Treatment of Neural Anosmia by Topical Application of Basic Fibroblast Growth Factor–Gelatin Hydrogel in the Nasal Cavity

An Experimental Study in Mice

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Objective: To investigate the effects of basic fibroblast growth factor (bFGF)–gelatin hydrogel on recovery of neural anosmia in mice.

Design: Anosmia was induced by intraperitoneal injection of 3-methylindole, 200 mg/kg. One week later, the animals underwent 1 of the following 3 procedures bilaterally: (1) group A: single-shot intranasal drip infusion of phosphate-buffered saline, (2) group B: single-shot intranasal drip infusion of bFGF, and (3) group C: placement of bFGF–gelatin hydrogel in the nasal cavity. The olfactory function of the animal was evaluated by the odor-detection test (ODT) 2 and 4 weeks later. Following the testing, the animal was killed, the thickness of the olfactory epithelium was measured, and the number of olfactory marker protein (OMP)-positive cells was counted.

Setting: Research installation.

Participants: Mice.

Intervention: The placement of bFGF–gelatin hydrogel in the nasal cavity.

Main Outcomes and Measures: An ODT, thickness of olfactory epithelium, the number of OMP-positive cells

Results: The ODT proved that neural anosmia recovered in group C but not in groups A and B. Histologically, olfactory epithelium became thicker and the number of OMP-positive cells increased in group C, while such functional and histologic recovery was poor in groups A and B. These findings suggested that placement of bFGF–gelatin hydrogel in the nasal cavity was an efficient way to facilitate recovery of neural anosmia.

Conclusions and Relevance: As a gelatin hydrogel degrades slowly in the body, bFGF is gradually released around the site of the lesion; thus, it constantly exerts its effects on neural regeneration.


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Olfactory receptor neurons (ORNs) are located in the olfactory epithelium and work as receptors of smell sensation. They are replaced on a continuous basis and in response to neural damage throughout life, suggesting the presence of neural stem cells and/or progenitor cells near the ORNs. Such a long-term persistent turnover of the neural cells is never seen in other sensory organs. Although ORNs are exposed to the outside environment in the nasal cavity, complete degeneration seldom occurs owing to their intrinsic recovery function. When regeneration fails, however, ORNs undergo degeneration, resulting in partial or total neural anosmia. In that sense, the clinical features and treatment modality of neural anosmia are completely different from those of respiratory anosmia. The latter could be treated simply by creating a root to pass smell molecules to the ORNs. This is accomplished by subsiding edema of the nasal mucosa and resecting a nasal polyp if one is present.

According to recent studies, various neurotrophic factors, such as nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3, have been proven to facilitate regeneration of ORNs. Basic fibroblast growth factor (bFGF) is 1 such neurotrophic factor. It was originally isolated from bovine brain and pituitary gland, where it stimulates fibroblast proliferation. Recently, bFGF was shown to potently promote neural regeneration, in addition to preventing death of injured neurons. This suggests the hypothesis that neural anosmia can be healed by use of bFGF. However, as was shown by Nishikawa et al, repeated application
of bFGF by intranasal drip infusion in mice did not promote proliferation of ORNs; it stimulated proliferation of only basal cells and supporting cells. This was probably because the action time of bFGF was very short when it was applied by intranasal drip infusion. In 1994, Tabata et al reported a new drug delivery system that involved embedding a medicine like bFGF in gelatin hydrogel that degrades slowly in the body after implantation. In our previous experimental study in guinea pigs, bFGF–gelatin hydrogel was found to be a promising medicine to promote recovery of transected facial nerve.10 The aim of the present study was to determine whether topical application of bFGF–gelatin hydrogel in the nasal cavity effectively facilitated healing of chemically induced neural anosmia in mice.

**METHODS**

**EXPERIMENTAL ANIMALS AND INDUCTION OF ANOSMIA**

Under approval by the ethics committee of Ehime University Graduate School of Medicine, the present study was conducted according to our institute’s guidelines for animal experimentation. Experiments were performed using 2.5-month-old female Balb/c mice (Charles River Laboratory Japan Inc), weighing 13 to 20 g. They were housed in an animal room at a temperature of 21°C to 23°C under a 12-hour light and dark cycle. Every effort was made to minimize the number of animals and their suffering.

Anosmia was induced by intraperitoneal injection of 3-methylindole (3-MI), 200 mg/kg. One week later, the animal was anesthetized with an intramuscular injection of ketamine hydrochloride, 35 mg/kg, and xylazine hydrochloride, 7 mg/kg. The mice then underwent 1 of the following 3 procedures bilaterally: (1) group A: single-shot intranasal drip infusion of phosphate-buffered saline (PBS), (2) group B: single-shot intranasal drip infusion of bFGF (10 µg), and (3) group C: placement of bFGF–gelatin hydrogel in the nasal cavity. Six animals were allotted to each group. Because olfactory epithelium is distributed widely in the nasal cavity in mice, we applied a small piece of bFGF–gelatin hydrogel via the nostril to the anterior portion of the lateral endoturbinate, which is thought to be the most sensitive site for the olfactory sensation in mice. It was difficult to place a piece of bFGF–gelatin hydrogel in the nostril while retaining enough space for respiration, but it could be accomplished by careful manipulation. In our preliminary experiment, gelatin hydrogel placed in the nasal cavity dissolved within 7 days. In this study, the day of intranasal administration of the test agent was defined as day 0.

**PREPARATION OF BFGF IMPREGNATED IN GELATIN HYDROGEL**

A gelatin with an isoelectric point of 4.9 was obtained from bovine bone collagen after alkaline treatment (Nitta Gelatin Co). By cross-linking with glutaraldehyde, the gelatin was prepared as a hydrogel and preserved in freeze-dried form until use. Human recombinant bFGF with an isoelectric point of 9.6 (Kaken Pharmaceutical Co) was applied to the gelatin hydrogel, and the solution was completely absorbed at 4°C for 12 hours. According to Tabata et al, the degradation time of the hydrogel was 14 days when implanted in rabbits. All procedures were conducted under sterile conditions.

**ODOR DETECTION TEST**

Olfactory function of the animals was assessed by odor detection test (ODT) using cycloheximide solution13 on days 14 and 28. This solution has a peculiar odor that makes the animal try to avoid it. Before induction of neural anosmia, the ODT was administered to the animals to verify that they would avoid water containing cycloheximide. The ODT was performed after the mice were deprived of water for 48 hours to make them thirsty. At the time of testing, 2 water bottles filled with either cycloheximide, 0.1%, solution or distilled water were set in the cage; the animal could freely access either bottle. Once the animal drank, the bottle was promptly removed to keep the animal thirsty. The next trial was then conducted by changing the positions of the bottles randomly. By observing the animals’ behavior, the percentage of correct selection of the bottle (distilled water) to whole trials was counted. Ten trials were performed for each test. All test procedures were recorded by video camera, and the validity of the results was later checked by other examiners.

**COMPARISON OF OLFACTORY EPITHELIUM THICKNESS**

After ODT on day 28, the animal was killed for histologic study. Under deep anesthesia with ketamine hydrochloride, 100 mg/kg, and xylazine hydrochloride, 20 mg/kg, the mouse was administered transcardial perfusion with saline, followed by 4% paraformaldehyde. Following decapitation, the skull bone, including the brain, was fixed with 4% paraformaldehyde at 4°C overnight. It was then decalcified with K-CX decalcification solution (Fukagawa, Tokyo, Japan) at 4°C for 7 days, dehydrated with a series of alcohol concentrations, and embedded in paraffin. The specimen was serially sectioned in 5-μm thicknesses and subjected to hematoxylin-eosin staining. The thickness of the olfactory epithelium was measured by use of the DP controller software package (version 03.02; Olympus). Measurements were made at 5 sites, and the mean values of the 3 groups were compared.

**COMPARISON OF OLFACTORY MARKER PROTEIN–POSITIVE CELLS**

A section of the specimen was immunostained with anti-olfactory marker protein (OMP) antibody. The specimen was deparaffinized by xylene and then dehydrated with a series of ethanol concentrations. The specimen was then placed in a citrate-buffered solution (pH 6.0) and heated at 120°C in a microwave oven for 10 minutes for antigen retrieval. Endogenous peroxidase activity was blocked by immersing the specimen in methanol solution mixed with 3% hydrogen peroxide for 20 minutes. After being washed in PBS, the specimen was incubated with rabbit polyclonal anti-OMP antibody at a dilution of 1:1000 (O三代es) at 4°C overnight. Following further washing in PBS, it was further reacted with hors eradish peroxidase–conjugated anti-rabbit IgG secondary antibody (Simplestain MAX-PO [Rabbit]; Nichirei) for 30 minutes at room temperature. After rinsing in PBS several times, it was then incubated with 3,3’-diaminobenzidine (DAB) (Simple Stain DAB; Nichirei) for 5 minutes to visualize OMP-positive cells. The number of OMP-positive cells per 1.0 × 104 µm² was counted at 3 separate sites, and the mean values of the 3 groups were compared.

**STATISTICAL ANALYSIS**

The results were compared using the Kruskal-Wallis test. All statistical analyses were performed with the IBM SPSS statistical software package. *P* < .05 was considered to indicate statistical significance.
RESULTS

ODOR DETECTION TEST

Results are summarized in Figure 1. The selection ratios in ODT on day 0 were similar among the 3 groups. On day 14, the ratios were 50%, 60%, and 73% in the PBS, bFGF, and bFGF–gelatin hydrogel groups, respectively (6 mice in each group). There was no statistically significant difference among the 3 groups. In contrast, on day 28, the ratios were 45%, 53%, and 82%, respectively. The ODT result in the bFGF–gelatin hydrogel group was significantly better than those in the other 2 groups (P < .05). These findings suggest that placement of bFGF–gelatin hydrogel in the nasal cavity facilitated recovery of olfactory function by day 28.

OLFACTORY EPITHELIUM THICKNESS

The mean (SD) epithelial thicknesses on day 28 was 15.0 (3.1) μm in the PBS group, 19.2 (4.1) μm in the bFGF group, and 34.9 (9.9) μm in the bFGF–gelatin hydrogel group (6 mice in each group), as shown in Figure 2 and Figure 3. Statistical analysis showed that the olfactory epithelium thickness in the bFGF–gelatin hydrogel group was the largest among the 3 groups (P < .05).

NUMBERS OF OMP-POSITIVE CELLS

The mean (SD) numbers of OMP-positive cells in the olfactory epithelium were 10.4 (2.9) in the PBS group, 25.4 (8.6) in the bFGF group, and 75.3 (14.7) in the bFGF–gelatin hydrogel group (6 mice in each group), as shown in Figure 4 and Figure 5. The number of OMP-positive cells in the bFGF–gelatin hydrogel group was the highest among the 3 groups (P < .05). This suggested that bFGF–gelatin hydrogel best facilitated growth of OMP-positive cells after chemically induced neural anosmia.

COMMENT

Experimental neural anosmia has often been induced by administering various olfactotoxic agents, such as zinc sulfate, 3-MI, and methimazole. In this study, we used 3-MI to impair ORNs of the olfactory epithelium, although it also caused bronchiolar insult. The olfactory epithelium is a ciliated pseudostratified columnar epithelium composed of 4 cell types: mature ORNs, immature ORNs, basal cells, and supporting cells. Mature ORNs are located in the middle layer of the epithelium and express OMP, a 19-kDa cytoplasmic protein. Immature ORNs are situated in the lower layer of the epithelium and move progressively to more superficial layers to become mature ORNs. The basal cells are located at the base of the epithelium and the supporting cells in the upper layer of the
epithelium. Severe ORN damage, such as by transaction of peripheral olfactory axons\textsuperscript{5} or by administration of a sufficient dose of 3-MI,\textsuperscript{14} causes loss of almost all OMP immunoreactive cells in about 1 week. In this study, the thickness of the olfactory epithelium and profile of OMP immunoreactive cells were used as an index of ORN damage induced by 3-MI.

Basic fibroblast growth factor is a 146-amino-acid single-chain polypeptide that is stored in the extracellular matrix of the nervous system as a complex combined with acidic polysaccharides; it stimulates fibroblast proliferation. Owing to recent advances in molecular biology, bFGF is now recognized as a multifunctional growth factor that facilitates angiogenesis, dilates blood vessels,\textsuperscript{17} prevents neuronal cell death, and promotes neuronal sprouting.\textsuperscript{18} In cases of peripheral nerve injury, bFGF has been shown to facilitate recovery from the nerve damage by preventing nerve degeneration and promoting neuronal regrowth.\textsuperscript{18} In an experimental study with guinea pigs, Nakamura et al\textsuperscript{19} reported that intraperitoneal injection of bFGF effectively facilitated regeneration of the olfactory epithelium. The effects were dose dependent: a higher dose of bFGF showed more distinct effects. As the presence of bFGF receptor was demonstrated in the olfactory epithelium,\textsuperscript{20} we speculated that bFGF applied in the nasal cavity might have coupled to the receptors and facilitated regeneration of ORNs. In the present study, we investigated the effects of bFGF by applying it in 2 different ways: as a single-shot intranasal drip infusion and as continuous application in a hydrogel. The results were markedly different. The latter effectively promoted recovery from neural anosmia, whereas the former had only a small beneficial effect, as reported by Nishikawa et al.\textsuperscript{8} Gelatin hydrogel is biologically inactive and is considered to be ineffective in terms of recovery from damage.\textsuperscript{21} These effects are likely due to sustained release of bFGF, as the hydrogel degrades slowly in the body. This new drug delivery system may be advantageous for continuous administration of a medicine and, therefore, the difficulties due to repeated administration of the medicine could be minimized by use of hydrogel. Many experimental studies have been performed concerning the safety of hydrogel in the body, and they show that this material does not cause adverse effects. Taba et al\textsuperscript{9} implanted a bFGF–gelatin hydrogel into an experimentally created bone defect of the skull in rabbits and demonstrated that the defect closed quickly without any adverse effects. Miyoshi et al\textsuperscript{12} demonstrated the beneficial effects of this conjugated agent on skin wound healing in mice and noted that the release of bFGF persisted for 2 weeks, sufficient time to facilitate wound healing. Nakae et al\textsuperscript{23} investigated the effects of bFGF–gelatin hydrogel in the streptozotocin-induced diabetic rat, and reported that the intramuscular introduction of this agent effectively improved the hypoalgesia and conduction velocity of the sciatic nerve. The animals experienced no adverse effects related to the use of hydrogel. Note that from our results, it was impossible to conclude whether bFGF
or the hydrogel carrier was responsible for the improvement of olfaction. Despite these findings, we believe that the bFGF-gelatin hydrogel can be safely applied in the nasal cavities of patients with peripheral neural anosmia. As mentioned in the Methods section, we did not use blind-observation procedures to assess the results so as to avoid judging bias. Instead, all of the processes in ODT were recorded with a video camera and reevaluated later by the coauthors. In the histologic examinations, all of the specimens were stocked and checked later by coauthors. In this way, the reliability of the test results was confirmed.

In conclusion, a bFGF-impregnated biodegradable hydrogel was demonstrated to facilitate regeneration of ORNs. Since the hydrogel degrades slowly in the body, the effects persist for an extended period owing to sustained release of bFGF. We believe intranasal administration of bFGF-gelatin hydrogel represents a promising treatment modality for neural anosmia.

Submitted for Publication: September 23, 2012; accepted December 19, 2012.

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Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Hakuba and Hato. Acquisition of data: Nota and Takahashi. Analysis and interpretation of data: Nota and Gyo. Drafting of the manuscript: Nota, Takahashi, Hakuba, and Hato. Critical revision of the manuscript for important intellectual content: Gyo. Statistical analysis: Nota. Obtained funding: Takahashi. Study supervision: Hakuba, Hato, and Gyo.

Conflict of Interest Disclosures: None reported.

Funding/Sponsor: This research was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Program for Enhancing Systematic Education in Graduate School).

Additional Contributions: We thank Yasuhiko Tabata, PhD, Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, for technical support of this project; Takaki Miwa, MD, PhD, Department of Otorhinolaryngology–Head and Neck Surgery, Kanazawa Medical University, for technical advice; and Takeshi Kiyoi, Integrated Center for Science, Ehime University, for assistance with histological staining.

REFERENCES


Figure 5. Numbers of olfactory marker protein (OMP)-positive cells. Results are presented as means ± 1 SD. The number of OMP-positive cells in the basic fibroblast growth factor (bFGF)-gelatin hydrogel group was significantly greater than in the other 2 groups (Kruskal-Wallis test, P < .05). PBS indicates phosphate-buffered saline.