The Effects of Cigarette Smoke on Airway Epithelium

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Abstract Interactions between macrophages and epithelial cells in the lung may promote a damaging inflammatory response to cigarette smoke toxins. A better understanding of the mechanisms involved in this response may prove useful in the development of treatments or preventative strategies for respiratory diseases associated with exposure to cigarette smoke. To examine this possibility, human epithelial cells (Calu-3), macrophages (RAW 264.7), and co-cultures of both cell types were incubated with cigarette smoke extract (CSE) for either 15 hours or 24 hours. Transepithelial Electrical Resistance (TER) was used to measure damage of the epithelium and the decrease in barrier function. TER was measured before the addition of CSE and macrophages, then again 24 hours afterwards. Results have shown that there is no significant difference in epithelial integrity and barrier function between epithelial cells that have been exposed to both CSE and macrophages compared to those that have been exposed to CSE alone. Results were expected to show that CSE would compromise the integrity of the airway epithelial barrier more significantly when macrophages were present than when CSE alone was present, because related studies had seen similar results. The only conclusion that can be made from results obtained in this study is that macrophages themselves do not have a significant impact on the damaging effects of cigarette smoke. However, the application of CSE and epithelial cell co-culture with macrophages may prove to be a useful model of *in vitro* cigarette smoke effects

Introduction

Tobacco smoke and other environmental toxins are known to be major risk factors for many respiratory diseases and associated concomitant infections (WHO 2002). Respiratory diseases constitute one of the largest causes of death worldwide and are mostly seen in children living in developing countries (Romieu *et al.* 2002). Many of these respiratory diseases can be linked to tobacco smoke inhalation, including chronic obstructive pulmonary disease and bronchial asthma (Thurlbeck 1990, Sjrostrand and Rander 1997, Sears 2000). According to the American Council on Science and Health, active smoking has been recognized as a major cause of disease and death for at least 40 years (1999). Moreover, accumulating research during the past 20 years has shown that exposure to secondhand smoke is also a threat to human health (ACSH 1999).

The inhalation of environmental tobacco smoke (ETS) is generally referred to as passive smoking or secondhand smoking, and there is now an extensive database on the health effects related to ETS (Samet 1999). Tobacco smoke is an environmental toxin that many people inhale everyday, whether they want to or not, and both ETS and active smoking cause a multitude of negative health impacts. There is now more knowledge on the toxic and carcinogenic components of ETS, and numerous epidemiological studies have examined the associations between ETS exposure and these negative health impacts (HHS 1986, EPA 1992). Because both ETS and active smoking are linked to many harmful health impacts, research needs to be done to help treat or perhaps prevent these destructive diseases.

Prevention is one way to reduce the damage done by cigarette smoke, but there also needs to be better understanding of the biological processes that instigate the harmful effects. Currently, the mechanism by which cigarette smoke stimulates lung epithelium to produce damaging inflammatory cytokines is poorly understood. Airway epithelium is a complex tissue with several different cell types, in which immune cells cooperate with epithelial cells to transform each other's response to pathogens by releasing cytokines and changing differentiation (Kaatinin *et al.* 1993). However, the interactions between resident intraepithelial immune cells and epithelial cells have yet to be studied in relation to airway epithelial cells. Furthermore, the role and participation of individual cell types in response to cigarette smoke is not known. Determination of the specific role of individual cell types in the airway epithelium will lead to a better understanding of the damaging effects of cigarette smoke and may become a basis for cell specific treatments.

The overall goal of this investigation is to explore the role of cell-cell interactions in airway epithelium and to measure the responses of different cell types to cigarette smoke. There is no way to account for the differences in ETS versus active smoking in my study, because the different exposure levels cannot be replicated *in vitro*. Instead, the goal of this study is to provide information that will lead to a better understanding of the mechanisms involved in lung damage caused by any type of exposure to cigarette smoke. The first specific aim of this project is to investigate the role of immune cells, or more specifically, the role of macrophages in the cellular response to cigarette smoke on airway epithelium. The first line of defense against inhaled substances are alveolar macrophages which keep the lungs clean by ingesting foreign particles, and are involved in the secretion of mediator molecules, including those involved in neutrophil recruitment (Lee *et al.* 2000). Research done by Tao and Erie has shown that Cigarette smoke and concomitant infections induce migration of immune cells, which subsequently results in a damaging inflammatory response and eventually a breakdown in the barrier function of airway epithelium (Tao *et al.* 2002, Erie *et al* 2000). I hypothesize that cigarette smoke toxins initially interact with macrophages already in the epithelium, which stimulates the release of cytokines that cause an influx of additional immune cells and the subsequent damaging inflammatory response (Lipscomb *et al.* 2002). Furthermore, if macrophages are not present, the inflammatory cytokine response to cigarette smoke by epithelial cells alone would be minimal or would require very high concentrations of cigarette smoke.

To test this hypothesis, epithelial cell cultures were grown on inserts that simulate actual lung conditions. Combinations of epithelial cell cultures, including macrophages and cigarette smoke extract, were then used to determine the role of macrophages in modulating the cellular response to cigarette smoke. Transepithelial electrical resistance (TER), which is an established technique to measure epithelial damage and barrier function, was used to test the influence of cigarette smoke and macrophages on the damage caused by the resulting inflammatory response (Zabner *et al.* 1998).

Methods

All research in this study was completed in Jonathan H. Widdicombe's laboratory at Children's Hospital Oakland Research Institute (CHORI). In order to examine the effects of

cigarette smoke on airway epithelial cells, cultures of human lung epithelial (Calu-3) cells were grown *in vitro* on filters that simulate the air-liquid interface found in the lungs. Calu-3 epithelial cell cultures were grown on inserts with 0.4 micron pore Costar filters (6-12 mm diameter inserts) for 15-35 days in Minimum Essential Medium (MEM)/F-12 with 10% heat inactivated fetal calf serum, fungizone and penicillin antibiotics at 37° C in a 5% CO_2 humidified incubator until confluence was reached and TER reached 200-1200 Ω x cm^2 (Tao 2002).

Macrophages were grown using the RAW 264.7 cell line on T-75 flasks for at least 15 days in Dubecco's modified Eagle's medium (DMEM)/F-12 with 10% heat inactivated fetal calf serum, fungizone, gentamicin, and penicillin antibiotics at 37° C in a 5% CO_2 humidified incubator. This cell line was established from the ascites of tumors induced in a male mouse. Dead cells were removed, and cell medium was changed 3 times a week. Because live macrophages are grown attached to the surface of the flasks, a sterile scraping tool was used to put cells into suspension for use in experiments. Cells were counted using a hemocytometer to determine the concentration in solution.

Cigarette smoke extract (CSE) was prepared by a modification of the method of Carp and Janoff (Carp and Janoff 1978). Two research cigarettes without filters that were produced for the University of Kentucky Research Foundation were combusted while attached to the tube of a vacuum. The smoke was bubbled through 50mL of phosphate buffered saline (PBS), and the resulting suspension was filtered through a 0.20 μm pore filter to remove bacteria and large particles. A final concentration of 50% CSE and concentrations of 135,000 and 250,000 macrophages were added to the chosen filters. These specific concentrations of macrophages were added because previous experiments conducted in Jonathan Widdicombe's laboratory have shown that lower concentrations do not produce significant results. RAW cell medium, macrophages, CSE, and CSE + macrophages were added to four groups of filters. As a measure of confluence of the epithelial layer, TER was measured with a Millicell ERS model "chopstick" voltohmmeter manufactured my Millipore Corporation, which characterizes the condition of tight junctions of epithelium. Damage done to epithelial cells is measured as a decrease in TER. TER was measured before addition of CSE and macrophages and then again 15 hours after.

Macrophages (RAWs), 50% CSE and RAW were all applied to the apical surface of the Calu-3 epithelial cells. A suspension of 0.25mL macrophages (5.4 x 10^5 cells/mL) was first added to two treatment groups: the "CSE + Macrophages" filters and the "Macrophage only"

filters. A solution containing 100% CSE was then added to the "CSE" and "CSE + Macrophages" treatment group. Finally, 0.5mL of RAW cell medium was added to the "RAW medium only" treatment group and 0.25mLs were added to the "Macrophage only" and "CSE only" treatment groups to bring the number of macrophages per filter to 135,000 and the concentration of CSE to 50%. The "Macrophage only" and "RAW medium only" treatment groups were the controls for this experiment. After incubating for 24 hours, macrophages and CSE were removed and TER was measured. Each treatment group contained five epithelial cell filters (n=5).

A second trial was conducted with a higher concentration of macrophages and shorter exposure time in order to compensate for the excessively high decrease in resistance shown in all filters in the first trial. Again, macrophages (RAWs), 50% CSE, and RAW medium were applied apically to Calu-3 epithelial cells. The only difference in procedure was that a suspension of 0.25mL macrophages (1.0×10^6 cells/mL) was added to the "CSE + Macrophages" and "Macrophage only" treatment groups, which resulted in 250,000 macrophages per filter. After incubating for 15 hours, macrophages and CSE were removed and TER was measured. Each treatment group contained four epithelial cell filters (n=4).

Analysis of TER results were performed using either the Kruskal-Wallis test for independent samples or the Mann-Whitney U test. Epithelial cell filters were placed into four treatment groups (cell medium only, macrophages only, CSE only, and CSE + macrophages) and the treatment group medians were compared to see if there was a significant difference.

Results

Table 1 shows that the resistance measurements of both treatment groups with CSE addition correspond to that of an empty filter ($<200\ \Omega \times cm^2$), which means that the resistance of these cells was effectively zero. Any cell filter with a resistance level less that $200\ \Omega \times cm^2$ is considered to be completely dead, and since the cells without CSE addition still had a resistance above $200\ \Omega \times cm^2$, this shows that barrier function was not completely destroyed in those cells. This may suggest that there was slightly more damage caused by the treatment groups with CSE compared to those without CSE addition. However, statistical analysis does not support this observation.

Addition (applied to cell filters)	**TER Average** (24 hrs after addition)
50% CSE + Macrophages	127.0 Ω x cm^2
50% CSE	149.4 Ω x cm^2
Macrophages only	240.0 Ω x cm^2
Cell medium only	210.6 Ω x cm^2

Table 1. Transepithelial Electrical Resistance Averages before and 24 hours after addition of macrophages and Cigarette Smoke Extract (CSE).

Overall, there was no significant difference observed between filters with or without macrophages in the first trial. Results of the Kruskal-Wallis test yielded an H-statistic of 2.291 with 3 degrees of freedom and a p-value of 0.5142. The p-value indicates that there are no significant differences between the medians of the four treatment groups. Figure 1 shows that all epithelial cells saw a large decrease in resistance, and those with CSE addition saw only a slightly higher decrease. Each bar in the figure represents the average decrease in resistance measured in five epithelial cell filters for each treatment group (n=5).

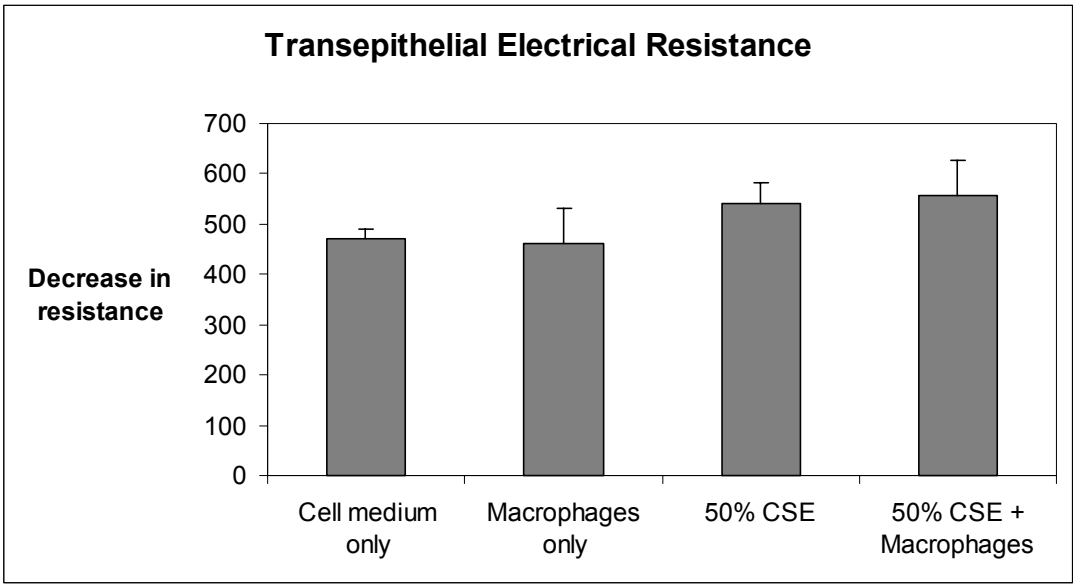

Figure 1. Averaged change in resistance of transepithelial electrical resistance measurements (n=5)

There was more observed variation between groups in the second trial, but there was still no significant difference observed between filters with or without macrophages. Results of the Kruskal-Wallis test yielded an H-statistic of 7.787 with 3 degrees of freedom and a p-value of 0.0506. The p-value indicates that there are no significant differences between the medians of

the four treatment groups. Figure 2 shows that the control groups saw a smaller decrease in resistance than in the first trial, and there was a larger observed degree of variation between the "CSE" and "CSE + Macrophages" groups. Again, each bar in the figure represents the average decrease in resistance measured in five epithelial cell filters for each treatment group (n=5).

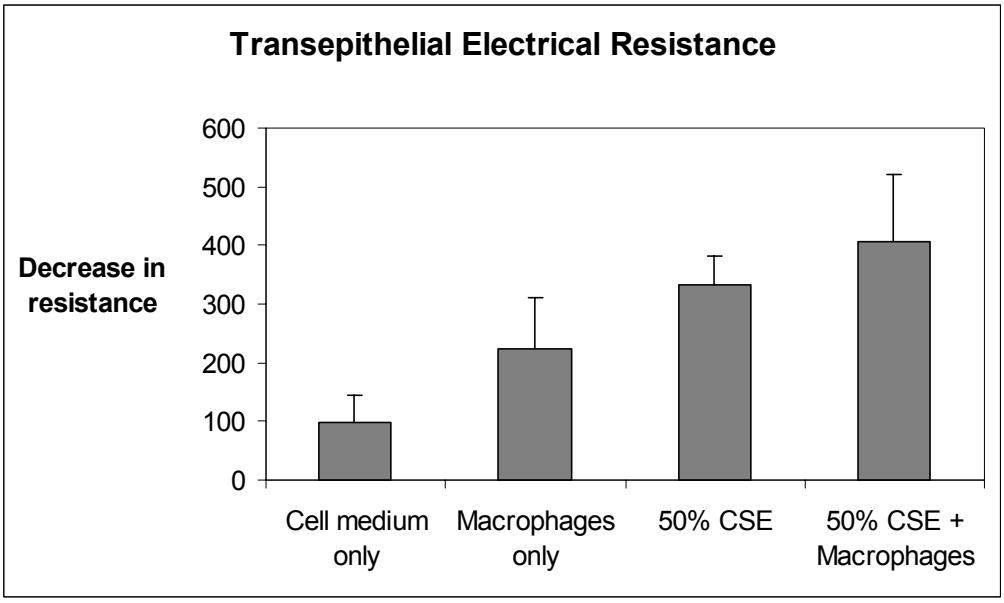

Figure 2. Averaged change in resistance of transepithelial electrical resistance measurements

In order to compare the non-control groups directly, a Mann-Whitney U test was performed on the "CSE" and "CSE + Macrophages" groups. The p-value of .7728 indicates that there was no significant difference between these two groups (n1=n2=4; u=7, u'=9).

Discussion

In the first trial, the filters with cell medium only and filters with macrophages only were controls, and should not have seen such a high decrease in resistance. This large decrease in resistance may have been due to a depletion of nutrients from the RAW medium that was applied to the apical side of the calu-3 cells. Calu-3's use a specific kind of cell medium that is usually applied to the basolateral side of the well, while leaving the apical side exposed to the air. After applying RAW medium to the apical side of the well for 24 hours, the medium turned yellow, which is a sign of a change in the pH level and is a visual indicator of when the cell medium is

depleted of nutrients. This may have had an adverse reaction that caused the calu-3s to die despite the fact that there was no CSE present.

After the addition of each treatment to their specific filters, the resistance measurements dropped to nearly zero in every group. The resistance measurements of both the "CSE + Macrophages" filters and "CSE only" filters correspond to completely dead cells, while the filters without CSE still had some resistance. This means that there was some additional damage done to the filters by CSE, because the filters without CSE were not completely destroyed. However, there was no statistically significant difference between the filters with macrophages compared to those without them. It would be impossible to tell if there was a difference, because of the extensive damage done by the CSE and perhaps by the depletion of nutrients and altered pH levels of the RAW medium.

In order to compensate for the large decrease in resistance, the filters in the second trial were exposed to their specific treatment group for 15 hours instead of 24 hours. The first trial did not have the number of macrophages that were previously used in other studies in our lab, which was found to be ideal for these types of experiments. Therefore, the concentration of macrophages was increased to 250,000 per filter in the second trial of the experiment.

In the control groups (cell medium only and macrophages only) there was a much smaller decrease in resistance than seen in the first trial. The filters with macrophages alone had a higher decrease in resistance than the cell medium only, which may be attributed to some cell-cell interactions with the epithelial cells that caused damage. Some decrease in resistance is expected in the controls, which may be due to handling during the testing procedure. The filters with CSE had a larger decrease in resistance, but there is no significant difference between filters with or without macrophages. Although there was a difference exposure time and concentration of macrophages in this trial, the results were essentially the same.

Results were expected to show that CSE compromises the integrity of the airway epithelial barrier more significantly when macrophages are present than when CSE alone is present. In this case, the medians of the "CSE" and "CSE + Macrophages" treatment groups would have had a statistically significant difference. This may indicate that a larger sample size is needed for a statistically significant result. Therefore, the only conclusion that can be made from the TER data collected is that the decrease in resistance associated with the addition of CSE is not due to the influence of macrophages. This may indicate that macrophages alone do not increase the

damage done to epithelial cells, and perhaps some other immune cell or another cell-cell interaction is responsible for the damage done by the inflammatory response.

In addition, because this model is not an exact reproduction of human lung physiology, there may be additional factors that are not represented. For example, macrophages are known to increase neutrophil recruitment in the lungs when foreign particles enter, but there were no neutrophils present in my model. Neutrophils are known to increase oxidative stress in the lungs, which corresponds with an increase in cell damage. Oxidative stress occurs when the body's antioxidant defenses are unable to metabolize damaging free radicals and other oxidative species. In response to oxidative stress, lung cells release inflammatory mediators and cytokines (TNF-α, IL-1 and IL-8), that are able to induce neutrophil recruitment and activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (Rahman 1998). Because all these factors are not incorporated into my model, it is possible there may still be some link between macrophages and cigarette smoke damage to the lungs, which further experimentation may discover.

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