The Protective Effect of a Topical Mucin Secretagogue on Ocular Surface Damage Induced by Airborne Carbon Black Exposure

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PURPOSE. Exposure to airborne particulate matter can induce ocular surface damage and inflammation. We evaluated the effects of a topical mucin secretagogue on the mitigation of ocular surface damage induced by exposure to airborne carbon black (CB).

METHODS. Sprague-Dawley rats were exposed to ambient CB for 2 hours twice daily for 5 days. Corneal staining score and tear lactic dehydrogenase (LDH) activity were measured to evaluate ocular surface damage. Serum immunoglobulin (Ig) G and IgE levels and the sizes of cervical lymph nodes were also measured. The expressions of interleukin (IL)-4, IL-17, and interferon (IFN)-γ were measured by Western blot analysis. Diquafosol tetrasodium was instilled six times a day for 5 days, and the extent of ocular surface damage was evaluated.

RESULTS. After exposure to airborne CB, the median corneal staining score and LDH activity were significantly increased. Serum IgG and IgE levels and the sizes of cervical lymph nodes were also significantly increased. Additionally, the expression of IL-4 and IFN-γ was elevated in the anterior segment of the eyeball. Furthermore, the expression of IL-4, IL-17, and IFN-γ was elevated in the cervical lymph nodes. When exposed to airborne black carbon, topical diquafosol tetrasodium significantly increased tear MUC5AC concentration and decreased tear LDH activity.

CONCLUSIONS. Exposure to airborne CB induced ocular surface damage and increased proinflammatory cytokines in the eyes and cervical lymph nodes. Topical mucin secretagogues seem to have a protective effect on the ocular surface against exposure to airborne particulate matters.

Keywords: air pollution, carbon black, particulate matter, ocular surface damage, mucin secretagogue

Air pollution has emerged as one of the most important issues in public health worldwide. Particulate matter (PM), also known as airborne particulate pollution, is a mixture of components such as nitrates, sulfates, organic chemicals, metals, soil, and dust particles.1,2 Numerous studies3–5 have demonstrated that these particles are linked with pulmonary and cardiac-associated morbidity and mortality. In recent years, a growing number of epidemiologic studies have shown that exposure to airborne PM is linked with an increased frequency of outpatient visits for ocular surface diseases, including nonspecific conjunctivitis,3 blepharitis,6 and pterygium.7

Air pollutants that contain ambient PM can impact the eye, causing ocular symptoms including irritation, burning, tearing, itching, and foreign body sensation.8 Previous studies have shown that ambient PM is linked with a reduction in tear-film breakup time.9,10 an increase in meibomian gland secretion,6 and goblet cell (GC) hyperplasia.11 However, treatment measures available to address PM-induced ocular problems have not been widely studied despite the increasing concerns regarding the influence of PM on ocular health.

Recently, some in vitro studies have shown that exposure to PM induces cytotoxicity and oxidative stress in cultured human corneal epithelial cells.12,13 In addition, Eom et al.14 have demonstrated that titanium dioxide (TiO2) nanoparticles, as a type of PM, damage the ocular surface, resulting in increased lactic dehydrogenase (LDH) level and inflammatory cell infiltration in rats. On the other hand, Torricelli et al.15 have reported that long-term exposure to air pollution increases ocular surface GC density and, consequently, prompts a rise in gel-forming MUC5AC messenger RNA level, which may be part of an adaptive response of the ocular surface to PM exposure. However, they also speculated that these adaptive mechanisms may be saturated by chronic PM exposure, accompanied by a reduction in GC count and MUC5AC level.15 Similarly, an animal experiment on rabbits has shown that the tear MUC5AC level increases after 1 day of exposure but returns to normal level after 5 days of exposure to TiO2 nanoparticles.16 This study has demonstrated that repetitive exposure to TiO2 nanoparticles seems to induce GC exhaustion, resulting in a reduction in the degree of ocular surface protection provided by MUC5AC against nanoparticles.16 Thus, anti-inflammatory and antioxidant treatment and improvement of ocular surface defense mechanisms such as the mucosal defense system may have
potential protective effects for the ocular surface against airborne PM exposure.

Diquafosol ophthalmic solution is a novel mucin secretagogue that has been introduced for the treatment of dry eye disease in Japan and South Korea.17,18 This drug is a P2Y2 purinergic receptor agonist that stimulates the receptors in ocular tissues and thus increases mucin (conjunctival GC stimulation) and the aqueous portion of the tear film (conjunctival epithelial cell stimulation).19 Diquafosol is reported to be beneficial in tear film stabilization and repair of corneal epithelial damage.20–22 In addition, Lee et al.23 recently have reported that diquafosol effectively decreases inflammatory markers in the lacrimal glands of a mouse dry eye animal model.

On the basis of these effects, we evaluated whether topical administration of diquafosol may have a potential protective effect against ocular injury caused by airborne PM exposure. To test this hypothesis, we established an airborne PM–exposed animal model using carbon black (CB) nanoparticles, which represent one of the best studied examples in particle toxicology and which have been commonly used to evaluate the effects of PM on biological functions.

MATERIALS AND METHODS

Thirty-six inbred male Sprague-Dawley rats, each weighing 250 to 300 g and aged between 6 and 8 weeks, were used in this study. The rats were randomly divided into four groups: a normal control group (n = 9), a CB group (n = 9), a saline-treated group (n = 9), and a diquafosol-treated group (n = 9) (see below). All procedures adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Approval for this animal study was obtained from the Korea University Animal Institutional Review Board, Seoul, South Korea.

Exposure to Airborne Carbon Black and Treatment With a Topical Mucin Secretagogue

A special exposure chamber with six fans was built to expose the ocular surface of rats in this study to airborne CB particles. CB powder (Printex35) was purchased from Orion Engineered Carbons (Piscataway, NJ, USA). CB powder was put in the exposure chamber, and the airborne particle concentration in the exposure chambers was adjusted for a PM amount less than 10 μm (PM10), as measured by an 831 Aerosol Mass Monitor (Met One Instruments, Inc., Grants Pass, OR, USA). The mean ± standard deviation of airborne CB particle concentration during the study period was 160 ± 36 μg/m³. We used CB because it represented the carbon core of PM and reduced the potential for confounding effects due to the chemical toxicity of other metals present in ambient PM.24 Additionally, a CB-exposed animal model has been previously used to investigate the effects of drug treatment25 or aerobic exercise26 on PM-induced lung injury.

Rats (n = 27) were exposed to airborne CB in the exposure chamber for 2 hours twice daily for 5 days. During the 5 days, the saline-treated group (n = 9) was instilled with 10 μL saline six times a day, while the diquafosol-treated group was instilled with 10 μL of 3% diquafosol tetrasodium, a topical mucin secretagogue, six times a day (Fig. 1). The temperature and relative humidity of the chamber were kept at 22 ± 2°C and 60 ± 10%, respectively, during the experiments.

General anesthesia was induced via intramuscular injection of xylazine hydrochloride (Rompun 2%, 1 mg/100 g body weight; Bayer, Leverkusen, Germany) and Zoletil (8 mg/100 g body weight; Virbac Laboratories, Carros, France) before corneal staining, tear sample collection, and euthanasia.

Corneal Staining

The bilateral corneal staining score (n = 6 for each group) was graded after the last exposure to CB particles with a slit lamp examination under general anesthesia by a single experienced ophthalmologist (BK) according to the National Eye Institute scoring scheme.27 The mean corneal staining score of both eyes of the individual rats was used for statistical analysis. Briefly, a fluorescein sodium–impregnated paper strip (Haag-Streit, Bern, Switzerland) was wetted with 5 μL sterile normal saline, and diluted dye was dropped onto the upper bulbar surface after retracting the upper lid. After the dye was placed, the rat’s eye was gently closed and opened five times, and the excessive tear fluid and dye were wiped away.
Corneal staining was observed by using slit lamp examination under cobalt blue illumination. Rats used for ocular surface staining were not used for tear sample collection, but were used in assessment of the size of superficial cervical lymph nodes as well as Western blot analysis for the expression of IL-4, IL-17, and IFN-γ.

**Tear Sample Collection**

Tear samples were collected under general anesthesia after the last exposure to CB particles, at 2 hours after the last instillation of saline or diquafosol. Tear samples (106 μL) were obtained from each eye by using the flush tear collection method, as previously described. To obtain eye-flush tears, 60 μL sterile normal saline was introduced into the space between the eye and the lateral canthus, the eye was manually blinked five times, and fluid was aspirated from the inferior meniscus by using a micropipette tip. In each collection of eye-flush tears, approximately 50 to 60 μL tear volume was collected. Sterile normal saline was instilled twice, and tear sampling was performed within 1 minute and stored at −20°C until analysis. LDH activity was measured in 100 μL diluted tear samples (n = 6 for each group) containing 6 μL eye-flush tears and 94 μL PBS (method described below). The remaining 100 μL undiluted eye-flush tears (n = 6 for each group) were used to measure MUC5AC level (method described below). The mean values were used to compare tear LDH activity and tear MUC5AC concentration among the four groups.

**Enzyme-Linked Immunosorbent Assay**

LDH activity and MUC5AC level were measured in six tear samples from each group. LDH activity was measured with the LDH ELISA (enzyme-linked immunosorbent assay) kit (CytoTox96 nonradioactive cytotoxicity assay; Promega, Madison, WI, USA), and the level of MUC5AC was measured with an ELISA kit for mucin 5 subtype AC (MyBioSource; San Diego, CA, USA). The blood samples for measuring the IgG and IgE levels in serum were collected from the abdominal aorta of four rats in each group under general anesthesia. The IgG level in serum (1,200,000) was measured with a rat IgG ELISA kit (ab189578; Abcam, Cambridge, UK), while the IgE level in serum (9:10) was measured with a rat IgG ELISA kit (ab189578; Abcam). The IgG level in serum was quantified by a blinded examiner (BRK) using ImageJ (1.43u, National Institutes of Health, Bethesda, MD, USA). First, primary antibodies were commercially obtained for IL-4, IL-17 (1:1000, orb 48034; Biorbyt, Cambridge, UK), and β-actin (1:10,000, No. 5125; Cell Signaling Technology, Danvers, MA, USA). The secondary antibody was an anti-rabbit IgG, HRP-linked antibody (1:10,000 for IL-4 and IFN-γ, and 1:20,000 for IL-17, No. 7074; Cell Signaling Technology).

**Western Blot Analysis**

The expression of IL-4, IL-17, and IFN-γ in the anterior segment of the eyelash and cervical lymph nodes was evaluated with Western blot analysis. The anterior segments of both eyes of each rat were homogenized together in Tper tissue protein extraction reagent containing a protease inhibitor mixture (Thermo Fisher Scientific, Waltham, MA, USA), and the tissue cell extracts (n = 3 for each group) were subjected to Western blot analysis for measuring the protein levels of IL-4, IL-17, IFN-γ, and β-actin, as previously described. Tissue homogenates from bilateral cervical lymph nodes from individual rats were also subjected to Western blot analysis for measuring protein levels of IL-4, IL-17, IFN-γ, and β-actin (n = 3 for each group). Protein concentration per lane in the Western blot was 50 μg for the anterior segment samples and 10 μg for the cervical lymph node samples. Primary antibodies were commercially obtained for IL-4, IL-17 (1:1000, ab9811 and 1:2000, ab79056, respectively; Abcam), IFN-γ (1:1000, orb 48034; Biorbyt, Cambridge, UK), and β-actin (1:10,000, No. 5125; Cell Signaling Technology, Danvers, MA, USA). The secondary antibody was an anti-rabbit IgG, HRP-linked antibody (1:10,000 for IL-4 and IFN-γ, and 1:20,000 for IL-17, No. 7074; Cell Signaling Technology).

**Statistical Analyses**

Statistical analyses were performed by using the Mann-Whitney U test in Statistical Package for Social Sciences version 20.0 (IBM Corp., Armonk, NY, USA). Values were expressed as the median and interquartile range (IQR). Results of Western blot analysis were expressed as a ratio to β-actin. Results were considered statistically significant at a P value less than 0.05.

**RESULTS**

**Corneal Staining**

After airborne CB exposure, the median (IQR) corneal staining score was significantly increased in the CB group (10.0 [8.3–11.8]; Figs. 2A, 2C) and the saline-treated group (8.0 [6.3–9.8]; Figs. 2A, 2D) compared with the normal control group (4.0 [2.5–4.8]; P = 0.004 and P = 0.006, respectively; Figs. 2A, 2B). The diquafosol-treated group demonstrated a significantly reduced corneal staining score (6.0 [5.3–6.0]; Figs. 2A, 2E) as compared with the CB group (P = 0.012), but no significant difference in comparison with the saline-treated group (P = 0.055).

**Tear Lactic Dehydrogenase Activity and MUC5AC Concentration**

After airborne CB exposure, tear LDH activity was significantly increased in the CB group (0.63 optical density [OD] [0.61–0.75]; Fig. 3A) and the saline-treated group (0.59 OD [0.54–0.60]; Fig. 3A) versus the normal control group (0.44 OD [0.43–0.45]; all P = 0.004; Fig. 3A). Diquafosol treatment significantly reduced tear LDH activity (0.48 OD [0.45–0.50]; Fig. 3A) as compared with the CB group (P = 0.006) and even the saline-treated group (P = 0.01).

Additionally, the tear MUC5AC concentration was significantly decreased in the CB group (2.79 ng/mL [2.36–5.26]; Fig. 3B) and the saline-treated group (3.21 ng/mL [2.12–3.87]; Fig. 3B) versus the normal control group (11.08 ng/mL [10.40–11.69]; all P = 0.004; Fig. 3B). Diquafosol instillation significantly increased tear MUC5AC concentration (6.79 ng/mL [5.70–8.77]; Fig. 3B) as compared...
with the CB group ($P = 0.004$) and the saline-treated group ($P = 0.004$).

Cervical Lymph Node Size

After airborne CB exposure, the median (IQR) size of cervical lymph nodes was significantly increased in the CB group (27.6 mm$^2$ [24.8–29.3]; Fig. 4) in comparison with the control group (20.1 mm$^2$ [18.6–20.5]; $P = 0.004$; Fig. 4). However, diquafosol instillation did not significantly change the size of cervical lymph nodes as compared with the CB group and the saline-treated group (Fig. 4).

Serum Immunoglobulin G and Immunoglobulin E Concentrations

After airborne CB exposure, the median (IQR) serum IgG level was significantly increased in the CB group (11.4 ng/mL [10.8–12.8]; Fig. 5A) and saline-treated group (10.4 ng/mL [9.0–11.4]; Fig. 5A) compared with the normal control group (3.9 ng/mL [2.5–5.5]; all $P = 0.009$; Fig. 5A). However, diquafosol instillation did not significantly change the serum IgG level as compared with the CB group and the saline-treated group (Fig. 5A).

However, the serum IgE level was significantly increased in the CB group (14.1 ng/mL [13.4–15.6]; Fig. 5B) and saline-treated group (10.4 ng/mL [9.9–10.5]; $P = 0.016$; Fig. 5B) compared with the normal control group (4.2 ng/mL [2.5–4.3]; all $P = 0.009$; Fig. 5B) after airborne CB exposure. Diquafosol treatment significantly reduced serum IgE level (5.2 ng/mL [4.3–6.0]; Fig. 5B) as compared with the CB group ($P = 0.009$) and even with the saline-treated group ($P = 0.016$).

Quantification of Cytokines by Western Blot Analysis

After airborne CB exposure, the level of IL-4 in the anterior segment of the eyeball was significantly increased in the CB group (1.0 [0.8–1.2]; Fig. 6A) versus the normal control group (0.3 [0.1–0.4]; $P = 0.028$; Fig. 6A). Diquafosol treatment significantly reduced the level of IL-4 in the anterior segment of the eyeball (0.4 [0.1–0.6]; Fig. 6A) as compared with the CB group ($P = 0.047$), but there was no significant difference in comparison with the saline-treated group (0.8 [0.4–0.8]; $P = 0.175$; Fig. 6A). In addition, the level of IL-4 in the cervical lymph node was significantly increased in the CB group (1.0 [0.9–1.1]; Fig. 6B) and the saline-treated group (0.9 [0.8–0.9]; Fig. 6B) compared with the normal control group (0.3 [0.3–0.6]; all $P = 0.009$; Fig. 6B). Diquafosol instillation did not significantly change the level of IL-4 in the cervical lymph nodes as compared with the CB group and the saline-treated group (Fig. 6B).

There was no significant difference in the level of IL-17 in the anterior segment of the eyeball between the groups (Fig. 6C). However, the level of IL-17 in the cervical lymph nodes was significantly increased in the CB group (1.5 [1.3–1.6]; Fig. 6D) and the saline-treated group (1.4 [1.2–1.5]; Fig. 6D) versus the normal control group (0.7 [0.6–0.8]; all $P = 0.016$; Fig. 6D). Diquafosol instillation did not significantly alter the level of IL-17 in the cervical lymph nodes as compared with the CB and the saline-treated groups (Fig. 6D).
In addition, the level of IFN-\(\gamma\) in the anterior segment of the eyeball was significantly increased in the CB group (2.2 [1.1–2.4]; Fig. 6E) and saline-treated group (2.0 [1.1–2.1]; Fig. 6E) compared with the normal control group (0.5 [0.2–0.8]; \(P = 0.028\) and \(P = 0.047\), respectively; Fig. 6E) after airborne CB exposure. However, diquafosol treatment did not significantly impact the level of IFN-\(\gamma\) in the anterior segment of the eyeball as compared with the CB group and the saline-treated group (Fig. 6E). In addition, the level of IFN-\(\gamma\) in the cervical lymph nodes was significantly increased in the CB group (1.3 [1.0–1.8]; Fig. 6F) and the saline-treated group (0.9 [0.8–1.3]; Fig. 6F) compared with the normal control group (0.5 [0.5–0.6]; \(P = 0.016\) and \(P = 0.047\), respectively; Fig. 6F). Interestingly, diquafosol instillation significantly decreased the level of IFN-\(\gamma\) in the cervical lymph nodes (0.7 [0.6–1.0]; Fig. 6F) as compared with the CB group (\(P = 0.047\); Fig. 6F), while there was no significant difference observed in the saline-treated group (\(P = 0.347\); Fig. 6F).

**DISCUSSION**

In the present study, we demonstrated that diquafosol as a mucin secretagogue inhibits LDH and proinflammatory cytokine release in ocular tissue in an experimental model of CB particle-induced ocular toxicity and inflammation, which may be associated with an increased mucosal defense response and a part of the anti-inflammatory effects following treatment. To our knowledge, this is the first study to evaluate the effect of drug treatment on PM-induced ocular injury.

In recent years, much research has focused on the direct or indirect toxic effects of PM. CB and TiO₂ are some of the best...
Thus, these results suggest that CB is similar in nature to TiO2, exposure, which is a representative cytokine of Th1 cytokines. Segments of the eyeball was significantly elevated by CB exposure to airborne TiO2 nanoparticles increases tear LDH activity, corneal staining score, serum IgG and IgE levels, and expression of inflammatory cytokines in the anterior segment of the eyeball, including especially type 2 cytokines like IL-4. Thus, this study has demonstrated that TiO2 particles damage the ocular surface, resulting in increased LDH level and inflammatory cell infiltration in rats, and the Th2 pathway may play a dominant role in the immune response following airborne TiO2 exposure to the ocular surface.14 However, there are currently no studies published that have investigated the effects of CB exposure on the ocular surface. CB is a major component of ambient PM generated by incomplete combustion of heavy petroleum materials.39 Previous research has shown that mouse intratracheal exposure to CB nanoparticles increases tear LDH activity, corneal staining score, serum IgG and IgE levels, and expression of inflammatory cytokines in the anterior segment of the eyeball, including especially type 2 cytokines like IL-4. Thus, this study has demonstrated that TiO2 particles damage the ocular surface, resulting in increased LDH level and inflammatory cell infiltration in rats, and the Th2 pathway may play a dominant role in the immune response following airborne TiO2 exposure to the ocular surface.14 However, there are currently no studies published that have investigated the effects of CB exposure on the ocular surface. CB is a major component of ambient PM generated by incomplete combustion of heavy petroleum materials.39 Previous research has shown that mouse intratracheal exposure to CB nanoparticles increases the level of LDH in bronchial alveolar lavage fluid25 and those of several cytokines like IL-5, IL-17A, and IFN-γ in lung tissue.40 To evaluate the effects of airborne CB exposure on the ocular surface, we examined the corneal staining score, the expression of inflammatory cytokines (IL-1, IL-17, and IFN-γ) in the anterior segment of the eyeball, and tear LDH activity. In addition, serum IgG and IgE levels were also considered to further evaluate the Th1 and Th2 pathways after CB exposure. Our results showed that airborne CB exposure increases corneal staining score, tear LDH level, corneal staining score, expression of IL-4 in the anterior segments of eyeball, and serum IgG and IgE levels, consistent with the findings of an animal experiment following TiO2 exposure.14 However, we also found that the expression of IFN-γ in the anterior segment of the eyeball was significantly elevated by CB exposure, which is a representative cytokine of Th1 cytokines. Thus, these results suggest that CB is similar in nature to TiO2, in that they both damage the ocular surface, leading to increased LDH level and inflammatory cell infiltration in rats, though the inflammmogenic effects of CB particles seem greater than those of TiO2 particles. In other words, the Th1 pathway seems to play a role in the immune response following airborne CB particle exposure to the ocular surface, separate from the Th2 pathway.

Diquafosol sodium, also known as a P2Y2 purinergic receptor agonist, is a mucin secretagogue. Previous studies have shown that P2Y2 activation can negatively regulate immunity and inflammation45 and could thus affect both mucosal inflammation and the healing process.42–44 An animal experiment involving rats has shown that diquafosol is effective in promoting corneal epithelial wound healing, and that this effect may result from ERK-stimulated cell proliferation and migration via P2Y2R-mediated [Ca2+]i elevation.22 However, wound healing is a complicated process regulated by various factors including inflammation. Recently, Lee et al.23 have reported that diquafosol effectively decreases the levels of inflammatory markers such as tumor necrosis factor α, ICAM-1, VCAM-1, and MMP-2 in the lacrimal glands of a mouse model with dry eye induced by subcutaneous scopolamine injection and exposure to environmental desiccating stress. On the other hand, P2Y2 activation is reportedly also associated with inhibition of cytotoxicity in NK cells in vitro.46 On the basis of these results, we can speculate that diquafosol may have potential protective effects on ocular surface cytotoxicity and inflammation caused by airborne PM exposure. As expected, we found that diquafosol treatment markedly improved CB-induced ocular surface damage in rat model, including with respect to the level of IL-4 in the anterior segments of the eyeball and tear LDH and the score of corneal staining. IL-4 is a T helper 2 (Th2) cytokine that induces differentiation of Th0 cells to Th2 cells and stimulates B cells to produce IgE. Interestingly, topical diquafosol treatment also markedly decreased serum IgE level in a CB-induced ocular surface damage rat model, which is supported by the significantly decreased expression of IL-4 present in the anterior segments following diquafosol treatment. Thus, these results suggest that...
Diquafosol improved the CB-induced ocular surface damage through reduction in cytotoxicity and inflammation, especially type 2 cytokines, and that this effect may be associated with P2Y2 activation and Th2 pathway inhibition. However, the mechanism of diquafosol that reduces CB-induced ocular surface damage may use another pathway, apart from these pathways. We aim to further investigate the underlying mechanism in other research.

Mucin is thought to play a very important role in tear film stability. Conjunctival GCs secrete MUC5AC, which stabilizes the tear film and decreases its surface tension. MUC5AC is reported to assist with removal of debris and cellular components from the tear film, contribute to the hydrophilicity of the tear film, and provide a protective barrier on the ocular surface. From these beneficial effects, Torricelli et al. have demonstrated that MUC5AC expression may act as an adaptive (or defensive) response of the ocular surface against PM exposure. Similarly, Eom et al. have revealed that cellular damage induced by a single (1-day) exposure to TiO2 nanoparticles increases tear MUC5AC level to protect the ocular surface and to clear nanoparticles, but then decreases to normal level after repetitive (5 days of) exposure to TiO2 nanoparticles, resulting in reduced ocular surface protection provided by MUC5AC against nanoparticles, which may be induced by GC exhaustion. However, our results revealed that 5 days of CB exposure seriously reduced tear MUC5AC level to a degree that was significantly lower than normal. Thus, these results suggest that the mucin (MUC5AC) adaptive (or defensive) mechanism may be seriously reduced by CB exposure, which may be also induced by GC exhaustion, as in TiO2 exposure, but to a degree that is greater than that associated with TiO2 particles. In addition, our findings indicated that diquafosol treatment markedly increased the tear MUC5AC level in a CB-induced ocular surface damage rat model, which is consistent with the outcomes of previous studies showing that diquafosol effectively stimulates MUC5AC.

**Figure 5.** The serum immunoglobulin (IgG and IgE) levels were evaluated by ELISA after 5 days of CB exposure and diquafosol treatment. Experimental groups are the same as in Figure 2. (A) An alteration of the serum IgG level after CB exposure and diquafosol treatment. (B) An alteration of serum IgE level in tears after CB exposure and diquafosol treatment. The asterisk indicates a P value < 0.05, as determined by the Mann-Whitney U test.
secretion in both normal and dry eye animal models. These results suggest that diquafosol as a mucin secretagogue improved the ocular surface adaptive (or defensive) mechanism against CB exposure, which may operate through increasing the functions of MUC5AC such as improvement of tear film stability and removal of debris components including PM. Thus, the mechanism of diquafosol that reduces ocular damage from CB-induced cytotoxicity and inflammation may be also related to these adaptive (or defensive) mechanisms.

Lymph node enlargement occurs as part of the body's natural immune response. Previous studies have found that TiO₂ exposure induces cervical lymph node cytokine secretion, including IL-4 and IL-17. Similarly, lung exposure of mice to CB particles by oropharyngeal aspiration for 24 hours induces the enlargement of peribronchial lymph nodes and increases the expression of IL-4, IL-5, and IFN-γ in the peribronchial lymph nodes. Our results showed that airborne CB exposure induces the enlargement of cervical lymph nodes and increases the expression of IL-4 and IL-17 in the cervical lymph nodes, which is consistent with the findings of an animal experiment following TiO₂ exposure. In addition, we also found that the expression of IFN-γ in the cervical lymph nodes was significantly elevated by CB exposure, which is further supported in that both the Th1 and Th2 pathways play a role in the immune response following airborne CB particle exposure to the ocular surface. Topical diquafosol...

**Figure 6.** Expression of inflammatory cytokines in the anterior segment of the eyeball and cervical lymph nodes was evaluated by Western blot analyses after 5 days of CB exposure and diquafosol treatment. Experimental groups are the same as in Figure 2. (A) Expression of IL-4 in each group's anterior segment of the eyeball. (B) Expression of IL-4 in each group's cervical lymph nodes. (C) Expression of IL-17 in each group's anterior segment of the eyeball. (D) Expression of IL-17 in each group's cervical lymph nodes. (E) Expression of IFN-γ in each group's anterior segment of the eyeball. (F) Expression of IFN-γ in each group's cervical lymph nodes. A representative example of a Western blot gel image is shown at the top left of each figure. β-Actin was used as an internal loading control for Western blot. The asterisk indicates a P value < 0.05, as determined by the Mann-Whitney U test.
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Introduction

treatment did not change the size of cervical lymph nodes or IL-4 level in the cervical lymph nodes in this study.

There are several limitations in the present study. First, the sample size was relatively small. Second, it is well known that diquafosol treatment can increase the tear volume on the ocular surface, 19,52 which may wash out CB nanoparticles and reduce the damage of ocular surface. However, we did not measure the change of tear volume after diquafosol treatment in this study. In addition, diquafosol treatment is reported to increase the GC density on the ocular surface. 33-55 The increase of tear MUC5AC concentration could be due to the increase of GC density in the diquafosol-treated group. However, we did not evaluate the change of GC density in this study. Further studies are needed to investigate the underlying mechanism of the protective effect of this mucin secretagogue on the ocular surface when exposed to airborne PM. Third, exposure to single CB nanoparticles does not accurately reflect the characteristics of the area where the actual PM is generated, since PM is a mixture of components like nitrates, sulfates, organic chemicals, metals, soil, and dust particles. 42,43 Recently, Gour et al. 40 have reported that the airway inflammatory effects of urban PM cannot be truly recapitulated by components of PM like CB alone and suggested the possibility that unidentified chemical or biological components of urban PM drive pathologic immune responses in the lungs. In addition, coal fly ash and diesel exhaust particles are also common constituents of airborne PM that are often used in studies to investigate the effects of PM on biological functions. 40,56,57 Thus, additional investigations are needed to compare the biological functions of these different types of airborne particles on the ocular surface with that of actual PM in order to better understand the effects of PM on the ocular surface.

In conclusion, CB exposure induced ocular surface damage and increased proinflammatory cytokines in the eyes and cervical lymph nodes in a rat animal model. Topical mucin secretagogue was effective in reducing ocular surface damage from CB exposure. Topical mucin secretagogues seem to have a protective effect on the ocular surface against exposure to airborne PM.

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