Damage to olfactory progenitor cells is involved in cigarette smoke-induced olfactory dysfunction in mice

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Abstract

Objectives: Exposure to cigarette smoke is a major cause of olfactory dysfunction. However, the underlying mechanisms by which cigarette smoke interferes with the highly regenerative olfactory nerve system remain unclear. To investigate whether cigarette smoke induces olfactory dysfunction by disrupting cell proliferation and cell survival in the olfactory epithelium (OE), we developed a mouse model of smoking that involved intranasal administration of a cigarette smoke solution (CSS).

Methods: Firstly, we explored the effects of CS on olfactory populations and olfactory sensitivity using histological analyses and behavioral testing with time. Secondly, we investigated the effects of CS on pro-inflammatory responses using histological analyses and quantitative real-time PCR analyses.

Results: Immunohistological analyses and behavioral testing showed that CSS administration over a period of 24 days reduced the number of olfactory marker protein-positive mature olfactory receptor neurons (ORNs) in the OE and induced olfactory dysfunction. These changes coincided with a reduction in the number of SOX2$^+$ ORN progenitors and Ki67$^+$ proliferating cells in the basal layer of the OE, an increase in the number of caspase-3$^+$ apoptotic cells, and an increase in the expression of mRNA for the inflammatory cytokines IL-1β and IL-6. Notably, the proliferating ORN progenitor population recovered following cessation of treatment with CSS, resulting in the subsequent restoration of mature ORN numbers and olfaction.

Conclusion: These results suggest that SOX2$^+$ ORN progenitors are targets of CSS-induced impairment of the OE, and that by damaging the ORN progenitor population and increasing ORN death, CSS exposure eventually overwhelms the regenerative capacity of the epithelium, resulting in reduced numbers of mature ORNs and olfactory dysfunction.

Objectives

Cigarette smoke represents a major source of exposure to toxic chemicals for humans and causes a diverse range of preventable illnesses. The numerous chemical irritants contained in cigarette smoke trigger the generation of reactive oxygen and nitrogen species and expression of inflammatory mediators such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF) in the respiratory tract. Because these mediators damage epithelial tissue and induce inflammatory responses in the respiratory tract.

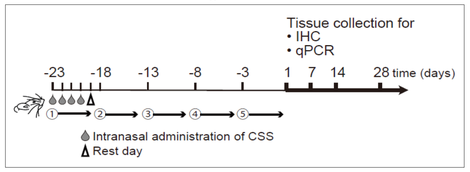
Olfaction is mediated by the olfactory system, which is composed of olfactory receptor neurons (ORNs) in the nasal cavity and the olfactory bulb in the forebrain. Olfactory dysfunction is associated with damage to ORNs and/or the olfactory bulb, which can occur due to a variety of causes, such as exposure to toxic chemicals, airway allergy, upper-airway viral infections, head trauma, and neurodegenerative diseases. Of note, cigarette smoke is a major cause of hyposmia and anosmia. Cigarette smoke decreases the thickness of the olfactory epithelium (OE) and increases apoptosis in the OE. However, ORNs have regenerative potential through the olfactory epithelial stem cell system, and thus the extent to which apoptosis causes olfactory dysfunction remains unclear.

In the basal layer of the OE, basal cells act as ORN stem and progenitor cells and give rise to mature ORNs expressing olfactory marker protein (OMP), which is expressed exclusively in mature ORNs. SOX2 is a transcription factor that is widely expressed in stem cell populations including in neural stem cells. In the OE, SOX2 is expressed by progenitor cells and regulates homeostasis. Accumulating evidence suggests that SOX2 expression is suppressed by inflammatory cytokines such as interleukin-6 (IL-6), resulting in a loss of stemness in multipotent cells. We hypothesized that cigarette smoke-induced inflammation might disrupt the olfactory progenitor cell system.

In the present study, we explored the effects of cigarette smoke on ORNs and ORN progenitors using a newly established mouse model of smoking that involved administration of a cigarette smoke solution (CSS). We also investigated the extent to which CSS-induced damage to the ORNs recovers following cessation of exposure to CSS.

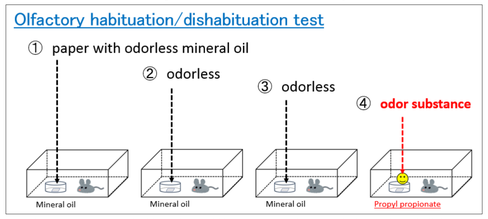
Methods

I. Mouse model of smoking



- C57BL/6 mice（8W, male)
- Cigarette smoke solution (CSS; 40 ml/40 cigarettes) .
- CSS mice were administered CSS (20 μl/ mouse/ time).
- Control mice received saline intranasally.
- CSS mice were sacrificed on days 1, 7, 14 and 28.

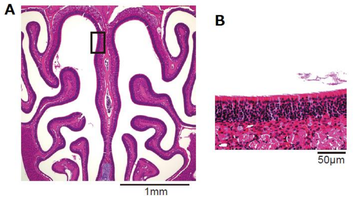
III. Behavioral testing to evaluate olfactory function



- Mice were presented with a piece of filter paper soaked in odorless mineral oil three times for 3 minutes, at 1 minute intervals.
- For the fourth exposure, the filter paper was soaked in the odorant propyl propionate instead of mineral oil.
- Mice with normal olfaction display gradually reduced durations of investigative behavior, but display reinstatement of investigative behavior in case with an odor substance.
- A lack of reinstatement indicates reduced or absent olfactory sensitivity.

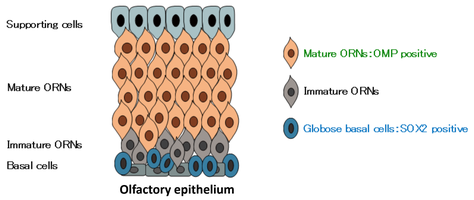
II. Histological analyses

- ***H&E staining***: Evaluation of whole tissue structure



* Representative images of the olfactory epithelium from untreated mice. (A, 40× magnification; B, 400× magnification)

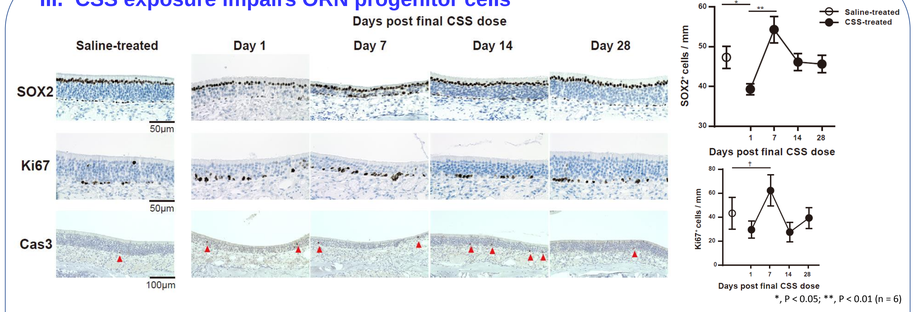
- ***Immunostaining*** (Primary antibodies)
- Olfactory marker protein (**OMP**): mature ORNs
- **SOX2**: olfactory progenitor cells
- **Ki67**: dividing cells
- cleaved capase-3 (**Cas3**): apoptotic cells



IV. Quantitative real-time polymerase chain reaction

Pro-inflammatory cytokines; ***IL-1β***, ***IL6***,

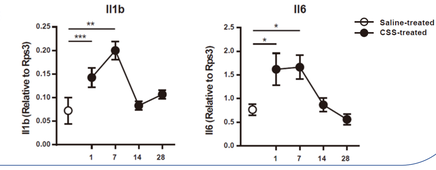
III. CSS exposure impairs ORN progenitor cells



*, P < 0.05; **, P < 0.01 (n = 6)

- The number of SOX2$^+$ ORN progenitors was around 20% lower than in saline-treated mice on day 1 after final CSS administration, was elevated on day 7, but had returned to saline-treated mouse levels by day 14.
- The number of Ki67$^+$ proliferating changed with a time course similar to that of SOX2$^+$ ORN progenitors.

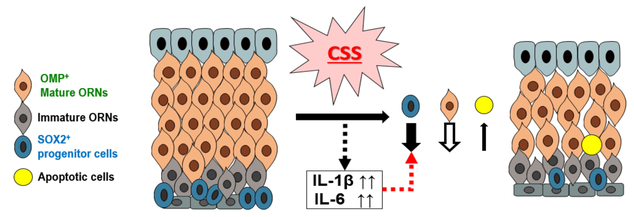
IV. CSS administration induces increases in IL-1β and IL-6 expression in the olfactory epithelium



*, P < 0.05; **, P < 0.01; ***, P < 0.001 (n = 4-6, Mann-Whitney U-test)

Discussions

- In this study, we demonstrate for the first time that long-term CSS administration damages not only OMP$^+$ mature ORNs but also SOX2$^+$ ORN progenitors in the OE.
- The numbers of SOX2+ ORN progenitors and Ki67+ proliferating cells increased transiently around 7 days after final CSS administration, preceding recovery of the OMP+ mature ORN population.
- Increased apoptosis is unlikely to be solely responsible for the reduction in ORN numbers.
- The reduction in mature ORN numbers was associated with olfactory dysfunction.

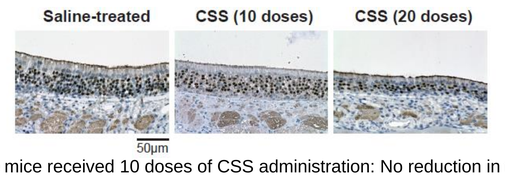


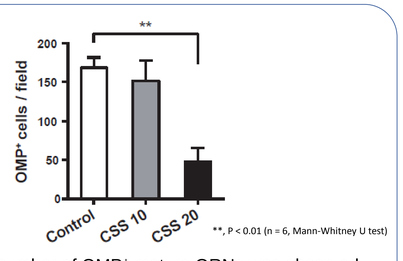
- Cigarette smoke could damage ORNs and their progenitors by inducing inflammation and immune responses in the OE. ⇒Similar to cigarette smoke toxicity in the lungs. (Hellermann GR, et al. 2002, Nyunoya T, et al. 2014)
- Inflammatory cytokines (IL-1β and IL-6) might be involved in reducing SOX2$^+$ ORN progenitor numbers, suppressing the maturation of ORNs from their progenitors or in inducing apoptosis. (Yoon DS, et al. 2014)

Results

I. CSS exposure impairs olfactory receptor neurons

Immunohistochemical staining of OMP$^+$ cells in the olfactory epithelium

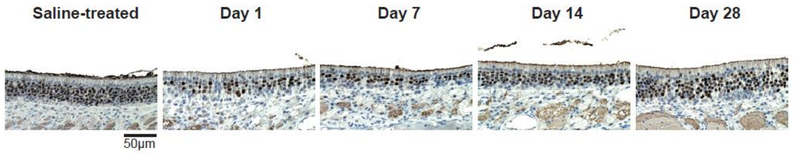


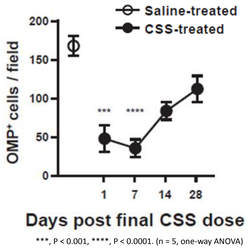


**, P < 0.01 (n = 6, Mann-Whitney U test)

- The mice received 10 doses of CSS administration: No reduction in the number of OMP$^+$ mature ORNs was observed.
- The mice received 20 doses of CSS administration: The number of OMP+ cells was around 70% less than that in saline-treated mice.

OMP$^+$ cells in the olfactory epithelium on various days following the final CSS dose



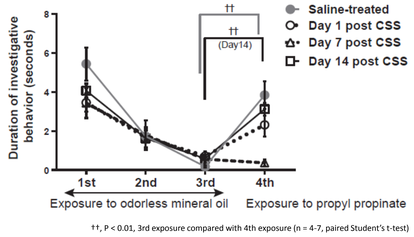


***, P < 0.001, ****, P < 0.0001. (n = 5, one-way ANOVA)

- The reduction in OMP+ ORN numbers became more severe on day 7 after the final intranasal administration of CSS, but then gradually recovered over days 14 to 28.

II. CSS exposure induces olfactory dysfunction

- In saline-treated mice, the duration of investigative behavior on the fourth exposure was significantly longer than that on the third trial, suggesting that the mice were capable of smelling the odorant.
- In CSS-treated mice, there was no significant difference in the duration of investigative behavior between the third and fourth trials on day 1 or day 7 after the final CSS administration, suggesting a decrease in olfactory sensitivity.
- The loss of olfactory sensitivity was most on severe on day 7 after the final CSS administration, but recovered by day 14.



††, P < 0.01, 3rd exposure compared with 4th exposure (n = 4-7, paired Student's t-test)

Conclusion

- We have demonstrated that by damaging the SOX2$^+$ ORN progenitor population and increasing ORN death, CSS eventually overwhelms the regenerative capacity of the epithelium, resulting in reduced numbers of mature ORNs and olfactory dysfunction. However the ORN progenitor population and olfaction recovered following cessation of exposure to CSS.
- Our findings provide a basis for future studies investigating the mechanisms underlying cigarette smoke-induced damage to ORN progenitor cells, and will help guide the development of preventive and therapeutic approaches for cigarette smoke-induced olfactory dysfunction.

Refersnces

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- R. Ueha, S. Ueha, et al. "Damage to olfactory progenitor cells is involved in cigarette smoke-induced olfactory dysfunction in mice." Am J Pathol 2016 **186**(3): 579-586.
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