Histochemical changes and apoptosis in degenerating taste buds of the rat circumvallate papilla*

Yasuo Ichimori, Katsura Ueda, Hiroyuki Okada, Shiho Honma, and Satoshi Wakisaka

Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan

Summary. The present study was designed to examine the histochemical changes and occurrence of apoptosis in taste buds of rat circumvallate papillae following bilateral transection of the glossopharyngeal nerve. Following transection of the glossopharyngeal nerve, the number of taste buds was not altered until postoperative day 3 (PO3), but decreased significantly thereafter. The number of cells within a taste bud, however, decreased significantly from PO2. In normal, uninjured animals, approximately 15.4%, 9.0%, and 7.7% of taste bud cells were labeled with antibodies for phospholipase C β2 subunit (PLCβ2), a marker for type II cells, neural cell adhesion molecule (NCAM), a marker for type III cells, and Jacalin, a marker for type IV cells, respectively. Following gustatory nerve injury, the ratio of cells expressing markers of type III and type IV decreased gradually from PO2, and Jacalin-labeled taste bud cells disappeared on PO3. Under normal conditions, immunoreactivity for single-strand DNA (ssDNA), a marker of apoptosis, was detected in the nuclei of PLC β2-immunoreactive cells and cells showing no labeling for PLCβ2, NCAM, or Jacalin. On PO1, the number of taste bud cells showing ssDNA immunoreactivity increased to double that of normal uninjured animals; these ssDNA-immunoreactive cells were also labeled with NCAM and Jacalin as well as PLCβ2. The present results suggest that denervation of the gustatory nerve causes apoptosis in all types of taste bud cells, resulting in the rapid degeneration of taste buds.

Address for correspondence: Prof. Satoshi Wakisaka, Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry, 1-8, Yamadaoka, Suita, Osaka, 565-0871 Japan
Tel: +81-6-6879-2871; Fax: +81-6-6879-2875
E-mail: wakisaka@dent.osaka-u.ac.jp

Introduction

The taste bud, a peripheral chemoreceptor perceiving gustatory stimuli, contains 50–80 spindle-shaped cells (taste cells) as well as proliferative round basal cells (progenitor cells). Morphologically, spindle-shaped taste cells are traditionally classified into dark and light cells. Histochemically, dark cells, or "type I cells", have an enzymic activity for carbonic anhydrase isoform II (CA II; Daikoku *et al.*, 1999) and nucleoside triphosphate diphosphohydrolase-2 (NTPDases-2; Bartel *et al.*, 2006), and immunoreactivity for glial glutamate/aspartate transporter (GLAST; Lawton *et al.*, 2000). Light cells are further divided into type II and type III cells based on the presence or absence of synaptic connections with gustatory nerves. Type III cells contain many synaptic vesicles and have synapses with gustatory nerves while type II cells do not (Murray *et al.*, 1967, 1969; Takeda and Hoshino, 1975; Yoshie *et al.*, 1990). Although type II cells do not have apparent synapses, both type II and type III cells are considered to act as taste receptor cells because of their histochemical properties. Type II cells are immunoreactive for α-gustducin (a taste-specific G protein), phospholipase C β2 (PLCβ2), and type III

inositol 1,4,5-triphosphate receptor (IP3R3), all of which are involved in the transduction cascade downstream of taste stimuli (Boughter *et al*., 1997; Cho *et al*., 1998; Yang *et al*., 2000b; Clapp *et al*., 2001, 2004; Miyoshi *et al*., 2001). Type III cells show neuronal features and are immunoreactive for protein gene product 9.5 (PGP 9.5), the synapse-associated protein of 25kDa (SNAP-25), a neural cell adhesion molecule (NCAM), and serotonin (5HT) (Nelson and Finger, 1993; Smith *et al*., 1993; Kanazawa and Yoshie, 1996; Yang *et al*., 2000a; Yee *et al*., 2001; Miura *et al*., 2005). Type IV cells are round, located at the basal portion of the taste buds, express sonic hedgehog mRNA (Miura *et al*., 2003, 2004), and bind to Jacalin (Taniguchi *et al*., 2005); these cells function as progenitor cells.

It is known that the development and maintenance of mammalian taste buds is highly dependent on gustatory innervation: nerve fibers penetrate into the gustatory epithelium prior to the formation of taste buds during development (Torrey, 1940; Farbman, 1965; AhPin *et al*., 1989; Wakisaka *et al*., 1996), and transection of the gustatory nerve results in the degeneration of taste buds (Farbman, 1969; Kennedy, 1972; Hosley *et al*., 1987). Although many studies have focused on the histochemical properties of each cell type in the taste buds of normal adult animals, few studies have attempted to elucidate the changes in the proportions of each cell type in degenerating taste buds caused by injury to gustatory nerves.

It is reported that the life span of taste buds is around 10 days; thus, a rapid continuous turn-over of taste bud cells occurs (Beidler and Smallman, 1965; Farbman, 1980). Although there is disagreement on the cell-lineage of taste bud cells, recent studies have shown that taste bud cells die by apoptosis under normal conditions and after denervation (Takeda *et al*., 1996; Zeng and Oakley, 1999; Zeng *et al*., 2000; Huang and Lu, 2001). Our previous study (Ueda *et al*., 2008) showed that apoptosis occurs in type II cells and probably also type I cells under normal conditions, as revealed by single-stranded DNA (ssDNA) immunohistochemistry. However, it is unknown whether other type(s) of cells die by apoptosis following the denervation of gustatory nerves. The present study therefore examined the histochemical changes and occurrence of apoptosis in degenerating taste buds of rat circumvallate papillae following gustatory denervation by combined ssDNA immunohistochemistry and histochemistry for cell type-specific markers.

Materials and Methods

All experimental procedures were reviewed and approved by the Intramural Animal Use and Care Committee at the Osaka University Graduate School of Dentistry.

Animals and tissue preparation

A total of 32 male Sprague-Dawley rats, weighing 200–250 g, were used in the present study. The animals were anesthetized with chloral hydrate (400 mg/kg, i.p., supplemented as necessary), and an incision was made on the midline of the ventral neck skin. The submandibular and major sublingual glands as well as the posterior half of the digastric muscles were retracted. The glossopharyngeal nerve was identified at the medial side of the hypoglossal nerve beneath the carotid artery and transected. Sutures were made by anatomical layers. The animals were allowed to survive for 1, 2, 3, 4, 6, and 8 days following axotomy (PO1, PO2, PO3, PO4, PO6, and PO8, respectively; at least n=4 for each period). Animals without any treatment (n=4) served as normal controls. After survival for appropriate periods, the animals were deeply anesthetized with chloral hydrate (600 mg/kg, i.p.), and transcardially perfused with 0.02 M phosphate-buffered saline (PBS; pH 7.2) followed by a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in a 0.1 M phosphate buffer (PB; pH 7.2). The circumvallate papillae were carefully removed and immersed in 4% paraformaldehyde in 0.1 M PB for at least 48 h. After soaking in 20% sucrose in PBS overnight for cryoprotection, the tissues were embedded in Tissue Tek OCT compound (Sakura Finetechnical, Tokyo) and frozen with liquid nitrogen. Frozen sections were cut transversely at a thickness of 16 μm, collected in cold PBS, and treated as free-floating sections.

Histochemistry

To label the taste buds, we applied lectin histochemistry for Ulex europaeus agglutinin-I (UEA-I) according to our previous study (Taniguchi *et al*., 2004). Briefly, floating sections were incubated with Texas Red (TR)-conjugated UEA-I (0.5 μg/m*l*; E-Y Laboratories, San Mateo, CA) for 2 h at room temperature. After a brief incubation with 4',6-diamino-2-phenylindole (DAPI) for 10 min and washing in PBS, sections were mounted onto gelatin-subbed glass slides and coverslipped.
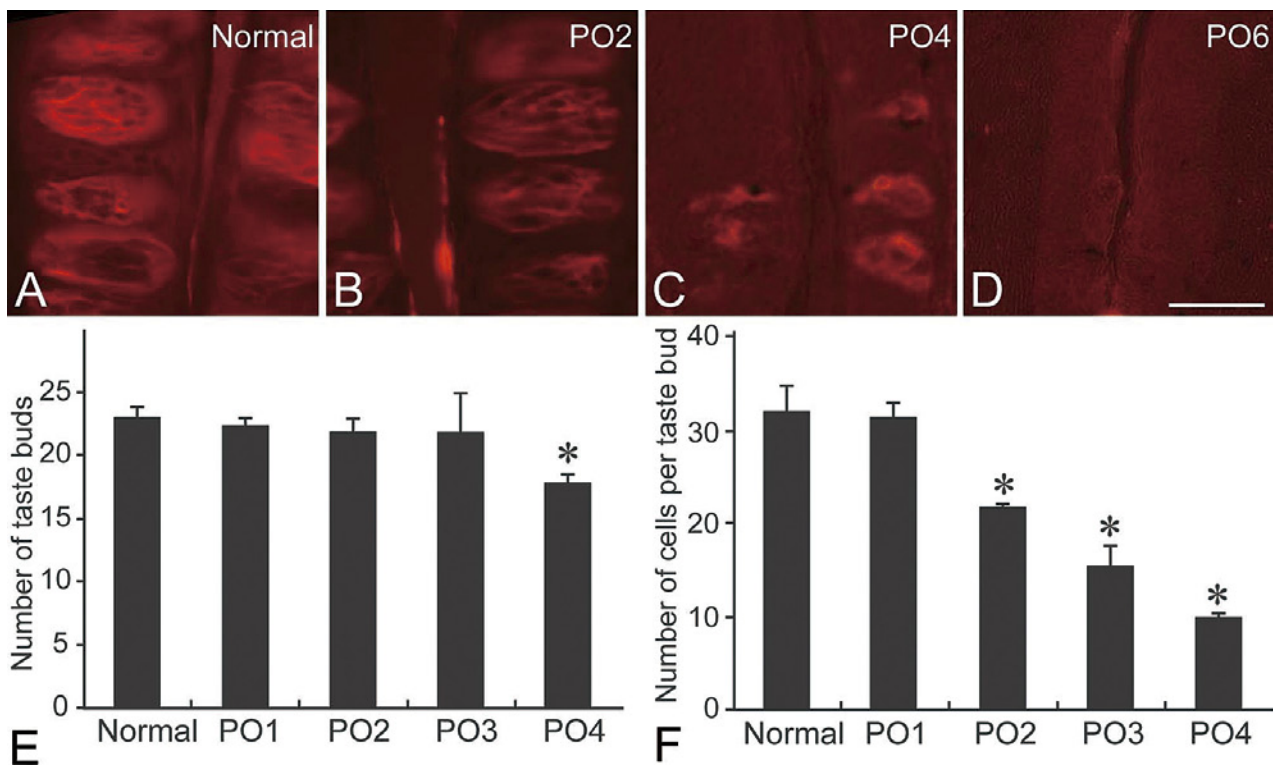
Fig. 1. A–D: Photographs of UEA binding in taste buds in normal animals and surgically injured animals on post-operative days (PO) 2, 4, and 6 following bilateral transection of the glossopharyngeal nerve. **A:** The binding of UEA-I is seen in the membranes of the entire taste buds of normal animals. **B:** On PO2, the number and morphology of UEA-I-labeled taste buds are almost identical to those in normal animals. **C:** The number of UEA-I-bound taste buds has decreased, and these taste buds show irregular outlines on PO4. **D:** No taste buds are detected on PO6. The scale bar in D represents 50 μm, and also applies to A–C. **E, F:** Temporal changes in the number of taste buds per section (**E**) and the number of cells per taste bud (**F**) following bilateral axotomy of the glossopharyngeal nerve. **E:** The number of taste buds is not changed until PO3, and is significantly decreased on PO4. **F:** The number of cells within a taste bud significantly decreases from PO2. * $P<0.01$ compared with normal uninjured controls.

Double-labeling experiments

Double-labeling experiments for ssDNA and PLCβ2 (a marker for type II cells), NCAM (a marker for type III cells), or Jacalin (a marker for type IV cells), were carried out as previously reported (Ueda *et al*., 2008). Briefly, sections were incubated with PBS containing 0.3% H_2O_2 to inactivate endogenous peroxidase activity for 30 min, and then incubated with a rabbit polyclonal antibody against ssDNA (1:500; DAKO, Copenhagen, Denmark) for 16–18 h at room temperature. After several rinses with PBS, the sections were incubated with biotinylated swine anti-rabbit IgG (1:500; DAKO) and subsequently with the ABC complex (Vector, Burlingame, CA) for 90 min each at room temperature.

Peroxidase activity was visualized by incubation with 0.04% 3-3'diaminobenzidine in 0.05 M Tris-HCl-buffered saline (TBS; pH 7.5) containing 0.003% H_2O_2 with nickel ammonium sulfate (0.08–0.1%) enhancement. After the detection of ssDNA as described above, the sections were incubated with rabbit polyclonal anti-PLCβ2 (1:500; Santa Cruz Biotechnologies, Santa Cruz, CA) or rabbit polyclonal anti-NCAM (1:1000; Chemicon International, Temecula, CA) for 16–18 h, and then with Alexa Fluor 488-conjugated anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR) for 90 min at room temperature. For the double-labeling of ssDNA and Jacalin, ssDNA-immunostained sections were incubated with biotinylated Jacalin (0.5 μg/ml; Vector) for 14–16 h and then with Alexa Fluor 488-conjugated streptavidin (1:500;
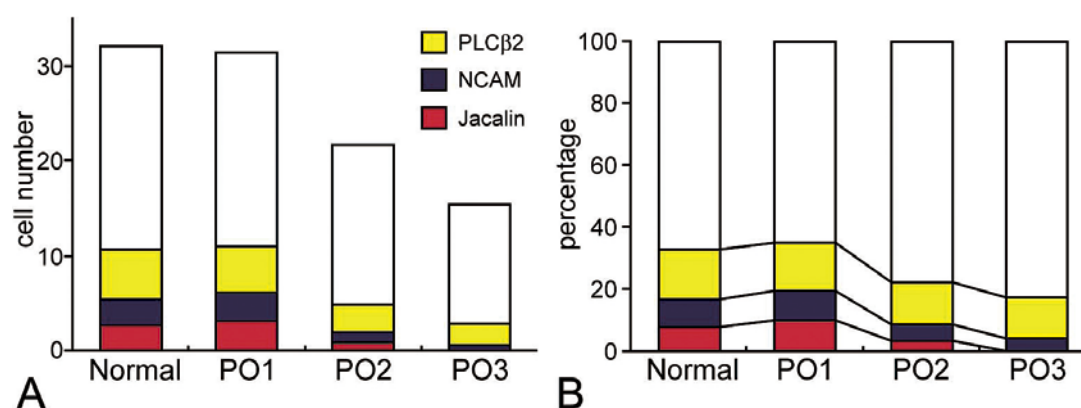
Fig. 2. Temporal changes in the numbers (**A**) and relative proportions (**B**) of cells showing PLCβ2 (marker for type II cell), NCAM (marker for type III cell), and Jacalin (marker for type IV cell) immunoreactivity following bilateral axotomy of the glossopharyngeal nerve.
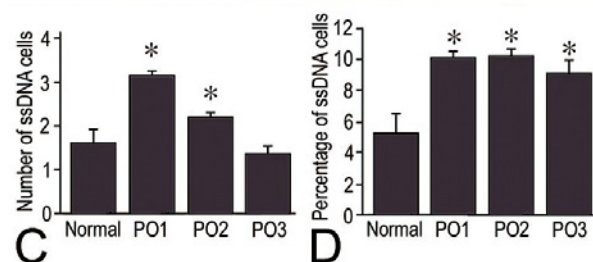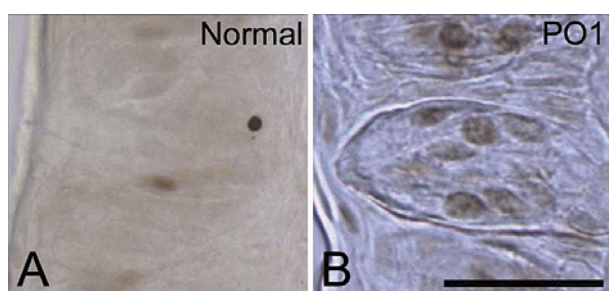
Fig. 3. A, B: Photographs of ssDNA immunoreactivity in the taste buds of normal animals (**A**) and injured animals at PO1 (**B**). **A:** A few ssDNA immunoreactive nuclei are detected in normal taste buds. **B:** Many cells with ssDNA immunoreactive nuclei are recognized on PO1. **C, D:** Temporal changes in the numbers (**C**) and relative proportions (**D**) of ssDNA-immunoreactive cells per taste bud following bilateral axotomy of the glossopharyngeal nerve. **C:** The number of ssDNA-immunoreactive cells increases drastically on PO1 and then decreases gradually. **D:** The percentage of ssDNA-immunoreactive cells is two times higher in injured animals on PO1 than in normal uninjured controls and remains constant thereafter. *$p<0.01$ compared with normal uninjured controls.

Molecular Probes) for 90 min at room temperature. All sections were mounted with Vectashield (Vector), and viewed under a fluorescence microscope (Axioskop 2 Plus).

For quantitative analysis, the total numbers of ssDNA-immunoreactive cells and those labeled for PLCβ2, NCAM, or Jacalin were counted in 15 randomly selected sections that were at least 40 μm apart from each other to avoid duplicate counting.

Results

Number of taste buds and taste bud cells in degenerating circumvallate papilla

In normal animals, UEA-I bound the membranes of entire taste buds, exhibiting an oval appearance (Fig. 1A). On PO1, the binding pattern of UEA-I was comparable to that in normal animals. On PO2, the number of taste buds labeled with UEA-I was not different from that on PO1 (Fig. 1B). Although the long axis of taste buds in injured animals, that is, the width of the trench epithelium, was comparable to that in normal animals, the short axis of taste buds became smaller than that in normal animals, resulting in a spindle-like appearance. On PO3, the number and morphology of taste buds were almost identical to those observed on PO2. On PO4, the number of taste buds decreased and those remaining had irregular outlines (Fig. 1C). On PO6 and thereafter, almost all taste buds disappeared, and the width of the trench epithelium became thicker (Fig. 1D).
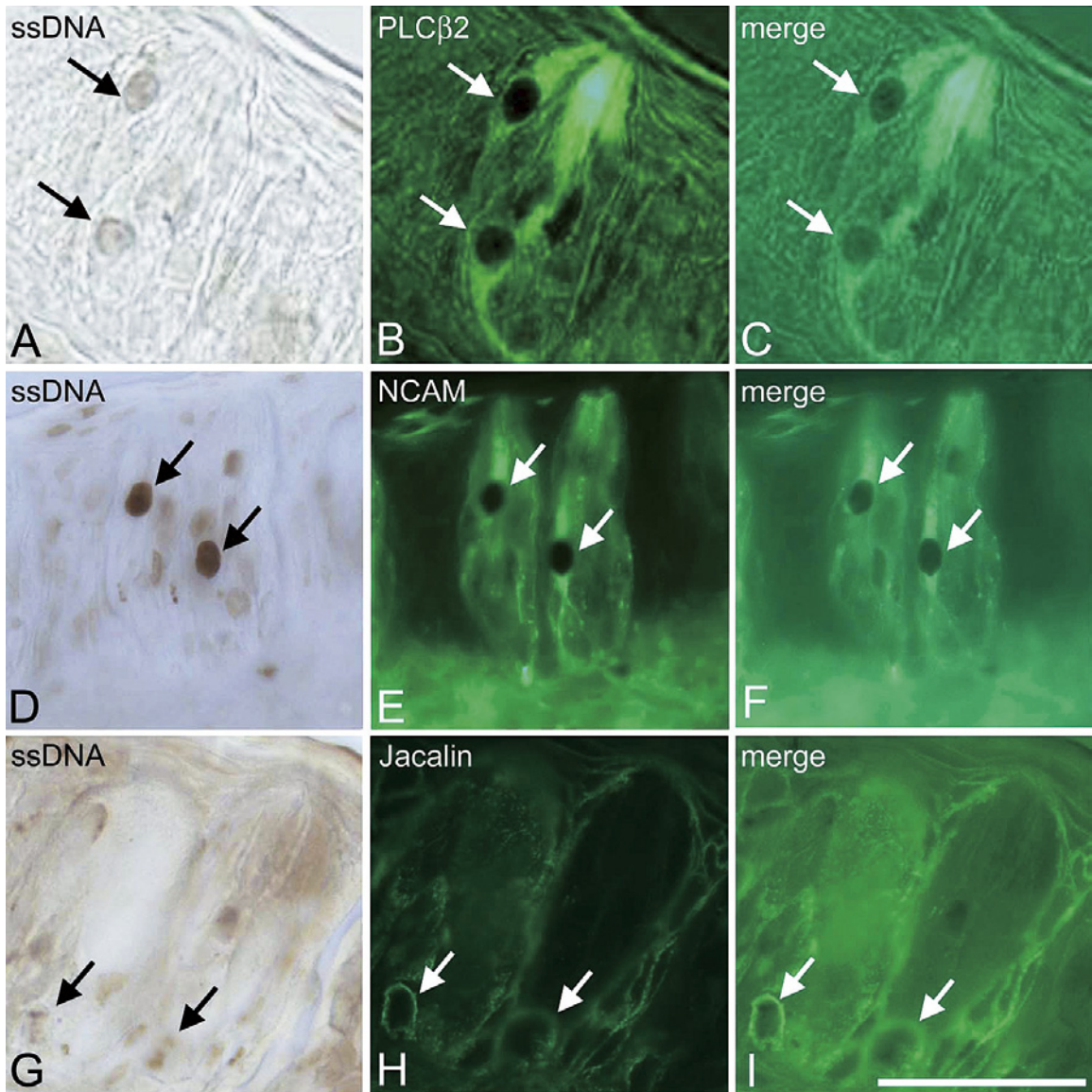
Fig. 4. Double-labeling for ssDNA (**A, D, G**) and PLCβ 2 (**B**), NCAM (**E**), or Jacalin (**H**), and the merged images (**C, F, I**) on PO2. A few ssDNA-immunoreactive nuclei are localized in taste cells showing immunoreactivity for PLC β 2 (**A**–**C**; arrows) or NCAM (**D**–**F**; arrows), and Jacalin (**G**–**I**; arrows). The images in each row are taken from the same section. The scale bar in I represents 50 μm and is applicable to all images.

Figures 1E and F show temporal changes in the numbers of taste buds per section (Fig. 1E) and the numbers of taste bud cells with nuclei per taste bud (Fig. 1F) following bilateral axotomy of the glossopharyngeal nerve. Under normal conditions, animals have 23.4 $\pm$ 1.3 (n=4) taste buds each containing 31.9 $\pm$ 2.8 (n=5) cells. Following denervation, the number of taste buds cells was not altered by PO3, being 22.8 $\pm$ 0.9 (n=4), 22.2 $\pm$ 0.6 (n=5), and 21.8 $\pm$ 1.0 (n=5) on PO1, PO2, and PO3, respectively. The number of taste buds on PO4 was significantly decreased to 17.1 $\pm$ 0.3 (n=5). By contrast, the number of cells within taste buds significantly decreased from PO2 (21.6 $\pm$ 0.4; n=5).
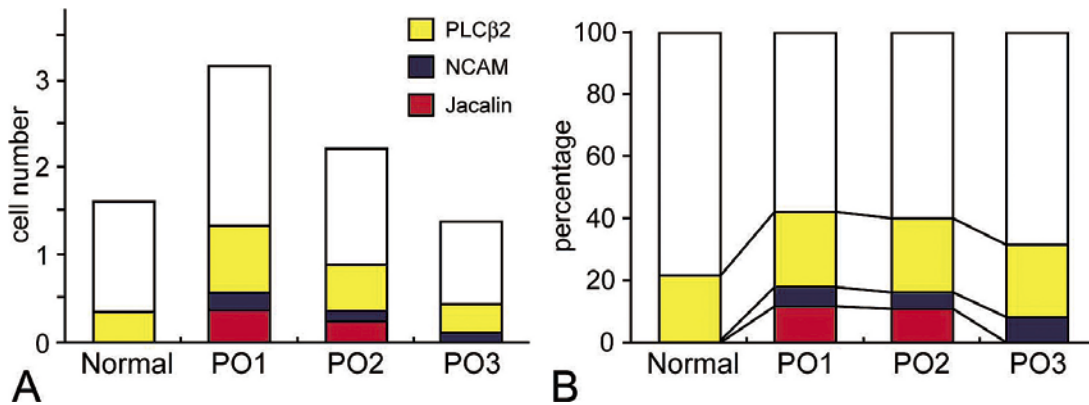
Fig. 5. Temporal changes in the number (**A**) and relative proportions (**B**) of ssDNA-immunoreactive cells immunoreactive for each cell type marker following glossopharyngeal nerve transection. After denervation, ss-DNA immunoreactive nuclei are also found in both subpopulations of NCAM-positive and Jacalin-positive cells.

Proportions of each cell type in degenerating circumvallate papillae

Figure 2 shows the temporal changes in the numbers (Fig. 2A) and relative proportions (Fig. 2B) of PLCβ2-, NCAM-, and Jacalin-labeled cells following bilateral transection of the glossopharyngeal nerve. In normal conditions, approximately 15.4%, 9.0%, and 7.7% of taste buds cells were immunoreactive for PLCβ2 and NCAM and labeled by Jacalin, respectively. There was no apparent difference in the proportions of each cell type marker between the normal condition and PO1. On PO2, the relative proportions of type III and type IV cells decreased, and type III cells could not be identified on PO3 (Fig. 2).

Apoptosis in denervating circumvallate papilla

Immunoreactivity for ssDNA was detected in the nuclei of intragemmal cells in the taste buds. Some ssDNA-immunoreactive nuclei were oval in shape, and others were indented — approximately 1.6 ± 0.3 ssDNA-immunoreactive cells were detected in a single taste bud of normal uninjured control animals (Fig. 3A, C). On PO1, the number of ssDNA-immunoreactive cells increased significantly to approximately 3.1 ± 0.1 cells per taste bud (Fig. 3B, C). ssDNA immunoreactive nuclei were mostly oval in shape. On PO2, approximately 2.1 ± 0.1 cells per taste bud exhibited ssDNA immunoreactivity — this number still being significantly greater than that

in normal animals. On PO3, this value became almost identical to that in normal animals. The ratios of ssDNA-immunoreactive cells to taste buds cells on PO1 increased to double that of normal animals and was constantly thereafter (Fig. 3D).

Double labeling showed that some PLCβ2-immunoreactive cells had ssDNA-immunoreactive nuclei under normal conditions: approximately 21.5 ± 4.8% of ssDNA-immunoreactive cells exhibited PLCβ2 immunoreactivity. Neither NCAM-immunoreactive cells nor Jacalin-labeled cells had ssDNA-immunoreactive nuclei (data not shown, but see Ueda *et al*., 2008).

Following bilateral gustatory nerve injury, approximately 22.8 ± 5.7% and 23.7 ± 4.6% of ssDNA-immunoreactive cells showed PLCβ2 immunoreactivity on PO1 and PO2, respectively (Fig. 4A–C, 5). Approximately 7.0 ± 1.2% and 6.2 ± 0.8% of ssDNA-immunoreactive cells exhibited NCAM immunoreactivity on PO1 and PO2, respectively (Fig. 4D–E, 5). Similarly, 13.2 ± 0.8% and 12.9 ± 1.2% of ssDNA-immunoreactive cells were labeled with Jacalin on PO1 and PO2, respectively (Fig. 4F–I, 5). On PO3, approximately 23.2 ± 3.2% and 8.9 ± 0.7% of ssDNA-immunoreactive cells showed PLCβ2 and NCAM immunoreactivity, respectively. Nearly 60–70% of ssDNA-immunoreactive cells did not show immunoreactivity for PLCβ2 or NCAM, or the binding of Jacalin (Fig. 5).

Discussion

In the present study, we examined the histochemical changes and occurrence of apoptosis in taste buds cells of degenerating rat circumvallate papillae following bilateral axotomy of the glossopharyngeal nerve, and found that Jacalin-labeled cells (type IV cells) began to show immunoreactivity for ssDNA, a sign of apoptosis, soon after gustatory denervation, and disappear by PO3. Moreover, NCAM-immunoreactive cells (type III cells), which do not show immunoreactivity for ssDNA under normal conditions, also began to express ssDNA immunoreactivity following gustatory nerve injury.

Technical consideration

Our previous report showed that UEA-I labels all taste buds in developing and normal animals (Taniguchi *et al*., 2004). We applied UEA-I lectin histochemistry to detect the taste buds in normal and denervated taste buds and found that UEA-I also binds to the membranes of entire degenerating taste bud cells. Thus, UEA-I lectin histochemistry is a useful method for labeling all taste bud cells of degenerating taste buds in the rat.

In the present study, we used PLCβ2, NCAM, and Jacalin as markers of type II, type III, and type IV cells, respectively. Previous studies have utilized α-gustducin as a marker of type II cells. However, it has been reported that α-gustducin labels a subpopulation of type II cells (Yang *et al*., 2000b). It has been established that PLCβ2 labels the majority of type II cells and does not label other types of cells. Similarly, type III cells have been reported to be immunoreactive for PGP 9.5 and 5HT (Yee *et al*., 2001); more recently, they were reported to also be immunoreactive for SNAP-25 (Yang *et al*., 2000a). A detailed histochemical analysis revealed 5-HT-immunoreactivity in a subset of type III cells, and PGP 9.5 was also observed in a subset of type II and type III cells (Yee *et al*., 2001). Yang *et al*. (2000a) reported that SNAP-25 is localized in type II cells. However, our previous study revealed that SNAP25 immunoreactivity is detected in a subset of both type II and type III cells (Ueda *et al*., 2006). It is now accepted that the majority of type III cells exhibit NCAM immunoreactivity. Therefore, we applied immunohistochemistry for PLCβ2 and NCAM to label type II and type III cells, respectively.

Type I cells have previously been shown to be immunoreactive for human blood antigen H (AbH) (Smith *et al*., 1994). However, our earlier report showed that AbH immunoreactivity was also detected in light cells, that is, type II and type III cells (Ueda *et al*.,

2003). Thus, in the present study, we did not perform immunohistochemistry for AbH. Because PLCβ2, NCAM, and Jacalin label the majority of type II, type III and type IV cells, respectively, we believe that most cells not labeled with these markers are type I cells.

We detected apoptotic taste buds cells by performing immunohistochemistry for ssDNA. The TUNEL method is widely used to detect apoptosis. Frankfurt *et al*. (1996) reported that TUNEL-stained nuclei were detected in necrotic areas in human tumor xenografts as well as in apoptotic cells, and that immunohistochemistry for ssDNA is more sensitive than the TUNEL method for detecting the earlier stages of DNA fragmentation. Thus, we believe that ssDNA immunohistochemistry is a useful and sensitive histochemical method for detecting apoptotic cells.

Histochemical changes and apoptosis in degenerating taste buds

As the life span of taste buds is reported to be around 10 days, a rapid, continuous turn-over of taste buds cells occurs (Beidler and Smallman, 1965; Farbman, 1980). Taste bud cells are thought to die by apoptosis, as demonstrated by TUNEL staining (Takeda *et al*., 1996; Huang and Lu, 2001) and immunohistochemistry for apoptosis-related molecules (bax, bcl-2; Zeng and Oakley, 1999; Zeng *et al*., 2000). More recently, we applied immunohistochemistry for ssDNA to demonstrate the presence of apoptotic cells in taste buds of normal rat circumvallate papillae (Ueda *et al*., 2008). Our present results showed that gustatory denervation causes an increase in the number of ssDNA-immunoreactive cells, which is consistent with the results of previous studies (Takeda *et al*., 1996; Huang and Lu, 2001). The average number of apoptotic taste bud cells per taste bud in the mouse circumvallate papilla is approximately five times higher on PO1 (Takeda *et al*., 1996), and approximately 15-times higher in the guinea pig vallate papilla (Huang and Lu, 2001) compared with that under normal conditions. Our present study showed an approximately 2-fold increase in the number of apoptotic cells following bilateral glossopharyngeal nerve denervation in rat. Although there are differences between experimental animals (rat *vs* mouse *vs* guinea pig) and detection methods (ssDNA immunohistochemistry *vs* TUNEL method), it is safe to say that the number of apoptotic cells increases rapidly after denervation, reaching a maximal level around PO1.

Although it is now established that gustatory nerve denervation causes the degeneration of taste buds, little is known about the histochemical changes in taste

bud cells during their degeneration. The present study demonstrated that taste buds disappear completely by PO6 following bilateral axotomy of the glossopharyngeal nerve. Although the number (and relative proportion) of each cell type is almost identical between normal taste buds and degenerating taste buds at PO1 (see Fig. 2A, B), ssDNA-immunoreactive nuclei were detected in all types of taste bud cells at PO1 (see Fig. 5A), indicating that gustatory denervation triggers cell death (apoptosis) in all types of taste bud cells.

The question arises whether all types of taste bud cells are homogeneously affected following glossopharyngeal nerve axotomy. Taste bud cells are divided into four types: spindle-shaped dark cells (type I cells), light cells (Type II and III cells depending on the appearance of synapses connection with gustatory nerves) extending from the basal portion to the surfaces of taste buds, and round-shaped progenitor cells (Type IV cells) at the base of the taste buds (Type IV cells). It is thought that taste bud cells constantly arise from local epithelial cells, which differentiate into progenitor cells (type IV cells) (Stone *et al*., 1995; Barlow and Northcutt, 1995). The appearance of ssDNA in Jacalin-labeled round cells, namely, type IV cells, from PO1, and the disappearance of Jacalin-labeled cells by PO3 following gustatory denervation, indicate that gustatory nerves secrete certain molecules involved in the initiation and differentiation of local epithelial cells into progenitor cells.

Type IV cells differentiate into other types of cells, such as the spindle-shaped cells, although there is disagreement over the cell lineage of taste bud cells. The present analysis showed that ssDNA-immunoreactive cells also expressed NCAM immunoreactivity — a marker of type III cells following gustatory denervation. In addition to the presence of synapse vesicles in type III cells, Yoshie *et al*. (1990) reported the presence of synapse vesicles at the axon terminals of gustatory nerves forming reciprocal synapses. Thus, it is speculated that type III cells receive some molecules from the axon terminals of gustatory nerves. Under normal conditions, ssDNA-immunoreactive cells display PLC β2 immunoreactivity, a marker for type II cells. Following denervation, the number of ssDNA-immunoreactive cells with the histochemical properties of type II cells almost doubled, indicating that type II cells are also affected by gustatory denervation. The present histochemical cal analysis showed that the number of type I cells, spindle-shaped cells negative for PLC β2, NCAM, and Jacalin,gradually decreased, but that the number of such cells that were also ssDNA-immunoreactive was slightly increased (see Fig. 5A), indicating that type I cells also depend on gustatory innervation. In summary, all types

of taste bud cells depend on gustatory nerve input, but type II, type III and type IV cells are more sensitive to the axotomy of the gustatory nerve than type I cells.

In conclusion, the present study showed that apoptosis occurs in all types of taste bud cell following gustatory denervation, supporting the idea that initiation of the development and maintenance of mammalian taste buds is highly dependent on the gustatory nerve. Further studies are required to elucidate what molecule(s) from the gustatory nerve contribute to the initiation of the development, maintenance, and differentiation of taste bud cells.

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