The Existence of Merkel Cells in the Lingual Connective Tissue of the Surinam Caiman, *Caiman crocodilus crocodilus* (Order Crocodilia)

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Summary. The tongue of the Surinam caiman (a reptilian species) was studied by light microscopy including immunohistochemistry for protein gene product 9.5 (PGP 9.5), and transmission electron microscopy. The connective tissue immediately under taste buds housed a cluster of cells immunoreactive for PGP 9.5. These cells synapsed on nerves, and their cytoplasm contained characteristic granules of 90 nm in the mean diameter, glycogen particles, and bundles of intermediate filaments. In light of these ultrastructural features, they were identified as Merkel cells. The Merkel cells were also surrounded by Schwann cells. These findings indicate that the present Merkel cell-neurite-Schwann cell complex is comparable to the avian Merkel corpuscle. On the basis of the granule localization in the cytoplasm, the caiman Merkel cell was presumed to be involved in not only mechanoreception but also endocrine or paracrine functions.

The oral mucosa is richly provided with various chemo- and mechano-receptors defined as taste buds (review: REUTTER and WITT, 1993), Meissner corpuscles (BYERS and YEH, 1984; TACHIBANA et al., 1987a), Vater-Pacini corpuscles (WATANABE and YAMADA, 1983, 1985; TACHIBANA et al., 1987b; TOYOSHIMA et al., 1987), Ruffini corpuscles (BEERTSEN et al., 1974; EVERTS et al., 1977; BYERS, 1985; MAEDA et al., 1987), Merkel cells (TOYOSHIMA and SHIMAMURA, 1991; RAMIERI et al., 1992; TOYOSHIMA et al., 1993a, b; review: TACHIBANA, 1995; GARZINO et al., 1996; HILLIGES et al., 1996), and free nerve endings (LUZARDO-BAPTISTA, 1973; RAMIERI et al., 1990; GARZINO et al., 1996; HILLIGES et al., 1996). Although histological and physiological studies have been carried out on these sensory apparatuses in all vertebrate classes, knowledge of these in the reptile class remains insufficient. One of the reasons for the neces-

sity of further investigations is that this animal class possesses certain groups of phylogenetic importance.

It is generally considered that the modern reptile group including the caiman (=Order Crocodilia) and modern birds (=Class Aves) derived from a common extinct group of reptiles (=Order Thecodontia), and that Order Crocodilia has inherited certain characteristics from Order Thecodontia. This phylogenetically close relationship between Order Crocodilia and Order Thecodontia gives credence to the notion that a knowledge of the former, e. g. caimans, will serve towards an understanding of the evolution of the sensory system. To our knowledge, however, only a limited amount of literature dealing with their sensory system is available (DÜRING, 1975; review: DÜRING and MILLER, 1979).

During the course of the histological examination of the caiman tongue, we encountered Merkel cells appearing exclusively in the connective tissue. The Merkel cell is generally intrinsic to the epidermis or oral epithelium (reviews: FUJITA et al., 1988; TACHIBANA, 1995) but is also known to occur in the connective tissue of certain species (DÜRING, 1975; TOYOSHIMA, 1989; TOYOSHIMA and SHIMAMURA, 1991; NARISAWA and HASHIMOTO, 1991; NARISAWA et al., 1992a, b). So far as we are aware, Merkel cells have never been demonstrated in the connective tissue of the reptilian tongue. This paper describes the histological findings of these cells as demonstrated by light and electron microscopy.

MATERIALS AND METHODS

Three Surinam caimans, *Caiman crocodilus crocodilus*, of 18–35 cm in total length were commercially

Fig. 1. Light micrograph of an apical part of the caiman tongue. A cluster of clear, ovoid cells (arrow) appear in the connective tissue under the taste bud (TB). Hematoxylin-eosin stain. $\times 400$

Fig. 2. Light-microscopic immunohistochemistry for PGP 9.5 in the caiman tongue. The cells in the connective tissue (arrow) and certain cells in the taste bud (TB) are immunoreactive. Differential interference contrast illumination. $\times 400$

purchased and used in this study. Before sacrificing by decapitation, the animals were anesthetized by chilling and with ethyl ether.

Light microscopy and immunohistochemistry

The tongue was removed, immersed in Bouin's solution, cut into tissue blocks, and fixed overnight in the solution at room temperature. The tissue blocks were then dehydrated through an ascending series of ethanol and embedded in paraffin. Serial sections, cut at $8\ \mu\text{m}$ in thickness, were mounted on glass slides, air-dried, and processed for the following procedures.

After hydration, some sections were stained mainly with either hematoxylin-eosin or Masson's trichrome.

For other sections, immunohistochemistry was performed with the avidin-biotin complex (ABC) method. The sections were incubated overnight at 4°C with the antiserum against protein gene product 9.5 (PGP 9.5) (UltraClone Limited, England) at a dilution of $1:8,000$ – $1:20,000$. After incubation, the sections were exposed to biotin-labeled anti-rabbit IgG (30 min, room temperature) and then to an avidin-biotin-peroxidase complex (30 min, room temperature; Histofine SAB-PO staining kit, Nichirei Corporation, Tokyo, Japan). The immunoreaction was visualized with 0.0125% diaminobenzidine tetrahydrochloride and 0.004% hydrogen peroxide in 0.05 M Tris buffer, pH 7.6.

The immunoreactive specificity was checked by the following controls: omission of the primary

antiserum, incubation of tissue sections with normal rabbit serum in place of the primary antiserum, and incubation of sections with the antiserum which had been preincubated for 24 h at 4°C with the antigen of 10 – $100\ \mu\text{g/ml}$.

Transmission electron microscopy

The excised tongues were immersed in 2.5% glutaraldehyde containing 2% paraformaldehyde in $1/15\ \text{M}$ phosphate buffer (pH 7.3), minced into small tissue specimens, and kept overnight in the fixative at room temperature. After being rinsed in the buffer, the specimens were post-fixed in 2% osmium tetroxide in the phosphate buffer for 2 h, dehydrated through a graded ethanol series and propylene oxide, and embedded in Epon 812. Ultrathin sections were made on an LKB microtome, mounted on copper grids, and double-stained in a saturated aqueous solution of uranyl acetate (20 min) and Reynolds' lead (13 min). The prepared sections were examined with a JEM-1200EX transmission electron microscope.

RESULTS

The tongue of the Surinam caiman was large and apically tapered, occupying the oral floor. The lingual dorsal surface was rather flat without forming the prominent papillae that commonly exist in mammals. The surface layer comprised a stratified squamous

Fig. 3. Electron-microscopic survey view of a connective tissue portion subjacent to a taste bud. Merkel cells (*M*), nerves (*N*), and Schwann cells (*S*) are illustrated. *TB* basal part of the taste bud. $\times 5,700$

epithelium, which housed taste buds predominantly in the apex of the tongue.

In the connective tissue immediately under the taste buds, there appeared a cluster of clear, ovoid cells (Fig. 1). These cells exhibited an immunoreactivity for PGP 9.5 (Fig. 2). A small part of taste bud cells, spindle-like in shape, were also immunostained for this protein, but the rest of epithelial cells were totally immunonegative (Fig. 2). Incidentally, the immunoreactions after the control procedures were all negative, which consequently confirmed the specificity of the immunostaining.

Under the electron microscope, the corresponding region revealed unique cells more or less elongated along the epithelial layer (Fig. 3). These cells possessed membrane-bound granules of 90 nm in the mean diameter in their cytoplasm (Fig. 4), contacted nerve fibers to form synapses (Fig. 5), and were surrounded by another cell element (Figs. 8, 9a). In consideration of these ultrastructural characteristics, especially the formation of synaptic contacts with

nerves, they were identified as Merkel cells. The cytoplasm of the Merkel cells contained glycogen particles and intermediate filaments in addition to the granules (Figs. 4, 6). Spine-like microvilli characterizing epidermal or epithelial Merkel cells were scarcely recognized in the present Merkel cells. No Merkel cells could be recognized in the epithelial layer.

The granules, round-shaped, measured 70–110 nm in diameter and contained a moderately or highly electron-dense material (Fig. 4). These granules were not filled with the dense material but possessed narrow, electron-lucent halos along the limiting membrane (Fig. 5). They were distributed over the cytoplasm, some of them being condensed towards the plasma membrane directly facing the nerve fibers (Fig. 5). Apart from the granules, a few Merkel cells included huge, lysosome-like granules (500–700 nm in diameter) filled with a homogeneously electron-dense or flocculent material (Fig. 6). The Merkel cells commonly also bore large amounts of glycogen particles in the cytoplasm (Figs. 6, 9a). These particles,

Figs. 4-6. Heavily granulated Merkel cells.

Fig. 4. The cytoplasm contains granules (*Gr*), glycogen particles (*Gl*), and bundles of intermediate filaments (*F*). Note that the endoplasmic reticulum (*arrowheads*) is inactive and sparse. $\times 30,000$

Fig. 5. Synapse between a Merkel cell and a nerve. The Merkel cell (*M*) gathers numerous granules near the synaptic membrane. The nerve (*N*) contains several mitochondria. $\times 46,000$

Fig. 6. Large, lysosome-like granules (*Gr*) occurring in the cytoplasm. The granules are filled with a highly or moderately electron-dense material. *Gl* glycogen particles. $\times 28,000$

Fig. 7. Lightly granulated Merkel cell (*M*). **a.** Survey view of the cell. **b.** Closer view of a part of the Golgi area (*G* in **a**). The Golgi apparatus is well developed with Golgi lamellae and contains maturing granules (*arrowheads*) on the trans-side. *N* nerves, *S* Schwann cell. **a:** $\times 11,000$, **b:** $\times 20,000$

angular or round in shape, tended to gather to each other, giving the cytoplasm a spotted appearance (Fig. 3). Another ultrastructural feature of the Merkel cell was the occurrence of a vast amount of intermediate filaments (Figs. 4, 6). These formed bundles extending in all directions through the cytoplasm (Fig. 4).

The Merkel cells observed did not always display identical features for their ultrastructures. They were divided into at least two categories: many cells containing large numbers of granules (Fig. 4), and the rest possessing only a few of them (Fig. 7a). The former cells generally showed degenerative aspects of rough endoplasmic reticulum and a Golgi apparatus. Lysosome-like granules occurred in small parts of this cell type (Fig. 6). In the latter cells, in contrast, the cytoplasm had developed both rough endoplasmic reticulum and Golgi apparatus (Fig. 7b). The endo-

plasmic reticulum was distributed throughout the cytoplasm and expanded its cisterns. The Golgi apparatus comprised well-developed lamellae and vesicles, and contained condensing and maturing granules on the trans-side (Fig. 7b).

A conspicuous feature of the Merkel cells was that they synapsed on nerve fibers. At the synaptic junction between the cell and the nerve, both plasma membranes were separated by a cleft of about 25 nm in distance and were characterized by a dense material on the plasmic surface—membrane thickening (Fig. 5). The granules in the Merkel cells consistently and specifically gathered at the synaptic zone. On the other hand, the axoplasm at the synaptic site accumulated mitochondria (Fig. 5).

Each Merkel cell was enveloped by a different type of cell, here called a supporting cell, which intervened between neighboring Merkel cells. These supporting

Figs. 8 and 9. Legends on the opposite page.

cells were wrapped in the basal lamina along the outer surface facing the connective tissue (Fig. 8). As these cells very often embraced nerve fibers on their outer side, they were considered Schwann cells (Figs. 8, 9a, b). The distal part of these cells covering the Merkel cells was usually thin, only showing mitochondria, microfilaments, and glycogen particles. The perinuclear region, also being poor in the amount of cytoplasm, contained rough endoplasmic reticulum and a Golgi apparatus in addition to the organelles appearing in the distal cytoplasm (Fig. 8).

Although the covering of the Schwann cells was almost complete, a few Merkel cells partly opened to the surrounding connective tissue through the basal lamina (Fig. 9a, b). In this case, the Merkel cells accumulated granules towards the plasma membrane devoid of the Schwann cell covering (Fig. 9b).

DISCUSSION

The present study is first to demonstrate that the lingual mucosa of the caiman possesses Merkel cells exclusively in the connective tissue under the epithelial portions containing taste buds. Merkel cells, as a member of the paraneuron family (review: FUJITA et al., 1988), share immunoreactivities for such neuron-specific substances as PGP 9.5 (DALSGAARD et al., 1989; RAMIERI et al., 1990, 1992; CRIVELLATO et al., 1994; HILLIGES et al., 1996) and neuron-specific enolase (e. g., GU et al., 1981; ZACCONE, 1986; TOYOSHIMA and SHIMAMURA, 1988; TACHIBANA et al., 1990). On the basis of this knowledge, the PGP 9.5-immunopositive cells occurring in the connective tissue should correspond to the Merkel cells which were identified under the electron microscope.

Taste buds have been known to include PGP9.5-immunoreactive cells in many species (e. g., HUANG and LU, 1996; KANAZAWA and YOSHIE, 1996; WAKISAKA et al., 1996; ASTBACK et al., 1997; YAMAMOTO et al., 1997); such cells in rat taste buds are identical with type III or gustatory cells (KANAZAWA and YOSHIE, 1996). A paper dealing with histological findings of the caiman taste bud is in preparation and will be published elsewhere.

Several works have demonstrated the constant existence of Merkel cells in subepithelial and sub-epidermal connective tissues (DÜRING, 1975; TOYOSHIMA, 1989; TOYOSHIMA and SHIMAMURA, 1991; review: DÜRING and MILLER, 1979). TOYOSHIMA and SHIMAMURA (1991) described the Merkel corpuscle, a gathering of Merkel cell-neurite complexes, in the lingual subepithelium of the finch (a species of bird). The lingual Merkel corpuscle, although it has no such correlation with taste buds as in the present case of the caiman, comprises flattened Merkel cells, nerve terminals, and Schwann cells, and forms a lamination of the disk-shaped Merkel cells arranged alongside each other very regularly (TOYOSHIMA and SHIMAMURA, 1991). Compared with the avian Merkel corpuscle, the Merkel cells in the caiman were disposed rather irregularly. Except for this difference in arrangement, however, the associated elements—neurites and Schwann cells—of the caiman Merkel cells are comparable to those of the avian Merkel corpuscle. Therefore, it seems reasonable to consider that the Merkel cell-neurite-Schwann cell complex in the present reptile tongue is one type of the Merkel corpuscle.

The term Merkel corpuscle was originally given to a dermal or subepithelial sensory apparatus existing in nonaquatic birds (ANDERSEN and NAFSTAD, 1968; SAXOD, 1978). In addition to the avian species, the caiman, *Caiman crocodilus*, also possesses a Merkel corpuscle-like apparatus in the dermis of the touch papilla, which is located and distributed in the skin of the upper and lower jaws (DÜRING, 1975). This apparatus also consists of Merkel cells, neurites, and Schwann cells (review: DÜRING and MILLER, 1979), and, consequently, is nothing else but the Merkel corpuscle. It is generally accepted that both Order Crocodilia and Class Aves have derived from Order Thecodontia, an extinct group of reptiles. Due to the phylogenetically close relationship between these two animal groups, they both likely share the characteristic sensory apparatus—Merkel corpuscle—in the dermis and in the mucosal tunica propria.

Merkel cells in all vertebrate classes are intrinsic components of the epidermis and epithelium. In mammals, moreover, these cells impermanently appear in the subjacent connective tissue during certain

Fig. 8. Perinuclear region of a Schwann cell covering a Merkel cell (M). The Schwann cell contains endoplasmic reticulum (ER) and microfilaments (arrow) in the cytoplasm. The cell embraces a nerve (N) and is wrapped in the basal lamina (arrowheads). Nu nucleus of the Schwann cell. $\times 37,000$

Fig. 9. Relation of a Merkel cell (M) to its Schwann cell (S) covering. The Merkel cell is incompletely covered and opens to the connective tissue within the extent between the arrowheads. To this portion the Merkel cell accumulates granules. N nerves. **b.** Enlarged view of a part of **a.** a: $\times 13,000$, b: $\times 26,000$

periods in embryonic or fetal stages (HASHIMOTO, 1972; MOLL et al., 1986). Recent studies of the human fetus skin by NARISAWA and his associates (NARISAWA and HASHIMOTO, 1991; NARISAWA et al., 1992a, b) have demonstrated the following. A great number of Merkel cells initially emerge in the epidermal layer. These cells then migrate to the dermis and begin to express the nerve growth factor receptor. At the point immediately after the migration, they have no connection with neurites, but finally come into contact with them growing towards the Merkel cells. Subsequently, the Merkel cells decrease in their population and vanish from the dermis. There is no proof yet whether they return to the epidermis. These findings strongly support the hypothesis that these cells are epidermal or epithelial in origin (e. g., MUNGER, 1965; TACHIBANA and NAWA, 1980; NAFSTAD, 1987).

Applying these findings to the Merkel corpuscle in question, there arises a possibility that Merkel cells, having differentiated in the epidermis or epithelium, shift in the connective tissue, where these cells persistently remain to form the corpuscle. To illustrate this possibility, will require further investigations using immunocytochemistry for other Merkel cell-specific or marker substances (cytokeratins, bioactive peptides, chromogranins, etc.) and examination of specimens from the animals at an adequate stage of development.

Merkel cells were first described under the name of Tastzellen (tactile or touch cells) by F. S. MERKEL in 1875. These cells have been widely considered to function in tactile sensation, as he speculated. In fact, they are distributed in various tactile-sensitive portions such as the vermilion border of the lip, snout, and sinus hair (review: FUJITA et al., 1988). Their ultrastructural features also support this presumed function. The spine-like microvilli towards neighboring keratinocytes have been regarded as the detectors of surrounding distortions. Very recently, TOYOSHIMA et al. (1998) applied immunohistochemistry for villin, an actin-crosslinking protein, to the rabbit hard palate, and beautifully demonstrated the microvilli densely radiating from the Merkel cell body.

The classical view that Merkel cells are involved in a slowly adapting mechanoreception has been derived from studies using the tactile domes or Haarscheiben (hair disks) of Pinkus which house numerous Merkel cell-neurite complexes (IGGO and MUIR, 1969). The Merkel corpuscle in the beak of geese conversely is a rapidly adapting mechanoreceptor (GOTTSCHALDT, 1974).

In an electron-microscopic study of the finch lingual Merkel corpuscle, TOYOSHIMA (1989) demon-

strated that the Merkel cells tend to concentrate granules in the cytoplasm towards the cell membrane facing both the nerve terminal and basal lamina. This study revealed granule discharges at both sites and also massive release of the granules at the synaptic portion after receiving a mechanical stimulus. Thus the author proposed dual functions—synaptocrinia and paracrinia/endocrinia—for the Merkel cell in the corpuscle (TOYOSHIMA, 1989). As the distribution patterns of the granules in the caiman Merkel cell were identical with those in the bird Merkel cell, the findings obtained in the present study support his proposal.

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