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Histochemical and ultrastructural analyses of the lubrication systems in the olfactory organs of soft-shelled turtle

Shoko NAKAMUTA[1)], Makoto YOKOSUKA[2)], Kazumi TANIGUCHI[3)], Yoshio YAMAMOTO[1,4)] and Nobuaki NAKAMUTA[1,4)]*

[1)]*Laboratory of Veterinary Anatomy, Iwate University, 3–18–8 Ueda, Morioka, Iwate 020–8550, Japan*
[2)]*Laboratory of Comparative Medicine, Nippon Veterinary and Life Science University, 1–7–1 Kyonancho, Musashino, Tokyo180–8602, Japan*
[3)]*Laboratory of Veterinary Anatomy, School of Veterinary Medicine, Kitasato University, 23–35–1 Higashi, Towada, Aomori 034–8628, Japan*
[4)]*United Graduate School of Veterinary Sciences, Gifu University, 1–1 Yanagido, Gifu, Gifu 501–1193, Japan*

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ABSTRACT. In general, the nasal cavity of turtles is divided into two chambers: the upper chamber, lined with the olfactory epithelium containing ciliated olfactory receptor cells, and the lower chamber, lined with the vomeronasal epithelium containing microvillous receptor cells. In the nasal cavity of soft-shelled turtles, however, differences between the upper and lower chamber epithelia are unclear due to the presence of ciliated receptor cells in both epithelia. In the olfactory organ of vertebrates, the surface of sensory epithelium is covered with secretory products of associated glands and supporting cells, playing important roles in the olfaction by dissolving odorants and transporting them to the olfactory receptors. Here, the associated glands and supporting cells in the olfactory organ of soft-shelled turtles were analyzed histochemically and ultrastructurally. The upper chamber epithelium possessed associated glands, constituted by cells containing serous secretory granules; whereas, the lower chamber epithelium did not. In the upper chamber epithelium, secretory granules filled the supranuclear region of supporting cells, while most of the granules were distributed near the free border of supporting cells in the lower chamber epithelium. The secretory granules in the supporting cells of both epithelia were seromucous, but alcian blue stained them differently from each other. In addition, distinct expression of carbohydrates was suggested by the differences in lectin binding. These data indicate the quantitative and qualitative differences in the secretory properties between the upper and lower chamber epithelia, suggesting their distinct roles in the olfaction.

KEY WORDS: associated glands, chemoreception, electron microscopy, reptile, supporting cells

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In general, tetrapods possess two distinct olfactory organs: the olfactory epithelium (OE) and the vomeronasal epithelium (VNE). They send axons to the main and accessory olfactory bulbs (OB), respectively [4, 32]. In most cases, the OE contains receptor cells bearing cilia on the tip of dendrites, and the VNE contains receptor cells bearing microvilli. The olfactory receptor cells express odorant receptors (OR), while the vomeronasal receptor cells express type 1 or type 2 vomeronasal receptors (V1R or V2R). The OR, V1R and V2R are the members of G-protein coupled receptor families and coupled with Gαolf, Gαi2 and Gαo, respectively [1, 3, 18]. Although it has been postulated that the OE detects general odorants and the VNE detects pheromones, such basic categorization has been under scrutiny as of late and reconsideration of their functional differences is required [35].

In turtles, ridges covered with the respiratory epithelium divide the nasal cavity into the upper and lower chambers. In some turtles including gopher tortoises and box turtles [12], Reeve's turtles [17, 34, 36] and red-eared sliders [22], it has been demonstrated that the OE and the VNE are distributed in the upper and lower chambers of nasal cavity and contain ciliated and microvillous receptor cells, respectively. Unique to birds, lizards, alligators and turtles, the olfactory receptor cells possess both cilia and microvilli [13, 20, 21]. In red-eared sliders, the main olfactory system, constituting the OE and the ventral part of the OB (the main OB), and the vomeronasal system, constituting the VNE and the dorsal part of the OB (the accessory OB), show different patterns of lectin binding, suggesting that the main olfactory system and the vomeronasal system have distinct functions [8]. On the other hand, receptor cells co-express Gαolf and Gαo both in the OE and the VNE of Reeve's turtles and red-eared sliders [22, 36]. From the electrophygiological studies on the VNE and the accessory OB of Reeve's turtles, it has been demonstrated that the vomeronasal system responds to general odorants without inherent behavioral significance [15, 16, 30, 31]. Furthermore, recordings from dissociated vomeronasal receptor cells demonstrate that the vomeronasal receptor cells of stinkpot turtles respond to a variety of complex natural odorants, including urine and musk, as well as to the odorants derived from the food pellets [7]. Thus,

*CORRESPONDENCE TO: NAKAMUTA, N., Laboratory of Veterinary Anatomy, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka, Iwate 020–8550, Japan. e-mail: nakamuta@iwate-u.ac.jp

functional differences between the OE and the VNE are not fully elucidated in turtles.

The soft-shelled turtles (family Trionychidae, *Pelodiscus sinensis*) are turtles highly adapted to the aquatic environment. They spend most of the time under water and use snorkel-like snout to breathe. Recently, we performed electron microscopic examinations of the olfactory organ of soft-shelled turtles and demonstrated the ultrastructural characteristics of receptor cells: unlike other turtles, ciliated receptor cells, not the microvillous receptor cells, are contained in the lower chamber epithelium as well as in the upper chamber epithelium [23]. The lack of obvious differences at the ultrastructure level between the receptor cells of upper and lower chamber epithelia led us to further investigation in the nasal cavity of soft shelled-turtles to elucidate some possible differences in the function of these epithelia.

In the olfactory organ of vertebrates, the surface of sensory epithelium is covered with the secretory products of the associated glands and supporting cells. These secretory products play important roles in the olfaction by dissolving odorants and transporting them to the olfactory receptors [11]. In this study, we analyzed the associated glands and supporting cells by transmission electron microscopy, periodic acid Schiff (PAS), alcian blue (AB) and lectin histochemistry to reveal the differences in the secretory properties of upper and lower chamber epithelia in the nasal cavity of soft-shelled turtles.

MATERIALS AND METHODS

Adult soft-shelled turtles (2 males and 4 females) were purchased from a local supplier TOBUN (Kamikita, Japan). All procedures were done in accordance with the Guideline for Care and Use of Animal Experiments and approved by the Animal Care and Use Committee at Iwate University. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (64.8 mg/kg). Two animals (1 male and 1 female) were perfused transcardially with Ringer's solution followed by Bouin's solution without acetic acid. The upper jaw was dissected, immersed in the same fixative solution overnight at 4°C and decalcified in 10% ethylenediamine tetra-acetic acid (EDTA) in 0.1 M phosphate buffer at 4°C for two weeks. The specimens were routinely embedded in paraffin and cut at 5–7 μm thick. Sections were stained with PAS or AB (pH 1.0 or pH 2.5), or processed for lectin histochemistry as described previously [19].

For transmission electron microscopy, samples were collected from four animals (1 male and 3 females) and processed as described previously [20].

RESULTS

The nasal cavity of soft-shelled turtles was divided into the upper and lower chambers (Fig. 1a and 1b). Both of the upper and lower chamber epithelia consist of three types of cells, the supporting cells, receptor cells and basal cells (Fig. 1c). Nuclei of the supporting, receptor and basal cells were situated in the apical, middle and basal layers of the epithelium, respectively. The associated glands (the Bowman's glands) were observed in the upper chamber epithelium, but not in the lower chamber epithelium (Fig. 1a–c). In addition, staining patterns for PAS and AB pH 2.5 differed between the two epithelia (Fig. 1d). PAS and AB pH 2.5 intensely stained the apical one-third of the upper chamber epithelium. In contrast, they stained the apical portion near the free border intensely and the supranuclear region of supporting cells less intensely in the lower chamber epithelium. Moreover, the upper chamber epithelium and the lower chamber epithelium were differently stained by AB pH 1.0: the upper chamber epithelium was positive for AB pH 1.0, but the lower chamber epithelium was negative. The Bowman's glands were positive for PAS, but negative for AB pH 1.0 and AB pH 2.5.

In the apical part of the upper chamber epithelium, the dendrites of the receptor cells bearing cilia on the tip (Fig. 2a, arrows) alternated with the cytoplasm of supporting cells bearing microvilli on the free border (Fig. 2a, Sp). In the supporting cells of upper chamber epithelium, supranuclear cytoplasm was filled with secretory granules (Fig. 2a). The secretory granules of supporting cells in the upper chamber epithelium, measuring 1–2 μm in diameter, showed a bipartite structure with the marginal part displaying moderate density and central part displaying low density (Fig. 2c). In addition, a core of high density was observed in some secretory granules (Fig. 2c, arrowheads). In the apical part of lower chamber epithelium, the dendrites of receptor cells bearing cilia alternated with the cytoplasm of supporting cells bearing microvilli (Fig. 2b, arrows). Most secretory granules were distributed near the free border of supporting cells in the lower chamber epithelium (Fig. 2b). The secretory granules of supporting cells, 0.5–1 μm in diameter, showed a bipartite structure of moderate and high densities (Fig. 2d). Secretory granules of high density, approximately 1 μm in diameter, were abundant in the glandular cells of the Bowman's gland (Fig. 2e).

Cellular expression of carbohydrate chains was visualized by histochemistry using lectins which can bind specifically to carbohydrate chains [2]. Staining patterns for lectins were evaluated in each region where the secretory granules were observed by transmission electron microscopy: the apical one-third of the upper chamber epithelium (Fig. 3a, top panels), the superficial portion (Fig. 3a, bottom panels, arrowheads) and supranuclear region (Fig. 3a, bottom panels, bidirectional arrows) of the lower chamber epithelium, and the glandular cells of the Bowman's gland (Fig. 3b). Eighteen lectins stained the secretory granules of supporting cells in the upper and/or lower chamber epithelium (Table 1). Among them, 14 lectins stained the supporting cells in the upper chamber epithelium and those in the lower chamber epithelium differently. Eleven lectins, including DSL, DBA, SBA, BSL-I, VVA, Jacalin, PNA, UEA-I, PSA, LCA and PHA-E, stained the supporting cells in the lower chamber epithelium more intensely than those in the upper chamber epithelium (Fig. 3a, left). Four lectins, including sWGA, RCA120, ECL and ConA, stained the supporting cells in the upper chamber epithelium and those in the lower chamber epithelium equally (Fig. 3a, middle). Three lectins, including

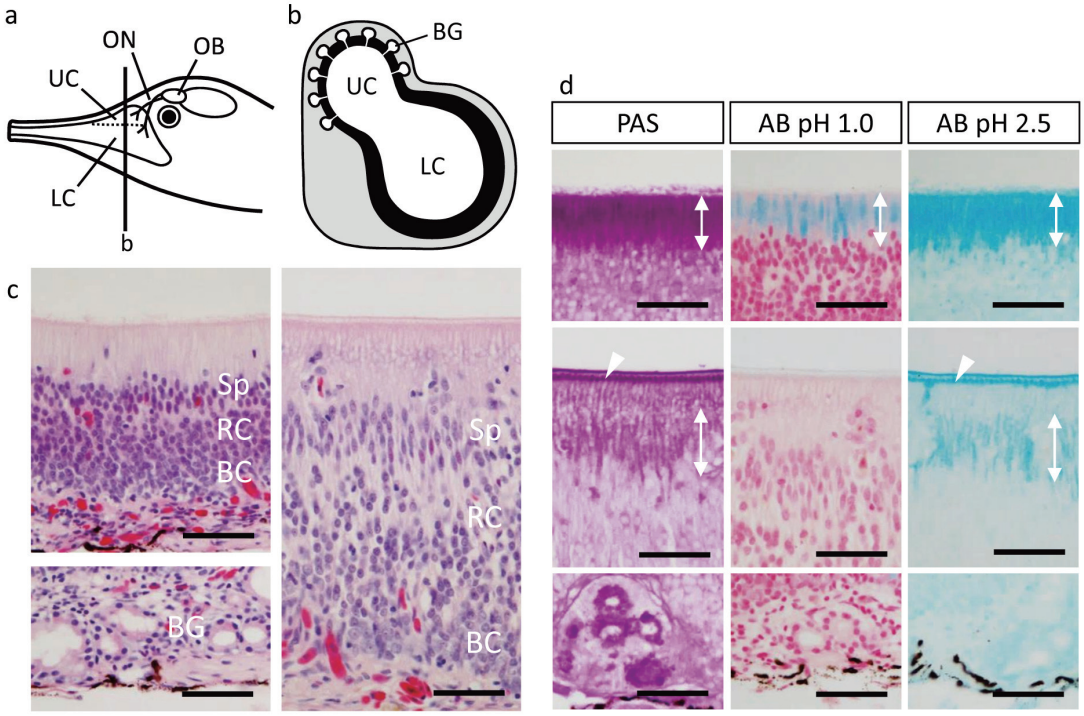

Fig. 1. The olfactory organs in soft-shelled turtle. (a) A schematic drawing of the sagittal view of head. UC, upper chamber; LC, lower chamber; ON, olfactory nerve; OB, olfactory bulb. Coronary section at line b is shown in b. (b) The right nasal cavity. Dorsal is top, and medial is left. BG, Bowman's gland. (c) HE stained sections showing upper chamber epithelium (upper left), the Bowman's glands (lower left, BG) and the lower chamber epithelium (right) in the nasal cavity of soft-shelled turtles. The nuclei of supporting cells (Sp), receptor cells (RC) and basal cells (BC) were situated in the apical, intermediate and basal layers of the upper and lower chamber epithelia. (d) PAS, alcian blue (AB) pH 1.0 and AB pH 2.5 stainings in the upper chamber epithelium (top panels), lower chamber epithelium (middle panels) and the Bowman's glands (bottom panels). The apical portion of the upper chamber epithelium was stained with PAS, AB pH 1.0 and AB pH 2.5 (bidirectional arrows). In the lower chamber epithelium, cytoplasm near the free border (arrowheads) was more intensely stained than the supranuclear region of the supporting cells (bidirectional arrows) with PAS and AB pH 2.5. The lower chamber epithelium was negative for AB pH 1.0. The Bowman's glands were positive for PAS, but negative for AB pH 1.0 and AB pH 2.5. The nuclei were counterstained with nuclear fast red in the sections stained with AB pH 1.0. Scale bars: 50 μm.

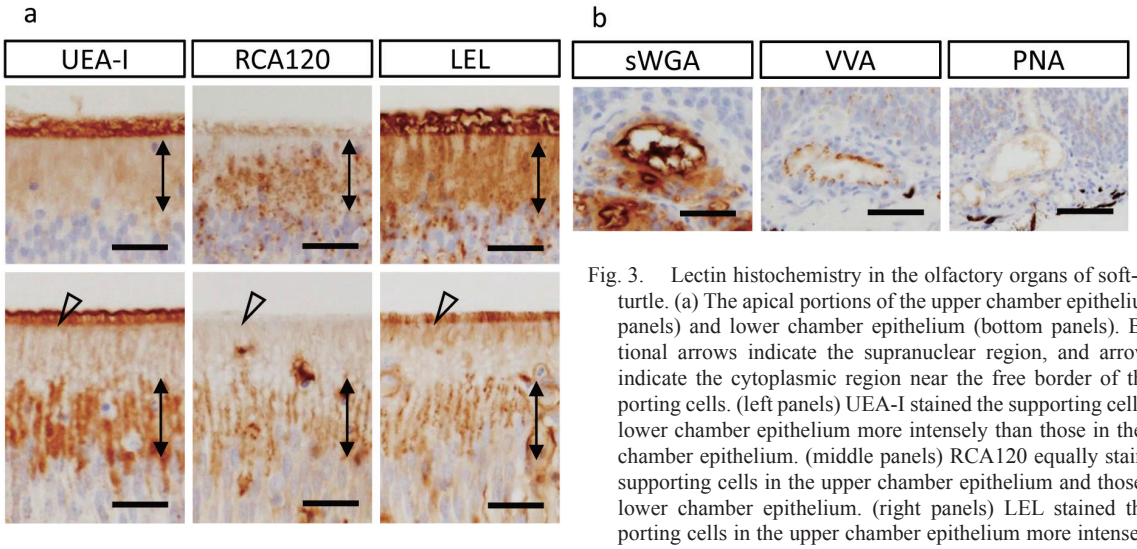

Fig. 3. Lectin histochemistry in the olfactory organs of soft-shelled turtle. (a) The apical portions of the upper chamber epithelium (top panels) and lower chamber epithelium (bottom panels). Bidirectional arrows indicate the supranuclear region, and arrowheads indicate the cytoplasmic region near the free border of the supporting cells. (left panels) UEA-I stained the supporting cells in the lower chamber epithelium more intensely than those in the upper chamber epithelium. (middle panels) RCA120 equally stained the supporting cells in the upper chamber epithelium and those in the lower chamber epithelium. (right panels) LEL stained the supporting cells in the upper chamber epithelium more intensely than those in the lower chamber epithelium. (b) Bowman's glands were stained intensely by sWGA, moderately by VVA and weakly by PNA. Scale bars: 25 μm.

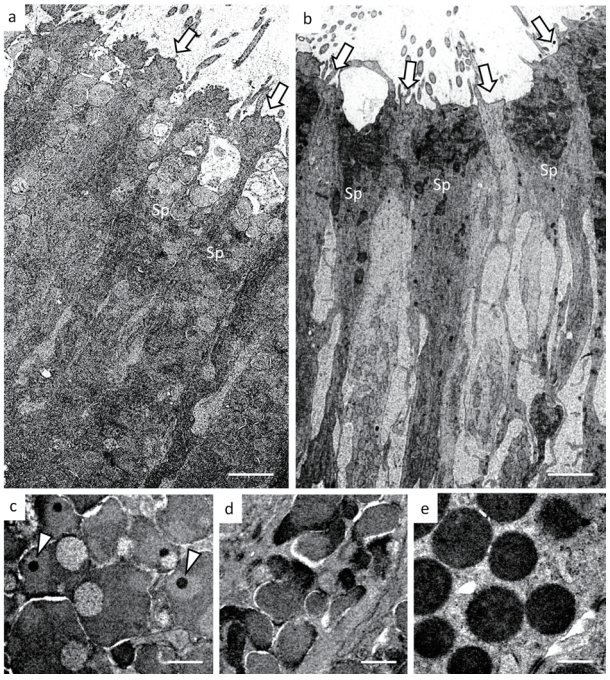

Fig. 2. Transmission electron micrographs of the olfactory organs of soft-shelled turtle. (a) Secretory granules filled the supranuclear region of the supporting cells (Sp) in the upper chamber epithelium. (b) Most secretory granules were distributed near the free border of supporting cells in the lower chamber epithelium. Arrows in a and b indicate receptor cells bearing cilia on the tip of the dendrites. (c) Secretory granules of the supporting cells in the upper chamber epithelium, 1–2 μm in diameter, consisted of the marginal part of moderate density and central part of low density. Some of the secretory granules contained a core of high density (arrowheads). (d) Secretory granules of the supporting cells in the lower chamber epithelium, 0.5–1 μm in diameter, consisted of two parts of moderate and high density. (e) The glandular cells in the Bowman's glands contained secretory granules of high density. Scale bars: 2 μm in a and b, 0.5 μm in c–e.

Table 1. Lectin binding patterns in the supporting cells of the upper and lower chamber epithelia and the Bowman's glands

Lectin (abbreviation)	Upper chamber epithelium	Lower chamber epithelium	Bowman's glands
Wheat germ agglutinin (WGA)	++	+	++
Succinylated wheat germ agglutinin (s-WGA)	+/–	+/–	++
Lycopersicon esculentum lectin (LEL)	++	+	+
Solanum tuberosum lectin (STL)	+	+/–	+
Datura stramonium lectin (DSL)	+	++	+
Bandeiraea simplicifolia lectin-II (BSL-II)	–	–	++
Dolichos biflous agglutinin (DBA)	–	+	++
Soybean agglutinin (SBA)	+/–	+	+
Bandeiraea simplicifolia lectin-I (BSL-I)	–	+/–	+/–
Vicia villosa agglutinin (VVA)	+	++	+
Sophora japonica agglutinin (SJA)	–	–	–
Ricinus communis agglutinin-I (RCA120)	++	++	+
Jacalin	–	+	+/–
Peanut agglutinin (PNA)	–	++	+/–
Erythrina cristagalli lectin (ECL)	+	+	+
Ulex europaeus agglutinin-I (UEA-I)	+/–	++	+
Concanavalin A (Con A)	++	++	++
Pisum satibum agglutinin (PSA)	–	+	+
Lens culinaris agglutinin (LCA)	–	+	+
Phaseolus vulgaris agglutinin-E (PHA-E)	–	++	+
Phaseolus vulgaris agglutinin-L (PHA-L)	–	–	+/–

–, negative staining; +/–, faint staining; +, moderate staining; ++, intense staining.

WGA, LEL and STL, stained the supporting cells in the upper chamber epithelium more intensely than those in the lower chamber epithelium (Fig. 3a, right). Twenty lectins, excluding SJA, stained the secretory granules in the glandular cells of the Bowman's glands (Table 1). Five lectins, including WGA, sWGA, BSL-II, DBA and ConA, stained the Bowman's glands intensely. Eleven lectins, including LEL, STL, DSL, SBA, VVA, RCA120, ECL, UEA-I, PSA, LCA and PHA-E, stained moderately, and 4 lectins, including BSL-I, Jacalin, PNA and PHA-L, stained weakly (Fig. 3b). Fourteen lectins stained the supporting cells of upper chamber epithelium and the Bowman's glands differently (Table 1).

DISCUSSION

As we demonstrated in this study, the supporting cells contained secretory granules both in the upper chamber epithelium and lower chamber epithelium in the nasal cavity of soft-shelled turtles. Supporting cells of the upper chamber epithelium contained secretory granules more abundantly than those of the lower chamber epithelium: the secretory granules filled the supranuclear region of the supporting cells in the upper chamber epithelium: whereas, most of the secretory granules were localized near the free border and were scarcely distributed in the supranuclear region of the supporting cells in the lower chamber epithelium. In addition, the upper chamber epithelium possessed the associated glands, but the lower chamber epithelium did not. These evidences suggest variable lubrication ability between the upper and lower chamber epithelia: secretory activity is higher in the upper chamber epithelium than in the lower chamber epithelium. In squamates, the presence or absence of associated glands and the amount of the secretory granules are different between the OE and the VNE [21, 27]. For example, the OEs of snakes, gekkos and scincomorpha associate with glands and contain abundant secretory granules in the supporting cells: whereas, their VNEs lack the associated glands and contain few secretory granules in the supporting cells [25, 26]. The lumen of squamate VNO is always filled with fluids, which have both an intrinsic source and, at least partially, an extrinsic source

(most likely the Harderian glands). In the case of soft-shelled turtles, the surface of the lower chamber epithelium is always in contact with extrinsic water, possibly leading to the lower secretory activity in the lower chamber epithelium as compared with the upper chamber epithelium.

In general, the secretory products of exocrine cells are classified into 3 types: serous type containing neutral mucopolysaccharides, mucous type containing acid mucopolysaccharides and seromucous type containing both neutral and acid mucopolysaccarides [29]. The secretory granules in the Bowman's glands of soft-shelled turtles have been suggested to be serous, because they were PAS-positive and AB-negative and showed high electron-density. The Bowman's glands, which present in all vertebrate species except fish and some amphibians, are generally serous in amphibians, reptiles and birds [11]. Also, in the soft-shelled turtles, the Bowman's glands contain serous secretory granules, suggesting that their secretory products play a significant role shared among non-mammalian Bowman's glands. On the other hand, the supporting cells of both upper chamber epithelium and lower chamber epithelium were positive for PAS and AB pH 2.5, suggesting that they contain mucous or seromucous secretory granules. By the electron microscopic observation, the supporting cells of both chamber epithelia have been demonstrated to contain secretory granules of heterogeneous electron-density, suggesting that they are seromucous. Furthermore, supporting cells of upper chamber epithelium and lower chamber epithelium were suggested to contain secretory granules of different compositions, because they exhibited inconsistent staining for AB pH 1.0.

In order to make further confirmation for the differences in the properties of secretory granules, we analyzed the expression of carbohydrate chains by lectin histochemistry in the area where secretory granules were distributed, and compared them between supporting cells of upper chamber epithelium and those of the lower chamber epithelium. Although 4 lectins stained the supporting cells of both chamber epithelia equally, 14 lectins stained the supporting cells of either upper or lower chamber epithelium more intensely. Among them, 3 lectins stained the supporting cells of upper chamber epithelium more intensely than those of the lower chamber epithelium, and 11 lectins stained the supporting cells of lower chamber epithelium more intensely than those of the upper chamber epithelium. Although electron-microscopic data indicated that the amount of the secretory granules in the supporting cells of lower chamber epithelium is apparently smaller than those of the upper chamber epithelium, most lectins stained the supporting cells of lower chamber epithelium more intensely than those of the upper chamber epithelium. Thus, it seemed that the differences in the lectin staining between them are not due to the differences in the amount of secretory granules but instead due to the differences in their carbohydrate expressions, i.e. the differences in the properties of secretory products.

In addition to the differences in staining patterns for PAS and AB or in the electron-density, the secretory granules in the Bowman's glands showed different binding patterns to lectins from the patterns of the supporting cells in the upper and lower chamber epithelia. For example, supporting cells of both epithelia were negative for BSL-II and PHA-L, whereas the Bowman's glands were positive. The supporting cells of both epithelia were weakly positive for s-WGA, whereas the Bowman's glands were intensely positive. These evidences suggest that the properties of secretory granules are different among them.

In this study, we revealed that the associated glands are present only in the upper chamber epithelium, that the supporting cells of upper chamber epithelium contain secretory granules more abundantly than those of the lower chamber epithelium and that the properties of secretory granules in the supporting cells are different between the upper and lower chamber epithelia in the nasal cavity of soft-shelled turtles. The fluid layer covering the surface of olfactory sensory epithelium plays important roles in olfaction as mentioned above, and the properties of fluid layer differ among functionally distinct epithelia. Considering the lubricating ability as one of the indices for olfactory functions, differences in the olfactory function are suggested by the differences in the amount and properties of secretory granules between the upper and lower chamber epithelia in the olfactory organ of soft-shelled turtles. In general, semi-aquatic turtles show sniffing behavior under water as well as on land. Even when turtles are under water and the lower chamber is filled with water, the upper chamber could be filled with air owing to the structure of nasal cavity. Thus, in the nasal cavity of semi-aquatic turtles, the upper and lower chamber epithelia could be considered as the olfactory organs that function in the air and under water, respectively [5, 28]. Also, in some amphibians, the olfactory organ that functions in the air is different from the olfactory organ that functions under water in the amount and properties of secretory granules. Two distinct sensory epithelia present in the main olfactory system of adult *Xenopus laevis*, the OE (functioning in the air) and the middle chamber epithelium (functioning under water), are such examples [9, 10]: the Bowman's glands are present, and the supporting cells contain abundant secretory granules in the OE; whereas, associated glands are not present, and the supporting cells contain few secretory granules in the middle chamber epithelium [14, 24]. The *Xenopus* OE functions under water in the larval stage and functions in the air during adulthood. During metamorphosis, the Bowman's glands are formed, the amount and size of the secretory granules increase, and their electron-density and lectin-binding patterns change in the supporting cells of the OE [6, 14, 33]. Also, in the olfactory organs of soft-shelled turtles, the upper and lower chamber epithelia seem to be the olfactory organs that function in the air and under water, respectively.

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