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Effect of intranasal administration of neurotrophic factors on regeneration of chemically degenerated olfactory epithelium in aging mice
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In the mammalian olfactory epithelium (OE), neurogenesis continues throughout the lifetime, by replacing olfactory receptor neurons (ORNs) lost by normal turnover in the postnatal period. However, this ability decreases with age and/or because of various toxic factors. To date, no effective treatment for olfactory dysfunction especially because of aging, is available in clinical practice. Here, we examined the effects of intranasal administration of fibroblast growth factor-2 and insulin-like growth factor-1 in gelatin hydrogel on the degenerated OE of aging mice induced by methimazole administration. These topical treatments led to increases in the number of olfactory marker protein-positive cells, which identified mature ORNs, resulting in the increased thickness of OE. These results indicate that both fibroblast growth factor-2 and insulin-like growth factor-1 promote the proliferation of basal cells and differentiation of immature ORNs into mature ORNs in the degenerated OE of aging mice. These agents might be promising candidates for the treatment of degenerated OE of aging humans. \textit{NeuroReport} 29:1400–1404 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.

Keywords: aging, neurotrophic factors, olfactory epithelium

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Introduction

The mammalian olfactory epithelium (OE) located in the nasal cavity is a specialized sensory epithelium involved in odor perception. In the OE, neurogenesis continues throughout the lifetime, replacing olfactory receptor neurons (ORNs) lost by normal turnover in the postnatal period [1,2]. This unique ability of the OE, which most other neural systems except the dentate gyrus and the subventricular zone do not have [3,4], decreases with age and/or because of various toxic factors including viruses, smoking [5], and environmental factors. In clinical practice, topical or oral corticosteroids are used for the treatment of olfactory dysfunction. These have been shown to be efficacious in the reduction of mucosal swelling and improving access of chemical substance to the ORNs in rhinosinusitis, but not effective in olfactory dysfunction resulting from damaged OE because of other factors except rhinosinusitis.

Here, we focus on OE regeneration by attempting to develop a strategy to promote it in a chemically damaged aging mouse model.

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reported the effects of neurotrophic factors on the regeneration of OE in adult mice [5,14–16].

In our previous study, in aging mice, intranasal administration of FGF2 promoted the production of immature ORNs, but not mature ORNs [14]. In this study, we assessed the effectiveness of topical application of FGF2 or IGF1 on methimazole (MMI)-damaged OE. We examined the expression of mRNA of FGF2 and Igf1, and their receptors, Fgfr1 and Igf1r, during regeneration in the damaged OE [15], to confirm the time of administration of FGF2 or IGF1. In an attempt to administer FGF2 or IGF1 more effectively, we included these factors in a gelatin-based hydrogel, which has the advantage of the sustained release of drugs [17].

Materials and methods

Animal preparation and methimazole injection

Male Institute of Cancer Research mice (7 months old) were used in this study. All mice were housed in acrylic cages with wood chip bedding and unlimited access to food/water at a temperature of 22 ± 1°C with a 12 h light/dark cycle. All the procedures were approved by the Animal Care Ethics Committee of Kobe University Graduate School of Medicine (registration no. P120912, P909116). The mice were injected intraperitoneally with 100 mg/kg body weight of MMI. Saline-injected mice served as controls. In this study, the day of MMI injection was defined as day 0.

DNA microarray analysis

The procedures are as described previously [15]. Total cellular RNA was isolated from the damaged OE, at 12 h, on day 1, 3, 7, and 14 (n = 10 in each subgroup) after MMI. It was quantified using spectrophotometry and adjusted to equal concentrations. DNA microarray analysis was carried out using CodeLink Mouse Whole Genome Bioarrays (Applied Microarrays, Tempe, Arizona, USA) according to the manufacturer’s protocol. The data were analyzed using Microarray Data Analysis tool version 3.2 supplied by the manufacturer (Filgen, Nagoya, Aichi, Japan).

Preparation of fibroblast growth factor-2-impregnated or insulin-like growth factor-1-impregnated gelatin hydrogels

Gelatin hydrogels were prepared just before administration by dripping 10 μl of a solution of saline or undiluted solution of 100 μg/ml human recombinant FGF2 (Kaken Pharmaceutical Co., Tokyo, Japan) or 10 mg/ml human recombinant IGF1 (Orphan Pacific, Tokyo, Japan) onto a 1 mm² sheet of freeze-dried gelatin hydrogel (Medgel Co., Tokyo, Japan).

Nasal instillation of topical agents

The mice were anesthetized by an intraperitoneal combined injection of midazolam, medetomidine chloride, and butorphanol (4, 0.3, and 5 mg/kg body weight). The saline (n = 4), or FGF2 (n = 5), or IGF1 (n = 4)-added gelatin hydrogel were applied on the right side of the nasal cavity on day 3, through a nostril, to the anterior portion of the lateral endoturbinate.

Tissue preparation

Mice were killed on day 18. Under deep anesthesia with isoflurane, mice were killed by cervical dislocation and then decapitated. Heads were fixed with 10% formaldehyde at room temperature for 24 h. The specimens were then decalcified in a decalcification solution (10% EDTA, pH 7.0) over 7 days, dehydrated through a graded alcohol and xylene series, and embedded in paraffin. The specimens were serially sectioned at 4 μm thickness.

Immunohistochemical staining

The sections were deparaffinized and rehydrated through a graded xylene and alcohol series. The sections were placed in 0.01 M of citric acid buffer solution and heated at 100°C in a microwave oven for 20 min for antigen retrieval. Endogeneous peroxidase was blocked with 3% hydrogen peroxide and nonspecific binding was blocked with 2.5% normal horse serum. The sections were incubated with the following anti-mouse polyclonal antibodies: GAP43 (1:500 dilution; MAB347; Chemicon International, Temecula, California, USA), which is expressed by immature ORNs, and olfactory marker protein (OMP) (1:900 dilution; 019-22291; Wakо, Osaka, Japan), which is expressed by mature ORNs, respectively, for 60 min. Antibody binding was visualized using the ImmPRESS (Filgen, Nagoya, Aichi, Japan) HRP anti-mouse or goat IgG (Peroxidase) Polymer Detection Kit (MP-7402 or MP-7405; Vector Laboratories, Burlingame, California, USA), respectively. Diaminobenzidine was used for coloration and nuclei were counterstained with hematoxylin.

Quantitative analysis

The OE on the nasal septum, in coronal sections through the olfactory bulbs, was observed at a magnification of ×200 under a Nikon microscope (Tokyo, Japan). The thickness of OE and the number of cells positive for GAP43 and OMP were examined. Three fields per each mouse were examined. To avoid variations between the regions of interest, the observation was restricted to the same areas of septal OE. The mean values of saline or FGF2-administered or IGF1-administered groups were compared statistically by one-way analysis of variance before being analyzed post-hoc by the Tukey–Kramer test. Differences were considered significant when the P value was less than 0.05.

Results

Transitional patterns of fibroblast growth factor-2 or insulin-like growth factor-1 mRNA expression during the regeneration of olfactory epithelium

The intensity ratio of mRNA for FGF2, normalized to the control, was greater than 1 at 12 h after MMI injection
(Fig. 1), indicating that the expression began to increase sometime between 0 and 12 h. It was still higher than the control level at 1 day, indicating that the expression continued to increase after 12 h to 1 day. On day 3, the ratio was less than 1, indicating that mRNA expression decreased below its control level between days 1 and 3. The mRNA of Fgfr1 was higher than the control level at 12 h, 1, 3, 7, and 14 days. The intensity ratio of mRNA for IGF1, normalized to the control, was below 1 at 12 h, and greater than 1 at 1, 3, 7, and 14 days (Fig. 1), indicating that the expression began to increase sometime between 12 h and day 1, although it may have peaked either before or after day 3. The mRNA level of Igf1r was higher than the control level at day 7 and 14, indicating that the expression began to increase sometime between days 3 and 7. These results suggested that FGF2 and IGF1 were required for regeneration of the OE within the first 7 days after excessive injury. Previous studies reported that neurotrophic factors such as FGF2 [18,19], IGF1, and BDNF [20,21] showed a considerable decrease with age in rodents. Taken together, we hypothesized that the administration of FGF2 and IGF1 in gelatin hydrogels, at 3 days after MMI injection, would yield the maximum effect on the regeneration of OE.

**Effects of fibroblast growth factor-2 or insulin-like growth factor-1 on the regeneration of degenerating olfactory epithelium in aging mice**

No significant differences were found in the number of GAP43-positive cells among saline or FGF2-administered or IGF1-administered groups. In contrast, significant differences were found in the number of OMP-positive cells and wall thickness among these groups. The OE was significantly thicker in the FGF2-administered or IGF1-administered group than that in the control group (Figs 2 and 3). Between the FGF2-administered and IGF1-administered group, the thickness of OE was not significantly different.

The number of OMP-positive cells increased in the FGF2-administered and IGF1-administered group compared with that in the control group (Figs 2 and 3). Between the FGF2-administered and the IGF1-administered group, the number of OMP-positive cells was not significantly different.

**Effects of fibroblast growth factor-2 or insulin-like growth factor-1 on the regeneration of olfactory epithelium depending on locations in the nasal cavity**

In this study, we placed gelatin hydrogels unilaterally (on the right side) to retain enough space for respiration. However, no significant differences were observed between the right and left sides of the nasal cavity in the thickness of OE, and the number of GAP43-positive or OMP-positive cells in each group (Fig. 4).

**Discussion**

In this study, we demonstrated the effect of intranasal administration of FGF2 or IGF1 on OE regeneration in aging mice. In the FGF2-administered and IGF1-administered group, use of gelatin hydrogels led to an increase in the number of OMP-positive cells, resulting in an increased thickness of OE, in contrast with our previous report, in which intranasal administration of FGF2 did not lead to differentiation of mature ORNs [14]. The most plausible explanation for the different results in these two studies is the use of gelatin hydrogels. In our previous study, FGF2 was administered intranasally in PBS solution twice a day. In contrast, in the present study, the continuous effect of FGF2 was achieved owing to gelatin hydrogel placement, which led to the controlled release of growth factors, the half-life of which is very short in its free form [17]. Second, the condition of OE was significantly different in the two studies. The OE was under normal conditions in our previous study. In contrast, the OE was excessively damaged by MMI in the present study. Just after the injection of
MMI, the whole OE degenerated and only basal cells were left. Three days after the injection of MMI, the OE was still thin; thus, the FGF2-administered might easily have reached the basal cells compared with the normal condition. In addition, processes of regeneration of ORNs between normal and excessively damaged conditions are obviously different [6]. The proliferation and differentiation of stem and progenitor cells in OE are stimulated and maintained by several secreted factors, and regulated by the feedback inhibitory signal expressed by ORNs under normal conditions [7]. In this study, the microarray data showed a higher level of FGF2 and IGF1 than the control in the OE after MMI damage, and regeneration of OE is promoted more after MMI damage than under the normal condition. This suggests that intranasal administration of FGF2 or IGF1 increases this regenerative capacity of progenitor cells. Interestingly, it was suggested that a part of the hydrogel in the nasal cavity may migrate to the opposite side through choanae, and that as found in this study, unilateral administration of gelatin hydrogel with FGF2 or IGF1 was effective on both sides of the OE. Taken together, our results suggest that a single intranasal administration of neurotrophic factors using gelatin hydrogel is effective for the treatment of degenerating OE in aged mice.

**Fig. 2**

Immunostaining for GAP43 or olfactory marker protein (OMP)-positive cells in the olfactory epithelium 18 days after the methimazole injection in saline, FGF-2-administered, or IGF1-administered groups. Yellow bars represent the thickness of olfactory epithelium from the apical to basal. Scale bar, 50 mm. FGF, fibroblast growth factor; IGF, insulin-like growth factor.

**Fig. 3**

Comparison of the wall thickness, number of cells positive for GAP43, or olfactory marker protein (OMP) in the olfactory epithelium (OE) 18 days after the methimazole injection among saline, FGF-2-administered, and IGF1-administered groups. Data were counted at 3 separate sites Fig. 2, and mean values of 3 groups were compared. *P < 0.05; **P < 0.01; NS. Results represent mean ± SD. n = 4 (saline, IGF-1), n = 5 (FGF-2), analysis of variance. FGF, fibroblast growth factor; IGF, insulin-like growth factor.
for clinical application. More detailed investigations should be performed targeting olfactory dysfunction following OE damage in the outpatient clinic. Theoretically, few side effects are expected in comparison with systemic administration. Our study provides a promising alternative therapeutic approach to targeting olfactory dysfunction following OE damage in the elderly. More detailed investigations should be performed for clinical application.

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Conflicts of interest
There are no conflicts of interest.

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