The glosopharyngeal nerve controls epithelial expression of Sprr2a and Krt13 around taste buds in the circumvallate papilla

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HIGHLIGHTS

• Sprr2a is expressed in the subsurface epithelium including the taste pore area.
• Krt13 expression is at a very low-level in the vicinity of taste buds.
• Denervation downregulates Sprr2a and upregulates Krt13 around the taste buds.
• The expression of Sprr2a and Krt13 around taste buds is controlled by taste nerve.

ABSTRACT

Tastants reach the tip of taste bud cells through taste pores which are openings in the epithelium. We found Sprr2a is selectively expressed in the upper layer of the epithelium surrounding taste buds in the circumvallate papilla (CV) where the epithelium is organized into taste pores. Sprr2a is a member of a small proline-rich protein family, which is suggested to be involved in the restitution/migration phase of epithelial wound healing. The expression of Sprr2a was restricted to the upper layer and largely segregated with Pch1 expression that is restricted to the basal side of the epithelium around the taste buds. Denervation resulted in the gradual loss of Sprr2a-expressing cells over 10 days similarly to that of taste bud cells which is in contrast to the rapid loss of Pch1 expression. We also found that denervation caused an increase of Keratin (Krt)13 expression around taste buds that corresponded with the disappearance of Sprr2a and Pch1 expression. Taste buds were surrounded by Krt13-negative cells in the CV in control mice. However, at 6 days post-denervation, taste buds were tightly surrounded by Krt13-positive cells. During taste bud development, taste bud cells emerged together with Krt13-negative cells, and Sprr2a expression was increased along with the progress of taste bud development. These results demonstrate that regional gene expression surrounding taste buds is associated with taste bud formation and controlled by the innervating taste nerve.

1. Introduction

Taste buds are an onion-shaped cell aggregate composed of a heterogeneous cell population. Taste cells possess neuronal properties, produce action potentials and release neurotransmitters selectively in response to particular taste stimuli [1,2]; also one type of cell (Type I cell) within the taste bud is glial-like, wraps neighboring cells and inactivates the taste cell neurotransmitter [3]. In spite of its neuronal and glia-like properties, taste buds are epithelial origin [4] and are maintained by continuous cell renewal throughout life [5–7]. Taste bud cells share precursor cells with surrounding epithelium, and keratin14 (Krt14)-positive basal epithelial cells residing in the vicinity of taste buds give rise to both taste buds and its surrounding epithelium [8].

The epithelial cells surrounding taste buds are specialized to form taste pores, openings of the surface layer of the keratinized epithelial cells through which tastants are able to reach the microvilli of taste cells. During development the organization of
the taste pore structure progresses along with the maturation of the taste bud [9] and depends upon taste nerve innervation [10]. Although the coordination of cell differentiation between the taste bud and its surrounding epithelium is important for the detection of taste stimuli, information on the molecules associated with the specification of the epithelial cells surrounding the taste buds is limited. The downstream molecules of Sonic hedgehog signaling, including Ptc1 [11] and GlI1 [12,13], are known to be selectively expressed in the surrounding epithelium. However, no molecular information directly linked to keratinocyte properties is available.

In this report, we compare the expression of Ptc1 with both the regional expression of Spr2a, a member of the small proline-rich protein family that contributes to the barrier function of squamous epithelia [14], and Krt13-negative cells that surround taste buds. The nerve-dependence and developmental expression of these molecules are assessed.

2. Materials and methods

2.1. Animals and nerve surgery

C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). Adult mice (8–12 weeks old) and newborn mice (0.5–10 days after birth) were used. All experimental procedures were approved by the institutional animal care and use committees of National Food Research Institute (NARO) and Kagoshima University before the onset of the experiments.

For nerve surgery, adult mice were anesthetized with pentobarbital sodium (50 mg/kg b.w. i.p.). The glossopharyngeal (GL) nerves were approached via a midline incision over the larynx, and were transected in the neck under the digastic muscle [15].

2.2. RNA extraction

Ringer’s solution containing 2.5 mg/ml Collagenase type IV and 2 mg/ml Elastase (Worthington Biochemical, Lakewood, NJ) was injected beneath a circumvallate papilla. After 20 min incubation at room temperature, about 7–10 mm square area of the tongue epithelium containing the CV was peeled off. The epithelium was dissected into the CV region and to its surrounding that is devoid of taste buds. Total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA).

2.3. Cloning and identification of Spr2a

Differential display (DD) analysis was conducted with total RNA extracted from the CV region of control adult mice and mice at 10 days after bilateral GL transection using a Beckman Coulter Fluorescence DD System (genomyxLRS). First strand cDNA synthesis and following Fluoro DD-PCR were performed with a HIEROGLYPH kit (Genomyx, Foster city, CA), and PCR products were separated on a 6% polyacrylamide gel for 2 h. Differentially expressed bands were recovered from the gel and cloned into pGEM-T vector (Promega, Madison, WI) followed by sequencing and in situ hybridization analysis. Spr2a was found among the 40 cDNA clones analyzed. Simian taste bud-specific gene (Stg) [16] and Keratin 13 (Krt13) were also observed among these clones.

2.4. Real-time PCR

Total RNA extracted from 6 adult mice was combined into one sample. cDNA was synthesized with 0.4 μg total RNA from the CV and its surrounding region using Superscript III (Invitrogen). Real-time PCR analysis was performed using specific primers for Ge-gustducin, Krt13, Ptc1, Spr2a and Stg, β-actin was used as an internal control. The list of primers is shown in Table 1. Real-time PCR reactions were performed using FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) on a LightCycler Nano (Roche Diagnostics). The PCR assay was repeated three times. Melting curve analysis after each assay confirmed the specificity of each primer set.

2.5. In situ hybridization

Tongues were excised, placed in embedding compound and rapidly frozen on dry ice. Tissues were sectioned at 5 μm for single-color and 10 μm for double-color detection. In situ hybridization was performed as described previously [15]. cRNA probes were transcribed in vitro with digoxigenin (Dig)- or fluorescent (Fluo) UTP using an RNA transcription kit (Roche diagnostics) from one of the following cDNA: Stg, Spr2a, Ptc1 and Krt13 (see Table 1 in detail). Single color detection was performed using BCIP/NBT (Roche Diagnostics) after hybridization with a Dig-labeled probe and immunoreaction with alkaline phosphatase (AP) conjugated anti-Dig antibody (Roche Diagnostics). For double color detection, a peroxidase (POD) conjugated anti-Fluo antibody (Roche Diagnostics) and an AP conjugated anti-Dig antibody were used after hybridization with Dig- and Fluo-labeled probes. HNPP/FastRed (Roche Diagnostics) was used as a substrate for AP, and Streptavidin-Alexa 488 (Molecular Probes, Eugene, OR) was used for detection of POD activity after reaction with Tyramide-biotin (Perkin-Elmer Life science, Boston, MA). In situ hybridization with sense probes was performed as a negative control, no specific signal was found with any of the sense probes. Images were observed using a Leica DM-IRB fluorescence microscope (Germany) and composed in Adobe Photoshop (San Jose, CA) by adjusting the brightness and contrast.

3. Results

3.1. Expression of Spr2a and Krt13 in the CV

Comparison of gene expression using differential display-RT-PCR in the CV epithelium between control mice and mice at 10 days post glossopharyngeal (GL) nerve transection provided Spr2a as a new candidate molecule that is expressed GL nerve-dependently. Stg, previously reported to be specifically expressed in taste buds [16], was also observed. However, Krt13 was negatively correlated
with GL innervation. Fig. 1A and B shows the result of in situ hybridization (ISH) of Sprr2a, Stg and Krt13. Sprr2a was expressed in the upper layer of the epithelium surrounding the taste buds. Stg was strongly expressed within taste buds as previously reported [16], but its weak expression was also noted in the uppermost layer of the epithelium. Krt13 expression was widely observed in the intermediate layer of the tongue epithelium, but not in the region adjacent to taste buds. Real-time PCR analysis confirmed the selective expression of Sprr2a and Stg in the CV (Fig. 1C).

3.2. Regional gene expression surrounding taste buds and its nerve dependence

The expression of Sprr2a was compared with that of Ptch1 by double-color ISH (Fig. 2a). Ptch1 expression was observed on the basal side of the epithelium surrounding taste buds as previously reported [11] and segregated with Sprr2a expression. Oval dark areas surrounded by the expression of Sprr2a and Ptch1 are corresponding to taste buds. Stg and Krt13 double detection showed that the cells adjacent to taste buds are Krt13-negative (Fig. 2b). Double-color detection of Stg and the combination of Sprr2a and Ptch1 demonstrated that both genes are expressed in the cells adjacent to, but not within, taste buds (Fig. 2c).

Transsection of the GL nerve resulted in the disappearance of taste buds as demonstrated by the decrease of Stg-expressing cells (Fig. 2b1–b3), accompanied with the loss of expression of Sprr2a and Ptch1 around taste buds (Fig. 2a1–a3). The time course of the decrease of Sprr2a after denervation was similar to that of Stg. At 12 days post surgery, a few remaining taste bud cells were observed scattered in the trench wall of the CV (Fig. 2b3), and Sprr2a expression was also detected in a few cells surrounding an Sprr2a-negative area corresponding to the remaining taste bud cells (Fig. 2a3). The decrease of Ptch1 expression was much more rapid than that of Sprr2a and Stg as reported previously [15]. Ptch1 was markedly decreased at 2 days and undetectable at 12 days post surgery. In contrast, immediately after denervation the expression of Krt13 markedly increased around taste buds. Although Krt13-negative cells were still noted adjacent to the thinning taste buds on the second day after surgery (Fig. 2b1), taste buds were closely surrounded by the cells expressing Krt13 intensely at 6 days (Fig. 2b2).

3.3. Expression of Stg, Krt13, Ptch1 and Sprr2a during taste bud development

Stg-expressing taste bud cells were rare at birth reflecting that taste buds generally develop after birth in the CV (Fig. 3a), and Sprr2a-expressing cells were rarely observed (Fig. 3c). In contrast, Ptch1 was intensely expressed in the basal layer of the CV epithelium (Fig. 3c). Weak expression of Stg was noted over the uppermost layer of the epithelium of the entire CV region at birth (Fig. 3a and b), but was restricted to the trench walls by 2.5 days after birth (Fig. 3d and e) as in adults (Fig. 1A). Intense Krt13 expression was clearly detected in the epithelium of the CV except for its top center region at birth (Fig. 3a), but was noted there by 2.5 days (Fig. 3d). The epithelial cells adjacent to Stg-expressing cells were consistently negative for Krt13 during development.

At 2.5 days after birth, several Stg-expressing cell clusters, developing taste buds, were evident (Fig. 3d), and Sprr2a-expressing cells emerged in the upper layer of the CV trench epithelium (Fig. 3f). By 10.5 days after birth, the expression pattern of Sprr2a, Krt13 and Ptch1 around taste buds became similar to that in adults (Fig. 3g–i), and the expression of Sprr2a and Ptch1 shaped dark oval area corresponded to taste buds (Fig. 3i).
4. Discussion

Our results provide the first molecular evidence that the stratified maturation of epithelial cells surrounding taste buds is associated with taste bud formation and controlled by the taste nerve. Epithelial tissues undergo continuous cell turnover, and the stratified maturation of epithelial cells progresses from the proliferating basal layer to the upper layer [17]. Although Pch1 and Gli1 was reported to be selectively expressed in the basal layer of the epithelium surrounding taste buds [11–13], the specificity of epithelial cell differentiation around taste buds remained unclear. Here we show the selective expression of Sprr2a in the middle to upper layer and Krt13-negative area in the middle layer, both of which were taste nerve-dependent.

Previous studies described the specific morphological properties of the epithelial cells sitting just around taste buds and referred these cells as marginal cells or type V cells [18]. Immunohistochemical studies showed a difference in keratin and CD44 within and external to taste buds [19,20]. However, antibodies staining the epithelium external to the taste buds were not able to show the difference between the marginal cells and the other epithelial cells outside taste buds, while the expression of two isoforms of CD44 (CD44v6 and 9) in the basally situated marginal cells was more intense than in other surrounding cells. Sox2 was recently reported to be expressed in not only taste bud cells but also perigemmal cells in the CV [21], while the selective expression of Sox2 is believed to be necessary for the cell-fate determination of bipotential precursors to taste bud cells [8,22,23]. The expression of Sox2 in perigemmal cells in the CV was shown to be dependent on gustatory innervation. This implies Sox2 expressing perigemmal cells share precursors with taste bud cells. However, no molecular information directly linked to keratinocyte specificity around taste buds was available.

Particular pairs of acidic and basic keratins are selectively expressed in the epithelium depending upon the tissue type and also upon the maturation stage [17]. In the oral mucosa, such as the tongue, Krt5/14 is expressed in the basal proliferating layer, and Krt4/13 occurs in the descendant suprabasal layer. However, although Krt14-expressing cells surrounding taste buds were shown to include bipotential precursors giving rise to both taste buds and surrounding epithelium [8], we here show that Krt13 is negative outside taste buds. The cells negative for Krt13 in the vicinity of taste buds are likely to share precursors with taste bud cells and may express Sox2. The rapid loss of Krt13-negative cells
after denervation seems to reflect the cessation of cell supply from the bipotential precursors and is consistent with the rapid cell turnover recently reported in this region [7]. Although Krt13 was uniformly expressed in the CV epithelium after taste bud degeneration, it is not clear if denervation caused the differentiation of Krt13-expressing cells from the Krt14-expressing bipotential precursor cells.

The stratification and cornification of the epithelium varies among tissues, providing different types of barriers. The small proline-rich proteins (Sprrs) are expressed in terminally differentiating keratinocytes and constitute cornified cell envelope precursors that crosslink with other proteins, such as loricurin [24]. The expression of Sprr2a, a member of Sprr family, around taste buds may contribute to a specific barrier function to maintain taste receptor cells viable by protecting them from various chemical and physical stresses in the oral cavity. A number of studies described the distribution and function of the Sprr family members [24], and it was recently reported that Sprr2a functions as a suppressor of p53-dependent transcriptional activity [25]. It is suggested that the function of Sprr2a as the suppressor contributes to epithelial mesenchymal transition, a process essential for the restitution/migration phase of epithelial wound healing. Sprr2a around taste buds may be involved in remodeling of the epithelium to generate and maintain the taste pore.

In contrast to the rapid loss of Krt13-negative area surrounding the taste bud after denervation, Sprr2a was detected even at 12 days after denervation similar to Stg. This suggests that the expression of Sprr2a is not directly regulated by the taste nerve. The expression of Sprr2a may be dependent on some factors from taste bud cells or alternatively retained autonomously during the life span of cells after being differentiated nerve-dependently from the bipotential precursors. Several promoter sequences were reported in the Sprr2a gene [24]. Identification of the Sprr2a promoter sequences responsible in this region may lead to the elucidation of the mechanisms that govern taste pore formation.

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