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Damage to Olfactory Progenitor Cells Is Involved in Cigarette Smoke–Induced Olfactory Dysfunction in Mice

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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). Immunohistological analyses and behavioral testing showed that CSS administration during 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 Ki-67^+ 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 after cessation of treatment with CSS, resulting in the subsequent restoration of mature ORN numbers and olfaction. 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. *(Am J Pathol 2016, 186: 579–586; http://dx.doi.org/10.1016/j.ajpath.2015.11.009)*

Cigarette smoke represents a major source of exposure to toxic chemicals for humans and causes a diverse range of preventable illnesses.[1,2] The numerous chemical irritants contained in cigarette smoke trigger the generation of reactive oxygen and nitrogen species and expression of inflammatory mediators, such as IL-1β, IL-6, and tumor necrosis factor, in the respiratory tract.[3] Because these mediators damage epithelial tissue and induce inflammatory responses in the respiratory tract, a long history of cigarette smoking significantly increases the risk of various pulmonary diseases, such as chronic obstructive pulmonary disease and lung cancer.[3,4] In addition to lower respiratory diseases, cigarette smoke causes upper respiratory disorders, such as nasal polyps[5] and chronic rhinosinusitis, and also impairs olfaction (hyposmia/anosmia).[6–8] However, the cellular and molecular mechanisms by which cigarette smoke disrupts olfaction remain largely unclear.

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.[9] Olfactory dysfunction is associated with damage to ORNs and/or the olfactory bulb, which can occur because of a variety of causes, such as exposure to toxic chemicals, airway allergy, upper-airway viral infections, head trauma, and neurodegenerative diseases.[10,11] Cigarette smoke is a major cause of

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hyposmia and anosmia.[6–8] Cigarette smoke decreases the thickness of the olfactory epithelium (OE)[12] and increases apoptosis in the OE.[11] However, ORNs have regenerative potential through the olfactory epithelial stem cell system,[13] and thus the extent to which apoptosis causes olfactory dysfunction remains unclear.

Two types of cells in the basal layer of the OE, globose basal cells and horizontal basal cells, act as ORN stem and progenitor cells and produce mature ORNs expressing olfactory marker protein (OMP), which is expressed exclusively in mature ORNs and modulates olfactory signal transduction.[14] Recently, it was suggested that globose basal cells are actively proliferating multipotent progenitor cells that display increased proliferation after OE damage,[15] whereas horizontal basal cells are quiescent stem cells that contribute to OE regeneration only after severe OE damage.[16,17] SOX2 is a transcription factor that is widely expressed in stem cell populations, including in neural stem cells, and that plays a role in maintaining cells in an undifferentiated state.[18] In the OE, SOX2 is expressed by progenitor cells and regulates homeostasis.[10,13,19–21] Accumulating evidence suggests that SOX2 expression is suppressed by inflammatory cytokines, such as IL-6, resulting in a loss of stemness in multipotent cells.[22] In addition, we recently demonstrated that $SOX2^+$ ORN progenitors are damaged by viral infection, alone or in combination with airway allergy, leading to the eventual loss of ORNs.[10] We, therefore, hypothesized that cigarette smoke–induced inflammation might also disrupt the olfactory progenitor cell system.

Herein, 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 after cessation of exposure to CSS. Using histological analyses, olfactory habituation/dishabituation tests, and quantitative RT-PCR (RT-qPCR) analyses, we found that cigarette smoke impairs not only OMP^+ mature ORNs, but also $SOX2^+$ ORN progenitors. Decreased numbers of ORNs and their progenitors were associated with olfactory disorder that gradually recovered as inflammatory cytokine levels decreased after cessation of exposure to CSS.

Materials and Methods

Mice

Eight-week-old male C57BL/6 mice were purchased from Saitama Experimental Animals (Saitama, Japan). Mice were housed in a temperature-controlled environment under a 12-hour light-dark cycle with access to food and water *ad libitum*. All animal experiments were conducted in accordance with institutional guidelines and with the approval of the Animal Care and Use Committee of the University of Tokyo (approval number P14-086; Tokyo, Japan).

Mouse Model of Smoking

The mouse model of cigarette smoking was adapted from a guinea pig model that used CSS.[23] CSS, prepared by bubbling a stream of the smoke of 40 Hi-Lite cigarettes (Japan Tobacco Inc., Tokyo) through 40 mL of saline, was purchased from CMIC Bioresearch Center Co, Ltd (Tokyo, Japan). We defined the day of final CSS administration as day 0. CSS (20 μL per animal per time) was administered intranasally once a day on days −23 to −19. This cycle of four consecutive CSS administrations, followed by 1 rest day, was then repeated, with cycles 2 to 5 conducted over days −18 to −14, −13 to −9, −8 to −4, and −3 to 0, respectively (Figure 1). Control mice received saline intranasally, according to the same schedule as CSS mice. CSS mice were sacrificed on days 1, 7, 14, and 28 after the final administration of CSS. Control mice were sacrificed on day 1 after the final administration of saline.

Tissue Preparation

Septal nasal mucosa was harvested on days 1, 7, 14, and 28 after the final intranasal administration of CSS for histological and RT-qPCR analyses. Immediately after sacrificing the mice, the nasal cavities were gently irrigated with 4% paraformaldehyde to minimize mechanical damage to the OE. The mandibles were discarded, and trimmed heads were skinned, fixed in 4% paraformaldehyde for a further 24 hours, and then decalcified for 7 days using Decalcifying Solution B (Wako Pure Chemical Industries, Ltd, Osaka, Japan). After decalcification, the tissues were dehydrated in a series of graded ethanol solutions, followed by embedding in paraffin.

Histological Analysis

All samples were cut at the level of the anterior end of the olfactory bulb, as described previously.[10] Paraffin sections (4 μm thick) were deparaffinized in xylene and rehydrated in ethanol before hematoxylin and eosin staining or immunostaining. Hematoxylin and eosin staining was used for evaluation of whole tissue structure. For immunostaining, deparaffinized sections were treated with 3% hydrogen

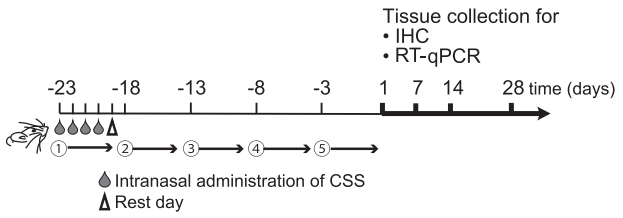

Figure 1 Experimental timeline. Mice were administered intranasally with cigarette smoke solution (CSS) between day −23 and 0 (five cycles, each including four daily doses of 20 μL per mouse and 1 rest day). Subsequently, the olfactory epithelium was collected for immunohistochemistry (IHC) and quantitative RT-PCR (qPCR) on the days indicated.

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