

Cigarette smoke extract enhances rhinovirus-induced toll-like receptor 3 expression and interleukin-8 secretion in A549 cells

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ABSTRACT

Background: Using A549 alveolar epithelial type II-like cells, we investigated the effects of cigarette smoke on the expression of rhinovirus (RV)-induced Toll-like receptor (TLR) 3, interleukin (IL)-6, and IL-8 secretion and viral replication.

Methods: Cigarette smoke extract (CSE) was prepared by smoking two commercial cigarettes with filters (0.9-mg of nicotine and 8.5-mg of tar per cigarette). Cells were infected with RV serotype 16 (RV-16) and cultured for 2 days (RV group), pretreated with 1, 2.5, or 5% CSE for 1 day before RV infection, and culture with CSE for 2 days after infection (RV plus CSE group), or were cultured in medium for 3 days (control group), treated with 1, 2.5, or 5% CSE for 3 days (CSE group). Changes in TLR3, IL-6, and IL-8 mRNA expression were assayed by real-time polymerase chain reaction and TLR3 protein expression were assessed by flow cytometry; IL-6 and IL-8 secretions were measured by ELISA; and RV replication was assessed by viral culture.

Results: CSE increased RV-induced TLR3 expression and RV-induced IL-8 secretion at lower concentrations in A549 cells. On the contrary, CSE dose dependently inhibited RV-induced IL-6 secretions and had no effect on RV replications.

Conclusion: Our results suggest that cigarette smoke may potentiate viral common cold symptoms by enhancing IL-8 secretion at lower concentrations, but not by increasing viral replication.

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Key words: A549 cells, cigarette smoke, common cold, IL-6, IL-8, rhinovirus, TLR3

Rhinovirus (RV) is the most frequent cause of self-limiting upper respiratory infections in children and adults, being responsible for 50–60% of common colds.^{1,2} In addition, RV has been reported to be the major risk factor associated with exacerbations of asthma and chronic obstructive pulmonary disease (COPD), suggesting that RV infection may be an important cause of lower airway disease.^{3–6}

RV infection triggers epithelial cell production of cytokines, including interleukin (IL)-6 and IL-8, which cause cold symptoms by inducing pyrexia and are important in the recruitment and activation of neutrophils, resulting in the release of the contents of their cytotoxic granules.^{7,8} RV infection also increases the expression in airway epithelial cells of Toll-like receptor (TLR) 3 mRNA and protein.⁹ TLR3, which binds viral double-stranded RNA (dsRNA), is important in the antiviral innate immune response.¹⁰

Cigarette smoke is the most important risk factor in the development of COPD.¹¹ Exposure to cigarette smoke significantly increases the expression of inflammatory cytokines, including IL-1 β and IL-8,^{12–14} which recruit increased numbers of neutrophils and macrophages in the airways; these cells cause respiratory inflammation by releasing proteases and free oxygen radicals, leading to injury of the parenchymal tissues and bronchioles.¹⁵ Several epidemiological studies have shown a relationship between cigarette smoke and longer duration of respiratory infections.^{16–18} Smokers also have a tendency to have longer-lasting and more serious cold symptoms than non-smokers,¹⁹ suggesting additive and/or synergistic interactions between cigarette smoke and viral infections. However, little is known about these interactions in the pathogenesis of respiratory infections.

We hypothesized that cigarette smoke may participate in the expression of TLR3 and cytokines during viral colds. We therefore investigated the effects of cigarette smoke on the expression of RV-

induced TLR3, IL-6 and IL-8 secretion, and viral replication in A549 alveolar epithelial type II-like cells.

MATERIALS AND METHODS

Cell Culture

A549 alveolar epithelial type II-like cells and the Medical Research Council (MRC) 5 human lung fibroblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA). A549 cells were grown in F-12K Nutrient Mixture (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM of L-glutamine, and 1.5 g/L of sodium bicarbonate. MRC-5 cells were grown in minimum essential medium (MEM; Gibco) supplemented with 10% FBS, 2 mM of L-glutamine, 20 mM of HEPES, 100 U/mL of penicillin, 100 μ L/mL of streptomycin, and 0.25 μ L/mL of fungizone.

Preparation of Cigarette Smoke Extract (CSE) Solutions

CSE was prepared by smoking two commercial cigarettes with filters (88 light; 0.9 mg of nicotine and 8.5 mg of tar per cigarette; Korea Tobacco & Ginseng, Seoul, Korea) with an automatic pump (Masterflex L/S Easy-Load II, Cole-Palmer Instrument Co., Vernon Hills, IL). The mainstream smoke was bubbled through 10 mL of 1% F-12K medium containing 25 mM of HEPES buffer, prewarmed to 37°C, at a constant flow of 260 mL/minute for 3.5 minutes. The CSE solutions were filtered through 0.2- μ m sterile filters (Acrodisc; Gelman Sciences, Ann Arbor, MI) to remove bacteria and large particles. Each CSE solution was standardized by measuring their absorbance at a wavelength of 340 nm before dilution and was used for dilution when optical density was in the range of 3.95 ± 0.05 . This solution was considered to be 100% CSE and was diluted with 5% F-12K medium to 1, 2.5, and 5% ($[\text{CSE solution (mL)}]/\text{total (mL)}] \times 100$) as a percentage of total volume. The resulting CSE was applied to A549 cell cultures within 30 minutes of preparation.

Cell viability after CSE treatment was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cytotoxicity of CSE as measured by absorbance of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

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(MTS) formazan products. A549 cells were treated with various concentrations of CSE (1, 2.5, 5, 7.5, 10, 12.5, 15, and 100%) for 3 days; subsequently, absorbance of MTS formazan products was measured at 490 nm. The absorbance is significantly decreased at 12.5% CSE concentration over cells treated with control media, suggesting the cytotoxicity of CSE to A549 cells at this concentration.

RV Serotype 16 (RV-16) Infection and Treatment with CSE

RV-16 was purchased from the ATCC and diluted with phosphate-buffered saline (PBS). A549 cells were plated at 2×10^5 cells/well in 12-well plates in 5% F-12K medium. Cells were cultivated at 37°C in a humidified 5% CO₂ incubator for 1 day. In the RV group, culture medium was aspirated on day 1, and the cells were infected with HRV-16 at a multiplicity of infection of 1 for 4 hours.²⁰ Thereafter, the viral solution was removed and the cells were rinsed with PBS and cultured at 33°C in a humidified 5% CO₂ incubator for 2 days.²¹ In the RV plus CSE group, the cells were pretreated with 1, 2.5, or 5% CSE for 1 day, infected with RV-16 as mentioned previously, and cultured in medium containing 1, 2.5, or 5% CSE at 33°C for 2 days. Control cells were cultured in medium alone for 3 days. In the CSE group, the cells were treated with 1, 2.5, or 5% CSE for 3 days. After cell culture, the supernatants were removed and stored at -70°C, and the cells were rinsed with PBS and harvested using 0.25% trypsin-EDTA (100 μ L/well).

Real-Time Polymerase Chain Reaction (PCR) for TLR3, IL-6, and IL-8

Each sample was lysed and total RNA was extracted with Trizol agent (Life Technologies, Rockville, MD) according to the manufacturer's protocol. Total RNA was quantified by spectrophotometry (Pharmacia, Uppsala, Sweden). DNase digestion was performed using DNA-free (Ambion, Inc., Austin, TX) as per the manufacturer's protocol. Two micrograms of total RNA was converted to cDNA using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). For each reaction, 4 μ L of 5 $\times$ first-strand buffer (50 mM of Tris-HCl, pH 8.3, 375 mM of KCl, and 15 mM of MgCl₂), 2 μ L of 0.1 M of dTT, 5 U of RNasin, 500 μ M of dNTP mix, 200 pmol of Oligo-dT, 25 U of Superscript II reverse transcriptase, and sterile water were added to the RNA to a volume of 20 μ L. This reaction was then incubated at 42°C for 1 hour. The finished cDNA products were stored in aliquots at -80°C until needed. Real-time quantitative PCR was performed in triplicate in a 96-well plate; each 20- μ L reaction consisted of 10 μ L of 2 $\times$ SYBR I Mix (Applied Biosystems, Foster City, CA), 2.5 μ L of 900-nM forward and reverse primers, and 1 μ L of cDNA.

Oligonucleotide PCR primer pairs were designed as follows:

TLR3, 5'-TTGCCCTGTATCTACTTTTGGGG-3' (forward) and 5'-GCGGCTGGTAATCTTCTGAGTT-3' (reverse); IL-6, 5'-TCTCCA-CAAGCGCCTTCG-3' (forward) and 5'-CTCAGGGCTGAGAT-GCCG-3' (reverse); IL-8, 5'-TTTGGCCAAGGAGTGCTAAAGA-3' (forward) and 5'-AACCCTCTGCACCCAGTTTC-3' (reverse).

After reverse transcription, the PCR reaction was performed in an ABI7900HT real-time sequence detection system programmed for 40 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C, and extension for 30 seconds at 72°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The mRNA levels of TLR3, IL-6, and IL-8 were calculated by using the comparative parameter threshold cycle and normalized to GAPDH.

Flow Cytometry for TLR3

A549 cells harvested as described previously were incubated with antibody to TLR3 (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at 4°C. After extensive washing in PBS, each cell preparation

was fixed in 200 μ L of 1% paraformaldehyde/PBS, and fluorescein was analyzed by single color flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ). The mean fluorescence intensity (MFI) was normalized relative to noninfected control values, after subtraction of background staining.

IL-6 and IL-8 Quantification

Immunoreactive IL-6 and IL-8 were quantitated using dual antibody ELISA kits (Biosource, Nivelles, Belgium) according to the manufacturer's protocol. The sensitivity limit of each kit was 10 pg·mL⁻¹. Data were expressed in picograms per milliliter and were derived by extrapolation from standard curves generated in parallel with each experiment.

Measurement of Viral Titer

MRC-5 cells were plated at 3×10^4 cells/well in 96-well microplates (Falcon Labware, Oxnard, CA) in MEM (Gibco). On the day of the experiment, 125 μ L of 5% MEM (Gibco) was removed from each well, 25 μ L of serial 10-fold dilutions of cell culture supernatants were added, and the cells were cultured at 33°C for 7 days. Viral cytopathic effects were examined using an inverted microscope. The amount of specimen required to infect 50% of the MRC-5 cells (50% tissue culture infection dose [TCID₅₀]) was determined, and the viral titer was expressed as TCID₅₀/mL.

Statistical Analysis

Differences between groups were analyzed using the Mann-Whitney *U* test. All data are expressed as mean values $\pm$ SD. A value of *p* < 0.05 was considered statistically significant.

RESULTS

Effects of CSE on TLR3, IL-6, and IL-8 mRNA Expression

Compared with the expression of TLR3 mRNA in control cells, there was an average 2.21-fold increase in TLR3 mRNA expression in the RV group (Fig. 1). CSE alone increased TLR3 mRNA expression with 3.72-fold at 5% CSE (*p* = 0.037) and CSE treatment also additively increased RV-induced TLR3 mRNA expression with 3.69-fold increase at 2.5% CSE compared with control cells (*p* = 0.05). However,

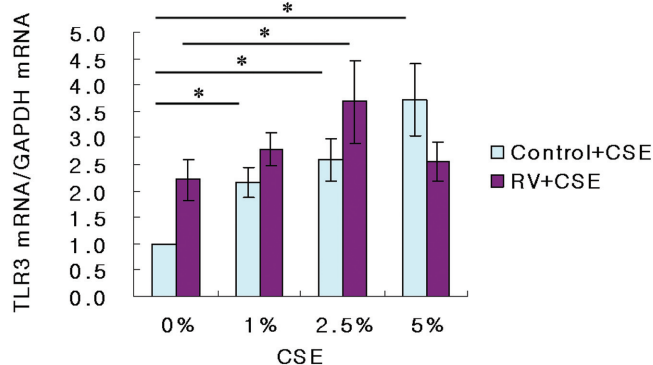


Figure 1. Effect of cigarette smoke extract (CSE) on Toll-like receptor (TLR) 3 mRNA expression in rhinovirus (RV)-16-infected A549 cells. Cells were treated with 1, 2.5, or 5% CSE for 3 days, or cells were pretreated with 1, 2.5, or 5% CSE for 1 day, rinsed with phosphate-buffered saline, infected with RV-16 for 4 hours (multiplicity of infection of 1), and incubated with 1, 2.5, or 5% CSE for 2 days. Results represent the mean $\pm$ SD of three independent experiments. Significant differences are indicated by an asterisk.

the expression of TLR3 mRNA decreased at 5% in comparison with 2.5% CSE concentration.

Compared with the expression of IL-8 mRNA in control cells, there was an average 5.41-fold increase in IL-8 mRNA expression in the RV group (Fig. 2). CSE alone slightly increased IL-8 mRNA expression at 1% CSE but its effect decreased at 5% CSE ($p > 0.05$). CSE (1%) increased the RV-induced IL-8 mRNA expression compared with the value in the presence of RV alone, with 1.12-fold greater ($p = 0.05$). CSE showed its maximal effect at 1% concentration. However, 5% CSE decreased RV-induced IL-8 mRNA expression, resulting in a 2.45-fold increase compared with control cells ($p = 0.05$).

RV infection significantly increased the expressions of IL-6 mRNA, 3.62-fold compared with control cells (Fig. 3). IL-6 mRNA expression was slightly increased by 1% CSE but its expression was decreased by 2.5 and 5% CSE ($p > 0.05$). However, CSE (1, 2.5, and 5%) progressively decreased RV-induced IL-6 mRNA expression in a dose-dependent manner ($p = 0.037$).

Effects of CSE on TLR3 Surface Protein Expression

RV infection significantly increased the MFI indicative of TLR3 from 3.63 to 6.69. CSE alone increased TLR3 protein expression from 3.63 to 6.19 at 5% CSE ($p = 0.016$) and CSE treatment also additively increased RV-induced TLR3 protein expression from 6.67 to 8.21 at 5% CSE ($p = 0.046$; Fig. 4). Incubation of A549 cells with RV plus CSE (2.5 and 5%) produced an increase in TLR3 surface protein compared with the value in the presence of RV alone, with 1.39 and 1.54 greater MFI, respectively.

Effect of CSE on IL-6 and IL-8 Secretion

RV infection significantly increased the mean concentration of IL-8 from 1203 pg/mL in control cells to 4029 pg/mL in the RV group (Fig. 5). CSE alone significantly increased IL-8 secretion at 1% CSE but its effect decreased at 5% CSE ($p = 0.008$). CSE (1 and 2.5%) significantly increased the RV-induced IL-8 secretion compared with the value in the presence of RV alone, with 1471 and 1077 pg/mL greater ($p = 0.008$), respectively. CSE showed its maximal effect at 1% concentration. However, CSE significantly decreased RV-induced IL-8 secretion at 5% concentration ($p = 0.008$).

RV infection also significantly increased the mean concentration of IL-6 from 140.3 pg/mL in control cells to 435.7 pg/mL in the RV group (Fig. 6). CSE alone decreased IL-6 secretion at 5% CSE ($p > 0.05$), and CSE also progressively decreased the RV-induced IL-6 secretion at 2.5 and 5% concentrations.

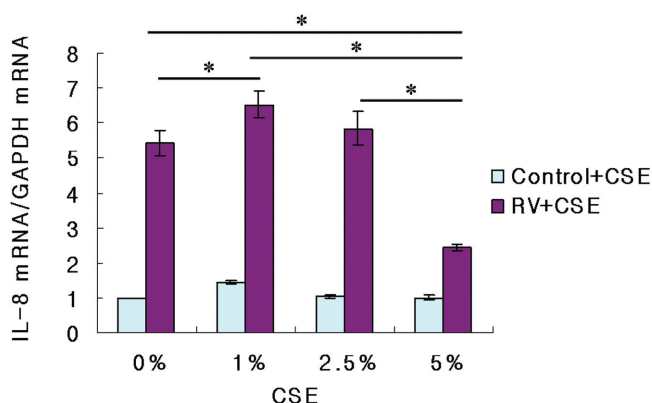


Figure 2. Effect of cigarette smoke extract (CSE) on interleukin (IL)-8 mRNA expression in rhinovirus (RV)-16-infected A549 cells. Cells were treated as described in the Fig. 1 legend. Results represent the mean $\pm$ SD of three independent experiments. Significant differences are indicated by an asterisk.

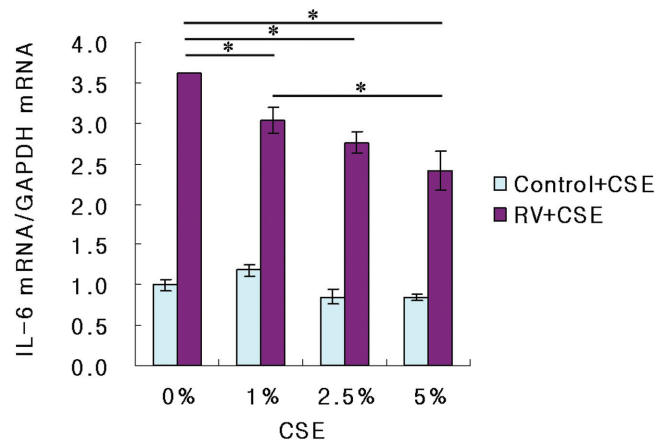


Figure 3. Effect of cigarette smoke extract (CSE) on interleukin (IL)-6 mRNA expression in rhinovirus (RV)-16-infected A549 cells. Cells were treated as described in the Fig. 1 legend. Results represent the mean $\pm$ SD of three independent experiments. Significant differences are indicated by an asterisk.

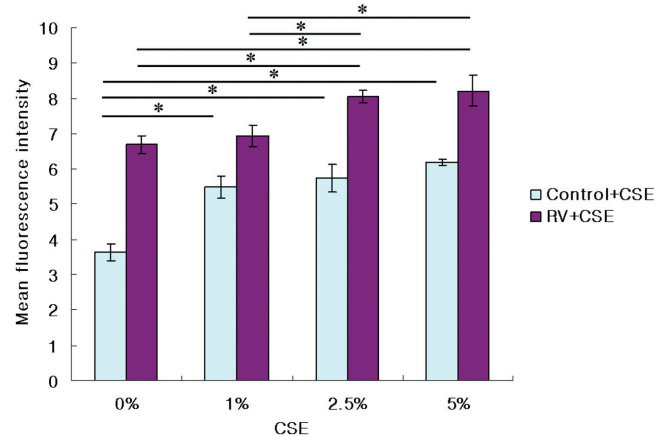


Figure 4. Effect of cigarette smoke extract (CSE) on Toll-like receptor (TLR) 3 surface protein expression in rhinovirus (RV)-16-infected A549 cells. Cells were treated as described in Fig. 1 legend, harvested, fixed, and incubated with antibody to TLR3 for flow cytometry. Each bar represents the mean $\pm$ SD of five experiments. Significant differences ($p < 0.05$) are indicated by an asterisk.

Effect of CSE on RV Replication

When the supernatants of RV-infected A549 cells were cultured with MRC-5 cells, the mean viral titer was $10^{2.08}$ TCID₅₀/mL (Fig. 7). In the RV plus CSE group, the viral titers were not significantly different from that of RV group ($p > 0.05$) irrespective of CSE concentrations, indicating that CSE treatment did not significantly affect RV replication.

DISCUSSION

In this study, we aimed to mirror the effect of continuous CSE exposure on RV infection in airway epithelial cells. Thus, we used continuous CSE exposures for 24 hours before and 48 hours after RV infection. This is the first *in vitro* study that attempts to explain the effects of cigarette smoke on RV infection.

TLRs are a family of receptors that recognize specific patterns of microbial components and induce antimicrobial innate immune responses.^{22,23} TLR3 has been shown to recognize dsRNA, a molecular

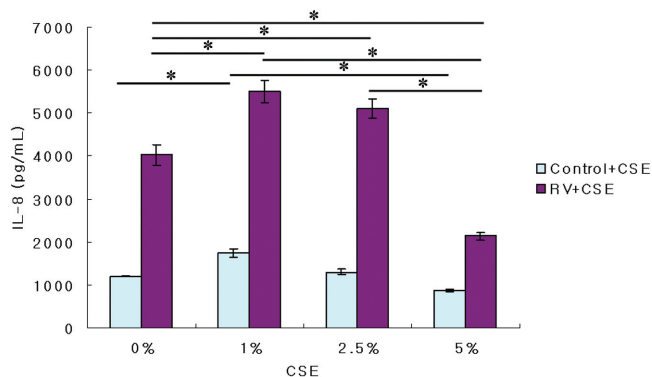


Figure 5. Effect of cigarette smoke extract (CSE) on interleukin (IL)-8 secretion in rhinovirus (RV)-16-infected A549 cells. Cells were treated as described in Fig. 1 legend, and their supernatants were harvested and assayed for IL-8 by ELISA. Each bar represents the mean $\pm$ SD of five experiments. Significant differences ($p < 0.05$) are indicated by an asterisk.

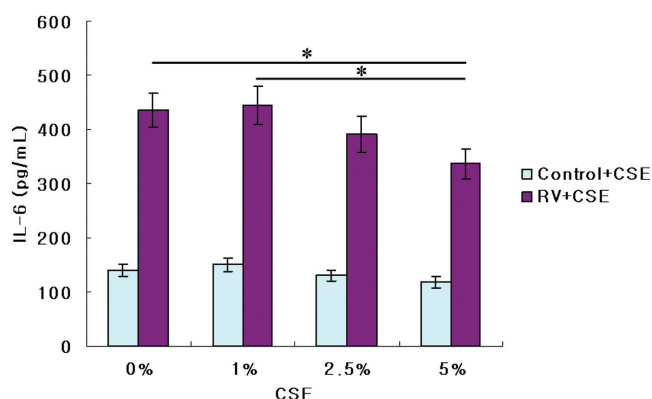


Figure 6. Effect of cigarette smoke extract (CSE) on interleukin (IL)-6 secretion in rhinovirus (RV)-16-infected A549 cells. Cells were treated as described in Fig. 1 legend, and their supernatants were harvested and assayed for IL-6 by ELISA. Each bar represents the mean $\pm$ SD of five experiments. Significant differences ($p < 0.05$) are indicated by an asterisk.

pattern associated with viral infection, and TLR3 binding of dsRNA has been shown to lead to the activation of both interferon-regulatory factor 3 and NF- κ B, contributing to the production of interferon β .^{10,22} RV infection has been reported to increase expression of TLR3 mRNA and surface protein and TLR3 has been found to mediate antiviral response *via* inhibition of RV replication.⁹ Using A549 cells, we have shown here that CSE up-regulated RV-induced TLR3 mRNA and surface protein. Although actively replicating RV has been shown to induce TLR3 expression, blocking of TLR3 using an anti-TLR3 antibody showed that this up-regulation of TLR3 inhibits RV replication.⁹ In this study, the mean viral titers according to CSE concentrations were not significantly changed and were not inversely proportional to the amount of CSE-induced TLR3 expression. This finding suggests that CSE may not have a significant effect on RV replication in A549 cells.

IL-8 plays an important role in the aggravation of RV-induced cold symptoms by recruiting neutrophils and releasing the contents of their cytotoxic granules.^{7,8} Cigarette smoke exposure leads to a significant increase in the secretion of IL-8,^{12–14} and the concentration of IL-8 correlates with neutrophil counts in bronchoalveolar lavage fluid of patients with COPD.¹⁵ On the contrary, some previous studies have reported the inhibitory effect of CSE and its component acrolein on the expression of IL-8 mRNA and IL-8 secretion in airway epithelial

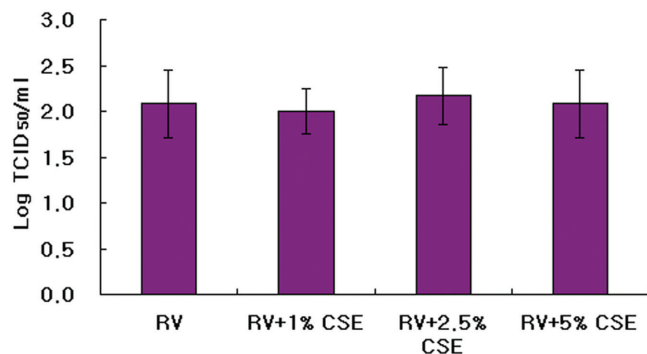


Figure 7. Effect of cigarette smoke extract (CSE) on rhinovirus (RV)-16 replication in A549 cells. Cells were pretreated with 1, 2.5, or 5% CSE for 1 day, rinsed with phosphate-buffered saline, infected with RV-16 for 4 hours (multiplicity of infection of 1), and incubated with 1, 2.5, or 5% CSE for 2 days. The infectious RV-16 in the supernatants was assayed by viral titration. Each bar represents the mean $\pm$ SD of five experiments. TCID₅₀, 50% tissue culture infection dose.

cells.^{11,24,25} Cigarette smoke also reduces cell viability by inducing the formation of blebs and free vesicles.²⁶ In this study, we found that CSE significantly increased IL-8 mRNA expression and IL-8 secretion in HRV-infected A549 cells at 1% concentration but progressively decreased them at 2.5 and 5% concentrations. A previous study shows that increasing dose of cigarette smoke leads to a “bell-shaped” response curve for IL-8 secretion in smooth muscle cells and the reason for this may be because of complex chemical components of cigarette smoke of >4000 kinds.²⁷ For instance, at higher CSE concentrations, some components reach to concentrations that have inhibitory effects on IL-8 secretion exceeding the stimulatory effects of other components. This finding suggests that continuous exposure to cigarette smoke during RV infection may result in an increase of neutrophil recruitment at a lower cigarette dose and a suppression of neutrophil influx at a higher dose.

In this study, we found that continuous CSE exposure inhibited the RV-induced expression of IL-6 mRNA and IL-6 secretion in a dose-dependent manner. This result is consistent with earlier reports showing that CSE can inhibit IL-6 production in human lung macrophage and mouse alveolar macrophage.^{28,29} However, this anti-inflammatory effect of CSE appears to be contrary to the increased IL-6 release in human bronchial epithelial cells.¹³ The discrepancy between these findings and the results presented here may have been caused by the different CSE concentrations, exposure time, and cell lines used in these studies.

Recently, there have been some reports about the relationship between TLR3 and cytokine secretions. IL-8 secretion is closely related to TLR3 expression, in that the increase in TLR3 potentiates the RV-induced secretion of IL-8.³⁰ In contrast, enhanced RV replication led to increased IL-6 secretion through a non-TLR3-mediated pathway, indicating that IL-6 secretion is related to viral replication but not to TLR3 expression.⁹ However, further studies are required to gain greater insight into the mechanism by which CSE-induced TLR3 influences on RV-induced IL-8 and IL-6 secretions.

In conclusion, we have shown here that CSE increased RV-induced TLR3 expression and RV-induced IL-8 secretion at lower concentrations in A549 cells. On the contrary, CSE dose dependently inhibited RV-induced IL-6 secretions and had no effect on RV replications. Our results suggest that cigarette smoke may potentiate viral cold symptoms by enhancing IL-8 secretion at a lower cigarette dose, but not by increasing viral replication.

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