Ann Allergy Asthma Immunol 110 (2013) 39–43

Contents lists available at SciVerse ScienceDirect

Effects of diesel exhaust particles on primary cultured healthy human conjunctival epithelium

Hiroshi Fujishima, MD [*,†]; Yoshiyuki Satake, MD [‡]; Naoko Okada, PhD [†,§]; Shinichi Kawashima, MD [†]; Kenji Matsumoto, MD, PhD [§]; and Hirohisa Saito, MD, PhD [§]

[*] Department of Ophthalmology, Tsurumi University School of Dental Medicine, Yokohama, Japan
[†] Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan
[‡] Department of Ophthalmology, Tokyo Dental College, Ichikawa, Chiba, Japan
[§] Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan

ARTICLE INFO

Article history:
Received for publication August 21, 2012.
Received in revised form October 11, 2012.
Accepted for publication October 23, 2012.

ABSTRACT

Background: Air pollution from road traffic is a serious public health problem. Epidemiologic studies have demonstrated adverse health effects associated with environmental pollution. Diesel exhaust is a major contributor to ambient particulate matter air pollution. We studied the effects of exposure to diesel exhaust particles on allergic conjunctivitis using cultured conjunctival epithelial cells obtained from healthy people.
Objective: To identify the factors involved in the human conjunctival epithelial response to diesel exhaust in vitro.
Methods: Healthy individuals underwent conjunctival biopsy, and the samples were incubated on conjunctival epithelial sheets. We investigated the effects of exposure to diesel exhaust using GeneChip arrays. The adhesion molecules and cytokines showing increased expression on GeneChip arrays were verified by real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay.
Results: The GeneChip array showed increased expression of adhesion molecules, cytokines, chemokines, and growth factors after exposure to diesel exhaust. Real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay confirmed that the expression of intercellular adhesion molecule 1 and interleukin 6, in particular, were significantly upregulated.
Conclusion: Our experimental data confirm that exposure to diesel exhaust particles increases inflammatory factor expression in human conjunctiva and thereby contributes to allergic conjunctival responses.

Introduction

Allergic conjunctivitis is the most common ocular surface allergic disease and affects more than 20% of the population.[1–3] As a result of changes in the living environment and other factors, the incidence of allergic diseases, including atopic dermatitis and pollinosis, is progressively increasing. The medical community has been challenged to develop effective therapies for these allergic diseases.[4] To better understand the pathogenesis of allergic disease, researchers are increasingly focusing on the effects of environmental factors, such as exposure to atmospheric pollutants, UV light, and viral infection. Epidemiologic studies have found that the increased incidence of allergic diseases is at least partly due to increased exposure to atmospheric pollutants.

The effects of diesel exhaust on the pathophysiology of allergic airway disease have been analyzed in animals and humans using in vitro and in vivo models. The components of diesel exhaust as environmental factors have received much attention in these studies. In fact, it was reported that diesel exhaust particles and carbon black can induce dust mite allergy in rats.[5] Exposure to diesel exhaust also enhances ozone-induced airway inflammation in healthy humans[6] and in people with asthma.[7] In the bronchial epithelium, diesel exhaust increases interleukin (IL) 8, growth-regulated oncogene (GRO) α, and IL-13 and activates several transcription factors (eg, nuclear factor–κB and activator protein 1) and mitogen-activated protein kinases (eg, p38 and c-Jun N-terminal kinase).[8,9] Holgate et al[10,11] exposed healthy and asthmatic individuals to diesel exhaust for 2 hours and determined messenger RNA expression in bronchial wash fluid. Stenfors et al[12] determined cytokine production in lavage fluid after exposing asthmatic and healthy individuals to diesel exhaust for 6 hours. These findings suggest that oxidant pollutants, including diesel exhaust particles, cause allergic inflammation by activating allergic inflammatory response elements.

In this study, we hypothesized that the ocular surface is exposed to diesel exhaust particles, which enhance the expression of

Reprints: Hiroshi Fujishima, MD, Department of Ophthalmology, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, Japan; E-mail: hfujishima6@gmail.com.
Disclosures: Authors have nothing to disclose.
Funding Sources: This study was supported by a research grant from Santen Pharmaceutical Co, Ltd, Osaka, Japan.

1081-1206/12/$36.00 - see front matter
http://dx.doi.org/10.1016/j.anai.2012.10.017

cytokines and growth factors, leading to allergic conjunctival inflammation. Using a GeneChip array, we first investigated the effects of exposure to diesel exhaust in vitro.

Methods

Culture of human conjunctival epithelial cells

This study was approved by the Ethics Committee of Tokyo Dental College (Chiba, Japan). All experiments were conducted in accordance with principles of the Declaration of Helsinki. Written informed consent was obtained from all volunteers before participation. Human conjunctival samples were collected from 7 healthy volunteers using scissors under 2% Xylocaine anesthesia. To establish primary conjunctival epithelial cells, samples were cultured in supplemented hormonal epithelial medium supplemented with 10% heat-incubated fetal bovine serum. Specimens were cultured in complete medium at 37°C in a humidified atmosphere supplemented with 5% carbon dioxide in air for a few weeks. Once the epithelial cells had grown, they were transferred to a flask and incubated. Subconfluent epithelial cells were subcultured in 96-well plates at 2×10^5 cells/mL. One day later, the cells were incubated with or without diesel exhaust particles (100 μg/mL) or tumor necrosis factor (TNF; 30 ng/mL). At the indicated times, culture supernatants were collected and the cytokine levels were determined. The conjunctival cells were also collected and gene transcription was measured. All experiments were performed in triplicate for each cell culture.

Genechip expression analysis

Human gene expression was examined using the U95A probe array (GeneChip; Affymetrix, Santa Clara, California), which contains an oligonucleotide probe set for approximately 10,000 full-length genes. Gene expression was determined according to the manufacturer's instructions and previous reports.[13] Briefly, total RNA (3-10 μg) was extracted from approximately 10^7 cells after incubation for 6 hours. Double-stranded complementary DNA was synthesized and subjected to in vitro transcription in the presence of biotinylated nucleoside triphosphates. The biotinylated complementary RNA was hybridized with a probe array at 45°C for 16 hours, stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, Oregon), and scanned using a Hewlett-Packard Gene Array Scanner. The fluorescence intensity of each probe was quantified using GeneChip Analysis Suite 5.0 software (Affymetrix). The expression level of individual messenger RNAs (mRNAs) was determined as the mean fluorescence intensity among the intensities for 6 pairs of probes (perfect-matched and single nucleotide-mismatched probes) consisting of 25-mer oligonucleotides. If the intensities of mismatched probes were very high, gene expression was judged to be absent even if a high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 software. The level of gene expression was determined as the average difference using the GeneChip software. The percentages of the specific average difference level vs the mean average difference level of 16 probe sets for housekeeping genes (β-actin and glyceraldehyde-3-phosphate dehydrogenase) were then calculated.

Real-time RT-PCR

Total RNA was obtained from isolated epithelial cells (>95% purity) using a total RNA isolation kit (RNeasy Mini Kit; Qiagen, Valencia, California) according to the manufacturer's protocol and was treated with DNase (Gibco-BRL) before undergoing reverse transcription. One microgram of each RNA sample was reverse transcribed using random hexamers and SuperScript III (Invitrogen, Carlsbad, California). The cDNAs were amplified and quantified on a LightCycler (Roche, Mannheim, Germany) using a QuantiTect SYBR Green PCR kit. The primers used for real-time PCR are listed in Table 1. To ensure equal loading and amplification, all products were normalized for glyceraldehyde 3-phosphate dehydrogenase transcription as an internal control.

Table 1
Primer Sequences

	Forward	Reverse
RANTES	ATCCTCATTGCTACTGCCCTCT	CAATGTAGGCAAAGCAGCAGG
ICAM-1	CTGTGTCCCCCTCAAAAGTCA	ATACACCTTCCGGTTGTTCCC
IL-8	GTCTGCTAGCCAGGATCCACAA	GAGAAACCAAGGCACAGTGGAA
IL-6	CAATAACCACCCCTGACCCA	GCGCAGAATGAGATGAGTTGTC
GRO-α	ATTCACCCCAAGAACATCCA	CACCAGTGAGCTTCCTCCTC
GRO-β	GCAGGGAATTCACCTCAAGC	AGCTTCCTCCTTCCTTCTGG
LARC	TGTCAGTGCTGCTACTCCACCT	CTGTGTATCCAAGACAGCAGTCAA

Abbreviations: GRO, growth-regulated oncogene; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; LARC, liver and activation-regulated chemokine; RANTES, regulated on activation, normal T-expressed, and presumably secreted.

ELISA

Human intercellular adhesion molecule (ICAM) 1, IL-6, IL-8, liver and activation-regulated chemokine (LARC), RANTES (regulated on activation, normal T-expressed, and presumably secreted), GRO-α, GRO-β, and GRO-γ concentrations were measured using ELISA kits (R&D Systems, Minneapolis, Minneapolis). The detection limits of these assay kits were approximately 3 pg/mL.

Statistical analysis

Paired t tests were conducted on log-transformed data to determine differences in supernatant cytokine content and cytokine expression between cells with or without exposure to diesel exhaust or TNF-α. Values are presented as mean $\pm$ SD, and $P < .05$ was considered statistically significant.

Results

Elevated transcripts in deactivated conjunctival cells

The optimal incubation time to measure the apparent increase of the mRNAs in the model was revealed as 6 hours after exposure among 1, 3, and 6 hours. In addition, 24 hours after exposure among 8, 24, and 48 hours was the optimal time to determine protein production.

We used a GeneChip array to examine the expression levels of approximately 12,000 genes in conjunctival cells exposed to diesel exhaust particles. The fold-increase in expression was determined by calculating the mean difference in expression in in activated cells relative to resting cells. The mean fold-increase was then determined for each sample. After eliminating the redundant transcripts, we selected the transcripts showing the greatest upregulation in the cells. An adhesion molecule (ICAM-1), chemokines (LARC, RANTES, GRO-α, GRO-β, and GRO-γ), cytokines (IL-6, IL-8, IL-1β), and growth factors (endothelial cell growth factor and epidermal growth factor) were upregulated by more than 3-fold compared with control cells and were selected for further analyses (Table 2).

Confirmation of mRNA expression in conjunctival epithelial cells by real-time RT-PCR

As shown in Figure 1, the gene expression of ICAM-1 (from 16,911.7 $\pm$ 4,914.3 to 28,930.9 $\pm$ 14,204.3 copies/ng of RNA; $P = .04$), IL-6 (from 1.73 $\pm$ 0.94 to 13.40 $\pm$ 11.02 copies/ng of RNA; $P = .02$), IL-8 (from 11,411.0 $\pm$ 7,907.8 to 73,509.9 $\pm$ 5,1729.9 copies/ng of RNA; $P = .003$), and LARC (from 9,780.5 $\pm$ 5,084.9 to 22,804.4 $\pm$ 11,346.3 copies/ng of RNA; $P = .03$) increased significantly after 6 hours of incubation with diesel exhaust particles compared with