

Dark-cell areas in the dog vestibular endorgans: an immunohistochemical study

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Summary. The stria vascularis in the cochlea and the dark-cell areas in the vestibular endorgans are structures involved in the production of endolymphatic fluid. This study investigated the dark-cell areas in the vestibular endorgans of the dog by classical staining and by immunohistochemistry (anti-Na,K-ATPase β 2 isoform, anti-cytokeratins (against cytokeratins 5 and 8), anti-vimentin and anti-S100A6) from birth to 110 postnatal days.

Using classical staining, it was not possible to discriminate dark cells from other epithelial cells lining the vestibular endolymphatic spaces. From birth, the Na,K-ATPase β 2 isoform was expressed in the lateral and basal cell membranes of a subset of cells located in the utricular wall, at the base of the cristae ampullaris and was identified as dark cells. From birth, anti-cytokeratins labelled all the cells forming the epithelial lining, including the dark cells, while anti-vimentin labelled the underlying mesenchymal cells. From postnatal day 10, anti-S100A6 labelled subepithelial cells exclusively located underneath the dark-cell areas and were identified as vestibular melanocyte-like cells. From birth, Fontana staining evidenced fine melanin granules in the subepithelial layer. The amount of melanin granules increased during the first month. Melanin distribution was closely associated with the region where S100A6-positive cells were located.

The cell-specific antigen expression in the dog dark-cell areas was clearly comparable to that of the dog stria vascularis previously described. The present investigation also suggested an earlier histological and immunohistological maturity in the dark-cell areas than in the stria vascularis of dogs.

This preliminary morphological description of the normal dark-cell areas in dogs by means of

immunomarkers may be instrumental in studying pathological processes involving the fluid-secreting structures in vestibular endorgans.

Key words: Dark-cell areas, Immunohistochemistry, Dog

Introduction

The mammalian inner ear is divided into the cochlea, concerned with hearing, and into a vestibular part concerned with equilibrium. Both contain a membranous labyrinth that contains compartments filled with endolymphatic fluid. The ionic composition of endolymph is characterized by high K^+ and low Na concentrations essential to support hearing and vestibular functions (Wangemann, 2002a,b).

The stria vascularis is a three-layered structure, which is actively involved in the production of cochlear endolymph and in the generation of a positive endocochlear potential (Marcus et al., 2002; Wangemann, 2002a). The normal histological aspect and the postnatal maturation of the dog stria vascularis have been recently investigated (Coppens et al., 2003).

Dark cells are specialized nonsensory epithelial cells involved in the production of vestibular endolymph (Kimura, 1969; Nicolas et al., 2001; Pitovski and Kerr, 2002; Wangemann, 2002a). Morphological and immunohistochemical studies in several species have demonstrated that dark-cell areas are located at the base of the cristae ampullaris and in the utricular wall (Kimura, 1969; Ichimiya et al., 1994; ten Cate et al., 1994; Nicolas et al., 2001; Peters et al., 2001; Pitovski and Kerr, 2002). No morphological data are available so far about the normal vestibular endorgans and about the dark cells in dogs, except for one electron microscopic study (Mount and Harrison, 1987).

Dogs, as well as other species, are affected by balance disorders that can be either congenital or

acquired during lifetime (Chrisman, 1980; Steel and Bock, 1983; Schunk, 1988; Wilkes and Palmer, 1992; Wangemann, 2002a). A morphological description of normal dark-cell areas and their postnatal maturation in dogs is therefore important to understand the basic mechanism and timing of spontaneous or acquired balance impairments.

Studies investigating the damage to dark cells in the mammalian inner ear due to genetic abnormalities or therapeutics remain highly relevant (Pikrell et al., 1993; Humes, 1999; Nicolas et al., 2001; Rauch, 2001; Watanabe et al., 2001; Wangemann, 2002a,b). Moreover, the dog has been proposed as a suitable animal model for the study of human inner ear diseases because inner ear size, inner ear lesions and susceptibility to ototoxics are similar in dogs and humans (Steel and Bock, 1983; Niparko et al., 1993; Harvey et al., 2001; Sockalingam et al., 2002).

Dark cells are morphologically and biochemically very similar to marginal cells of the stria vascularis in that they display characteristics of fluid transport tissue (Kimura, 1969; Nicolas et al., 2001; Wangemann, 2002a,b). The Na,K-ATPase enzyme, expressed in ion and fluid transporting tissues, is composed of two subunits, α and β , with 3 isoforms for the α subunit ($\alpha 1$, $\alpha 2$, $\alpha 3$) and 2 isoforms for the β subunit ($\beta 1$, $\beta 2$). The antiserum against Na,K-ATPase $\beta 2$ isoform specifically labels marginal cells of the stria vascularis in several mammals, including dogs, and dark cells of the vestibular endorgans in rodents (Ichimya et al., 1994; Mc Guirt and Schulte, 1994; Schulte and Steel, 1994; ten Cate et al., 1994; Peters et al., 2001; Coppens et al., 2001, 2003).

Dark cells are epithelial cells, and studies in rodents and humans have evidenced the expression of cytokeratins in the epithelial cells limiting the vestibular endolymphatic spaces, while the underlying mesenchymal cells express vimentin (Anniko et al., 1989; Schulte and Adams, 1989; Anniko and Arnold, 1990; Kuijpers et al., 1991, 1992).

Melanocyte-like cells, derived from the neural crest, are associated with the stria vascularis and dark-cell areas (Kimura, 1969; Steel and Barkway, 1989; Cable et al., 1994; Kikushi et al., 1994; Peters et al., 1995; Masuda et al., 1994, 1995, 2001). Previous immunohistochemical studies have demonstrated that the antiserum against the S100A6 protein reacts with melanocyte-like cells in the dog stria vascularis (Coppens et al., 2001, 2003), leading to the assumption that this antiserum may also react with melanocyte-like cells in the dog vestibular endorgans. The S100A6 is a protein member of the multigenic EF-hand subfamily of calcium-binding proteins (Heizmann et al., 2002).

The purpose of this preliminary morphological investigation was to observe the normal aspects of dark-cell areas in dogs during the postnatal period by using both classical staining and immunohistochemistry. Based on the functional and histological similarities between the stria vascularis and dark-cell areas, Na,K-ATPase $\beta 2$,

cytokeratins 5 and 8, vimentin, and S100A6 antibodies were used because these antibodies have provided useful information about the dog stria vascularis (Coppens et al., 2001, 2003). Fontana staining was used to visualize the melanin granule distribution in the dog vestibular endorgans.

Material and methods

Puppies

Inner ears were collected from beagle puppies (males and females) of the breeding colony of our faculty. The dogs were cared for according to the principles of the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the University Ethical and Animal Care Committee (reference LA 1230324/4). The investigations were performed in accordance with the principles of the Declaration of Helsinki. Twenty-two puppies, aged from birth (0 PD) to postnatal day 110 (110 PD) were randomly selected from ten different litters. The ages for histological examination were: birth (2 puppies), 1 PD (2 puppies), 3 PD (one puppy), 5 PD (2 puppies), 8 PD (one puppy), 9 PD (one puppy), 10 PD (one puppy), 12 PD (2 puppies), 14 PD (one puppy), 18 PD (2 puppies), 24 PD (one puppy), 27 PD (one puppy), 31 PD (2 puppies), 91 PD (one puppy), 95 PD (one puppy) and 110 PD (one puppy).

Procedure

The puppies were deeply anesthetized with 4 mg/kg intramuscular xylazine (Rompun, Bayer Belgium) followed by 30 mg/kg intraperitoneal or intravenous pentobarbital (Nembutal, Sanofi Belgium). They received 7500 IU/kg intrasplenic or intravenous heparin (Léo, Belgium). They were transcardially perfused with 0.1 molar (M) phosphate-buffered saline (PBS) followed by 4% buffered paraformaldehyde in 0.1 M PBS. The thoracic aorta of the puppies more than one month of age was clamped before perfusion. The temporal bones were immediately dissected, the bullas were opened, and the samples were immersed in the same fixative at 4 °C for 24 to 48 hours. The temporal bones were decalcified for 10 to 143 days in 0.12 M ethylene-diamine-tetraacetate (EDTA) (Titriplex III, Merck-Belgolabo, Belgium) in solution in 0.01 M PBS, pH 7.3 or in 5% formic acid (for the left inner ear of the 10 PD, 17 PD, 91 PD and 110 PD puppies) for 18 to 50 days. Immunostaining results have been found to be similar with EDTA or formic acid procedures (Coppens et al., 2003). The decalcified samples were embedded in paraffin wax and cut (6 to 9 μ m thick) in a plane parallel to the modiolus of the cochlea. Sections were dewaxed, hydrated, and every 15th section was stained with either haematoxylin-eosin or cresyl violet to localize the sacculus, utricle and cristae ampullaris. For each vestibular compartment, 4 to

Dark-cell areas of dogs

6 sections were stained with the Fontana technique counterstained with eosin.

Immunohistochemistry

Dewaxed sections were treated for 30 minutes with methanol containing 1% peroxide solution to inhibit endogenous peroxidase activity, then washed in 0.05 M Tris buffered saline, pH 7.4 (TBS). Sections for cytokeratins-labelling were pre-treated with the proteinase K (ready-to-use, Dako, Netherlands) for 2 minutes. The sections were incubated in a humid chamber for 30 minutes at 37 °C with 1/20 normal horse serum (NHS) in TBS. Sections were covered for 18 hours at 4 °C with antibodies diluted in TBS containing 2% bovine serum albumin (BSA) (Sigma; Belgium), i.e., polyclonal rabbit NaKA/TPase ($\beta 2$ subunit) antiserum (Upstate Biotechnology, USA), 1/1500; monoclonal mouse vimentin antiserum (clone V6630; Sigma, Belgium), 1/1400; monoclonal mouse cytokeratin antiserum against Cytokeratins 5 and 8 (RCK102; Monosan, USA), 1/10; goat polyclonal anti-S100A6 (Ilg et al., 1996), 1/4000. The primary immune antiserum was revealed by the avidin-biotin procedure as follows: a 30 min incubation in biotinylated anti-rabbit/mouse

(Na,K-ATPase, cytokeratins and vimentin) or anti-goat (S100A6) gamma globulin (Vector Labs, USA) diluted to 1/100 in TBS with BSA 1%; followed by a 30 min incubation with avidin-biotin-peroxidase complex (ABC) standard kit reagent (Vector Labs, USA). The intermediate washings were done using TBS. The staining was performed in diaminobenzidine/ H_2O_2 solution (liquid DAB substrate pack from Biogenex, USA). The immunolabelled sections were not counterstained. Adjacent sections were chosen to study the immunodistribution of each antiserum, and to allow comparison between the immunodistribution of each of them.

Control sections, with the primary antibody omitted, remained unlabelled. Specificity of the immunomarkers was previously verified (Coppens et al., 2003).

Results

Classical staining (cresyl violet and haematoxylin-eosin)

The sacculus, the utricle and the semicircular canals were limited by a multi-layered membrane, separating the endolymphatic fluid-filled spaces from the surrounding perilymph (Fig. 1a).

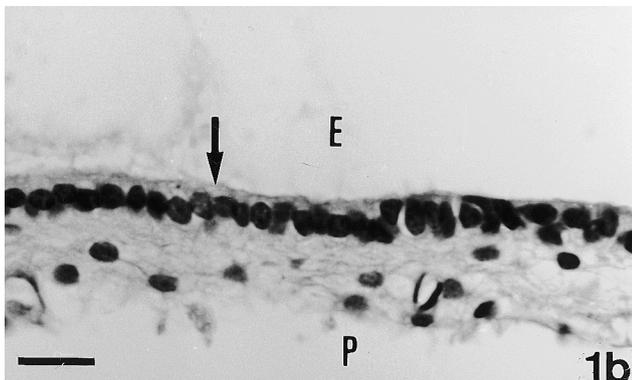
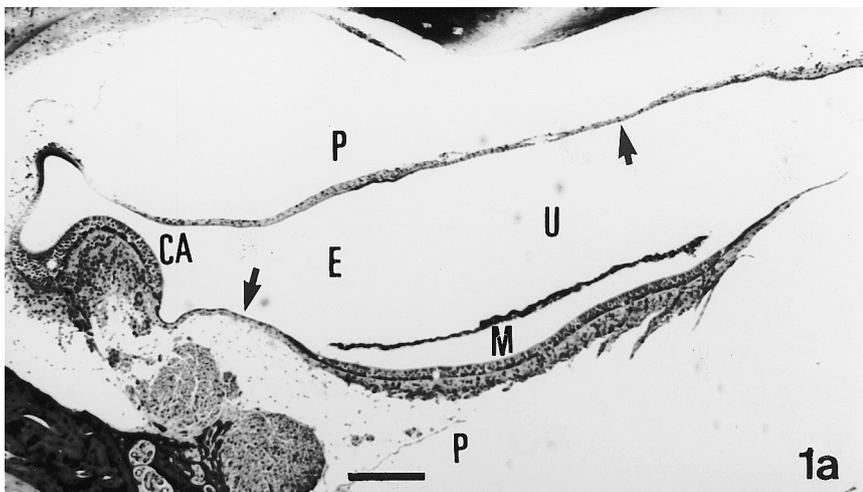


Fig. 1. a. Classical staining (haematoxylin-eosin) of the dog semicircular canal and utricle: a membrane (arrows) limits the semicircular canal, containing the crista ampullaris (CA), and the utricle (U), containing the macula (M), and separates endolymphatic spaces (E) from the surrounding perilymph (P). It is not possible to discriminate the dark-cell areas. Scale bar: 250 μ m. **b.** Classical staining (cresyl violet) at the base of the crista ampullaris: a multi-layered membrane separates the endolymphatic space (E) from the surrounding perilymph (P); a layer of cuboidal cells (arrows) borders the endolymphatic space. Scale bar: 15 μ m.

Dark-cell areas of dogs

The endolymphatic fluid-filled spaces were lined by a continuous layer of cuboidal to squamous epithelial cells (Fig. 1b). It was not possible to discriminate the dark cells from other nonsensory epithelial cells by classical staining.

The overall morphological picture of the vestibular endorgans was mature from birth as evidenced by classical staining detecting no morphological changes in the dog vestibular endorgans throughout the postnatal period.

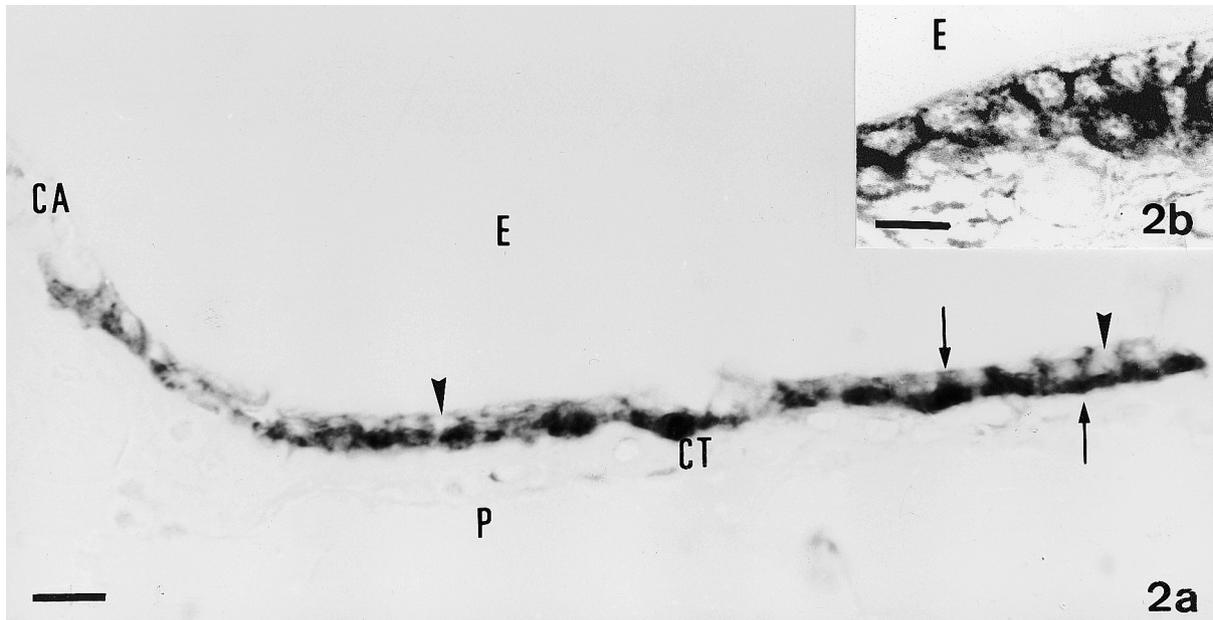


Fig. 2. a. Na,K-ATPase b2 immunostaining at 91PD at the base of the crista ampullaris (CA); labelled dark cells are situated at the base of the crista ampullaris (CA); labelled dark cells form a simple layer of cuboidal cells; intense Na,K-ATPase-staining is localized in the basal and lateral cell membranes (arrows), see also insert; the luminal surface (arrowheads) limiting the endolymphatic space (E) do not show Na,K-ATPase-positivity; the underlying connective tissue (CT) bordering the perilymph (P) is unlabelled. Scale bar: 15 μm . **b.** insert: tangential cut in Na,K-ATPase β 2-immunostained dark cells. Scale bar: 13 μm .

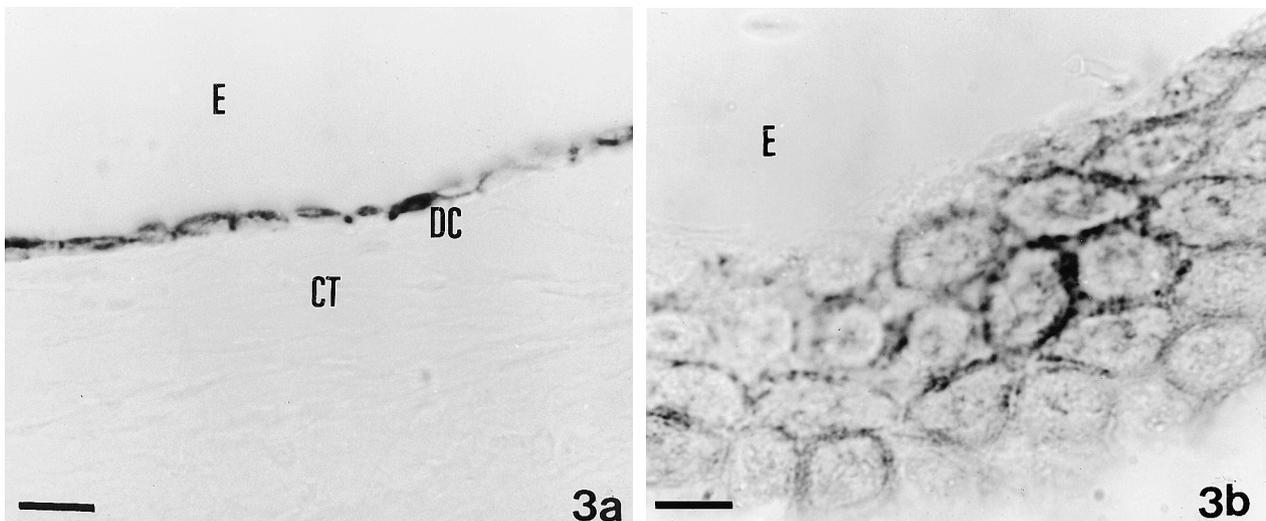
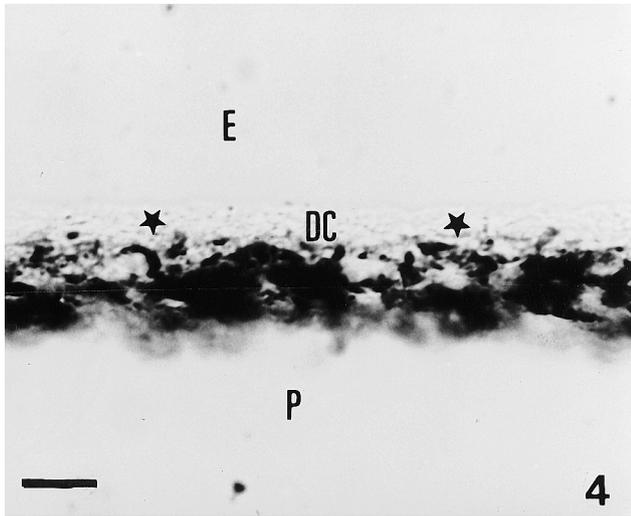


Fig. 3. a. Cytokeratins 5 and/or 8 immunolabelling at 91PD in dark-cell areas: the apical pole of epithelial dark cells (DC) close to the endolymphatic space (E) is labelled; the underlying connective tissue (CT) is unlabelled. Scale bar: 15 μm . **b.** Cytokeratins immunolabelling, tangential cut in dark cells: cytokeratins form an intracellular network situated close to the cell borders evidencing lateral limits of cells. Scale bar: 10 μm .

Dark-cell areas of dogs

Na,K-ATPase $\beta 2$ isoform immunolabelling

In the semicircular canals, Na,K-ATPase $\beta 2$ isoform immunolabelling was observed in a subset of cells located at the base the crista ampullaris (Fig. 2a). In the utricle, immunolabelling extended over the wall opposite the macula utriculi. No labelling was seen in the sensory epithelial cells, in the immediate periphery of the utricular macula or in the wall of the sacculus.

The immunostained dark cells formed a single layer of cuboidal cells. The Na,K-ATPase $\beta 2$ isoform was

Fig. 4. Vimentin immunolabelling at 91PD in dark-cell areas: the epithelial dark cells (DC, stars) limiting the endolymphatic space (E) are unlabelled; mesenchymal cells constituting the subepithelial connective tissue show strong immunolabelling; immunolabelled mesenchymal cells border the perilymph (P). Scale bar: 15 μm .

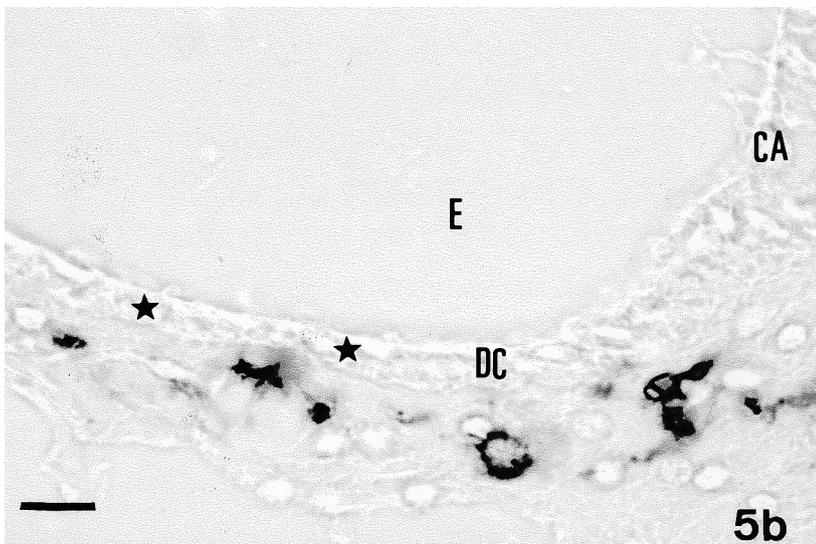
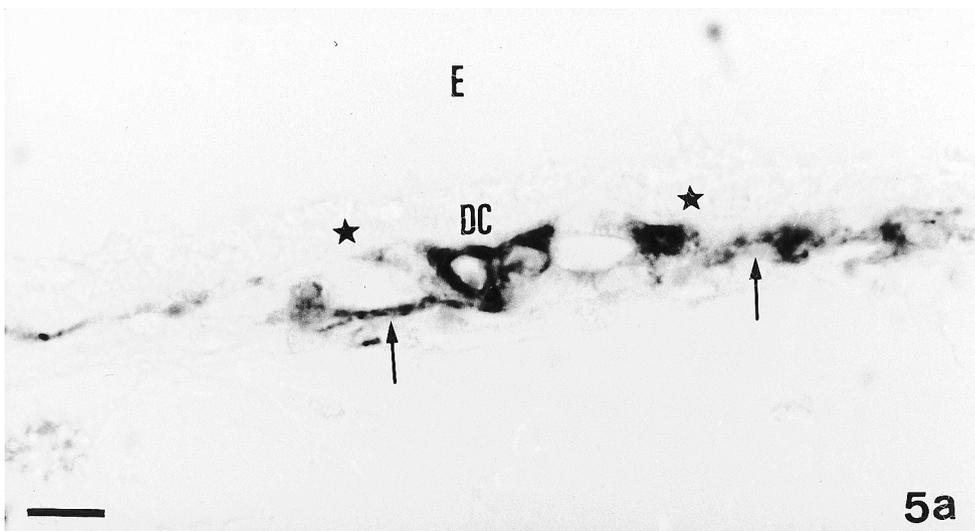


Fig. 5. a. S100A6 immunolabelling at 91PD in dark-cell areas: S100A6-immunolabelled melanocyte-like cells are scattered in the subepithelial layer underneath unlabelled dark cells (DC, stars) that limit the endolymphatic space (E); melanocyte-like cells send out thin cellular processes (arrows). Scale bar: 13 μm . **b.** S100A6 immunolabelling at 10PD in dark-cell areas at the base of the crista ampullaris (CA). Scale bar: 13 μm .

Dark-cell areas of dogs

localized in the basal and lateral cell membranes, while the luminal surface remained immunonegative (Fig. 2a,b).

The dark cells were found to be Na,K-ATPase-positive from birth onwards.

Cytokeratins 5 and 8 immunolabelling

Strong cytokeratin expression was detected along the whole nonsensory epithelium lining the endolymphatic spaces (Fig. 3a). Cytokeratins were found close to the luminal surface of the epithelial cells including the dark cells. In tangential cuts, cytokeratins formed a regularly organized intracellular network situated close to the lateral cell borders (Fig. 3b).

The underlying connective tissue and the cells bordering the perilymphatic space remained unlabelled. The expression remained unchanged from birth to 110 PD.

Vimentin immunolabelling

The mesenchymal cells underlying the dark cells and

the cells bordering the perilymphatic spaces of the vestibular labyrinth showed strong vimentin immunolabelling (Fig. 4). The epithelial dark cells were immunonegative.

The immunolabelling remained unchanged from birth to 110 PD.

S100A6 immunolabelling

The S100A6-positive cells were located in the same region as the Na,K-ATPase-positive dark cells, which were confined to the base of the cristae and to the utricular wall that faced the maccula utriculi. The sacculus lacked both S100A6 and Na,K-ATPase $\beta 2$ isoform immunolabelling.

S100A6-labelled cells were situated under the dark cells, scattered between the underlying mesenchymal cells and constituted a non-continuous layer in the dark-cell areas subepithelial layer (Fig. 5a). No S100A6 reactivity was observed in the epithelial dark cell layer. The S100A6 antiserum defined a granular cytoplasmic pattern and S100A6-positive cells displayed a dendritic appearance with well-developed cell processes.

No S100A6-positivity was evidenced in newborn puppies and sparse S100A6-labelled cells appeared at 10 PD (Fig. 5b). Initially, the S100A6-labelled cells were rare and dispersed, but reached a definitive adult-like distribution during the first month of life.

Fontana staining

Fontana staining revealed melanin pigments as dark-blue to black granules.

Melanin granules were observed at the base of each crista and in the utricular wall, mainly in the area opposite to the maccula utriculi. The sacculus lacked melanin granules.

The melanin-containing cells were subepithelially located, i.e., underneath the areas of the dark cells (Fig.

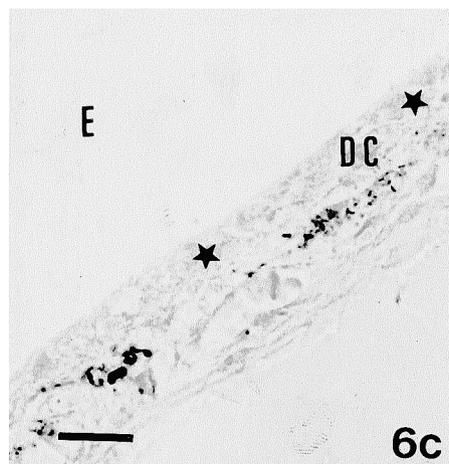
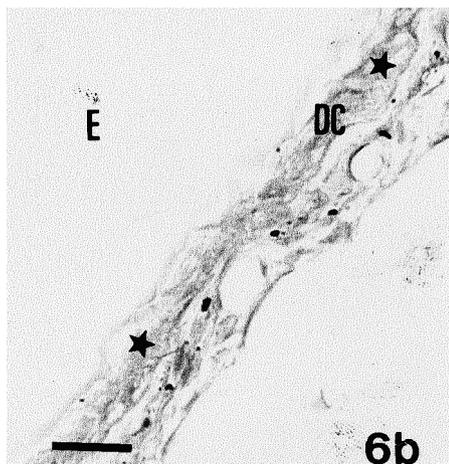
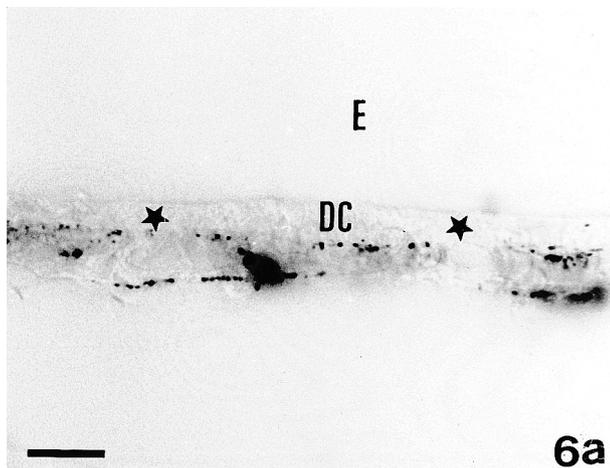


Fig. 6. a. Fontana at 91PD in dark-cell areas. Melanin granules are scattered in the subepithelial layer underneath dark cells (DC, stars) that limit the endolymphatic space (E). Scale bar: 15 μm . b. Fontana at 0PD in dark-cell areas. Scale bar: 14 μm . c. Fontana at 31PD in dark-cell areas. Scale bar: 14 μm .

Dark-cell areas of dogs

6a). Adjacent sections showed that melanin granules mostly colocalized with the S100A6-immunodistribution.

From birth, Fontana staining revealed rare, small melanin granules scattered in the utricular wall and sometimes in the wall of the semicircular canals, next to the cristae ampullaris (Fig. 6b). The number of melanin granules increased during the first month (Fig. 6c).

Discussion

This study is the first report documenting the immunodistribution of cytokeratins, vimentin, Na,K-ATPase and S100A6 in the vestibular endorgans of the dog inner ear. This panel of antibodies allowed the identification of the cell types that constituted dark-cell areas.

In the mammalian inner ear, the endolymphatic spaces forming the vestibular endorgans are limited by a simple epithelium. Dark cells are epithelial cells that are mainly involved in the vestibular endolymph production, secreting potassium towards the endolymphatic fluid (Kimura, 1969; Nicolas et al., 2001; Wangemann, 2002a). Dark-cell areas are located at the base of the cristae ampullaris in each semicircular canal and in the utricular wall, whereas the sacculus lacks dark cells (Kimura, 1969; Nicolas et al., 2001; Pitovski and Kerr, 2002). Up to now, dark-cell areas and their location have not been studied in dogs.

In the present study, all of the nonsensory epithelial cells, including epithelial dark cells, lining the endolymphatic spaces expressed cytokeratins, as previously described in other species (Anniko et al., 1989; Anniko and Arnold, 1990; Kuijpers et al., 1991, 1992). Since the antiserum against cytokeratins recognizes both 5 and 8 cytokeratins, it could not be stated whether 5 and/or 8 forms were expressed in the dog vestibular endorgans. In this study using the light microscope, the Na,K-ATPase $\beta 2$ isoform was expressed in the lateral and basal cell membranes in a subset of epithelial cuboidal cells located at the base of the cristae ampullaris and in the utricular wall opposite to the utricular macula. The Na,K-ATPase $\beta 2$ immunolabelling and the location of this subset of cells in dogs mainly coincided with that of dark cells in other species (Kimura, 1969; Mc Guirt and Schulte, 1994; Schulte and Steel, 1994; Ichimiya et al., 1994; ten Cate et al., 1994; Peters et al., 2001; Wangemann, 2002a,b).

The dog sacculus was devoid of Na,K-ATPase $\beta 2$ expression, suggesting that the dog sacculus was devoid of dark-cell areas. Morphological, electrochemical and biochemical studies in laboratory mammals have proposed that the saccular endolymph derives from the cochlea through the canal reuniens (Kimura, 1969; Sellick and Johnstone, 1975; Pitovski and Kerr, 2002). Indeed, in cochleosaccular deafness in dogs, as well as in other mammals, the stria vascularis degenerates, leading to the collapse of the cochlear duct and sacculus without abnormalities in the utricle or semicircular

canals (Steel and Bock, 1983; Cable et al., 1994).

In mammals, neural crest-derived melanocyte-like cells are located in the dark-cell areas (Kimura, 1969; Kikushi et al., 1994; Masuda et al., 1994, 1995, 2001; Peters et al., 1995; Pitovski and Kerr, 2002). In the present study, the S100A6 antiserum stained cells that were scattered within the dark cell subepithelial layer in the utricle and semicircular canals of dogs. On the other hand, Fontana staining disclosed a clear-cut correlation between melanin granule distribution and S100A6-positive cells. A previous immunohistochemical report has evidenced that S100A6 antiserum is a valid marker of melanocyte-like cells in the canine stria vascularis (Coppens et al., 2001, 2003). In the dog vestibular endorgans, based on S100A6 expression, location of immunostained cells and melanin granule colocalisation, the S100A6-positive cells were interpreted as melanocyte-like cells.

This finding leads the authors to assume that anti-S100A6 would be a marker of the neural crest derived melanocyte-like cells in both vestibular endorgans and cochlea of dogs. This hypothesis is further strengthened by a number of previous reports describing the expression of S100A6 in neural crest-derived cells in various tissues and in melanoma (Böni et al., 1997; Coppens et al., 2001; Fullen et al., 2001; Heizmann et al., 2002). Nevertheless, the functional implication of S100A6 expression in melanocyte-like cells of the dog inner ear is undecided, as the roles of the S100A6 calcium-binding proteins remains elusive and under investigation (Heizmann et al., 2002).

As mentioned before, the stria vascularis and dark-cell areas contribute to the production of inner ear endolymph and morphological investigations in mammals have shown that both structures show a similar general histological pattern (Kimura 1969; Nicolas et al., 2001; Wangemann, 2002a,b). In a previous study, immunohistochemistry made it possible to distinguish the constituting cell-types of the canine stria vascularis (Coppens et al., 2003). The present study evidenced a similar immunohistochemical picture in canine dark-cell areas. Epithelial dark cells, as well as epithelial marginal cells of the stria vascularis, expressed cytokeratins 5 and/or 8 and showed Na,K-ATPase $\beta 2$ immunolabelling in their basal and lateral cell membranes; melanocyte-like cells were found S100A6-positive and the underlying mesenchymal basal cells were vimentin-positive in both structures.

Stria vascularis and dark-cell areas, however, differ from a morphological and developmental point of view in dogs. In the dog stria vascularis, immunostainings made it possible to delineate numerous processes sent from the basal cell membrane of marginal cells and multiple slim digitations that closely surrounded the melanocyte-like cells (Coppens et al., 2003). In the dog vestibular endorgans, the basal cell membrane processes of dark cells were found to be less developed and the S100A6-positive melanocyte-like cells were not closely surrounded by dark-cell digitations. This morphological

variation correlates with the different functional role played by melanocyte-like cells. The melanocyte-like cells in the stria vascularis are involved in recycling the cochlear endolymphatic ions, but these cells also play a major role in generating the high positive endolymphatic potential in the cochlear duct (Marcus et al., 2002; Wagemann, 2002a,b). In the vestibular endorgans, the melanocyte-like cells are mainly involved in ion and material transport, but they do not generate such a high positive endolymphatic potential (Cable et al., 1994; Masuda et al., 2001; Marcus et al., 2002; Wagemann, 2002b).

Previous studies have suggested that the melanocyte-like cells may also have a protective role in the inner ear under certain pathological conditions. Studies in rodents have pointed out that if the melanocyte-like cells are commonly situated in the subepithelial layer in normal animals, the contact between dark cells and melanocyte-like cells becomes significantly closer, and melanin production is increased in experimentally-injured rodent inner ears (Jahnke et al., 1991; Yoshira et al., 1994). Morphological descriptions of adult human vestibular endorgans have also evidenced some intraepithelial melanocytes in patients suffering from vestibular tumour (Masuda et al., 1994, 1995, 2001). It has therefore been suggested that melanocyte-like cells in the vestibular endorgans connect more closely with dark cells under certain pathological conditions (Jahnke et al., 1991; Yoshira et al., 1994; Masuda et al., 1995). In the present description, neither melanin granules nor S100A6-positivity were observed in the dark-cell layer proper, leading to the conclusion that the melanocyte-like cells are subepithelially located in the dog dark-cell areas. The present investigation was undertaken on healthy young dogs without clinical balance disorder. Contacts with physical, chemical or biological agents, which might damage the puppy inner ear, was ruled out in the breeding conditions. Consequently, it cannot be excluded that intraepithelial melanocytes-like cells could appear or that melanin pigments could be transferred to epithelial dark cells in older dogs or under pathological conditions.

The dog stria vascularis is morphologically and immunohistochemically immature at birth (Coppens et al., 2001, 2003). The Na,K-ATPase immunolabelling of marginal cells changes and basal membrane processes of marginal cells increase towards the basal cell layer during the first three weeks of life; S100A6 immunolabelling clearly marks the intermediate cells from 24 PD; the amount of melanin increases beyond the first month; and basal cells become elongated during the first month (Coppens et al., 2001, 2003). These morphological changes may be correlated with the positive endocochlear-potential increase occurring during the first month of life in altricial species with an immature cochlea at birth. In contrast, only minor morphological changes were observed in puppy vestibular endorgans during the studied period: the S100A6-immunolabelling became clearly detectable as

soon as 10 PD and the amount of melanin increased during the first month of life. This observation suggests that the dark-cell areas in dogs are mature earlier than the stria vascularis and this might offer explanations for the different susceptibility of the cochlea and vestibule to physical and chemical insults in newborns and very young puppies (Chrisman, 1980; Wilkes and Palmer, 1992; Pikrell, 1993).

As dark-cell areas may be involved in hereditary or acquired vestibular defects, a study, such as this preliminary one, may form a basis for further investigations into the mechanisms leading to vestibulopathies (Humes, 1999; Watanabe et al., 2001; Wagemann, 2002a,b). Further investigation of the canine genome would provide information about the genetic origin of hearing and balance defects in this species.

A detailed description of the normal dog vestibular endorgans may also be helpful should the dog be used as an animal model for inner ear insults and therapeutic approaches in humans. An important current direction in inner ear therapeutic research and otoprotection lies in the possibility to instil neurotrophins, growth factors or stem cells in the inner ear (Feghali et al., 1998; Li et al., 2003). The next step towards this goal will be the use of animal models. In this context, dogs offer advantages when compared to rodents. The longevity of dogs should allow the study of the long-term effects of regeneration/repair treatments or otoprotection. The inner ear size of dogs permits easier surgical access; the use of a minipump or cochlear implant for humans might be tested on dogs without major technical adaptations.

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Dark-cell areas of dogs

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