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ORIGINAL ARTICLE

Cigarette Smoke Differently Alters Normal and Ovalbumin-Sensitized Bronchial Epithelial Cells from Rat

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Background. Smoking is a common habit in the general population, even in asthmatic patients. Bronchial epithelial cells are the first cellular elements exposed to environmental stimuli such as cigarette smoke. These cells produce a wide range of mediators involved in inflammation and remodeling processes. However, the effects of chronic smoke exposure on the release and production of these mediators remain unclear. **Objectives.** To investigate the effects of repeated exposure to cigarette smoke extract on mediator released by bronchial epithelial cells isolated from control and asthmatic rats. **Methods.** Bronchial epithelial cells were isolated from normal (NRBE) and asthmatic rats (ARBE) (ovalbumin (OVA)-sensitized rat). Cells were exposed to cigarette smoke extract (CSE) obtained by impacting cigarette smoke with an AGI-30. A concentration of 3% CSE was added in the medium daily, for 5 consecutive days. Supernatant was recovered at baseline and after the 5 days. Levels of macrophage chemoattractant protein (MCP)-1, interleukin (IL)-10, vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF), IL-1 α , and interferon (IFN)- γ were measured using enzyme-linked immunosorbent assay (ELISA). **Results.** TNF, IL-1 α , and IFN- γ were lower than the detection limit of our methods. At the baseline, NRBE produced less MCP-1 but more IL-10 and VEGF when compared with ARBE. CSE exposure reduced NRBE IL-10 production but did not significantly alter MCP-1 and VEGF production. Interestingly, bronchial epithelial cells of asthmatic rats responded differently to CSE. MCP-1 level was decreased and VEGF increased after CSE exposure, whereas IL-10 level did not change in ARBE. **Conclusion.** Cells isolated from asthmatic rats produced distinct levels of mediators compared with cells isolated from control rats. Furthermore, these cells react differently to CSE exposure.

Keywords bronchial epithelial cells, cigarette smoke extract, VEGF, IL-10, MCP-1

INTRODUCTION

In the bronchial tract, bronchial epithelial cells are the first cells exposed to inhaled irritants such as cigarette smoke. Airway epithelium plays an important role in the preservation of homeostasis and the protection of mucosa (1, 2). Epithelial cells are involved in the repair process after mucosal damage and secrete a variety of mediators influencing inflammation and cell recruitment (3). Bronchial epithelial cells have various functions such as fluid regulation, ion transport, mucus secretion, and mucociliary clearance (1, 3). Furthermore, bronchial epithelial cells are an important source of anti- and pro-inflammatory mediators, suggesting a potential role in the immune response (4–9).

Interleukin (IL)-10, macrophage chemoattractant protein (MCP)-1, and vascular endothelial growth factor (VEGF) are some of the mediators produced by bronchial epithelial cells and are involved in different processes after injury. IL-10 is a regulatory cytokine in the production of TGF- β by bronchial epithelial cells (10) and an inhibitory cytokine suppressing the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-1 β , IL-6, IL-8, and macrophage inflammatory protein (MIP)-1 α and increasing the release of IL-1Ra and MCP-1 by macrophages, monocytes, and fibroblasts (8). MCP-1 is a chemokine sponta-

neously produced by bronchial epithelial cells (9), and its production is increased following cellular damage (11). This cytokine induces the recruitment of macrophages in the airway lumen (12). VEGF is involved in angiogenesis, remodeling, and vascular endothelial cell proliferation (13, 14). It is also an inflammatory mediator inducing monocyte and neutrophil recruitment (15). A good balance in the production of these mediators is important to maintain lung homeostasis.

The effects of a single exposure of cigarette smoke extract on bronchial epithelial cells have been investigated using an *in vitro* model. Epithelial cells exposed to cigarette smoke produced an increased concentration of IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), MCP-1, G-CSF, and leukotriene (LT)-B₄ (16, 17). In these studies, alveolar and bronchial epithelial transformed cell lines were used. The function and the response of transformed cell lines may differ from cell isolated from primary culture. The effects of chronic cigarette smoke exposure on primary culture of bronchial epithelial cells have never been studied.

To our knowledge, the effects of CSE on cells isolated from normal and ovalbumin (OVA)-sensitized rats, a well known asthma model (18), have not been investigated. This study characterizes the responses of bronchial epithelial cells isolated from normal (NRBE) and OVA-sensitized rat (ARBE) after a 5 days repeated exposure to cigarette smoke on the production of MCP-1 (macrophage chemoattractant), IL-10 (inhibitory cytokine), VEGF (angiogenesis/remodeling factor), and IL-1 α , TNF, and IFN- γ (pro-inflammatory cytokines).

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MATERIALS AND METHODS

Cell Culture

Bronchial epithelial cells were isolated from Sprague Dawley rats (NRBE) (Harlan-Sprague Dawley, Indianapolis, IN, USA) and challenged-OVA-sensitized Brown Norway rats (ARBE) (Charles River, St. Constant, QC, Canada). The tracheobronchial tract was isolated from rat, the pulmonary section was removed, and cells were isolated after serial digestion (19). Cells were resuspended in complete Dulbecco's modified Eagle's medium (DMEM) with Ham's F12 (Invitrogen, Burlington, ON, Canada) in a 3:1 proportion and placed in 6-wells plate at 37°C for 24 hours. Medium was changed every 2 days until confluence. The Laval University Animal Care Committee approved the experimental protocol in accordance with the guidelines of the Canadian Council on Animal Care.

CSE Preparation

CSE was prepared from two 2R4F reference cigarettes (Kentucky Tobacco Research and Development Center, Lexington, KY, USA) using a modified syringe-driven apparatus method and an AGI-30 as previously described (20). A negative control was obtained by impacting ambient air in PBS for 14 min (air control). Optical density was measured for CSE and air control samples. Bronchial epithelial cells were exposed to 3% CSE solution and air control within 30 minutes of preparation. At the baseline and at day 5, supernatant was recovered and conserved for further analysis. Optical density was measured for both CSE and control air each day before the exposure to verify the reproducibility of the method by the absorbance of the extract.

Fresh medium (2 mL) was added to confluent cells for 24 hours and 1 mL of supernatant was recovered to determine mediator baseline level. Then, 1 mL of fresh medium containing 3% CSE or control air solution was added. Every day for 5 days, 1 mL of supernatant was recovered and 1 mL of fresh medium containing 3% CSE or air control solution was added for the next exposure.

Cell Viability and Cytotoxicity Assay

Cell viability and total cell count were assessed by trypan blue method after each day of exposure. LDH cytotoxicity assay (Biovision, Mountain View, CA, USA) was performed to assess cytotoxic effects of cigarette smoke on bronchial epithelial cells. Supernatants were recovered after cigarette smoke exposure. Measures of LDH activity were performed according to manufacturer protocol.

ELISA

MCP-1, IL-10, TNF, and IFN- γ release were measured by BD OptEIA assays (BD Biosciences, San Diego, CA, USA). The sensitivity of the assays was 15 pg/mL, 4 pg/mL, 10 ng/mL, and 10 ng/mL for MCP-1, IL-10, TNF, and IFN- γ , respectively. VEGF and IL-1 α release was measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions (R&D systems, Minneapolis, MN, USA). The sensitivity of the VEGF and IL-1 α kits was 8.4 pg/mL and 1.43 pg/mL, respectively. Data were obtained using a microplate reader VERSA max and with SoftMax Pro software v.5.0.1 (Molecular Devices).

Statistical Analysis

Paired *t* test was used to compare exposed and control groups. *t* test was used for the comparison of NRBE and ARBE. A difference was considered significant at *p* value < 0.05 (StatView, SAS Institute v.5.0.1, Cary, NC, USA).

RESULTS

Reproducibility of CSE Samples

Fresh CSE was performed each day of exposure. Optical density (OD) determining extract opacity of CSE was measured to evaluate the reproducibility of the method. Optical density of the CSE was similar in all samples (0.2702 ± 0.0162 , represented by mean $\pm$ SEM), showing this reproducibility.

Cell Viability

Cell viability was assessed daily for 5 days to evaluate the toxicity of CSE. No significant differences were observed between CSE exposed and control cells (*p* > 0.05) (Table 1), showing that 3% CSE was not toxic to bronchial epithelial cells. No significant differences in cell viability were observed between the baseline and day 5 (*p* > 0.05). Moreover, total cell count was performed and no significant differences were found between air control cells and cells exposed to CSE every day during 5 days (*p* > 0.05) (data not shown).

To further investigate CSE cytotoxicity, LDH measurement was obtained after 24 hours of exposure. No cytotoxic effects were observed after 3% CSE exposure. No significant differences were observed between air control and exposed cells (Table 2).

Mediator Measurements

To determine the effect of chronic CSE exposure, levels of TNF, IL-1 α , IFN- γ , MCP-1, IL-10, and VEGF were measured in cell-free supernatants at the baseline and at day 5. Production of TNF, IL-1 α , and IFN- γ by bronchial epithelial cells was lower than the limit of detection of the kit and was not detectable in cell-free supernatants of all samples.

Production of MCP-1 at baseline and after 5 days was significantly higher in ARBE than NRBE (*p* < 0.05) (Figure 1). Interestingly, ARBE spontaneously produced 222 times more MCP-1 than NRBE, suggesting that bronchial epithelial cells are altered in asthma. Furthermore, these cells differently responded to CSE exposure. A nonsignificant increase of MCP-1 release was observed in NRBE after CSE exposure compared with air control cells (*p* > 0.05), whereas a significant inhibition of MCP-1 release was observed in ARBE (*p* < 0.05).

TABLE 1.—Percentage of cell viability during 5 days.

Days	Air control	CSE sample
Baseline	95.5 $\pm$ 0.7	95.1 $\pm$ 0.5
1	90.6 $\pm$ 1.3	90.0 $\pm$ 1.4
2	86.6 $\pm$ 4.0	91.6 $\pm$ 1.6
3	84.8 $\pm$ 2.8	85.6 $\pm$ 2.9
4	85.9 $\pm$ 4.2	87.1 $\pm$ 3.2
5	87.6 $\pm$ 2.5	90.6 $\pm$ 1.8

n = 8.

Mean $\pm$ SEM.*p* > 0.05 for all data.

TABLE 2.—Measures of cytotoxicity assay.

Sample	OD _{mean}	% Cytotoxicity
Minimum control	0.625	—
Maximum control	2.370	—
Air control samples	0.523	−5.8
CSE samples	0.534	−5.2

n=5.

OD= Optical density.

% Cytotoxicity = test sample − minimum control / maximum control − minimum control.

In contrast to MCP-1, IL-10 production was significantly lower at baseline and after 5 days in ARBE supernatants compared with NRBE ($p < 0.05$) (Figure 2). At baseline, NRBE produced 5 times more IL-10 than ARBE. The difference in IL-10 production was increased after 5 days of culture with NRBE producing 20 times more IL-10 than ARBE ($p < 0.05$). CSE exposure significantly inhibited (60%) IL-10 production in NRBE ($p < 0.05$), but CSE did not modulate IL-10 in ARBE ($p > 0.05$).

At baseline, VEGF production was lower in ARBE compared with NRBE ($p < 0.005$) (Figure 3). However, in both groups, the concentration of VEGF was increased over time (baseline versus day 5), but this increase was not significant. CSE exposure did not alter VEGF production in NRBE but significantly increased VEGF release in ARBE ($p < 0.005$).

DISCUSSION

The effects of cigarette smoke on bronchial epithelial cells after a single exposure have already been published (21–23). Chronic exposure to cigarette smoke induces various patterns of inflammation in mice (24). This is the first study to expose bronchial epithelial cells from both normal and asthmatic rats to CSE for 5 consecutive days. We have herein demonstrated interesting differences in MCP-1, IL-10, and VEGF production by NRBE and ARBE exposed to CSE.

Bronchial epithelial cells can produce TNF, IL-1 α and IFN- γ after cigarette smoke or environmental oxidant

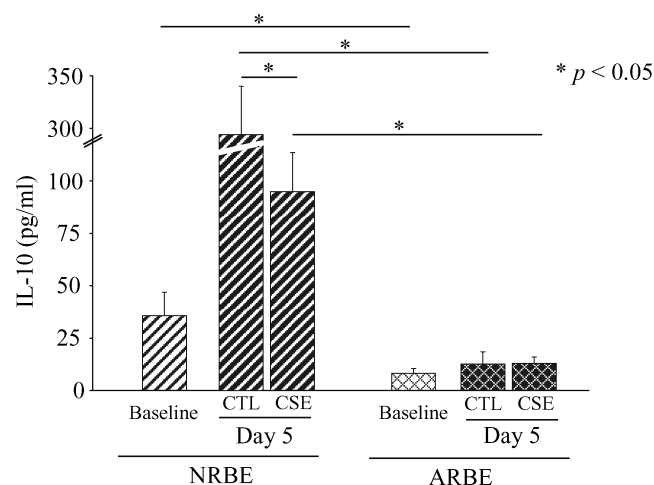


FIGURE 2.—Production of IL-10 by NRBE and ARBE after CSE exposure. Production of IL-10 in ARBE was lower compared with NRBE. CSE significantly inhibited IL-10 production in NRBE (hatched bar), but no difference was observed in ARBE (grid bar). Mean $\pm$ SEM. CTL = air control cells; CSE = cells exposed to cigarette smoke extract. NRBE $n = 10$, ARBE $n = 6$, $*p < 0.05$.

exposure (24–26). In these studies, cytokine production was evaluated using mRNA level. In our model, protein level of these cytokines were lower than the detection limit, suggesting that longer time may be required to detect these proteins in supernatants.

MCP-1 is a chemoattractant mediator for monocytes and macrophages (17, 27). Our data showed that a tremendous production of MCP-1 by ARBE compared with NRBE. IL-4 and IL-13, well known for their role in asthma, have been shown to stimulate MCP-1 production in human epithelial cell line (28). Elevated production of MCP-1 by ARBE may represent the inflammatory pattern observed in asthma. Indeed, higher expression of MCP-1 was observed in bronchial tissues of asthmatic subjects compared with non-asthmatic

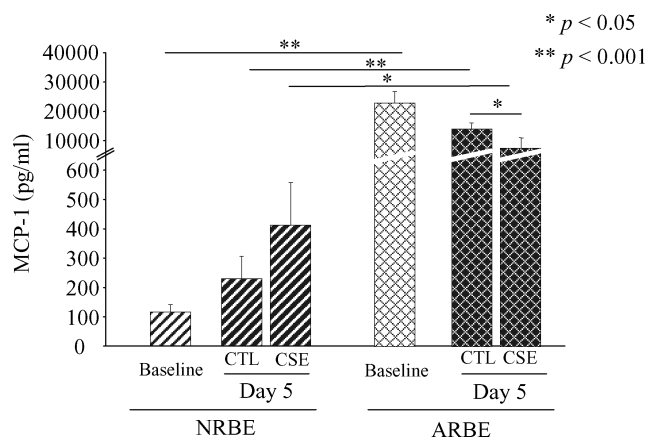


FIGURE 1.—Production of MCP-1 by NRBE and ARBE after CSE exposure. ARBE produced significant more MCP-1 than NRBE. CSE did not affect MCP-1 production in NRBE (hatched bar) but decreased its production in ARBE (grid bar). Mean $\pm$ SEM. CTL=air control cells; CSE=cells exposed to cigarette smoke extract. NRBE $n = 10$, ARBE $n = 6$, $*p < 0.05$, $**p < 0.001$.

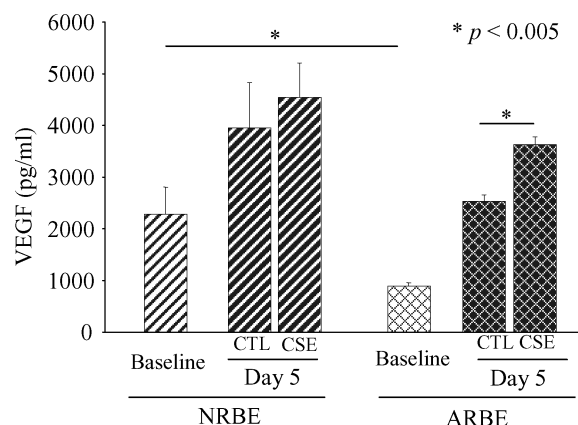


FIGURE 3.—Production of VEGF by NRBE and ARBE after CSE exposure. At the baseline, production of VEGF by ARBE was lower than NRBE. ARBE showed an increased concentration of VEGF after 5 days of CSE exposure (grid bar). Production of VEGF was not significantly different in NRBE after CSE exposure (hatched bar). Mean $\pm$ SEM. CTL = air control cells; CSE = cells exposed to cigarette smoke extract. NRBE $n = 10$, ARBE $n = 6$, $*p < 0.005$.

subjects (29). Interestingly, our results suggest that freshly isolated bronchial epithelial cells conserved their asthmatic phenotype *in vitro* by producing a higher level of MCP-1. In contrast to other studies, we observed a nonsignificant increase of MCP-1 release by NRBE exposed to CSE (17, 23). An increased expression of MCP-1 is usually associated with leukocyte infiltration (30), and since cigarette smoke is known to induce the attraction of alveolar macrophages in airways (7, 31), an increase was expected. This difference may be explained, at least in part, by the cellular type (cell lines) exposed to CSE and the duration of exposure used in other studies. It is possible that primary cells need prolonged exposure to tobacco smoke to induce changes observed *in vivo*. Interestingly, CSE exposure reduced MCP-1 production in ARBE showing the distinct responsiveness of asthmatic rats bronchial epithelial cells to cigarette smoke compared with control cells. In bronchial biopsies of asthmatic smokers, no increase in alveolar macrophage number was observed compared with non-smoking asthmatics (32), suggesting that MCP-1 is not increased in asthmatic smokers as observed in ARBE. Although neutralization of MCP-1 production in a mouse model reduced airway hyperresponsiveness and inflammation (33), MCP-1 level was still 35 times higher in ARBE than in NRBE exposed to CSE. Our data suggest that the reduction of MCP-1 level by CSE in ARBE may not be reflected by a reduced inflammation *in vivo*.

IL-10 is known to inhibit inflammatory responses (34). In our study, the production of IL-10 by NRBE was higher than ARBE, suggesting a defect in asthmatic subjects bronchial epithelial cells to produce IL-10. Indeed, IL-10 concentration in sputum and its expression in bronchial epithelium are reduced in asthmatic compared with non-asthmatic subjects (35, 36). As observed with MCP-1, bronchial epithelial cells isolated from asthmatic rats conserved their asthmatic phenotype with their low production of IL-10, which could not be further inhibited by CSE. In contrast, CSE exposure inhibited IL-10 production in NRBE, which may contribute to the inflammatory process observed in smokers. Levels of IL-10 in sputum from asthmatic, chronic obstructive pulmonary disease (COPD), and healthy smokers are lower compared with control subjects, suggesting that inhibition of IL-10 induces inflammatory patterns in lung diseases (35). Inhibition of IL-10 by cigarette smoke exposure may promote the inflammatory process by reducing the inhibition of pro-inflammatory cytokine release. Furthermore, IL-10 can regulate TGF- β production (10), which is involved in the growth and differentiation of bronchial epithelial cells (37). Reduced level IL-10 following cigarette smoke exposure may decrease TGF- β production resulting in the inhibition of bronchial epithelial cell growth as observed in the smoker airways.

VEGF is involved in angiogenesis factor and attraction of neutrophils and monocytes (38, 39). To our knowledge no study ever compared the production of VEGF by primary cultured bronchial epithelial cells with an *in vitro* smoking model. In our study, the baseline production of VEGF in ARBE was lower than NRBE. Similar data were observed after 5 days of culture, but the difference was not significant. Some studies showed an increased VEGF concentration in asthmatic sputum (40), whereas no differences were observed in BAL of asthmatic subjects compared with con-

trol subjects (41). Given that VEGF is an autocrine survival factor for airway epithelial cells (42), epithelial desquamation observed in asthma may be influenced by VEGF concentration. CSE exposure did not alter VEGF production in NRBE, but increased it in ARBE compared with air control cells. This distinct response may influence the inflammatory pattern induced by bronchial epithelial cells. Indeed, VEGF in the presence of TNF and IFN- γ can promote neutrophil infiltration by increasing IL-8 production (15, 43). Thus, the increased VEGF production caused by cigarette smoke exposure in asthmatic cells may promote neutrophil infiltration observed in the airways of asthmatic smokers. A longer CSE exposure may be necessary to influence VEGF production in normal airways. Indeed, Suzuki *et al.* showed that a short exposure of mice (10 days) to cigarette smoke did not alter VEGF production of bronchial epithelial cells but increased it after 4 weeks of exposure (44). Asthmatic cells may be more sensitive of cigarette smoke effects given the inflammatory state observed in asthma.

Bronchial epithelial cells isolated from an animal with allergic asthma showed a pattern of mediator production different to bronchial epithelial cells isolated from control rats. This animal model reproduces the inflammatory process observed in asthma (45). The difference in the production of mediators by NRBE and ARBE may reflect the inflammatory process observed in human studies. Moreover, bronchial epithelial cells isolated from this rat model conserved, *in vitro*, their inflammatory pattern observed *in vivo*. Finally, CSE modulates mediator production by bronchial epithelial cell after 5 days of exposure, suggesting that these cells are involved in the inflammatory pattern observed in smoking subjects. Further analysis should be performed to better understand the role of bronchial epithelial cells in the inflammatory process in asthma and smoking status.

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