

Cell-type specific occurrence of apoptosis in taste buds of the rat circumvallate papilla*

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Summary. The present study employed immuno-histochemistry for single-stranded DNA (ssDNA) to detect apoptotic cells in taste buds of the rat circumvallate papilla. Double-labeling of ssDNA and markers for each cell type — phospholipase C β 2 (PLC β 2) and α -gustducin for type II cells, neural cell adhesion molecule (NCAM) for type III cells, and Jacalin for type IV cells — was also performed to reveal which types of cells die by apoptosis. We detected approximately 16.8% and 14.0% of ssDNA-immunoreactive nuclei among PLC β 2-immunoreactive and α -gustducin-immunoreactive cells, respectively, but rarely found ssDNA-immunoreactive cells among NCAM-immunoreactive or Jacalin-labeled cells, indicating that type II cells die by apoptosis. We also applied double labeling of ssDNA and human blood group antigen H (AbH) — which mostly labels type I cells as well as other cell types — and found

that approximately 78% of ssDNA-immunoreactive cells were labeled with AbH, indicating that apoptosis also occurs in type I cells. The present results revealed that apoptosis occurs in both type I cells (dark cells) and type II cells (light cells), suggesting that there are two major cell lineages (dark cell and light cell lineages) for the differentiation of taste bud cells. In summary, type IV cells differentiate into dark and light cells and type III cells differentiate to type II cells within the light cell line.

Introduction

Taste buds, chemoreceptors that mediate taste, contain 50–80 spindle-shaped cells (taste cells) as well as proliferative round basal cells (progenitor cells). In mammals, taste buds are mostly found in the three lingual papillae, i.e. the fungiform, foliate, and circumvallate papillae, but are also present in the epithelium of the posterior portions of the palate, larynx and pharynx. Elongated taste cells are traditionally classified into dark and light cells. Dark cells (also called type I cells) have many electron-dense granules in the apical portion and are considered to be supporting cells. They exhibit the enzymatic activity of carbonic anhydrase isoform II (CA II; Daikoku *et al.*, 1999) and nucleoside triphosphate diphosphohydrolase-2 (NTPDases-2; Bartel *et al.*, 2006) as well as immunoreactivity for human blood group antigen H (AbH; Smith *et al.*, 1994) and the glial glutamate/aspartate transporter (GLAST; Lawton *et al.*, 2000). Light cells, which have an electron-lucent cytoplasm, are further divided into type II and type III cells based on the presence or absence of synaptic connections with gustatory nerves. Specifically, type III cells contain many synaptic vesicles and have synapses

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with gustatory nerves, while type II cells do not (Murray *et al.* 1967, 1969; Takeda and Hoshino 1975). Although type II cells do not have apparent synapses, both type II and type III cells are considered to act as taste receptor cells, due to their histochemical properties. Briefly, type II cells are immunoreactive for α -gustducin (a taste-specific G protein), phospholipase C β 2 (PLC β 2), and type III inositol 1,4,5-triphosphate receptor (IP3R3), all of which are involved in the transduction cascade of bitter stimuli (Boughter *et al.* 1997; Cho *et al.* 1998; Yang *et al.* 2000a, b; Clapp *et al.* 2001, 2004), while type III cells show neuronal features and are immunoreactive for protein gene product 9.5 (PGP 9.5), neural cell adhesion molecule (NCAM), and serotonin (5HT) (Nelson and Finger 1993; Smith *et al.* 1993; Kanazawa and Yoshie 1996; Yee *et al.* 2001). Type IV cells are round, located at the basal portion of the taste buds, express sonic hedgehog mRNA (Miura *et al.* 2003, 2004), bind to Jacalin (Taniguchi *et al.* 2005), and function as progenitor cells. Although the histological properties of the different types of cells have been elucidated, the relationships among the cell types regarding their cell lineage remain uncertain. There are two major hypotheses for the cell lineage of taste bud cells: the "one cell-line theory" where all spindle-shaped cells are derived from the same progenitor cells (Delay *et al.* 1986), and the "multiple cell-line theory" in which the different types of taste cells have their own progenitor cells, meaning that there would be at least three types of progenitor cells that differentiate into types I, II, and III cells, respectively.

The lifespan of taste cells is thought to be approximately 10 days (Beidler and Smallman 1965; Farbman 1980); and therefore, continuous cell differentiation and cell death are expected to be necessary to maintain the shape of the taste buds (Mistretta 1989). Although recent studies have shown that taste bud cells die by apoptosis (Takeda *et al.* 1996, 2000; Zeng *et al.* 1999, 2000; Huang and Lu 2001), it remains unknown which types of cells so die. The present study was thus designed to elucidate which types of cells undergo apoptosis, using immunohistochemistry for single-stranded DNA (ssDNA) to label apoptotic cells combined with immunohistochemistry for specific markers for type I (AbH), type II (PLC β 2, α -gustducin), type III (NCAM), and lectin histochemistry for type IV (Jacalin) cells in the taste buds of the rat circumvallate papilla.

Materials and Methods

All experimental procedures were reviewed and approved by the Intramural Animal Use and Care Committee at

Osaka University Graduate School of Dentistry.

Animals and tissue preparation

A total of 5 male Sprague-Dawley rats, weighing 200–250 g, were purchased from Nihon Doubutsu (Osaka, Japan). 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 16 μ m thickness, collected in cold PBS, and treated as free-floating sections. For methodological comparison between ssDNA immunohistochemistry and the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling (TUNEL) method, the small intestine — where rapid cell turn-over occurs and many apoptotic cells are present — was also sectioned at 16 μ m thickness and treated as free-floating sections.

Immunohistochemistry for ssDNA

We performed the avidin-biotin-complex (ABC) method for ssDNA immunohistochemistry to detect apoptotic cells. After washing in PBS, sections were incubated with PBS containing 0.3% H₂O₂ to inactivate endogenous peroxidase activity for 30 min, and then incubated with a rabbit polyclonal anti-ssDNA antibody (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 then 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% H₂O₂ with nickel ammonium sulfate (0.08–0.1%) enhancement. The immunostained sections were mounted on gelatin-subbed glass slides, counterstained with methyl green, dehydrated through an ascending series of ethanol, cleared in xylene, and cover-slipped with Permount (Fisher Scientific, New Jersey, NJ). The sections were observed under a light microscope (Axioskop 2 plus; Carl Zeiss, Jena, Germany), and images were captured with a CCD camera (Axio Cam; Carl Zeiss).

TUNEL histochemistry

Sections from the small intestine was also stained by a commercially available staining kit (*In Situ* Cell Death Detection Kit, Roche Diagnostics GmbH, Mannheim, Germany) according to the protocol provided by the manufacture.

Double-labeling experiment

Double-labeling experiments for ssDNA and AbH (a marker for type I cells), PLC β 2 or α -gustducin (markers for type II cells), NCAM (a marker for type III cells), and Jacalin (a marker for type IV cells) were also carried out. After the detection of ssDNA by the ABC method as described above, the sections were incubated with mouse monoclonal anti-AbH (1:500; clone 92-FR-A2; DAKO), rabbit polyclonal anti- PLC β 2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti α -gustducin (1:500; Santa Cruz Biotechnology), or rabbit polyclonal anti-NCAM (1:1000; Chemicon International, Temecula, CA) for 16–18 h, and subsequently with Alexa Fluor 488-conjugated anti mouse IgM (1:100; Molecular Probes, Eugene, OR) for AbH, or Alexa Fluor 488-conjugated anti-rabbit IgG (1:500; Molecular Probes) for PLC β 2, α -gustducin, or NCAM, for 90 min at room temperature. For double-labeling of ssDNA and Jacalin, ssDNA-immunostained sections were incubated with biotinylated Jacalin (0.5 μ g/ml; Vector) for 14–16 h, and then with Alexa Fluor 488-conjugated streptavidin (1:500; Molecular Probes) for 90 min at room temperature. All sections were mounted onto gelatin-subbed glass slides, cover-slipped with Vectashield (Vector), and viewed under a fluorescence microscope (Axioskop 2 plus).

For a quantitative analysis, the total numbers of ssDNA-immunoreactive cells and those labeled with AbH, PLC β 2, α -gustducin, NCAM, or Jacalin were counted in 15 randomly selected sections that were at least 40 μ m apart from each other to avoid duplicate counting.

Results

Apoptotic cells in small intestine

For methodological comparison, we applied ssDNA immunohistochemistry and the TUNEL method to adjacent sections from the small intestine where rapid cell turnover takes place and many apoptotic cells are present. The immunoreaction for ssDNA was detected at the tip of the intestinal villi (Fig. 1A). The comparison of adjacent sections stained by ssDNA immunohistochemistry (Fig.

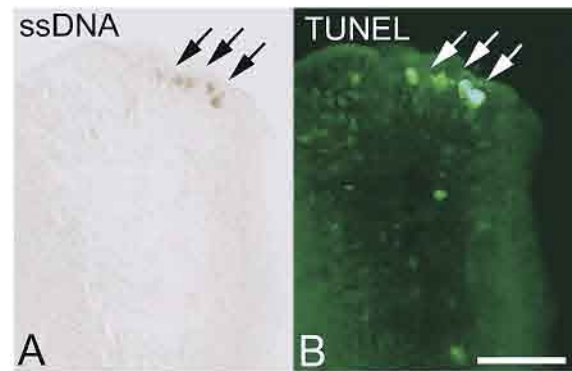


Fig. 1. Apoptotic cells in the tips of intestinal villi in the small intestine (arrows) as demonstrated by ssDNA immunohistochemistry (A) and the TUNEL method (B) taken from adjacent sections. There is no apparent difference between ssDNA immunohistochemistry (A) and the TUNEL method (B). Scale bar in B represents 25 μ m; and is applied to A.

1A) and the TUNEL method (Fig. 1B) showed that there is no apparent difference in the number and distribution of ssDNA-immunoreactive nuclei and TUNEL positive nuclei.

ssDNA in taste buds

Immunoreactivity for ssDNA was restricted to the nuclei of intragemmal cells located at the middle to apical portions of the taste buds and extragemmal epithelial cells of the trench wall (Fig. 2, 3). The shapes of immunoreactive nuclei varied; some nuclei were dense and shrunk (Fig. 2A, 3D) and others appeared oval (Fig. 2D,G, 3A). Among 100 taste buds, a total of 118 nuclei exhibited ssDNA immunoreactivity. Most taste buds had a low number (0–2 per taste bud profile) of ssDNA-immunoreactive nuclei, but a few taste buds contained a high number (3–5 per taste bud profile).

ssDNA and specific types of taste bud cells

Immunoreaction for AbH was strongly detected at the cell surface of spindle-shaped taste bud cells, and the cytoplasm also displayed faint immunoreactivity. Rounded basal cells lacked immunoreactions (Fig. 2B, C). Double labeling with ssDNA showed approximately 77.9% (162/208) of ssDNA-immunoreactive cells exhibit AbH-immunoreactivity (Fig. 2A–C).

Immunoreactivity for PLC β 2 or α -gustducin was detected within the cytoplasm of a subpopulation of

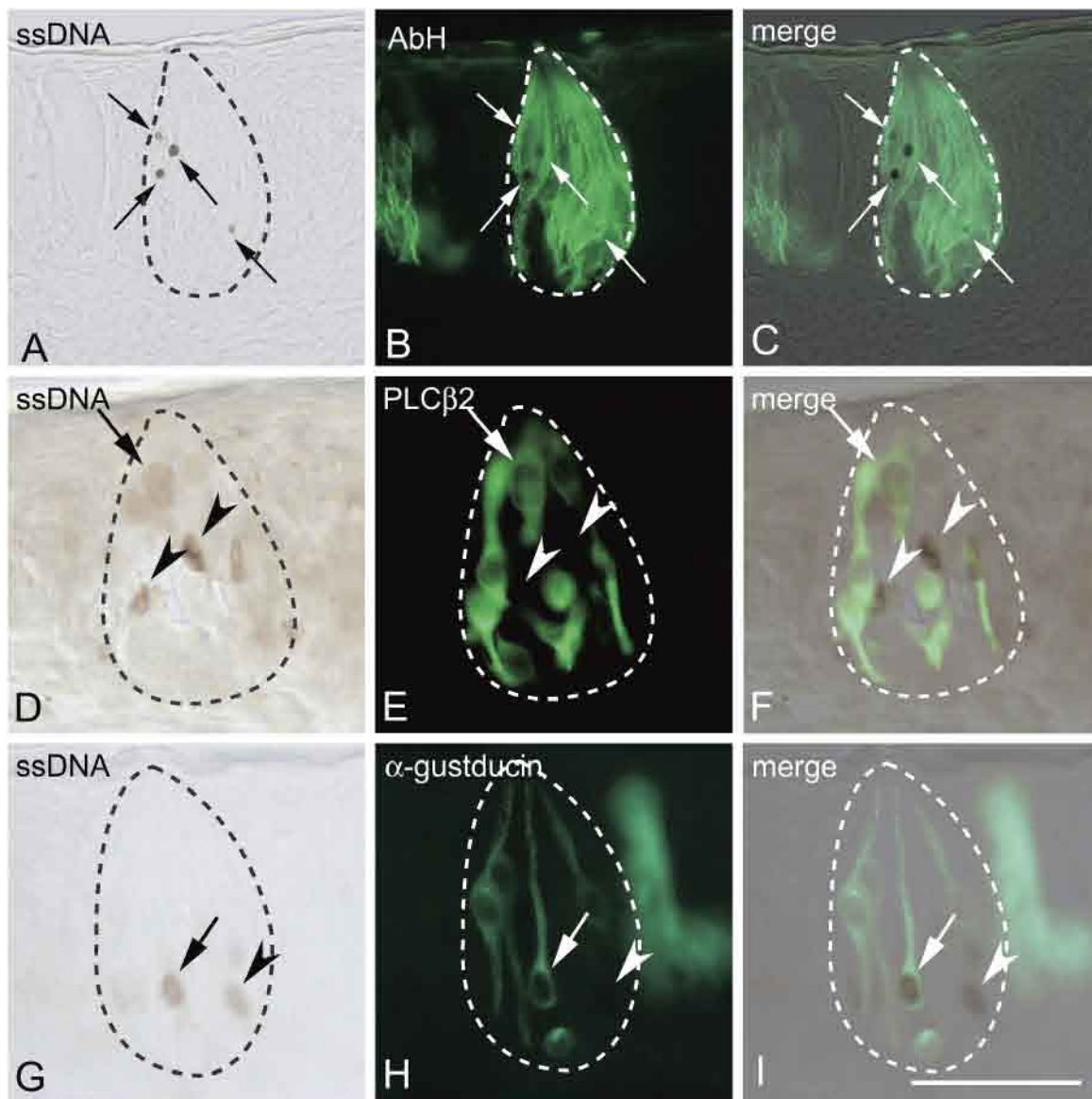


Fig. 2. Double-labeling for ssDNA (**A**, **D**, **G**) and markers for type I cells [AbH (**B**)] or type II cells [PLC β 2 (**E**) and α -gustducin (**H**)], and the merged images (**C**, **F**, **I**). **A–C**: Most ssDNA-immunoreactive nuclei (**A**) are present in taste bud cells immunoreactive for AbH (**B**, **C**). **D–F**, **G–I**: A few ssDNA-immunoreactive nuclei are localized in taste cells showing immunoreactivity for PLC β 2 (**D–F**; arrow) or α -gustducin (**G–I**; arrows), but most of them lack immunoreactivity for PLC β 2 or α -gustducin (**F**, **I**; arrowheads). Photographs in each row are taken from the same section, and a taste bud is indicated by the dotted line. The scale bar in **I** represents 50 μ m, and is applicable to all photographs.

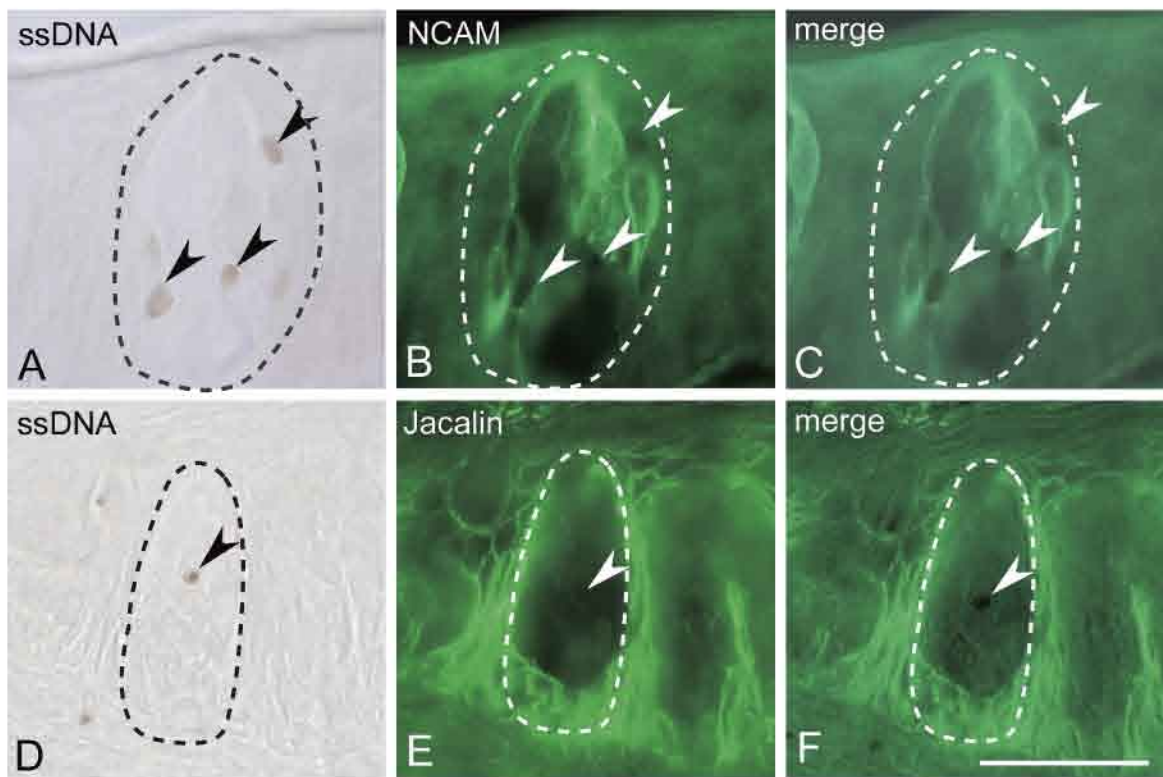


Fig. 3. Double-labeling for ssDNA (**A**, **D**) and markers for type III cells [NCAM (**B**)] or type IV cells [Jacalin (**E**)], and the merged images (**C**, **F**). There are many NCAM-immunoreactive cells in the taste buds (**B**) and Jacalin-labeled cells in the basal portion of taste buds (**E**), but no ssDNA-immunoreactive cells (arrowheads in **A–C**, **D–F**) overlap with the NCAM-immunoreactive and Jacalin-labeled cells (NCAM: **A–C**; Jacalin: **D–F**). The images in each row are taken from the same section and a taste bud is indicated by the dotted line. The scale bar in **F** represents 50 μm , and is applicable to all images.

spindle-shaped intragemmal cells (Fig. 2E, F, H, I). A greater number of taste cells appeared to be PLC β 2-immunoreactive rather than α -gustducin-immunoreactive (compare Fig. 2E with Fig. 2H). Double-labeling with ssDNA revealed that approximately 16.8% (22/231) and 14.0% (24/171) of ssDNA-immunoreactive cells were PLC β 2-immunoreactive and α -gustducin-immunoreactive, respectively (Fig. 2D–F and 2G–I, respectively).

Immunoreactivity for NCAM was found in the cytoplasm of some spindle-shaped intragemmal cells as well as in intragemmal and extragemmal nerve fibers (Fig. 3B, C). No ssDNA-immunoreactive cells displayed NCAM-immunoreactivity (0%; 0/159; Fig. 3A–C).

Jacalin labeling was found on the cell surface of rounded basal cells of the taste buds as well as surrounding extragemmal epithelial cells (Fig. 3E, F). No

ssDNA-immunoreactive cells were labeled with Jacalin (0%; 0/128; Fig. 3D–F).

Discussion

The present report is the first to detect apoptotic cells within taste buds using ssDNA immunohistochemistry. There are other histochemical methods for detecting apoptotic cells, i.e. the TUNEL method, immunohistochemistry for apoptosis-associated molecules such as Bax, Bcl, p53 and caspases, and the ultrastructural detection of apoptotic cells. Immunohistochemistry for apoptosis-associated molecules is also useful for detecting cells undergoing apoptosis, but these molecules appear in a stage-specific manner during apoptosis. For example,

Bax-immunoreactive cells were observed in taste bud cells of mice at 5–7 days after the injection of BrdU (Zeng *et al.*, 1999, 2000), suggesting that Bax appears at the early stages of apoptosis since the lifespan of taste bud cells is thought to be approximately 10 days (Beidler and Smallman 1965; Farbman 1980). The TUNEL method is widely used for the detection of apoptosis. However, Frankfurt *et al.* (1996) reported that TUNEL-stained nuclei were detected in necrotic areas in human tumor xenografts as well as in apoptotic cells, suggesting that both necrotic and apoptotic cells were labeled by the TUNEL method. Immunohistochemistry of ssDNA is a new method to detect the apoptosis. During the process of apoptosis, DNA fragmentation is triggered by caspase-3 (Enari *et al.*, 1998) activated by other cell death-regulatory genes, such as caspase-8, caspase-9, and Bcl-2 (Cheng *et al.*, 1997; Fuchs *et al.*, 1997; Kitahara *et al.*, 1997), which in turn are activated by p53 and Bax. Previous immunohistochemical studies have shown that immunoreactivity for ssDNA is highly co-localized with TUNEL-positive apoptotic cells, and that ssDNA immunohistochemistry is more sensitive than the TUNEL method for detecting earlier stages of DNA fragmentation (Frankfurt *et al.* 1996; Kawashima *et al.*, 2004; Ito *et al.*, 2006). Indeed, we found the identical distribution of ssDNA-immunoreactive nuclei and TUNEL-labeled nuclei in the villus tips of the small intestine. In the taste buds, we found that nuclei labeled by the ssDNA antibody varied in shape: some nuclei were oval and seemed to be intact, and other nuclei had shrunk. We think that cells with ssDNA-immunoreactive oval nuclei represent the early stage of apoptosis, and that cells with ssDNA-immunoreactive shrunk nuclei represent the late, or final stage of apoptosis. Thus we believe that ssDNA immunohistochemistry labeled apoptotic cells from the early stage to late stage of apoptosis, and that ssDNA immunohistochemistry is a useful and sensitive histochemical method for detecting apoptotic cells.

In the present study, we found approximately 1.2 ssDNA-immunoreactive nuclei per taste bud in 16- μ m sections, which is much higher than previously reported values (Takeda *et al.* 1996; Huang and Lu 2001). Specifically, Takeda *et al.* (1996) detected approximately 0.13 TUNEL-positive nuclei per taste bud in mouse circumvallate papillae using 6- μ m paraffin sections, while Huang and Lu (2001) reported approximately 0.6 TUNEL-positive nuclei per taste bud in guinea pig vallate papillae in 20- μ m sections. When these values are applied to a section of 16- μ m thickness, they become 0.35 and 0.5, respectively. There are two possible explanations for the differences in these results: species differences (rat, mouse and guinea pig) and methodological differences (ssDNA

immunohistochemistry and TUNEL method). Frankfurt *et al.* (1996) reported that ssDNA immunohistochemistry is more sensitive than the TUNEL method for detecting apoptotic cells.

In the present study, we used PLC β 2 and α -gustducin as markers for type II cells (Boughter *et al.* 1997; Cho *et al.* 1998; Yang *et al.* 2000a, b; Clapp *et al.* 2001, 2004). Although we did not perform quantitative analyses of PLC β 2 and α -gustducin-immunoreactive cells, the number of PLC β 2-immunoreactive cells appeared to be greater than the number of α -gustducin-immunoreactive cells. Previous studies have shown that PLC β 2 immunoreactivity is present in almost all cells showing immunoreactivity for IP3R3, another molecule localized in type II cells, while α -gustducin-immunoreactivity is only observed in a subset of IP3R3-immunoreactive cells (Huang *et al.*, 1999; Miyoshi *et al.*, 2001; Clapp *et al.* 2001, 2004), indicating that almost all – if not all – α -gustducin-immunoreactive cells are PLC β 2-immunoreactive. In addition, a recent study (Ma *et al.*, 2007) revealed that approximately 31.8% and 18% of taste bud cells in rat circumvallate papillae showed immunoreactivity for PLC β 2 and α -gustducin, respectively. The present double-labeling for ssDNA and markers of type II cells revealed that approximately 16.8% and 14.0% of ssDNA-immunoreactive cells showed immunoreactivity for PLC β 2 and α -gustducin, respectively, indicating that type II cells with immunoreactivity for both PLC β 2 and α -gustducin are candidate cells for the late stages of apoptosis. In addition, our present results showed that a small percentage of ssDNA-immunoreactive cells were immunoreactive for PLC β 2, but not α -gustducin. At present, we cannot determine whether this is due to a methodological error or the presence of very few PLC β 2-immunoreactive cells with no immunoreactivity for α -gustducin undergoing apoptosis.

It is clear that NCAM-immunoreactive cells do not exhibit immunoreactivity for ssDNA, suggesting that taste cells expressing NCAM immunoreactivity do not die by apoptosis. Since it is well established that NCAM is present in type III cells, these cells do not appear to undergo apoptosis. Miura *et al.* (2005) reported that approximately 20% of NCAM-immunoreactive cells expressed α -gustducin mRNA in adult mice. In addition, we previously reported the possible existence of transitional cells between type II and type III cells, since synapse-associated proteins are present in PLC β 2-immunoreactive and α -gustducin-immunoreactive cells as well as NCAM-immunoreactive cells (Ueda *et al.*, 2006). These lines of evidence suggest that type III cells may differentiate into type II cells. In addition to type III cells,

ssDNA-immunoreactive nuclei were not present in Jacalin-labeled round cells at the basal portion of taste buds, presumably type IV cells (Taniguchi *et al.*, 2005). It is reasonable that type IV cells do not die by apoptosis, as these cells are thought to be progenitor cells.

It is worth noting that the majority (>80%) of ssDNA-immunoreactive cells were not labeled by PLC β 2 and α -gustducin (markers for type II cells), NCAM (a marker for type III cells), or Jacalin (a marker for type IV cells). We performed double-labeling with ssDNA and AbH, and found that approximately 78% of ssDNA-immunoreactive cells showed immunoreactivity for AbH. Smith *et al.* (1995) first described AbH as a marker for type I cells. We previously, however, reported that AbH immunoreactions were also detected in light cells, i.e. type II and type III cells (Ueda *et al.*, 2003). We think that ssDNA-immunoreactive cells with AbH immunoreactivity also display PLC β 2, a marker for type II cells. Even if all ssDNA-immunoreactive type II cells (or PLC β 2-immunoreactive cells) are also labeled with AbH, approximately 61% of ssDNA-immunoreactive cells — 78% (ssDNA-immunoreactive/AbH-immunoreactive cells) minus 17% (ssDNA-immunoreactive/PLC β 2-immunoreactive cells) — are not labeled with PLC β 2, and these ssDNA-immunoreactive cells are most likely to be type I cells. Previous quantitative studies showed that type I cells constitute about 50% of taste cells within the taste buds of rabbits (Murray *et al.*, 1973) and mice (Delay *et al.*, 1986). However, a recent report revealed that approximately 31.8%, 15.9%, and 14.3% of taste cells per taste bud in rat circumvallate papillae exhibit immunoreactivity for PLC β 2, 5HT, and PGP 9.5, respectively (Ma *et al.*, 2007). It has also been reported that, though both 5HT and PGP 9.5 are present in type III cells, the population of 5HT-immunoreactive cells differs from that of PGP 9.5-immunoreactive cells. Thus, rough calculations indicate more than half (62%) of taste cells were immunoreactive for molecules contained in light cells (type II and type III cells) in rat taste buds. Although there are species differences, it is safe to say that nearly 50% of taste cells are type I cells. Thus, it is likely that a majority of ssDNA-immunoreactive cells without labeling with markers of type II cells (PLC β 2 and α -gustducin) are type I cells.

Generally, disintegrated small pieces of apoptotic cells — called apoptotic bodies are phagocytosed by neighboring cells or macrophages. A previous ultrastructural study (Takeda *et al.*, 1996) showed that type I cells in denervated mouse circumvallate papillae contained condensed and fragmentary nuclei in their cytoplasm. Similar ultrastructural profiles have been demonstrated in normal taste buds (Farbman 1969;

Fujimoto and Murray 1970; Farbman *et al.*, 1985). Thus, the question arises whether type I cells undergo apoptosis or type I cells phagocytose neighboring apoptotic cells. We found that there are two shapes of ssDNA-immunoreactive nuclei, i.e. oval and shrunken — the former is thought to be intact nuclei representing the early stage of apoptosis, and the latter is thought to represent final stage of the apoptosis. It is likely that most of cells having oval ssDNA-immunoreactive nuclei are type I cells undergoing apoptosis, and those having shrunken ssDNA-immunoreactive nuclei are type I cells at the final stage of apoptosis and/or phagocytosing apoptotic bodies from neighboring apoptotic cells. A further ultrastructural analysis is now in progress to clarify this issue.

It has been established that type IV cells differentiate into spindle-shaped taste cells (types I, II, and III cells), but the exact cell lineage of taste cells remains obscure. There are two theories: the one-cell line theory and multi-cell line theory. Our present results have shown that both type I and type II cells die by apoptosis, indicating that there are two major cell lines in taste buds.

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