

Glutathione Redox Regulates Airway Hyperresponsiveness and Airway Inflammation in Mice

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Glutathione is the major intracellular redox buffer. We have shown that glutathione redox status, which is the balance between intracellular reduced (GSH) and oxidized (GSSG) glutathione, in antigen-presenting cells (APC) regulates the helper T cell type 1 (Th1)/Th2 balance due to the production of IL-12. Bronchial asthma is a typical Th2 disease. Th2 cells and Th2 cytokines are characteristic of asthma and trigger off an inflammation. Accordingly, we studied the effects of the intracellular glutathione redox status on airway hyperresponsiveness (AHR) and allergen-induced airway inflammation in a mouse model of asthma. We used γ -Glutamylcysteinylethyl ester (γ -GCE), which is a membrane-permeating GSH precursor, to elevate the intracellular GSH level and GSH/GSSG ratio of mice. *In vitro*, γ -GCE pretreatment of human monocytic THP-1 cells elevated the GSH/GSSG ratio and enhanced IL-12(p70) production induced by LPS. In the mouse asthma model, intraperitoneal injection of γ -GCE elevated the GSH/GSSG ratio of lung tissue and reduced AHR. γ -GCE reduced levels of IL-4, IL-5, IL-10, and the chemokines eotaxin and RANTES (regulated on activation, normal T cell expressed and secreted) in bronchoalveolar lavage fluid, whereas it enhanced the production of IL-12 and IFN- γ . Histologically, γ -GCE suppressed eosinophils infiltration. Interestingly, we also found that γ -GCE directly inhibited chemokine-induced eosinophil chemotaxis without affecting eotaxin receptor chemokine receptor 3 (CCR3) expressions. Taken together, these findings suggest that changing glutathione redox balance, increase in GSH level, and the GSH/GSSG ratio by γ -GCE, ameliorate bronchial asthma by altering the Th1/Th2 imbalance through IL-12 production from APC and suppressing chemokine production and eosinophil migration itself.

Keywords: glutathione redox; mouse; bronchial asthma; Th1/Th2 balance; eosinophils

Bronchial asthma is a typical helper T cell type 2 (Th2) disease. The Th cells found in individuals with asthma are primarily Th2; therefore, it has been hypothesized that Th2 cells play a pivotal role in the pathogenesis of this disease. Through the release of Th2 cytokines, such as IL-4, IL-5, and IL-13, orchestrate the recruitment and activation of the primary effector cells of the allergic response: the mast cells and the eosinophils. Activation of the effector cells results in the release of a plethora of inflammatory mediators that individually or in concert induce changes in airway wall geometry, airway hyperresponsiveness

CLINICAL RELEVANCE

We demonstrated that changing glutathione redox by γ -GCE ameliorates bronchial asthma by three mechanisms. This study provides *in vivo* evidence that regulation glutathione redox can be a novel approach for the treatment of allergic diseases.

(AHR), and airway inflammation and produce the symptoms of the disease (1).

Glutathione is the most abundant nonprotein sulfhydryl compound in almost all cells. This tripeptide plays a significant role in many biological processes. It also constitutes the first line of the cellular defense mechanism against oxidative injury along with SOD, ascorbate, vitamin E, and catalase, and is the major intracellular redox buffer in ubiquitous cell types (2, 3). Recent findings suggest that the intracellular redox status regulates various aspects of cellular function, and that glutathione is important in immune modulation (4, 5). For instance, the glutathione S-transferase has been reported to modulate asthma susceptibility (6–8). We have shown that glutathione redox status, namely the balance between intracellular reduced (GSH) and oxidized (GSSG) glutathione, in murine antigen-presenting cells (APC) plays a central role in determining which of the reductive and oxidative APC predominate during immune status, and the balance between reductive and oxidative APC regulates Th1/Th2 balance through production of IL-12 (9–12). We further reported the molecular mechanism for glutathione redox status regulation of IL-12 production in human APC, demonstrating that p38 mitogen-activated protein kinase (MAPK) positively and c-jun N-terminal kinase (JNK) negatively regulates LPS-induced IL-12 production, and the increase in the GSH/GSSG ratio induced by GSH precursor enhances IL-12 production during the mediation of both enhanced p38 MAPK activation and suppressed JNK activation (13, 14). In addition, we have also shown that exposure of human alveolar macrophages to the Th1 cytokine IFN- γ or the Th2 cytokine IL-4 either increases or decreases the GSH/GSSG ratio, respectively, which regulates Th1/Th2 balance through IL-12 production (15). Thus, the ability to generate a Th1 or Th2 type response has turned out to depend not only on T cells but also on the intracellular glutathione redox status of APC, and we surmised that the increase in the intracellular GSH levels in APC may improve the imbalance in Th1/Th2 responses and ameliorate AHR and allergen-induced inflammation in a mouse asthma model.

In the present study we examined whether changing glutathione redox status determines the skewing either to Th1 or Th2 cytokine responses during allergen-induced immune responses, and effects on AHR and allergen-induced bronchial inflammation in a mouse asthma model. To increase GSH levels and the

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GSH/GSSH ratio, we used γ -Glutamylcysteinylethyl ester (γ -GCE), a membrane-permeating GSH precursor, because it has been demonstrated that γ -GCE is transported into cells more rapidly than GSH itself and that the SH-containing ester, after being hydrolyzed by esterase, is converted to GSH by glutathione synthetase (16–18). We demonstrate that the rise of the GSH/GSSG ratio in lung by γ -GCE administration ameliorates bronchial asthma by not only improving Th1/Th2 balance but also by inhibiting eosinophil migration. Our observations indicate the clinical potential of glutathione redox for treating bronchial asthma.

MATERIALS AND METHODS

In Vitro Cell Cultures and γ -GCE Pretreatment

Human monocytic THP-1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Life Technologies, Rockville, MD) with 10% fetal bovine serum (Equitech-Bio, Ingram, TX) and maintained in humidified 5% CO₂/95% air. For cytokine production, subconfluent cells were washed and resuspended with in fresh RPMI–0.5% bovine serum albumin (BSA). THP-1 cells ($2.0 \times 10^5/500 \mu\text{l}$) were incubated with γ -GCE (0–5 mM) for 60 minutes, and then stimulated with 10 ng/ml LPS for 24 hours. Thereafter, the supernatants were used for enzyme-linked immunosorbent assay (ELISA). For measurement of the glutathione concentrations, THP-1 cells ($2.0 \times 10^5/500 \mu\text{l}$) were incubated with γ -GCE (0–5 mM) for 60 minutes. After the treatment, the glutathione concentrations of cells were measured as described previously (14).

Mice and Administration of γ -GCE

Female BALB/c mice (SPF; 6 wk old), purchased from Charles River Japan (Tsukuba, Japan), were sensitized with intraperitoneal injections of either saline or 20 μg ovalbumin (OVA) (Grade V; Sigma-Aldrich, St. Louis, MO) plus 2.25 mg aluminum hydroxide on Days 1 and 14. On Days 28–30, mice received aerosol challenge containing either saline or 1% OVA for 15 minutes per day. γ -GCE solution (100 mg/kg body weight/day; Teijin Pharma, Tokyo, Japan) was administered intraperitoneally on Days 25–30. All animals received human care in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86023, revised 1985).

Measurement of Airway Responsiveness in Anesthetized and Conscious Mice

On Day 31, 24 hours after the last aerosol challenge, AHR was measured in anesthetized mice as described previously (19). Mice were intraperitoneally anesthetized with urethane (25%). Briefly, anesthetized mice were tracheostomized and placed on a Harvard ventilator, then placed inside whole-body plethysmographs (Buxco Electronics, Inc., Troy, NY) to measure airway resistance (Raw). Increasing doses of methacholine (0–100 mg/kg) were administered intravenously.

In conscious mice, airway responsiveness was measured by recording the enhanced pause (Penh) values (using whole-body plethysmography [Buxco Electronics, Inc.]) obtained in response to inhaled methacholine (0–40 mg/ml).

Bronchoalveolar Lavage and Cell Counting

After the measurement of AHR, the lungs were lavaged with saline through the tracheotomy. Bronchoalveolar lavage fluid (BALF) collection and total and differential cell counts were performed as described previously (19). Samples were centrifuged, and supernatants were stored at -80°C for ELISA.

Histologic Analysis

After the last challenge, mice were anesthetized with urethane and killed for the preparation of lung sections as described previously (20).

Lungs were fixed with 10% formalin, and tissue sections were stained with hematoxylin and eosin (H&E) to determine the eosinophil count.

Cytokine and Chemokine Measurements

Concentrations of mouse IL-4, IL-5, IL-10, and RANTES (regulated on activation, normal T cell expressed and secreted) in BALF supernatants were determined using the Bio-Plex Suspension Array System (Nippon Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer's instructions (21). Concentrations of mouse eotaxin, IL-12(p70), and IFN- γ in BALF and human IL-12(p70) in cell supernatants were determined using ELISA kits (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Measurement of Glutathione Concentrations in Lung Tissue

After the last OVA challenge, the lungs of the mice were removed, washed with cold saline, weighed, and immediately frozen in liquid nitrogen and stored at -80°C until assay. The lungs were homogenized on ice using a Polytron homogenizer (Kinematica Inc., Newark, NJ), in a homogenization solution consisting of 2 ml of 0.1% Triton X per 100 mg lung tissue. After homogenization, 300 μl of 0.1 M HCl and 300 μl of 50% sulfosalicylic acid were added, and the mixture was centrifuged at 4°C at 13,000 rpm for 20 minutes. The supernatant was used for assay of the GSSG-reductase 5',5'-dithio-bis (2-nitrobenzoic acid) recycling procedure to measure the intracellular GSH and GSSG contents, as described (14).

Chemotaxis Assay

To study the chemotactic effects of γ -GCE, we used cell suspensions of BALF collected from OVA-sensitized mice. The chemotaxis assay was performed in a 24-well Chemotaxicell chamber (Kurabou, Kurashiki, Japan). Eotaxin (0–20 ng/ml) diluted in medium was placed in the bottom wells. The cells, after being incubated with γ -GCE (0–20 mM) diluted in RPMI 1640 with 0.1% BSA for 60 minutes at 37°C , were placed in the top wells of the chamber (2.5×10^5 cells/200 μl), which were separated from the bottom wells by a polycarbonate filter (pore size 5 μm). The plate was incubated at 37°C with 5% CO₂ for 60 minutes, then the filters were removed and stained with Diff-Quick (International Reagents Co., Kobe, Japan). The number of infiltrating cells was taken as the mean of cell counts in five immersion fields.

To study the chemotactic effects of γ -GCE *in vivo* administration, we used cell suspensions of BALF obtained from OVA-sensitized mice or OVA-sensitized/ γ -GCE-treated mice after the last OVA challenge. Cells were resuspended in RPMI 1640 medium, loaded into the top wells (2.5×10^5 cells/200 μl) of a 24-well Chemotaxicell chamber, and tested for chemoattraction to the medium or BALF supernatant from OVA-sensitized mice or OVA-sensitized/ γ -GCE-treated mice. The cells infiltrating into the bottom wells were counted in total, and differential cell counts were performed as described previously (20).

Flow Cytometry of Chemokine Receptor 3 Expression

After the last OVA challenge, BALF was performed with Ca²⁺- and Mg²⁺-free PBS with 0.5% BSA from three groups of mice: non-sensitized mice, OVA-sensitized mice, and OVA-sensitized/ γ -GCE-treated mice. Red blood cells (RBC) were lysed using ammonium chloride lysis buffer. The BALF cells (1.5×10^5 cells/100 μl) were washed and stained for 30 to 45 minutes at 4°C with CCR3-FITC single monoclonals (5 $\mu\text{g}/\text{ml}$; R&D Systems, Abingdon, UK). To prevent non-specific binding to Fc receptors, 2.4G2 blocking reagent (10 $\mu\text{g}/\text{ml}$) was added to the monoclonal antibody mix. Cells were analyzed on a FACS Calibur (Becton and Dickinson Immunocytometry Systems, Franklin Lakes, NJ) flow cytometry system using Cell Quest software (Becton and Dickinson Immunocytometry Systems).

Statistical Analysis

Values are expressed as mean $\pm$ SE. Differences among groups were analyzed using ANOVA together with *post hoc* Bonferroni analysis. *P* values $< 0.05/m$ (in which *m* is the number of comparisons) were considered to be significant.

RESULTS

Effect of γ -GCE on Intracellular GSH/GSSG Ratio and LPS-Induced IL-12(p70) Production in Human Monocytic THP-1 Cells

Incubating THP-1 cells for 60 minutes with 0.05–5 mM γ -GCE promoted a significant and dose-dependent increase in the intracellular GSH/GSSG ratio (Figure 1A). *In vitro* γ -GCE elevated both of GSH and GSSG levels in THP-1 cells, but the elevation of the GSH levels were larger than that of GSSG levels (data was not shown). Thus, the ratio of GSH/GSSG was significantly increased. IL-12(p70) protein was undetectable in unstimulated THP-1 cells but detectable after incubation with LPS (10 ng/ml, 24 h). Figure 1B shows that γ -GCE significantly and dose-dependently enhanced IL-12(p70) production in THP-1 cells stimulated with LPS, and then IL-12 production was proportional to the GSH/GSSG ratio.

Effect of γ -GCE on Airway Hyperresponsiveness

We sensitized and challenged mice using a conventional method. Mice immunized with OVA/alum and then challenged for 3 days with an OVA-containing aerosol were more sensitive to methacholine than were nonsensitized mice (who received saline rather than OVA). In OVA-sensitized mice, intravenous injection of methacholine elicited a dose-dependent increase in Raw over the dose range 3.3 to 100 mg/kg. The responses to methacholine shown by OVA-sensitized/ γ -GCE-treated mice were characteristic of Raw, with a right-shifted curve and decreased maximal reactivity (Figure 2A). To investigate lung function in mice, we measured the Penh value which is an indicator of Raw. In OVA-sensitized mice the Penh value was increased by exposure to methacholine, but this phenomenon was significantly inhibited by γ -GCE (Figure 2B). This result accorded with those obtained from Raw measurements using an invasive method (Figure 2A).

Numbers of Inflammatory Cells in BALF and Histology of the Lung

Mice immunized with OVA showed significant increases in the number of eosinophils, macrophages and lymphocytes in BALF cells relative to nonsensitized mice. The increases in these cells, in particular the increase in the number of eosinophils, were significantly reduced in OVA-sensitized/ γ -GCE-treated mice

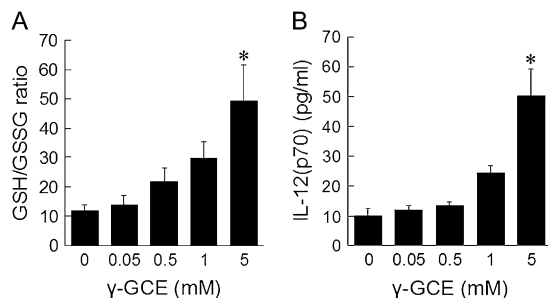


Figure 1. Effect of γ -GCE on human THP-1 monocytes. After incubation of THP-1 monocytes with γ -GCE for 60 minutes, the ratios of intracellular GSH/GSSG were determined by enzyme assay (A). After the cells incubated with γ -GCE were stimulated with LPS (10 ng/ml) for 24 hours, IL-12(p70) production in culture supernatants was evaluated by ELISA (B). Data are mean $\pm$ SE of at least four experiments. * P < 0.05 compared with control according to ANOVA with Bonferroni correction.

(Figure 3A). We next performed a histologic analysis of lung sections taken from the three groups of mice: nonsensitized, OVA-sensitized, and OVA-sensitized/ γ -GCE-treated mice (Figures 3B–3G). OVA-sensitized mice showed marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared with nonsensitized mice (Figures 3B and 3D). As expected, eosinophils constituted the majority of infiltrating inflammatory cells in OVA-sensitized mice (Figure 3E, arrowheads). Eosinophils were not observed in the airways of nonsensitized mice (Figure 3C). Injection of γ -GCE attenuated the eosinophil-rich leukocyte infiltration as compared with nontreated OVA-sensitized mice (Figures 3E and 3G). Furthermore, OVA-sensitized mice developed marked goblet cell hyperplasia, whereas nonsensitized mice did not (Figures 3B and 3D). The OVA-induced goblet cell hyperplasia was substantially reduced by γ -GCE (Figure 3F).

Glutathione Concentration of Lung Tissue

Concentrations of GSH, GSSG, and ratio of GSH/GSSG in lung tissues are compared in four groups of mice: nonsensitized mice, nonsensitized/ γ -GCE-treated mice, OVA-sensitized mice, and OVA-sensitized/ γ -GCE-treated mice (Table 1). In OVA-sensitized mice, the GSH level was suppressed and the GSSG level was increased compared with those of nonsensitized mice. Accordingly, OVA sensitization decreased the GSH/GSSG ratio of lung tissue. On the other hand, γ -GCE treatment to OVA-sensitized mice not only increased GSH levels, but also decreased GSSG levels in lung tissue. Consequently, the GSH/GSSG ratio of lung tissue was significantly elevated in OVA-sensitized/ γ -GCE-treated mice. Thus, the GSH/GSSG ratio is more sensitive than the glutathione concentrations to express glutathione redox status *in vivo*.

Cytokine and Chemokine Levels in BALF

We measured the levels of the cytokines IL-12(p70) and IFN- γ and the chemokine eotaxin in BALF using an ELISA kit. We were unable to measure the levels of IL-4, IL-5, IL-10, and RANTES using this method, because their concentrations were under the detectable limits of the ELISA kit. Therefore we instead used the Bio-Plex Suspension Array System for

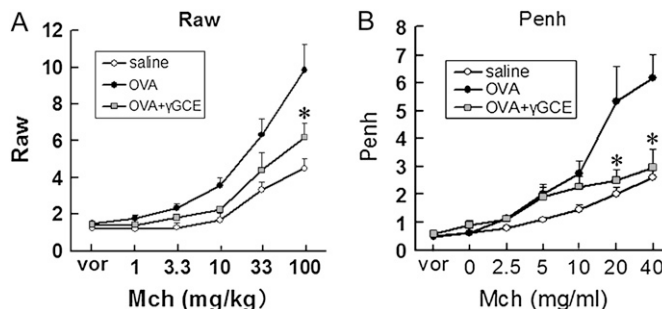


Figure 2. Effect of γ -GCE on AHR in an OVA-induced murine asthma model. Mice were sensitized and aerosol challenged with OVA. In anesthetized mice, airway responsiveness was determined by measuring Raw change after intravenous methacholine administration in three groups of mice (A). In conscious mice, airway responsiveness was determined by measuring the changes in Penh values associated with methacholine inhalation in three groups of mice (B). Saline, nonsensitized control mice; OVA, OVA-sensitized mice; OVA+ γ -GCE, OVA-sensitized/ γ -GCE-treated mice; Mch, methacholine. Data are mean $\pm$ SE for 6 or 21 mice per group. * P < 0.05 compared with OVA-sensitized mice according to ANOVA with Bonferroni correction.

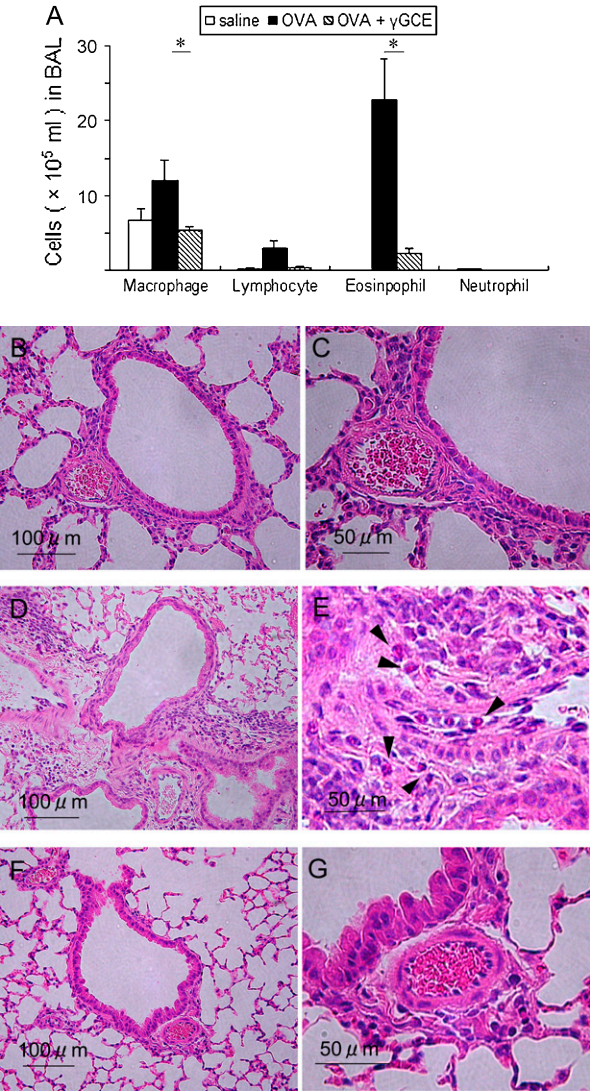


Figure 3. Inhibitive effect of γ -GCE on eosinophil infiltration into the lung. BALF or lung tissues were obtained from nonsensitized mice (saline and B, C), OVA-sensitized mice (OVA and D, E), and OVA-sensitized/ γ -GCE-treated mice (OVA+ γ -GCE and F, G) after last challenge. (A) Effects of γ -GCE on cell counts in BALF. Data are mean $\pm$ SE for at least six mice per group (* P < 0.05 according to ANOVA with Bonferroni correction). (B–G) Lung tissue sections were obtained from formalin-fixed, paraffin-embedded lung tissue prepared and stained with H&E. (D and E) Eosin-positive cells were taken as eosinophils (indicated by arrow-heads in E). Original magnification: B, D, and F, $\times 200$; C, E, and G, $\times 400$.

measurement (21). In OVA-sensitized mice concentrations of IL-4, IL-5, IL-10, eotaxin, and RANTES in BALF were increased compared with those in nonsensitized mice, although in OVA-sensitized/ γ -GCE-treated mice these concentrations were significantly lower than those in OVA-sensitized mice (Figures 4A and 4B). However, the concentrations of the Th1 cytokine IFN- γ and IL-12(p70) were lower in OVA-sensitized mice than in nonsensitized mice, whereas levels of IFN- γ and IL-12(p70) were significantly increased in OVA-sensitized/ γ -GCE-treated mice (Figures 4A and 4B). These data indicate that the Th1/Th2 imbalance, which is biased toward Th2 in OVA-sensitized mice, was improved by the administration of γ -GCE.

Moreover, γ -GCE inhibited the production of chemokines, such as RANTES and eotaxin.

TABLE 1. EFFECTS OF γ -GCE ON GSH/GSSG RATIOS IN LUNG TISSUES

	GSH (nM)	GSSG (nM)	GSH/GSSG
Saline	20.26 $\pm$ 1.00	14.13 $\pm$ 0.83	1.45 $\pm$ 0.07
γ -GCE	22.69 $\pm$ 1.04	11.53 $\pm$ 1.30	2.12 $\pm$ 0.30
OVA	13.68 $\pm$ 3.39	16.87 $\pm$ 0.49	0.83 $\pm$ 0.22
OVA/ γ -GCE	19.68 $\pm$ 2.05	12.22 $\pm$ 1.23*	2.07 $\pm$ 0.49*

Definition of abbreviations: γ -GCE, nonsensitized/ γ -GCE-treated mice; GSH, intracellular reduced glutathione; GSSG, oxidized glutathione; OVA, OVA-sensitized mice; OVA+ γ -GCE, OVA-sensitized/ γ -GCE-treated mice; saline, non-sensitized control mice.

After last challenge, lung tissues were collected and glutathione concentrations were measured by enzyme assay. Data are mean $\pm$ SE for at least six mice per group.

* Statistically significant difference (P < 0.05) compared with OVA-sensitized mice, according to ANOVA with Bonferroni correction.

Effect of γ -GCE on Eosinophil Chemotaxis

γ -GCE suppressed Th2 cytokines and chemokines, eotaxin, and RANTES (Figures 4A and 4B), which are involved in the recruitment of eosinophils. However, the total effect of γ -GCE on eosinophil recruitment was much larger than that which could be caused by cytokine and chemokine suppression. Therefore, we hypothesized that γ -GCE can suppress eosinophil chemotaxis directly. To establish whether γ -GCE directly modulates eosinophil chemotactic response to chemoattractants, we performed *in vitro* chemotaxis experiments using BALF cells of OVA-sensitized mice, which had increased eosinophils. Pretreatment

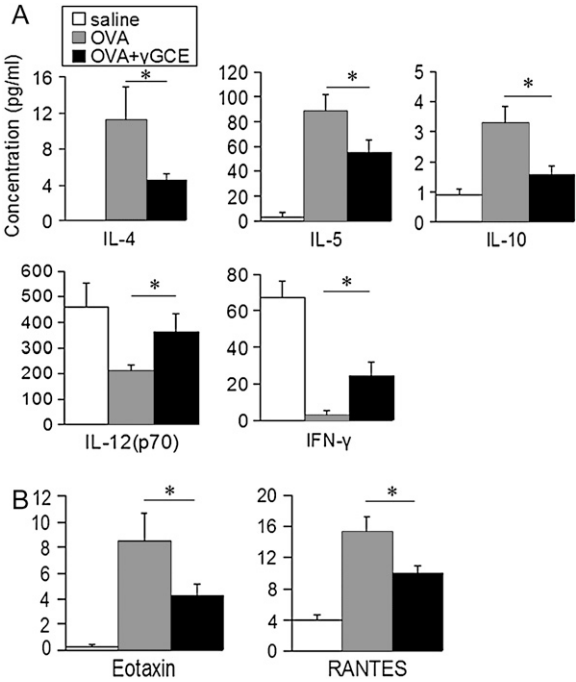


Figure 4. Elevated cytokines and chemokines levels in BALF. Effects of γ -GCE on (A) cytokine and (B) chemokine levels in BALF. Cytokine and chemokine production in BALF supernatants were measured after last challenge. IL-4, IL-5, IL-10, and RANTES concentrations were determined using Bio-Plex. Mouse eotaxin, IL-12(p70), and IFN- γ concentrations were assessed by ELISA. Data are mean $\pm$ SE for at least six mice per group. * P < 0.05 according to ANOVA with Bonferroni correction. Saline, nonsensitized control mice; γ -GCE, nonsensitized/ γ -GCE-treated mice; OVA, OVA-sensitized mice; OVA+ γ -GCE, OVA-sensitized/ γ -GCE-treated mice.

with γ -GCE of the mice BALF cells significantly inhibited eotaxin-induced eosinophil chemotaxis dose-dependently (Figure 5).

Subsequently, to confirm the anti-chemotactic effect of γ -GCE *in vivo* administration, we performed a chemotaxis assay using BALF cells obtained from OVA-sensitized mice and OVA-sensitized/ γ -GCE-treated mice, and their BALF supernatants as the chemoattractant. In the same supernatant group obtained from OVA-sensitized mice, eosinophil chemotaxis was inhibited by using the cells obtained from OVA-sensitized/ γ -GCE-treated mice compared with using that from OVA-sensitized mice (Figure 6, lanes 5 and 2). Furthermore, in the same OVA-sensitized mice cell group, eosinophil chemotaxis was suppressed by using the supernatant obtained from OVA-sensitized/ γ -GCE-treated mice compared with using that from OVA-sensitized mice (Figure 6, lanes 2 and 3). These findings showed that γ -GCE inhibits eosinophils chemotaxis as well as chemoattractant production, such as eotaxin and RANTES. On the other hand, for OVA-sensitized/ γ -GCE-treated mice BALF cells, differences in eosinophil chemotaxis were observed depending on whether we used BALF supernatant from OVA-sensitized mice or OVA-sensitized/ γ -GCE-treated mice in the bottom chamber (Figure 6, lanes 5 and 6). This reveals that γ -GCE does not have a cytotoxic effect.

CCR3 Expression on Eosinophils

Because our data showed that γ -GCE suppresses eosinophil chemotaxis, we examined whether γ -GCE decreases the expression of eotaxin receptor CCR3. We obtained BALF cells from the three groups (nonsensitized mice, OVA-sensitized mice, and OVA-sensitized/ γ -GCE-treated mice) and incubated them with anti-CCR3 antibody.

In this fluorescence-activated cell sorter (FACS) dot-plot data, granulocytes were recognized as highly granular cells, and in this model almost all cells within this gate were eosinophils, which were defined as cells expressing the eotaxin receptor CCR3. Lymphocytes were identified as FSC^{low}/SSC^{low} , and macrophages as FSC^{high}/SSC^{high} cells. FACS dot-plot data showed no eosinophils in nonsensitized mice, and an increased percentage of eosinophils in OVA-sensitized mice, but a decreased percentage of eosinophils in OVA-sensitized/ γ -GCE-

treated mice (Figure 7A). These data provide support for previous data regarding inflammatory cell numbers in BALF (Figure 3). FACS fluorescence data showed that eotaxin receptor CCR3 expression was not suppressed in OVA-sensitized/ γ -GCE-treated mice (Figure 7B). These data suggest that γ -GCE inhibits eosinophil migration without suppressing eotaxin main receptor CCR3 expression.

DISCUSSION

The bulk of the evidence from patients with asthma and from mouse models of allergen-induced airway disease identifies α/β^+ , $CD4^+$ Th2 cells as having a pivotal role in mediating the inflammation that is central to asthma (22–24). There are many exceptions to this rule, but the immunopathological hallmark of allergic disease is the infiltration of affected tissue by Th2 cells (25, 26).

In an earlier study, we have shown that intracellular glutathione redox status, the ratio of GSH/GSSG, in murine APC determines whether reductive or oxidative APC predominate, leading to regulation of the Th1/Th2 balance through production of IL-12 (9). Our group further reported that Th1 cytokine IFN- γ and Th2 cytokine IL-4 increases and decreases the GSH/GSSG ratio, respectively, and that this ratio influences LPS-induced IL-12 production from alveolar macrophages (15). Thus, the ability to generate a Th1 or Th2 response is dependent on glutathione redox status of APC. Therefore, we examined whether changing glutathione redox status controls Th2 disease, such as bronchial asthma.

In the present study, we used γ -GCE to elevate intracellular GSH levels *in vivo* and showed that intraperitoneal injection of γ -GCE to OVA-sensitized mice not only increased GSH amount but also decreased GSSG amount of lung, resulting in significant elevation of the GSH/GSSG ratio (Table 1). Some enzymes that bear an accessible thiol essential for activity can form protein-mixed and intramolecular disulfides by reacting with small disulfide moieties, incubating those of glutathione, namely GSSG (27). Conversely, GSH, which can reduce a wide variety of disulfides by transhydrogenation, is a major reductant of cellular protein disulfides (27). These enzyme activities depend on protein S-thiolation/dethiolation, that is, the oxidation of protein sulfhydryls to mixed disulfides and their reduction back to sulfhydryls (28). Therefore, the balance of the

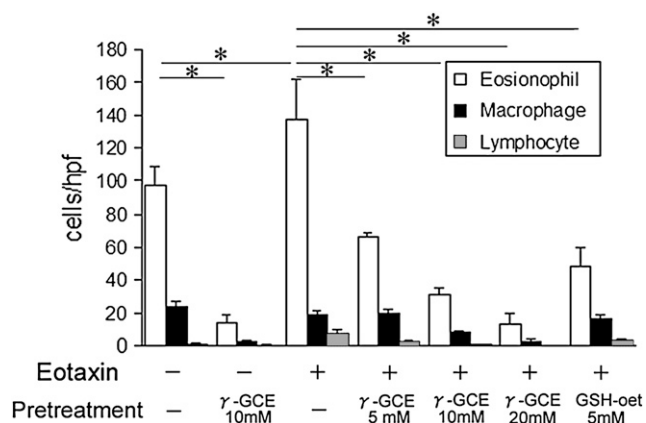


Figure 5. Dose-dependent inhibition of eotaxin-induced chemotaxis of mouse BALF eosinophils by γ -GCE *in vitro*. Cells collected from OVA-sensitized mice BALF were incubated with γ -GCE (0–20 mM) for 60 minutes. An eotaxin (10 ng/ml)-induced chemotaxis chamber assay was performed, and then the filters were removed and stained with Diff-Quick. The number of infiltrating cells was taken as the mean of counts in five immersion fields. Data are mean $\pm$ SE of six experiments. * $P < 0.05$ according to ANOVA with Bonferroni correction.

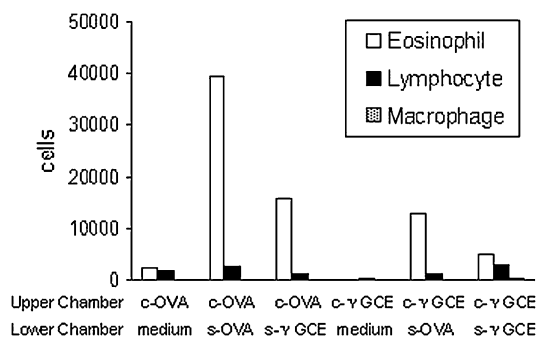


Figure 6. Inhibition of eosinophil chemotaxis in BALF cells of OVA-sensitized/ γ -GCE-treated mice. A chemotaxis chamber assay was performed. Cells were prepared from BALF of OVA-sensitized mice (c-OVA) or OVA-sensitized/ γ -GCE-treated mice (c- γ GCE). BALF supernatants, which were obtained from OVA-sensitized mice (s-OVA) or OVA-sensitized/ γ -GCE-treated mice (s- γ GCE), were put into the lower chamber and used as a chemoattractant. The numbers of cells that migrated into the lower chambers are shown here (representative of three experiments).

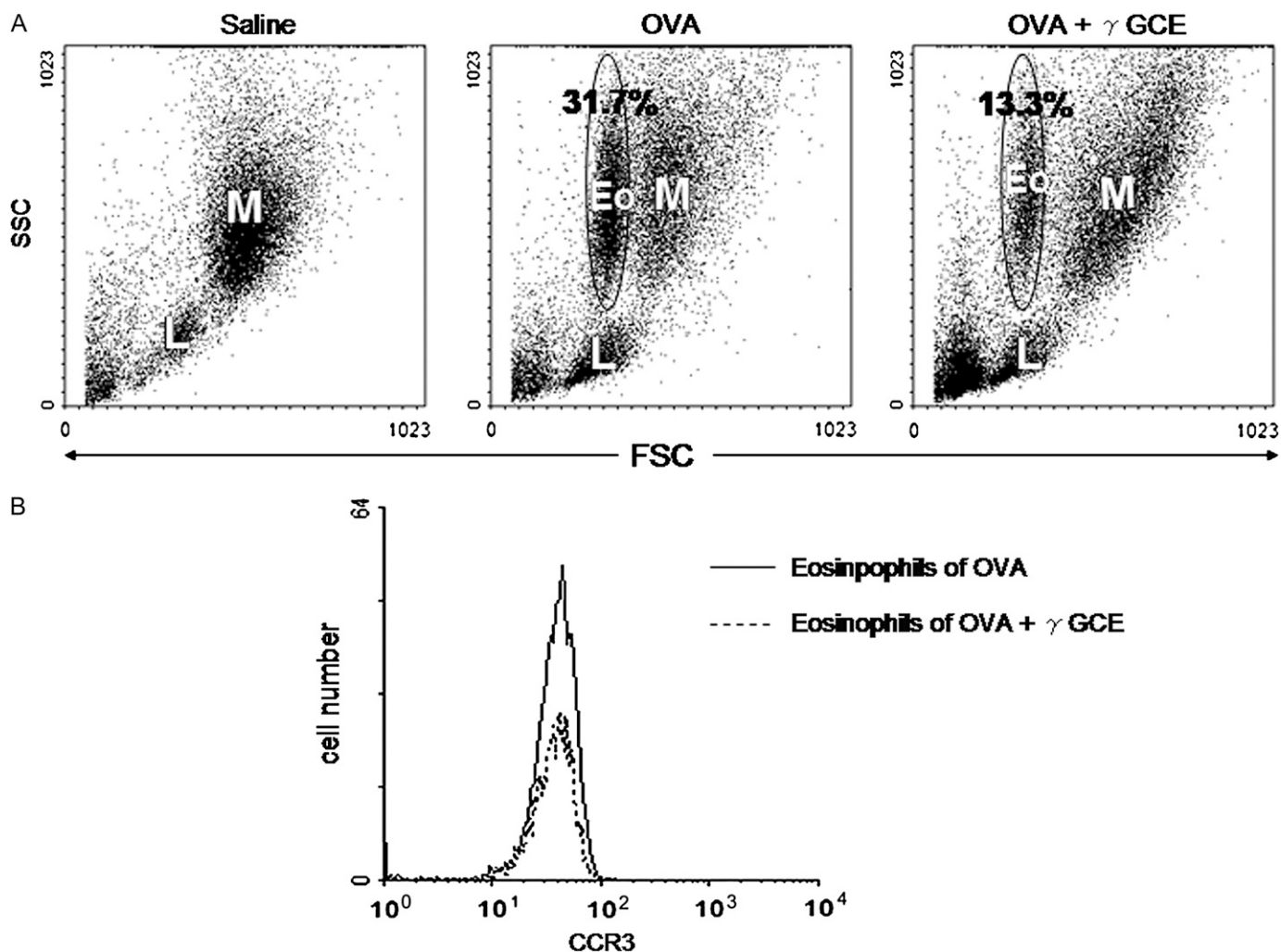


Figure 7. Effect of γ -GCE on CCR3 expression in mouse BALF eosinophils. (A) BALF cells collected from nonsensitized mice (saline), OVA-sensitized mice (OVA), and OVA-sensitized/ γ -GCE-treated mice (OVA + γ -GCE) were incubated with anti-mouse CCR3-fluorescein antibody in the presence of an Fc receptor block (to prevent nonspecific binding), and then sorted on a FACS Calibur flow cytometry system. Lymphocytes (L), granulocytes (mainly eosinophils in this experiment) (Eo), and macrophages (M) were sorted by FSC and SSC. The annotated numbers indicate the percentages of cells in each region. The data shown are representative of three independent experiments with similar results. (B) CCR3 expression in eosinophils. Histogram (log scale fluorescence) shows CCR3 expression in OVA-sensitized/ γ -GCE-treated mice (dotted line) versus OVA-sensitized control mice (solid line) in gated eosinophils (representative of three different mice).

reaction from cellular thiol to disulfide, incubating that of glutathione, must be able to regulate the activity of these enzymes (27). For example, Klatt and colleagues demonstrated that changes in the GSH/GSSG ratio provide the potential to oxidase c-jun sulfhydryls, and that protein S-thiolation/dethiolation, which is specifically targeted to the cysteine residue located in the DNA binding site of the protein, may account for the reversible glutathione redox status regulation of c-jun DNA binding (29). Moreover, our group previously showed that the changes of GSH/GSSG ratio oppositely regulates JNK and p38 MAPK, resulting in control of IL-12 production from human macrophages (13, 14). These findings support that changes of the GSH/GSSG ratio by γ -GCE administration improved Th1/Th2 imbalance through IL-12 production. However, there is an idea that GSH level is important rather than GSH/GSSG ratio, from the point of view that redox signaling is due to reactions of H₂O₂. Since the ratios seem to be affected by many factors, including rates of GSH oxidation, GSSG reduction, protein thiol/disulfide exchange, and GSH synthesis, GSH levels may be possible to be regarded as an index of glutathione redox status.

Actually, GSH/GSSG ratio and GSH level have a similar tendency. We need more consideration in future which is critical ratio or GSH content in our study.

Furthermore, we demonstrated that γ -GCE ameliorated bronchial asthma in a mouse model of OVA-induced asthma, which is the classical and widely used animal model of human asthma and characterized by increased AHR, eosinophilia, and Th2 cytokines. Changes in Raw and Penh values in response to intravenously administered and inhaled methacholine, respectively, were used to measure AHR. Our present study revealed that γ -GCE administration significantly reduced Raw (Figure 2A) and Penh (Figure 2B) values compared with nontreated OVA-sensitized mice. The lung histologic findings and cell differentiation counts of BALF indicated that γ -GCE administration significantly inhibited eosinophil infiltration, which was increased in OVA-sensitized mice (Figure 3). In addition, the cytokines measurement in BALF found that IL-4 and IL-5 levels were decreased, whereas IFN- γ and IL-12 levels were increased in OVA-sensitized/ γ -GCE-treated mice (Figure 4A). In an *in vitro* assay, elevation of the GSH/GSSG ratio by

γ -GCE enhanced LPS-induced IL-12 production, from THP-1 monocytic cells (Figure 1). These findings indicate that the administration of γ -GCE prevents AHR and antigen-induced airway inflammation through increasing intracellular GSH concentration in APC of the lung and shifting the Th1/Th2 balance to Th1 due to the enhancement of IL-12 production. In fact, OVA sensitization decreased the GSH/GSSG ratio in the lung and γ -GCE improved the ratio (Table 1), suggesting that oxidative APC induced by OVA sensitization convert to reductive type by γ -GCE administration. Recent findings showed that endocrine-disrupting chemicals promote Th2 polarization indirectly via the depletion of glutathione in APC and subsequent modulation of IL-10 and IL-12 production, and consequently increase the number of eosinophils and the levels of IL-5 in BALF in mice (30). This report supports our finding that elevate the intracellular GSH level and GSH/GSSG ratio by administration of γ -GCE ameliorates bronchial asthma through improving Th1/Th2 imbalance in a mouse asthma model. On the other hand, it was reported that administration of thioredoxin, which is one of redox-regulatory molecules, suppressed AHR and cells number in BALF, and up-regulated expression of IL-1 family, which is Th1-like cytokine, in the lungs of OVA-sensitized and -challenged mice (31). This report did not find prevention of Th2 development and up-regulation of Th1 cytokine production, such as IFN- γ and IL-12, which were demonstrated in the present study. Therefore, we consider that regulation of the Th1/Th2 balance through IL-12 production is not the common effect to redox-regulatory molecules but the characteristic effect of glutathione.

Interestingly, the eosinophil count of BALF from OVA-sensitized/ γ -GCE-treated mice was more markedly reduced compared with the degree of reduction in Th2 cytokine production due to changing the Th1/Th2 balance. Next, we investigated the direct effect of γ -GCE on eosinophils and found that γ -GCE suppressed the chemokine-induced chemotaxis of eosinophils. In fact, pretreatment of γ -GCE *in vitro* dose-dependently suppressed eotaxin-induced eosinophil chemotaxis (Figure 5), and administration of γ -GCE *in vivo* inhibited eosinophil chemotaxis, which was enhanced by OVA sensitization (Figure 6). These results indicate that the administration of γ -GCE to mice with asthma inhibited the chemokine-induced migration of eosinophils from the bloodstream into the lung. However, FACS fluorescence data showed that γ -GCE failed to decrease the expression of the main eotaxin receptor CCR3 (Figure 7). These findings are consistent with the report that thioredoxin suppresses chemokine-induced chemotaxis of neutrophils, macrophages, and lymphocytes without affecting chemokine receptor expression in experimental autoimmune myocarditis model (32), suggesting that anti-chemotactic activity is a conventional effect of redox-regulatory molecules, such as glutathione and thioredoxin. In addition, we examined the chemokine production analyses because RANTES and eotaxin have been known to be involved in the recruitment of eosinophils, and showed that RANTES and eotaxin levels in BALF were significantly reduced by γ -GCE administration (Figure 4B). In chemotaxis assay, the use of BALF supernatant from OVA-sensitized/ γ -GCE-treated mice as a chemoattractant reduced eosinophil chemotaxis compared with that from nontreated OVA-sensitized mice (Figure 6), indicating that γ -GCE inhibits production of eosinophil chemoattractant, such as RANTES and eotaxin, in BALF. Taken together, we believe that the increase in the GSH/GSSG ratio by γ -GCE leads to suppression of not only chemokine production but also eosinophil migration itself, resulting in improvement of antigen-induced airway inflammation in an allergic mouse model.

The present study demonstrated that administration of γ -GCE elevates GSH level and GSH/GSSG ratio in the lung,

and ameliorates AHR and eosinophilic airway inflammation by altering the Th1/Th2 balance and suppressing chemokine production and eosinophil migration in a mouse asthma model. Therefore, we first provided *in vivo* evidence that the use of modulators of glutathione redox reactions, such as γ -GCE, offers a novel approach for the treatment of bronchial asthma, especially severe or steroid-resistant asthma. In addition, these reagents may aid the development of new therapeutic strategies for improving the Th1–Th2 balance in “Th1” and “Th2” diseases, such as autoimmune and allergic diseases, as well as in other conditions.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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