performed according to a method described previously (Kim et al., 2009). Briefly, 10 μl of a mixture of NF- κ B oligonucleotide (1.75 pmol/ μl), T4 polynucleotide kinase 10 $\times$ buffer, [α - ^{32}P]ATP (10 μCi ; 3,000 Ci/mmol), nuclease-free water, and T4 polynucleotide kinase (5–10 U/ μl) 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 eluted 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 were already incubated with a gel shift binding 5 $\times$ 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 poly (di-dC)] for 10 min. Each reaction was mixed with 10X gel loading buffer (1 μl) and electrophoresed on 6% polyacrylamide gels. The radioactive bands on the gels were analyzed using Phosphor Imager FLA-2000 (Fujifilm, Tokyo, Japan).

2.13. Statistical analysis

Unless otherwise noted, experimental data are presented as mean $\pm$ S.E.M. of $n=8$ and multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Scheffe's *post hoc* test using the SPSS statistic program (SPSS Inc., Chicago, IL). The PCR and blots are representative of four independent experiments ($n=4$). P values <0.05 were regarded as significant.

3. Results

3.1. Effect of 8-oxo-dG on inflammatory cell infiltration, goblet cell hyperplasia, VCAM-1 expression and collagen deposition in lung tissues

Firstly, the effect of 8-oxo-dG on inflammation was assessed by histological examination. Fig. 1A shows H & E stained lung tissues. Control and general sensitization mice showed almost no inflammatory changes. In contrast, ovalbumin mice showed typical inflammatory reactions including infiltration of inflammatory cells into peribronchial and perivascular areas as well as epithelial thickening. 8-oxo-dG (6, 30 or 60 mg/kg) dose-dependently reduced these inflammatory changes. The infiltrating inflammatory cells of each group are quantified in the histogram.

Fig. 1B shows the immunohistochemistry of VCAM-1. 8-oxo-dG (6, 30 or 60 mg/kg) reduced severely and dose-dependently increased expression of VCAM-1 (brown color) in ovalbumin mice. The expression in each group was quantified and shown in the histogram.

Fig. 1C presents PAS staining for goblet cell hyperplasia. Ovalbumin mice showed the largest number of goblet cells (pink-stained dot indicated by an arrow) and the greatest epithelial thickness, both of which were dose-dependently decreased by 8-oxo-dG (6, 30 or 60 mg/kg). In the histogram, the numbers of goblet cells in each group were quantified as the ratio of PAS-positive cells/total inflammatory cells in 10 sites of $100\mu\text{m} \times 100\mu\text{m}$ areas.

Fig. 1D shows van Gieson for collagen deposition (pink color). Collagen deposition was markedly enhanced in lung tissues of ovalbumin mice and 8-oxo-dG (6, 30 or 60 mg/kg) reduced the collagen deposition dose-dependently.

3.2. Effect of 8-oxo-dG on expressions of VCAM-1 and Muc5ac, and collagen levels

The results from the histological examination in Fig. 1 were confirmed by examining mRNA or protein amounts of VCAM-1, Muc5ac and collagen in lung tissues. As shown in Fig. 2A and B, the mRNA or protein

amounts of VCAM-1 and Muc5ac were increased in lung tissues of ovalbumin mice and these increases were inhibited by 8-oxo-dG dose-dependently. Collagen amounts were also enhanced significantly in lung tissues of ovalbumin mice ($17.2 \pm 0.94 \mu\text{g/ml}$) compared to those of control ($3.1 \pm 0.42 \mu\text{g/ml}$) and general sensitization mice ($3.4 \pm 0.26 \mu\text{g/ml}$). 8-oxo-dG (6, 30 or 60 mg/kg) decreased $17.2 \pm 0.94 \mu\text{g/ml}$ of ovalbumin mice significantly to $13.8 \pm 0.94 \mu\text{g/ml}$; $11.2 \pm 0.58 \mu\text{g/ml}$; $7.2 \pm 0.55 \mu\text{g/ml}$, respectively. A percentage decreasing by each dose of 8-oxo-dG was 19.8%, 34.9% and 58.9%, respectively, compared to those of control and general sensitization mice (Fig. 2C).

3.3. Effect of 8-oxo-dG on expressions of mRNA and protein levels of matrix metalloproteinase (MMP)-2 and -9

mRNA levels of MMP-2 and -9 were much increased in lung tissues of ovalbumin mice compared to control and general sensitization mice, which were decreased by 8-oxo-dG. And, mRNA levels of tissue inhibitor of metalloproteinase (TIMP)-1 and -2, which were decreased in lung tissues of ovalbumin mice, were recovered by 8-oxo-dG (Fig. 3A).

We also examined the effect of 8-oxo-dG on protein amounts of MMP-2 and -9. In Fig. 3B, ovalbumin mice showed an increased amount of MMP-2 ($86.4 \pm 7.90 \text{ ng/ml}$) and MMP-9 ($20.4 \pm 1.21 \text{ ng/ml}$), compared to those of control mice ($5.2 \pm 1.21 \text{ ng/ml}$ for MMP-2; $1.1 \pm 0.56 \text{ ng/ml}$ for MMP-9) and of general sensitization mice ($5.8 \pm 0.38 \text{ ng/ml}$ for MMP-2; $0.9 \pm 0.17 \text{ ng/ml}$ for MMP-9). 8-oxo-dG (6, 30 or 60 mg/kg) reduced the increased level of MMP-2 of OVA mice by 35.0%, 45.0% and 72.9%, respectively (to $56.2 \pm 2.92 \text{ ng/ml}$ for 6 mg/kg; $47.5 \pm 4.17 \text{ ng/ml}$ for 30 mg/kg; $23.4 \pm 3.57 \text{ ng/ml}$ for 60 mg/kg), and also reduced the increased level of MMP-9 by 25.0%, 47.5% and 61.8%, respectively (to $15.3 \pm 3.65 \text{ ng/ml}$ for 6 mg/kg; $10.7 \pm 0.74 \text{ ng/ml}$ for 30 mg/kg; $7.8 \pm 0.99 \text{ ng/ml}$ for 60 mg/kg).

3.4. Effects of 8-oxo-dG on formation of nitro-tyrosine

Fig. 4 shows that formation of nitrotyrosine was markedly increased in ovalbumin mice compared to control and general sensitization mice, indicating that oxidative stress was increased in lung tissue of ovalbumin mice. However, this increase of nitrotyrosine was significantly and dose-dependently inhibited by 8-oxo-dG (6, 30 or 60 mg/kg).

3.5. Effects of 8-oxo-dG on activation of STAT1 and NF- κ B

Phosphorylation of STAT1 on serine 727 was markedly increased in nuclear extract of lung tissues of ovalbumin mice, compared to those of control and general sensitization mice. However, these increased phosphorylation levels of STAT1 were inhibited by 8-oxo-dG (Fig. 5A).

Activation of NF- κ B was measured by its capacity to bind to DNA. Here again, binding of this protein was markedly increased in ovalbumin mice compared to those of control and general sensitization mice. This increased binding efficiency was inhibited by 8-oxo-dG (6, 30 and 60 mg/kg) dose-dependently (Fig. 5B).

3.6. Effects of 8-oxo-dG on protein levels of cytokines

Next, the effect of 8-oxo-dG on protein levels of these cytokines in lung tissues was tested. As shown in Table 1, the protein levels of all cytokines measured were significantly elevated in lung tissues of ovalbumin mice compared to those of control and general sensitization mice. However, 8-oxo-dG (6, 30 or 60 mg/kg) significantly and dose-dependently reduced these elevated levels.

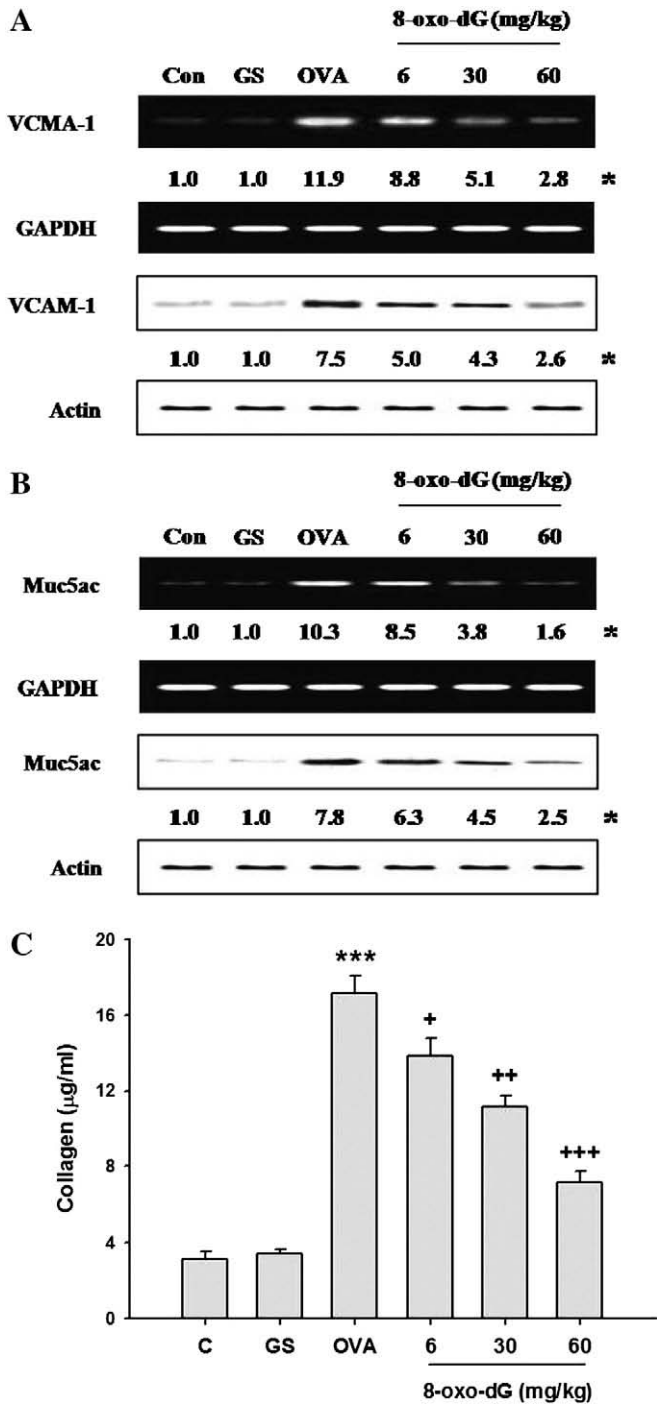


Fig. 2. Effect of 8-oxo-dG on expressions of VCAM-1 and Muc5ac, and collagen levels in lung tissues of ovalbumin-challenged allergic mice. Experimental details and group symbols used are described in Fig. 1. mRNA and protein expressions of VCAM-1 and Muc5ac or amounts of total secreted collagen were determined in the homogenized lung tissues using RT-PCR, western blotting, and Sircol assay kit, respectively. Upper and lower panels, mRNA and protein expression, respectively. A, expression of mRNA and protein of VCAM-1. B, expression of mRNA and protein of Muc5ac. C, amount of total collagen (n = 8). *, Numbers below the band are the ratio of band density of VCAM-1 or Muc5ac versus control and that of actin protein. The bands are representative of four independent experiments (n = 4). ***, P < 0.001 versus control or general sensitization mice; +, P < 0.05; **, P < 0.01; ***, P < 0.001 versus ovalbumin mice.

3.7. Effects of 8-oxo-dG on activation of Rac

We tested the effect of 8-oxo-dG on activities of Rac1 and 2 in lung tissues of allergic mice. As shown in Fig. 5C, Rac1 and 2 were markedly

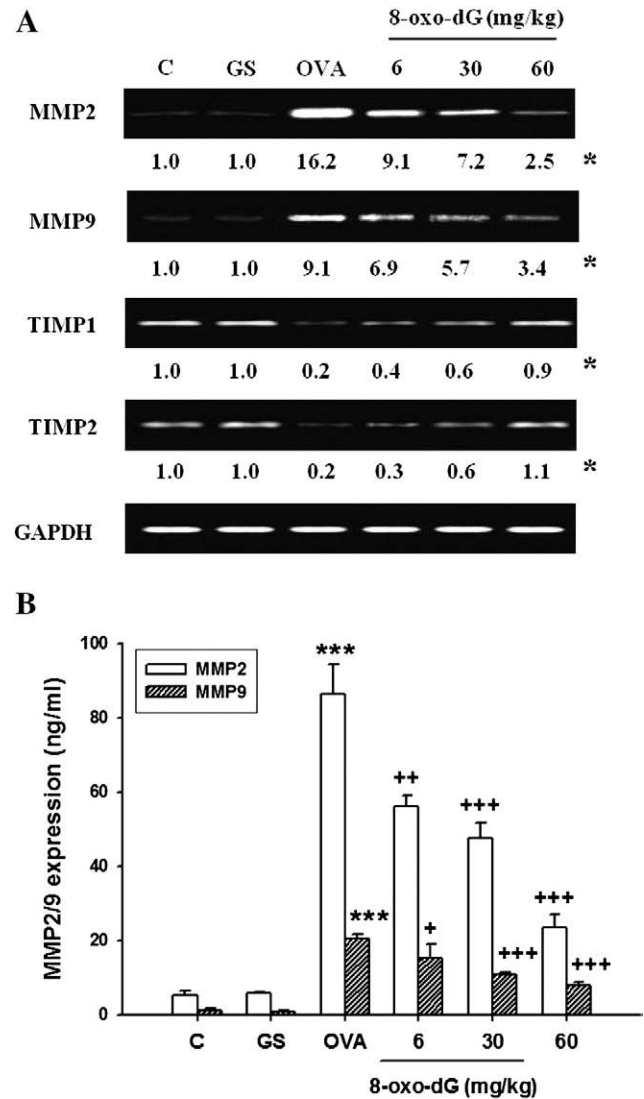


Fig. 3. Effects of 8-oxo-dG on the expressions of MMP-2/-9 or TIMP-1/-2 mRNA and protein in the lung tissues of ovalbumin-challenged allergic mice. Experimental details and group symbols used are described in Fig. 1. Each mRNA or protein expression of MMPs and TIMPs was analyzed by RT-PCR and ELISA, respectively. A, Expressions of MMP-2/-9 mRNA. *, Numbers below the band are the ratio of band density of MMP-2/-9 or TIMP-1/-2 versus control and that of GAPDH mRNA. The bands are representative of four independent experiments (n = 4). B, Amounts of MMP-2/-9 protein. The data for protein indicate mean ± S.E.M. of n = 8.

activated in lung tissues of ovalbumin mice compared to control and general sensitization mice. However, this activation was dose-dependently inhibited by 8-oxo-dG (6, 30 or 60 mg/kg).

4. Discussion

Allergic asthma is associated with airway hyperresponsiveness, airway inflammation and remodeling (Blacchiere et al., 2010; Leigh et al., 2004; Locke et al., 2007). We previously reported that 8-oxo-dG inhibited airway hyperresponsiveness by suppressing allergy-associated immune responses (Ro et al., 2009). In the present study, we studied its effects of the inflammatory and remodeling responses in the lung tissue of allergic ovalbumin mice histologically and biochemically. The histological symptoms of inflammation shown by ovalbumin mice include peribronchial infiltration of inflammatory cells (Fig. 1A), increased immunohistochemical staining of VCAM1 (Fig. 1B), hyperplasia of mucus-secreting goblet cells (Fig. 1C) in

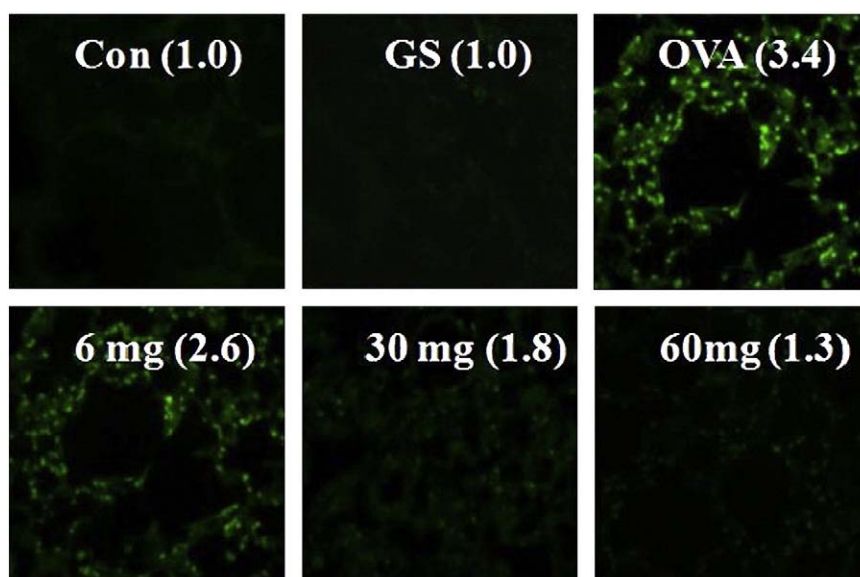


Fig. 4. Effects of 8-oxo-dG on formation of nitrotyrosine in lung tissues of ovalbumin-challenged allergic mice. Experimental details and group symbols used are described in Fig. 1. This figure indicates reactive oxygen species generation in lung tissue sections using primary anti-nitrotyrosine antibody and anti-rabbit IgG antibody fluorescence conjugated secondary antibody. Numbers of parenthesis are the ratios of fluorescence intensity of each versus that of control and general sensitization mice.

bronchial walls and collagen deposition (Fig. 1D). The biochemical symptoms were the activation or increased amounts of various inflammation-mediating proteins including VCAM-1 (Fig. 2A), Muc5ac (Fig. 2B), STAT1 (Fig. 5A) and NF- κ B (Fig. 5B). The ovalbumin mice also showed the tissue remodeling symptoms in lung tissues, which include increased levels of collagen (Fig. 2C), nitrotyrosine (Fig. 4A) and MMP-2/-9 levels (Fig. 3) and decreased expression of TIMP-1/-2 (Fig. 3A). The inflammatory and remodeling symptoms were of allergic nature since the levels of IL-4, IL-5, IL-8, IL-13, TNF- α and IFN- γ were increased in lung tissue (Table 1), which are the typical cytokines to induce inflammation in allergic condition (Howarth et al., 2005; Nath et al., 2007). In this allergic lung tissue, Rac was also activated (Fig. 5C). Surprisingly, however, inflammatory mediators like cytokines and various molecules related to airway inflammation and remodeling as well as Rac activation were suppressed significantly by 8-oxo-dG, suggesting that suppression of these symptoms were mediated by Rac inactivation. This conclusion is consistent with the recent finding that Rac plays an essential role in immune complex-mediated acute inflammatory lung injury (Dooley et al., 2009).

The migration of inflammatory cells from blood into the airway occurs through binding to specific adhesion molecules like VCAM-1, which directs the extravasation of inflammatory cells into the inflamed sites (Choi et al., 2010), and chemokines like IL-8 (Gibson et al., 2003). VCAM-1 expression is enhanced by IL-4, IL-13 and TNF- α (Wills-Karp and Karp, 2004) via NF- κ B activation (Tu et al., 2001). The inflammatory cells such as eosinophils and neutrophils are mainly recruited into asthmatic lung tissues through IL-5, IL-8 and VCAM-1 (Kim et al., 2009; Zhu and Gilmour, 2009). Thus, it is expected that inhibition of inflammatory cells recruited into lung tissues by 8-oxo-dG may reduce lung tissue inflammation (Fig. 1A and B, Fig. 2A, Table 1).

In allergic condition, Rac is activated and the activated Rac is then inducing activation of NF- κ B a transcription factor (Kim et al., 2007a), probably by increasing oxidative stress. Activated NF- κ B is then promoting expression of Th2-inflammatory cytokines such as IL-4, IL-5 and IL-13 as well as Th1 cytokines such as IFN- γ and TNF- α , as evidenced by involvement of these cytokine in the pathogenesis of asthma and atopic diseases (Howarth et al., 2005; Smart and Kemp, 2002) and increase of their levels in lung tissues of an asthmatic human (Zimmermann et al.,

2003) and animals (Kim et al., 2009; Secor et al., 2008; Zosky et al., 2008) including our ovalbumin mice (Table 1). IL-4 and IL-13 stimulates goblet cell hyperplasia and Muc5ac gene (mucus production) in bronchial epithelial cells (Kasaian and Miller, 2008; Nath et al., 2007; Tagaya and Tamaoki, 2007). TNF- α mediates recruitment of inflammatory cells such as eosinophils and neutrophils during airway inflammation (Lukacs et al., 1995). Thus, suppression of Rac activation by 8-oxo-dG in ovalbumin mice is expected to be linked to a sequential suppression of NF- κ B activation, production and expressions of Th2 and Th1 cytokines and inflammatory reaction.

Importantly, however, the results in Figs 2 and 3 in the present study suggest that this compound has the capacity to suppress tissue remodeling of airway and lung occurring in allergic asthma. MMP-9 and TIMP-1 are the most important factors to induce the remodeling in asthma (Sands et al., 2009; Tagaya and Tamaoki, 2007). In asthmatic condition, tissue levels of TNF- α or IL-13 are increased, which then induce expression and activation of MMPs or an imbalance of active MMPs and TIMPs (Giolamo et al., 2006; Sergejeva et al., 2005), induce all proteins of extra-cellular matrix including collagen and mucin hypersecretion, and then initiate tissue remodeling (Ko et al., 2005). Thus, inactivation of Rac by 8-oxo-dG in allergic condition is also expected to decrease the levels of TNF- α or IL-13, normalize activity of MMPs and balance of active MMPs and TIMPs and eventually stop the tissue remodeling.

Although it is not easy to provide direct evidence for Rac-inactivation as a mechanism of anti-inflammatory and anti-tissue remodeling action, the data in the previous and present studies seem to be supporting this assumption. In the previous study, 8-oxo-dG was shown to inhibit chemotaxis (Lee et al., 2009), production of reactive oxygen species and nitric oxide (NO), and release of pro-inflammatory cytokines (Kim et al., 2006a; Lee et al., 2009) in phagocytes. All of which are Rac-linked functions (Abdel-Latif et al., 2004; Kim et al., 2006a; Weiss-Haljiti et al., 2004; Yamauchi et al., 2004). In the present study, we observed inhibitions of these Rac-linked functions in the inflamed lung tissue, which were leukocyte infiltration (Fig. 1A), and production of reactive oxygen species and NO supported by nitrotyrosine formation (Fig. 4), which is produced by an attack of peroxynitrite (ONOO^-) formed by interaction of superoxide anion ($\text{O}_2^{\bullet-}$) and NO, which are produced in inflamed tissues, on tyrosine residues in proteins (Kim et al., 2005), and the increase in amounts of

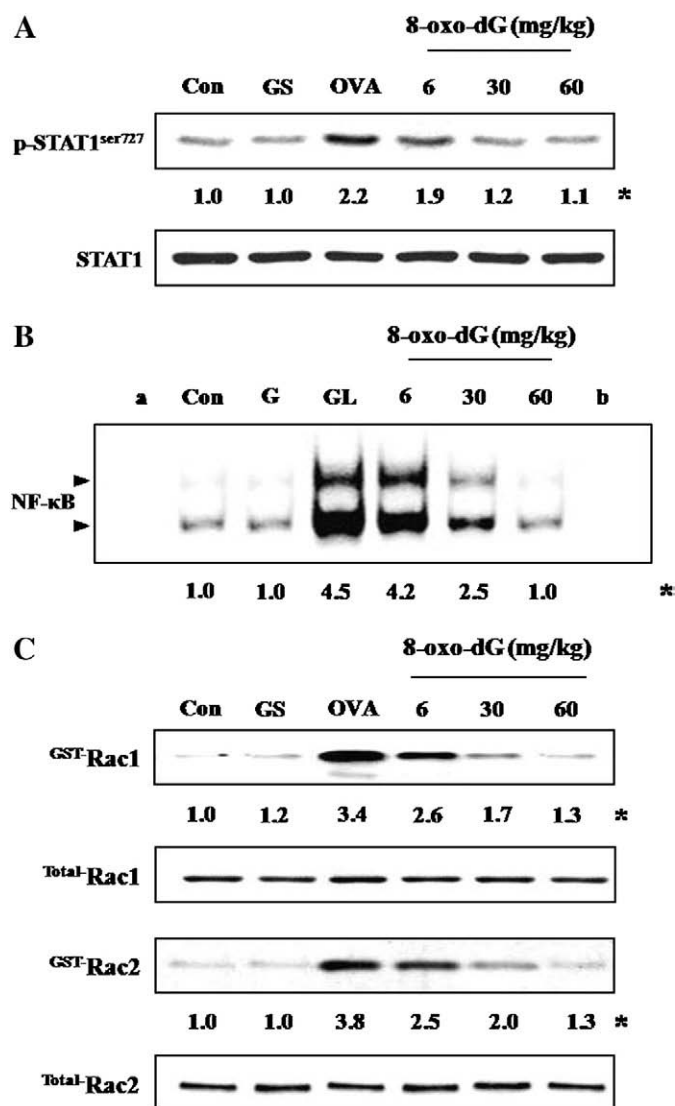


Fig. 5. Effects of 8-oxo-dG on activation of Rac, STAT1 and NF-κB in lung tissues of ovalbumin-challenged allergic mice. Experimental details and group symbols used are described in Fig. 1. The activities of Rac1 and 2 were assayed by affinity precipitation of activated Rac1 and 2, which were then immunoblotted. Phosphorylated STAT1 on serine 727 were assayed in nuclear extracts using immunoblotting. Activated NF-κB transferred into the nucleus was assayed using EMSA. In each band determinant, amount of total protein was determined and used as a loading control. A, B, and C, activities for STAT1, NF-κB, and Rac1 and 2, respectively. *, The numbers below each band are the ratios of band density of STAT1, NF-κB or Rac family versus those of control mice and total protein. a, negative control. b, competition. The bands are representative of four independent experiments (n = 4).

cytokines (Table 1). The Rac-inactivation as an anti-inflammatory and anti-tissue remodeling mechanism of 8-oxo-dG is consistent with the recent report (Dooley et al., 2009) showing a critical role of Rac2 in immune complex-mediated lung injury.

In the present study, 8-oxo-dG blocked all the parameters tested, suggesting that 8-oxo-dG suppresses a key mediator(s) or pathway(s) triggering activation of various proteins, which are concerted to mediate the inflammatory reaction and lung tissue remodeling. We propose that key mediators are reactive oxygen species and NO as initiators of inflammation and tissue remodeling. Rac in inflammatory cells within inflamed tissues is activated, thereby stimulating the production of reactive oxygen species and NO (Fig. 4). The resulting oxidative stress induces inflammation and tissue remodeling by activating NF-κB (Fig. 5B) and STAT1 (Fig. 5A), which are key regulators of inflammation (Covert et al., 2005). Under these

Table 1

Effects of 8-oxo-dG on cytokine levels in lung tissues of ovalbumin-challenged allergic mice^a.

Group	Cytokine production (pg/ml)					
	IL-4	IL-5	IL-8	IL-13	TNF-α	IFN-γ
Con	90 ± 2.4	375 ± 6.6	120 ± 2.1	699 ± 35.6	1066 ± 38.8	108 ± 3.5
GS	93 ± 4.4	369 ± 14.1	116 ± 5.3	721 ± 13.0	1067 ± 53.3	112 ± 7.4
OVA	330 ± 14.7 ^b	689 ± 9.9 ^b	381 ± 10.5 ^b	1737 ± 63.6 ^b	2159 ± 52.2 ^b	402 ± 1.6 ^b
8-oxo-dG (mg/kg)						
6	278 ± 4.8 ^c	508 ± 7.6 ^d	307 ± 4.8 ^c	1417 ± 32.4 ^c	1799 ± 15.1 ^d	316 ± 5.1 ^e
30	206 ± 1.5 ^d	489 ± 10.4 ^d	266 ± 3.8 ^d	1132 ± 24.9 ^d	1628 ± 11.2 ^d	214 ± 4.7 ^e
60	126 ± 1.0 ^e	411 ± 6.0 ^e	216 ± 4.3 ^e	878 ± 27.2 ^e	1227 ± 19.3 ^e	161 ± 2.9 ^e

^aExperimental details and group symbols used are described in Fig. 1. Individual cytokine levels in lung tissues were determined by ELISA. Data are mean ± S.E.M. of n = 8. ^b, P < 0.001 versus control mice; ^c, P < 0.05; ^d, P < 0.01 and ^e, P < 0.001 versus ovalbumin mice. 8-oxo-dG, 8-hydroxy-deoxyguanosine.

circumstances, inactivation of Rac by 8-oxo-dG would decrease production of reactive oxygen species and NO and then the above sequential interactions cease, which then lead to suppression of inflammatory reaction and tissue remodeling.

The incidence of allergic disorders is increasing, but therapeutic measures against them are still unsatisfactory. Inflammation in bronchi and lung parenchyma is one of principal targets in treatment of pulmonary asthma (Levine, 2003). Steroids are the agents most commonly used but their usage is very limited due to many side effects.

In conclusion, our data suggest that 8-oxo-dG may reduce expression of inflammatory cytokines and molecules related to remodeling by regulating Rac activity upstream cascade reactive oxygen species/NO and NF-κB, which are related to the production of cytokines and molecules, and show that 8-oxo-dG suppresses inflammation and remodeling in the allergic lung tissues and could be a potential therapeutic for allergic disorders. In contrast to 8-oxo-dGTP, 8-oxo-dG is not incorporated into DNA (Kim et al., 2006b; Kim and Chung, 2006), thus it not mutagenic. dG is salvaged for DNA synthesis.

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