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Sonic Hedgehog in Nasal Mucus is a Biomarker for Smell Loss in Patients with Hyposmia

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Abstract

Title: Sonic hedgehog in nasal mucus is a biomarker for smell loss in patients with hyposmia

Background: Many chemical moieties have been identified in nasal and olfactory mucus related to cellular activity, cell signaling and olfaction. Sonic hedgehog (Shh) has been identified as a growth factor in taste buds but not in olfactory receptor tissues. We wished to determine if Shh were present in nasal mucus and, if present, does it relate to smell function and smell loss (hyposmia).

Methods and findings: Shh was evaluated in nasal mucus in 14 normal volunteers and 44 patients with smell dysfunction of several etiologies. Nasal mucus was collected over a 1-4 day period in a 50 ml plastic container, transferred to a 12 ml plastic tube, centrifuged at 18,000 rpm for 45-55 minutes, the supernatant transferred to PCR tubes and frozen at 20°C until analyzed. All samples were analyzed by use of a sensitive spectrophotometric ELISA. Shh was found in nasal mucus in all normal subjects and in hyposmia patients. Levels in hyposmia patients of several etiologies were significantly lower than in normal. Levels decreased as subjects aged.

Conclusions: This is the first systematic demonstration of Shh in nasal mucus in normal subjects and in hyposmia patients. Its presence is consistent with its role as a cell signaling moiety and growth or transcription factor related to olfactory receptor function. Its measurement in lower than normal concentrations in hyposmic patients may indicate that it can serve as a biomarker for smell loss in these patients. Its measurement can help to identify patients with hyposmic on an objective basis and help to define the biochemical parameters of their smell loss. Further studies can assist in determination of the specific role for this moiety in olfaction.

Introduction

Investigators have identified several chemical moieties in nasal mucus [1,2], in olfactory mucus and in mucus from olfactory mucosa [3-8] and related its presence to regulation of cellular activity, cell signaling in the olfactory mucosa [3-8] or in pathology of the upper airways [9-12]. Levels of one of these moieties, olfactomedin, identified as an olfactory glycoprotein [3], was lower than normal in animals with loss of smell (hyposmia); increasing these levels was associated with what was characterized as normal olfactory function [4]. However, there are many moieties in nasal mucus in humans not identified through these and other analyses [13]. Chief among these are adenylyl cyclases [14,15] and their downstream metabolites cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [16,17]. These latter moieties were found decreased in hyposmic patients compared to normal subjects [16,17]. Sonic hedgehog (Shh) has been characterized as a critical factor in cellular growth and development of taste receptors [18,19] with its absence associated with inhibition of growth and development of receptors of these sensory organs [20]; however, it has not been identified to play a role in olfaction per se.

Based upon these [18-20] and other studies [21] we and other before us recognized that moieties in nasal mucus, including several cytokines [21] and several drugs [22], could act as regulators of cellular function and cellular signaling [21] and cytokines could be identified as so-called growth or transcription factors [23] which stimulated stem cells in the olfactory epithelium to induce stimulation, growth and perpetuation of olfactory receptors to maintain olfactory function [24]. Inhibition of secretion of these nasal mucus moieties initiated smell loss [24] whereas treatment which increased these moieties improved or even restored olfactory function to or towards normal [24].

Because of the large number of hyposmic patients in the US [24] these studies generated our interest to search for other nasal mucus moieties that might act as growth factors involved with maintaining olfactory function in humans. This search resulted in a preliminary report in which Shh was identified in nasal mucus [25].

In this study we report substantive studies which identify the presence of Shh in nasal mucus of normal subjects and of patients with hyposmia.

Methods

Subjects

Normal Subjects: Fourteen volunteers with normal smell and taste function were studied. These subjects were four men, 10 women, aged 44-82 y, 61 ± 3y (Mean ± SEM) who were either patients who presented to The Taste and Smell Clinic in Washington, DC for evaluation of symptoms unrelated to smell loss or who were employees of the Taste and Smell Clinic who volunteered for the study. Subjects were selected in a consecutive manner and included all subjects who volunteered for the study. All subjects stated that they had normal smell function. Olfactometry by use of systematic tests of smell function [24] was within normal limits for each subject.

Patients: Forty-four patients, aged 10-88 y, 56 ± 3 y (Mean ± SEM) who presented to The Taste and Smell Clinic in Washington, DC for evaluation and treatment of smell dysfunction were also subjects of the study. Patients reported presence of smell loss six months to 10 years (5 ± 2 y) prior to their initial clinic visit. Patients were selected consecutively from patients evaluated at The clinic from 2012-2013. Patients were 24 men, aged 12-88 y, 54 ± 4 y and 20 women, aged 10-84 y, 51 ± 5 y. All patients stated that they had a smell loss. Olfactometry by use of systematic standardized tests of smell function indicated significant smell acuity impairment with increased detection and recognition thresholds (loss of sensitivity) and decreased magnitude estimation for each of four odors (pyridine, nitrobenzene, thiophene and amyl acetate) [24]. One patient not only had acuity loss but also dysgeusia (distorted taste sensitivity) and oropyrosis [26].

Patients exhibited six etiologies related to their smell loss: post-influenza-like hyposmia (PIHH) [27] (10 patients), allergic rhinitis [28] (15 patients), congenital hyposmia [29] (nine patients), head injury [30] (eight patients), post general anesthesia [31] (one patient) and dysgeusia and oropyrosis [26] (one patient). Patients were not taking any medications at the time this study was performed.

Physical examination of the upper airways by use of anterior rhinoscopy or nasal endoscopy was within normal limits in each patient. Computer tomography or magnetic resonance imaging of brain in each patient revealed the presence of olfactory clefts and bulbs in each patient.

Measurements of Shh in nasal mucus were performed in all normal subjects and hyposmic patients.

Study protocol was approved by the Chesapeake Institute Review Board. Each patient and subject agreed to participate in the study and signed an informed consent participation form.

Biochemical procedures

Patients and volunteers collected all nasal mucus spontaneously produced over a period of 1-4 days into a 50 ml plastic tube. Patients collected all nasal mucus generated spontaneously over each waking hour over each 24 hour period using continuous collection into the 50 ml plastic tube. All samples were refrigerated overnight for collections longer than 24 hours.

Each sample was transferred to a 12 ml plastic tube and centrifuged in a Sorvall RC5C Plus centrifuge at 18,000 rpm for 45-55 minutes. Supernatant was transferred to PCR tubes and stored at 20°C until analyzed.

Each sample was analyzed by use of a sensitive spectrophotometric ELISA technique obtained from Abcam Inc. (Cambridge, MA). Analysis of duplicate samples agreed within 5%. All analyses were made independent of the knowledge of the status of any subject. Only after all samples were analyzed were results tabulated and samples classified in relationship to subject status.

Results were analyzed such that mean ± SEM of nasal mucus Shh levels were obtained with results of normal subjects and hyposmic patients compared using Student t test with $p<0.05$ considered significant. Each set of values was obtained independently and not evaluated or compared until each set was completely analyzed.

Results

Shh was found to be present in nasal mucus in all normal volunteers and in all patients with hyposmia (Table 1). Mean Shh levels in hyposmic patients were significantly lower than in normal subjects. These levels were less than 2% those found in normal subjects (Table 1).

Table 1: Sonic Hedgehog in nasal mucus in normal subjects and in patients with Hyposmia.

Subjects	Sonic Hedgehog*
Normals (14)	7538 ± 1105^{+}
Patients (44)	149 ± 2^{A}
( ) Subject Number; * In Pg/Ml; $^{+}$ Mean ± SEM with respect to normal; ^{A} P<0.001	

Mean Shh levels in patients with various etiologies related to the cause of their smell loss varied widely (Table 2).

Table 2: Sonic Hedgehog in nasal mucus in patients with Hyposmia classified by etiology of smell loss.

Subjects	Sonic Hedgehog*
Etiology of Hyposmia (44)	
Post-Influenza-Like Hyposmia (10)	1537 ± 159$^{+,A}$
Allergic Rhinitis (15)	34 ± 2^{A}

Congenital Hyposmia (9)	180 ± 12[A]
Head Injury (8)	1396 ± 252[A]
Dysgeusia With Oropyrosis (1)	226
Post General Anesthesia (1)	1.3
( ) Subject number; * in pg/ml; + Mean ± SEM; with respect to normal levels (Table 1) [A] p<0.001	

The patient with hyposmia following general anesthesia exhibited the lowest level of any patient followed in order by patients with allergic rhinitis, congenital hyposmia, the patient with dysgeusia and oropyrosis, head injury and PIHH. Mean Shh levels of patients were significantly lower than in normal subjects.

Characterized by age there is a consistent decrease in nasal mucus Shh in hyposmic patients as they age (Table 3).

Table 3: Sonic Hedgehog in nasal mucus in patients with Hyposmia categorized by age.

Age [Y]	Sonic Hedgehog*
10-30 (9)	235 ± 97[+]
31-50 (9)	53 ± 25
51-70 (17)	36 ± 19
71-90 (9)	23 ± 14
( ) Subject Number; * In Pg/Ml; + Mean ± Sem	

Discussion

Hedgehog proteins belong to a family of extracellular signaling molecules involved in regulation of multiple physiological processes including invertebrate and vertebrate embryo development [32]. Vertebrate organisms express multiple forms of hedgehog. In mammals there are three hedgehogs-Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) [33]. Shh plays an important role in several developmental processes involving induction of dopaminergic [34] and cholinergic neurons [35], development of the retina [36] and in activating neural stem cells [37].

Shh is synthesized as a 45 kD precursor protein that is cleaved auto catalytically to yield a 20 kD N-terminal fragment with a cholesterol molecule covalently attached to the C-terminal glycine and a 25 kD C-terminal fragment [38]. Its crystal structure has been determined [39]. It is structurally homologous to several zinc-dependent hydrolases [40] and it contains one zinc atom coordinated by two histidines and a glutamate residue [41] similar to the presence of zinc in the structure of the taste bud growth factor gustin (carbonic anhydrase (CA) VI) [42]. Elimination of zinc from either Shh or CA VI inhibits their respective activity [41,42]. CA VI and zinc components have also been shown to play a role in adenylyl cyclase metabolism by acting as a potent activator of phosphodiesterase [43].

Shh has been shown to play a regulatory and signaling role in organisms as diverse as drosophila [33] and Caenorhabditis elegans [44] as in mammalian systems [18-21]. It has been shown to play a regulatory and structural role in multiple sensory systems including hearing [45], involving the cochlea [46], vision, involving retinal receptors [47], taste, involving basal cell function of taste receptors [18,19] and growth of taste papillae [20]. Disruption of Shh signaling causes inhibition of growth and patterning of these sensory receptors [18-20]. While there have been no studies dealing with Shh in olfactory function per se multiple studies involving the primary essential role of Shh in cilia assembly and regulation have been published [48,49]. Interference of Shh signaling in these systems inhibited cilia function. Shh has also been shown to play a role in neurogenesis and functional plasticity in multiple neuronal circuits [50] including coordination of growth of patterning of several brain structures, including the cerebellum [51] and olfactory bulb [52].

Results of the present study indicate that Shh is present in nasal mucus in both normal subjects and in patients with hyposmia. Levels of nasal mucus Shh in hyposmic patients were significantly lower than in normal subjects. Levels of nasal mucus Shh decrease with age. These results are consistent with reports of decreased olfactory acuity as people age [53]. Measurements of lower than normal nasal mucus levels of Shh in hyposmic patients may serve as a marker for the presence of smell loss in hyposmic patients.

The study has several limitations. The number of normal subjects and hyposmic patients studied was relatively small. In addition, results within each subject category were variable. However, despite the relatively small number of subjects and their variability differences between normals and patients were significant and reflect the presence of Shh in nasal mucus in both groups of subjects.

Low levels of nasal mucus Shh are present in patients with the major pathologies responsible for hyposmia; as such, low levels of nasal mucus Shh may reflect a chemical biomarker by which these patients can be identified. This measurement is important since it can assist in the diagnosis of hyposmia in an objective manner.

Conclusion

This is the first systematic demonstration of Shh in nasal mucus. Its presence is consistent with its role as a cell signaling moiety and growth factor involved in olfactory function. Nasal mucus Shh may serve as a chemical biomarker to identify patients with hyposmia of various etiologies on an objective basis and to assist in defining the biochemical cause of their loss.

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There are no sources of funding to declare.

Conflict of Interest

Robert I. Henkin is a member of the board of directors of Cyrano Therapeutics. None of the other authors has a conflict of interest, financial or otherwise with respect to the publication of this manuscript

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