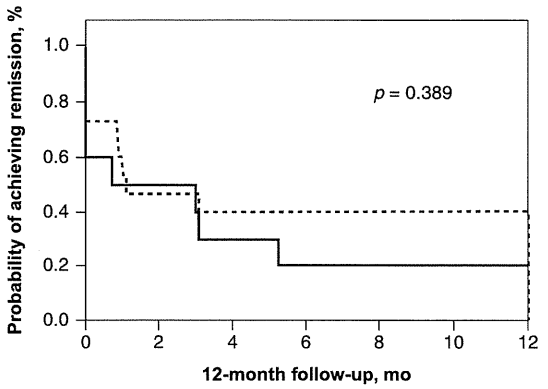

Figure 3. Kaplan–Meier estimates for the probability of achieving remission of definitive spells during the 12 month follow-up period. The black line indicates the eight patients treated by the TMM device (TM group). The dotted line indicates the nine patients treated by the Meniett device (ME group). Achievement of remission (6 consecutive months with absolutely no definitive spells) occurred quickly, within the first 3 months (60%) in both groups. There was no significant difference in the time course of the reduction in definitive spells between the two groups ($p > 0.05$).

In the 7–12 month period after treatment, 2 ears (MD, $n = 2$) showed significant improvement, 13 ears (MD, $n = 9$; DEH, $n = 4$) had stable hearing, and 1 MD ear showed significant hearing deterioration. The distribution of hearing outcomes according to the three grade evaluation system (improved; unchanged; and deteriorated hearing) in the TM group was not statistically different from the ME group during both the 1–6 month and the 7–12 month periods after treatment ($p > 0.05$).

In the TM group, the mean hearing level (i.e. PTA) in the 6 months before treatment was 69.0 ± 27.4 dB, while in the 1–6 month period after treatment it was 71.7 ± 26.6 dB. The mean PTA in the 7–12 month period after treatment was 72.2 ± 26.4 dB. In the ME group, the mean PTA in the 6 months before treatment was 58.8 ± 23.1 dB, while in the 1–6 month period after treatment the mean PTA was 57.8 ± 25.5 dB. The mean PTA in the 7–12 month period after treatment was 52.4 ± 28.0 dB. Figure 4 indicates that the hearing level after treatment had not changed in either group ($p > 0.05$), and the time course of change in the hearing level in the TM group was not significantly different to that of the ME group ($p > 0.05$).

In the TM and ME groups, no complications were directly attributable to either device.

Discussion

In this study, the effects of pressure treatment were compared between the TM and ME groups. Regarding vertigo outcomes in particular, all the patients in both groups experienced either complete or substantial control in the 7–12 month period after treatment. The distribution of the NV according to the JSER guideline was not significantly different between the two groups. In addition, the time course of vestibular symptoms (i.e. the frequency of vertigo per month and the period required for remission) was also not significantly different between the two groups. These findings suggested that alongside the Meniett device, the TMM device may provide a 1 year period of relief, or reduction in definitive spells in the majority of patients with intractable MD and DEH. Hearing outcomes remained stable in the majority of patients (TM group, 92%; ME group, 81%) in the 7–12 month period after treatment. The distribution of hearing outcomes according to the JSER guideline was not significantly different between the two groups. These findings suggested that both the Meniett and TMM devices may induce insignificant changes to hearing in the majority of ears treated in patients with MD and DEH.

Table III. Change in hearing outcomes in the post-treatment period in the TM group (TMM device) and ME group (Meniett device).

	TM group		ME group	
Hearing	1–6 months	7–12 months	1–6 months	7–12 months
Improved	0	0 (0%)	2 (13%)*	2 (13%)*
Unchanged	12 (100%)†	11 (92%)†	12 (74%)*	13 (81%)
Deteriorated	0	1 (8%)	2 (13%)	1 (6%)
Total	12 (100%)	12 (100%)	16 (100%)	16 (100%)

Values are given as number of ears (percentage).
*In 1 of 11 patients with MD patients who used the ME device bilaterally, hearing outcomes in both ears were classified as improved.
†In 1 of 10 patients and 1 of 2 patients with DEH who used the TMM device bilaterally, hearing outcomes in both ears were classified as unchanged.
Chi squared test: TM group vs ME group 1–6 months after treatment, $p > 0.05$; 7–12 months after treatment, $p > 0.05$.

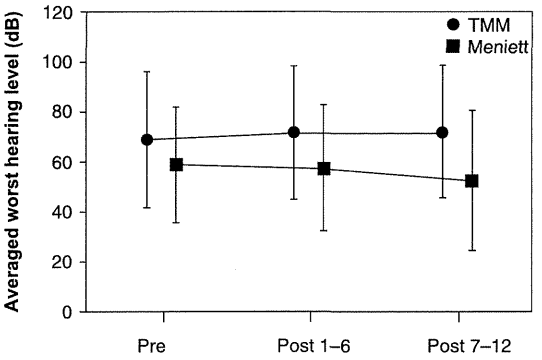

Figure 4. Change in averaged worst hearing level. The black circle indicates the TM group (TMM device), while the black square indicates the ME group (Meniett device). The worst hearing level after treatment was not reduced significantly in either group ($p < 0.01$).

The external ear canal pressure changes produced by the TMM device are transmitted to the inner ear by two routes: (1) via the ossicular chain and (2) via the middle ear cavity. The amplitude of pressure transfer through the intact tympanic membrane to the inner ear is significantly smaller than the pressure transmitted directly via the middle ear and the round window [15]. Our TMM device produces a biphasic pressure pulse, which is almost twice as large as the monophasic pressure pulse produced by the Meniett device. It is possible that the larger pressure pulse from the TMM device might be helpful in compensating for the attenuation of pressure changes in the transfer process, resulting in similar effects from pressure treatment in both the TM and ME groups.

The pressure changes transmitted to the inner ear are immediately stabilized by the inner ear pressure release routes, mainly by the patency of the cochlear aqueduct. The calculation of time constants related to pressure stabilization time courses could establish the most effective pressure pulse frequency for influencing the inner ear status. Densert et al. showed that low frequency sine pressure pulses from 5 to 15 Hz were far superior in influencing the inner ear hydrodynamic balance than square-wave pressure pulses [16]. There were no significant differences in the perilymphatic response to varying sine wave frequencies. In 1995, Densert et al. [17] used the 9 Hz frequency of alternating pressure. Subsequently, the frequency was adjusted to 6 Hz, which is equal to the Meniett device [2,6]. In the case of the TMM and Meniett devices, the pressures used are 7 Hz and 6 Hz, respectively. Under these frequencies, it is possible that the pressure pulse might be transmitted to change inner ear pressure efficiently. Animal studies are needed to measure inner ear pressure during the transmission of complex oscillating ear canal pressures to the intact tympanic membrane as they are changed by the TMM device.

The application of pressure changes to the inner ear has induced a change in the summating potential/action potential (SP/AP) amplitude ratio in the electrocochleograms (EcochGs) of patients with MD. Densert et al. [17] described that the effect of a very short duration of pressure treatment with the Meniett device in patients with MD induced a significant reduction in the abnormal SP/AP amplitude ratio. There was no difference in the EcochG responses of MD patients treated by a false pulse generator, in which the external appearance of the unit was the same as the Meniett device but there was no pressure pulse output. Storms et al. [18] reported that a transient application of pressure to the ear canal with amplitude ranging from –60 to +40 cmH_2O in patients with MD induced a significant reduction in the SP/AP amplitude ratio. Changes in EcochG parameters from positive pressure were not different to those generated by negative pressure. There was no significant effect of ear canal pressure changes on EcochG parameters for the normative subjects. The TMM device is a positive/negative pressure pulse generator within amplitude from –20 to +20 cmH_2O and it is possible that the pressure pulse provided by the TMM device might influence inner ear status, resulting in the EcochG responses becoming more normal.

The mechanism whereby external ear pressure application by the TMM device might affect inner ear physiology is not understood. Like the Meniett device, intermittent pressure pulses might directly improve drainage of the endolymph to the endolymphatic duct and sac [6]. An alternative possibility is that low-intensity stimuli may lead to hormone production from the endolymphatic sac, which controls the secretion and absorption of endolymph [19]. Circulatory flow of the endolymphatic fluid through the inner ear membranes and connecting blood vessels, or other mechanisms activating turnover of the endolymph, might be responsible for the beneficial effects of ear pressure applications on the inner ear in endolymphatic hydrops [20].

The effect of the Meniett device on definitive spells has been documented in a number of studies within the first 3 months following treatment [3,5,6]. Odkvist et al. [6] reported that a significant improvement in the frequency of definitive spells occurred from a 2-week-long, multicentre, placebo-controlled study. Dornhoffer and King [5] described that nine patients responded to the Meniett device within 4 weeks. Gates et al. [3] reported that patients probably achieved remission of definitive spells in

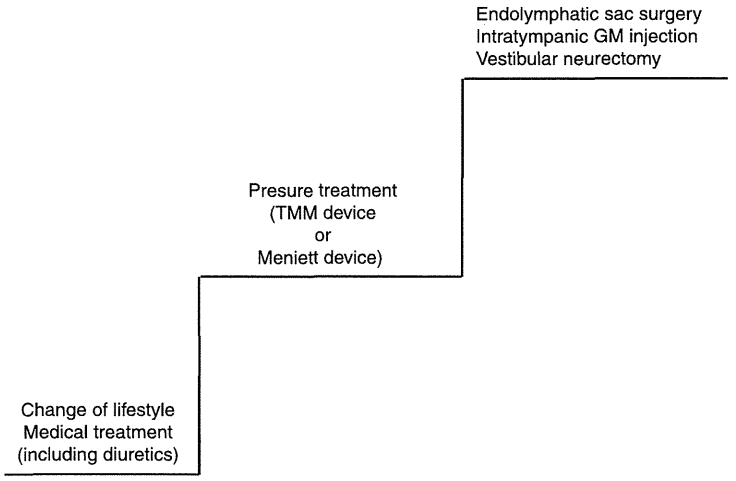

Figure 5. Treatment algorithm for Meniere's disease at the University of Toyama.

2.8 months on average. In this study, the period requiring a probable achievement of definitive spells remission in the ME group was an average of 23 days after treatment, while in the TM group the average was 45 days after treatment. In addition, 60% of patients probably achieved remission of definitive spells within the first 3 months following treatment in both groups. Based on our study, we consider that the use of the TMM device should be continued for at least the first 3 months following the initial treatment.

The patency of tympanostomy tubes is a prerequisite for successful treatment by the Meniett device. The management of tubes requires periodic otologic attention for occlusion and extrusion. Other potential tubal problems are post-tympanostomy tube otorrhea and permanent perforation of the tympanic membrane. Barbara et al. [4] documented that occlusion or early extrusion of the tympanostomy was experienced by 10 of 36 patients. Densert and Sass [2] reported that middle ear infections were observed in 5 of 37 patients. Gates et al. [3] mentioned that intolerance for the tympanostomy tube is a limiting factor for treatment by the Meniett device. In contrast, in the case of the TMM device the patient is able to start treatment immediately without the insertion of a tympanostomy tube. The pressure pulse is simply applied to the intact tympanic membrane. Our experience since 2007 has shown that 2 of 12 consecutive patients selected the Meniett device, while the remaining 10 patients chose the TMM device. Even if insertion of a tympanostomy tube is the most minimally invasive procedure, the majority of our patients did not prefer this method. Because this study reports that the efficacy of vestibular symptoms in the TM group is similar to the ME group, the TMM device might be a worthwhile option before use of tympanostomy tubes.

In this study, in the 7–12 month period after treatment, 11 of 12 ears were recorded with unchanged hearing, and 1 ear displayed reduced hearing in the TM group. In the ME group, the number of ears with improved, unchanged, and deteriorated hearing in these studies is summarized as 2 (13%), 13 (81%), and 1 (6%), respectively. These findings suggest that, at the moment, the changes to hearing induced in the majority of treated ears in patients with MD by both the TMM and Meniett devices might be insignificant according to the JSER treatment outcome criteria.

Many important questions still remain unanswered, such as: who is the ideal candidate for the TMM device, how long should it be used, and when should it be discontinued? Undoubtedly, further clinical assessments over longer periods as well as basic research into the mechanisms of action are needed to better understand the clinical value of the TMM device for neuro-otologic use.

Conclusion

We recommend the TMM device for patients whose vestibular symptoms are poorly controlled by lifestyle change and medical therapy. Moreover, we recommend employing TMM devices before the use of tympanostomy tubes is considered (Figure 5).

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ORIGINAL ARTICLE

Localization of prostanoid receptors in the mouse inner ear

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Abstract

Conclusion: EP4, EP2, and IP prostanoid receptors exert an otoprotective function and FP may be important for fluid homeostasis in the inner ear. *Objective:* To investigate the expression of prostanoid receptors in the normal mouse inner ear. *Methods:* CBA/J mice were used in this study. The localization of prostanoid receptors, i.e. DP, EP1, EP2, EP3, EP4, FP, IP, and TP, in the inner ear, i.e. the cochlea, vestibular end organs, endolymphatic sac, was studied by immunohistochemical techniques. *Results:* The EP4, IP, and FP prostanoid receptors were found to be abundantly distributed in many inner ear structures, i.e. stria vascularis, inner and outer hair cells, spiral ganglion cells, vestibular sensory and ganglion cells, and the endolymphatic sac. EP2 and EP3 are also localized in the inner ear whereas DP, EP1, and TP are only weakly expressed.

Keywords: *Immunohistochemistry, otoprotection, fluid homeostasis*

Introduction

The prostaglandins (PGs) are autacoids that exert their effects via specific cell membrane receptors of the 7 TM G-protein coupled metabotropic type [1]. The pharmacological classification and molecular biological characterization of these receptors have been reviewed in several articles [1]. At least eight different prostanoid receptors (PRs) exist, namely DP (PGD2), EP1 (PGE2), EP2 (PGE2), EP3 (PGE2), EP4 (PGE2), FP (PGF2α), IP (PGI2), and TP (TxA2), the most abundant endogenous ligand for each receptor is indicated in parentheses. In the inner ear, the distribution of EP1, EP3, EP4, and FP has been demonstrated in both guinea pig and human cochlea [2,3], but the other PR types have not been investigated.

Likewise, relatively little is known about the expression and distribution of cyclo-oxygenase (COX) enzymes in the inner ear [2,4]. These are crucial for the biosynthesis of PGs, as they catalyse conversion of arachidonic acid to the PG endoperoxides PGG2 and PGH2, which are important intermediates in the formation of prostaglandins.

In clinical use, prostaglandin E1 (PGE1) is often chosen as a second-choice treatment for acute sensorineural hearing loss (SNHL) [5]. PGE1 is usually administered to improve local blood circulation. In an experimental study in guinea pigs, an increased blood supply to the cochlea was observed following local PGE1 application [6], but the actual mechanisms underlying the effect of PGE1 on the inner ear have not yet been elucidated [3].

The purpose of the present study was to investigate the expression and localization of the eight PRs (DP, EP1, EP2, EP3, EP4, FP, IP, TP), as well as the two COX isoenzymes (COX1, COX2) in the inner ear to elicit more information about the functional significance of PRs in the inner ear.

Material and methods

We used six healthy, otomicroscopically normal, 8-week-old CBA/J mice with body weights in the range 20–25 g and a normal Preyer's reflex. The care and use of the animals was approved by

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the Animal Experimentation Committee, Hiroshima University School of Medicine (permit no. A06-149) and was carried out in accordance with the Guide to Animal Experimentation, Hiroshima University and the guidelines of the Committee on Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine. The animals were deeply anesthetized with pentobarbital and fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4. The temporal bones were excised and immersed in the same fixative for a further 1 h before decalcification with 0.1 M buffered Na-EDTA for 14 days. The specimens were frozen in OCT mounting medium (Sakura Finetechnical Co. Ltd, Tokyo, Japan), serially sectioned on a cryostat at 4 μm, and mounted on glass slides. After pretreatment with blocking serum, the specimens were incubated with a rabbit polyclonal antibody (I) to COX1 (Cayman Chemical, Ann Arbor, MI, USA) diluted 1:50; (II) to COX2 (Cayman Chemical) diluted 1:300; (III) DP (GenWay Iiotech, Inc., CA, USA) diluted 20 μg/ml; (IV) to EP1 (Acris Antibodies, Herford, Germany) diluted 17 μg/ml; (V) to EP2 (Acris Antibodies) diluted 10 μg/ml; (VI) to EP3 (Acris) diluted 4 μg/ml; (VII) to EP4 (Acris) diluted 3 μg/ml; (VIII) to FP (GenWay) diluted 11 μg/ml; (IX) to IP (Cayman) diluted 1:50; or (X) to TP (LifeSpan Biosciences Inc., Seattle, WA, USA) diluted 2 μg/ml in 0.3% Triton X-100-containing phosphate-buffered saline (PBS) at 4°C for 48 h. The specimens were then washed in PBS and incubated for1 h with Alexa Fluor 488 goat anti-rabbit secondary antibodies (1:500; Molecular Probes, Eugene, OR, USA). The sections were washed and coverslipped with DakoCytomatia Fluorescent Mounting Medium (DakoCytomatia, CA, USA). They were then viewed in a Nikon fluorescence microscope (Eclipse E600) equipped with an appropriate filter set. Fluorescence analog images were obtained with an intensified digital color charge-coupled device camera (C4742-95; Hamamatsu Photonics) and stored as digital images using IP Lab Spectrum software (version 3.0; Signal Analytics Corporation). Control sections were incubated with PBS instead of the primary antibodies; the results in these sections were invariably negative.

Results

Distribution of COX

In the cochlea, an immunofluorescent reaction to COX1 was observed in stria vascularis, especially in the basal cells (Figure 1), spiral prominence, spiral ligament, outer hair cells (OHCs), inner hair cells (IHCs), and some supporting cells of the organ of Corti (Figure 2) and in the spiral ganglion cells (Figure 3). In the vestibular end organs, immunofluorescence was noted in the vestibular sensory cells. The vestibular ganglion cells displayed immunofluorescence, more intensely in the nuclei (Figure 5). In the endolymphatic sac (ES), immunofluorescence was noted in the ES epithelial cells (Figure 6).

Generally speaking, there was no staining reaction to COX2 (Figures 1, 5, and 6), while only faint staining was observed in the spiral ganglion cells (Figure 3).

Distribution of prostanoid receptors

DP. In the cochlea, weak fluorescence for DP was observed in the basal cell layer of stria vascularis (Figure 1) and in the spiral ganglion cells (Figure 3). In the vestibular end organs, faint immunofluorescence was noted in the sensory cells (Figure 4) and in the cytoplasm of vestibular ganglion cells (Figure 5). In the ES, only faint immunofluorescence was noted in the ES epithelial cells (Figure 6).

EP1. In the cochlea, weak immunofluorescence for EP1 was found in the basal cell layer of stria vascularis (Figure 1), OHCs, IHCs (Figure 2), and the cytoplasm of spiral ganglion cells (Figure 3). In the vestibular end organs, faint immunofluorescence was noted in the apical layer of sensory cells (Figure 4) and the cytoplasm of vestibular ganglion cells

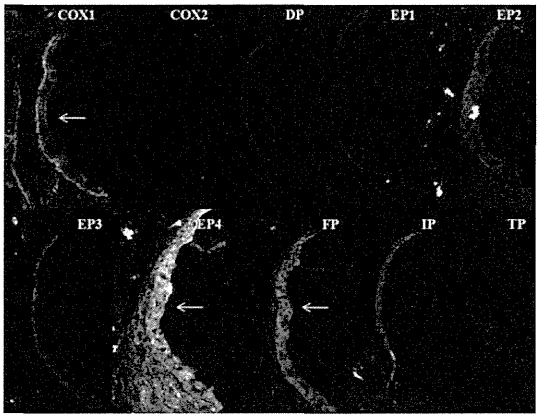

Figure 1. Stria vascularis (arrows) shows moderate immunofluorescence for COX1 and EP3, weak fluorescence for DP and EP1, especially in the basal cells, and also for EP2, EP4, FP, and IP.

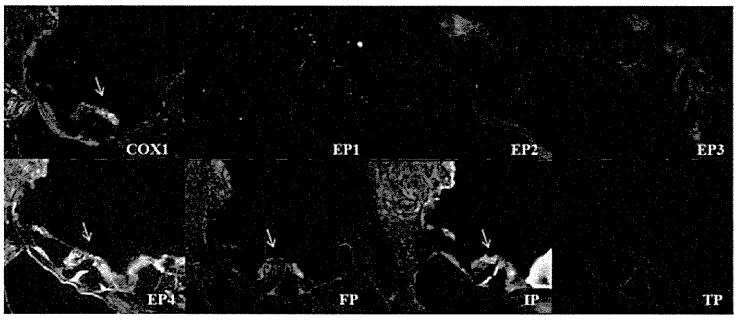

Figure 2. Outer hair cells (OHCs), inner hair cells (IHCs) (arrows), and some supporting cells of the organ of Corti show marked immunofluorescence for EP4, FP, and IP. Moderate fluorescence for EP2, EP3, and COX1 is evident in OHCs, IHCs, and some supporting cells.

(Figure 5). In the ES, immunofluorescence was faint in the apical border of ES epithelial cells (Figure 6).

EP2. In the cochlea, immunofluorescence for EP2 was observed in stria vascularis (Figure 1), the spiral prominence, OHCs, IHCs, some supporting cells of the organ of Corti (Figure 2), and the cytoplasm of spiral ganglion cells (Figure 3). In the vestibular end organs, immunofluorescence was noted in the vestibular sensory cells, dark cells, and in nerve fibers in the subepithelial space (Figure 4). Vestibular ganglion cells showed immunofluorescence for EP2 (Figure 5). In the ES, immunofluorescence was noted in ES epithelial cells (Figure 6).

EP3. In the cochlea, immunofluorescence for EP3 was observed in the basal cell layer of stria vascularis (Figure 1), OHCs, IHCs, some supporting cells of the organ of Corti (Figure 2), and in spiral ganglion cells (Figure 3). In the vestibular end organs, immunofluorescence was weak in the apical layer of the vestibular sensory and dark cells, and in nerve fibers in the subepithelial space (Figure 4). Vestibular ganglion cells showed weak immunofluorescence for EP3 (Figure 5). In the ES, immunofluorescence was noted in the ES epithelial cells (Figure 6).

EP4. In the cochlea, marked immunofluorescence for EP4 was observed in stria vascularis (Figure 1), the spiral prominence, spiral ligament, OHCs, IHCs, some supporting cells of the organ of Corti (Figure 2), and in spiral ganglion cells (Figure 3). In the vestibular end organs, immunofluorescence was intense in the vestibular sensory and dark cells, and in nerve fibers in the subepithelial space (Figure 4). The cytoplasm of the vestibular ganglion cells and nerve fibers showed immunofluorescence for EP4 (Figure 5). In the ES, immunofluorescence was noted in ES epithelial cells (Figure 6).

FP. In the cochlea, marked immunofluorescence for FP was observed in stria vascularis (Figure 1), OHCs, IHCs, some supporting cells of the organ of Corti (Figure 2), and in spiral ganglion cells (Figure 3). In the vestibular end organs, immunofluorescence was intense in the vestibular sensory and dark cells, and in

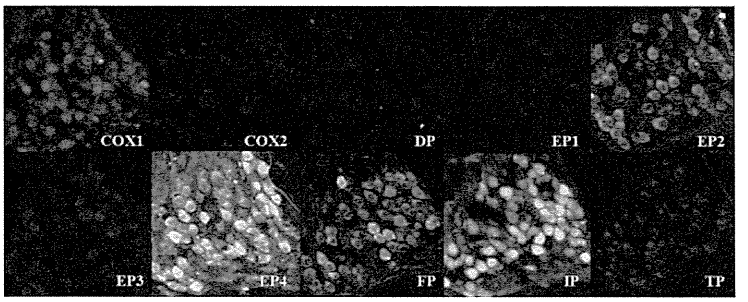

Figure 3. Marked fluorescence for EP4, FP, and IP, while moderate fluorescence for EP2, EP3, and COX1 is evident in spiral ganglion cells.

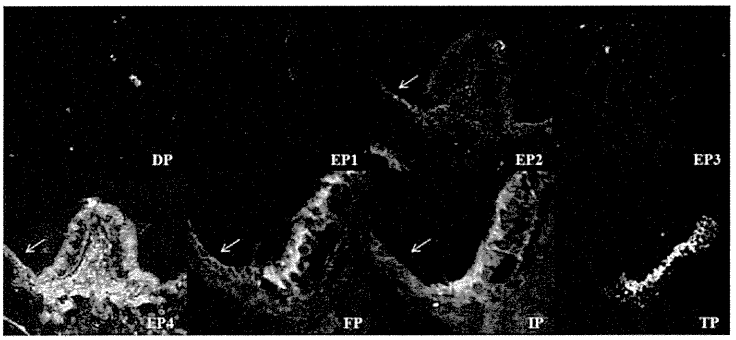

Figure 4. In the vestibular end organs, intense immunofluorescence for EP4, FP, and IP is evident in the vestibular sensory cells, and in nerve fibers in the subepithelial space. Vestibular dark cells (arrows) also show fluorescence for EP4, FP, and IP. Immunofluorescence for TP is evident in vestibular sensory cells.

nerve fibers in the subepithelial space (Figure 4). The cytoplasm of the vestibular ganglion cells and nerve fibers showed immunofluorescence for FP (Figure 5). In the ES, immunofluorescence was noted in ES epithelial cells (Figure 6).

IP. In the cochlea, immunofluorescence for IP was observed in stria vascularis (Figure 1), in OHCs, IHCs, some supporting cells of the organ of Corti (Figure 2), and the spiral ganglion cells (Figure 3). In the vestibular end organs, immunofluorescence was intense in the vestibular sensory and dark cells, and in nerve fibers in the subepithelial space (Figure 4). The vestibular ganglion cells were immunofluorescent, especially intense in the nucleus (Figure 5). In the ES, immunofluorescence was noted in ES epithelial cells (Figure 6).

TP. In the cochlea, weak immunofluorescence for TP was observed in stria vascularis (Figure 1), in OHCs, IHCs, some supporting cells of the organ of Corti (Figure 2), and in the cytoplasm of spiral ganglion cells (Figure 3). In the vestibular end organs, immunofluorescence was noted in the apical layer of sensory cells (Figure 4) and the cytoplasm of vestibular ganglion cells (Figure 5). In the ES, no significant immunoreactivity was noted (Figure 6).

Discussion

In the present study, we investigated the localization and expression of the eight PRs and two COX enzymes in the inner ear. We found that EP4, IP, and FP were abundantly distributed in many structures in the IE. EP2 and EP3 too were localized in the IE, while DP, EP1, and TP showed only weak expression.

SNHL is one of the most frequent disabilities. Once hearing has been lost, it is rarely recovered, because the mammalian auditory system (particularly the sensory cells) has a limited ability for regeneration. Clinically, there are no curative therapeutic options

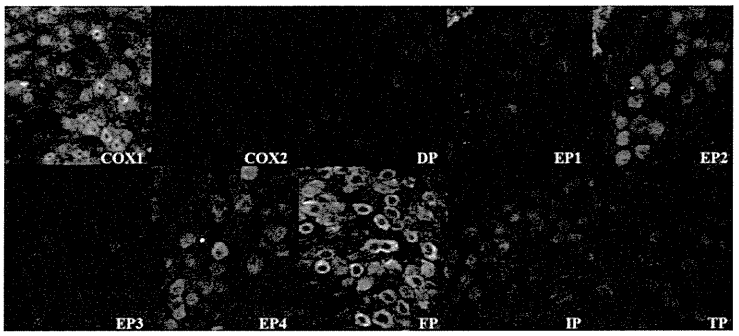

Figure 5. Marked fluorescence for EP4, FP, and COX1, and moderate fluorescence for EP2 and IP are evident in the vestibular ganglion cells.

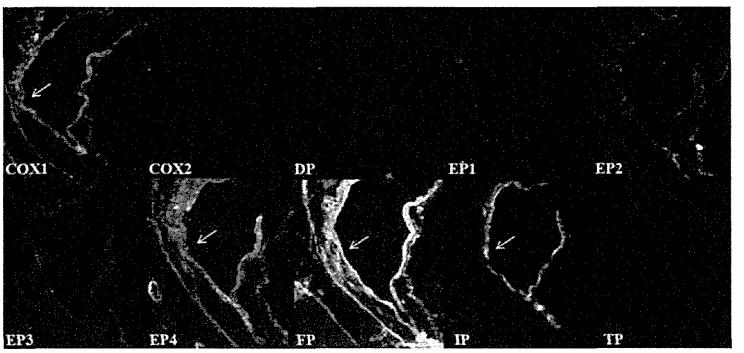

Figure 6. Intense fluorescence for EP4, FP, IP, and COX1, while moderate fluorescence for EP2 and EP3 is evident in the ES epithelial cells (arrows).

for chronic SNHL, and the cure rate for acute SNHL is also limited. Systemic application of corticosteroids has been accepted as the primary treatment of choice for acute SNHL, although its efficacy has not been substantiated [7]. In general, about half of patients with SNHL fail to respond to the systemic use of corticosteroids; other options for the treatment of acute SNHL have therefore been sought. In clinical use, PGE1 has often been a secondary choice treatment for acute SNHL, although its clinical efficacy remains controversial [5]. Usually, PGE1 is applied to improve local blood circulation. An experimental study using guinea pigs demonstrated an increased blood supply to the cochlea following local PGE1 application [6], while the actual mechanisms underlying the effects of PGE1 on the inner ear have not yet been elucidated [3].

The physiological actions of PGE1 are mediated by the PRs, i.e. IP and the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4 [1]. The present study revealed that the expression of EP4 is most abundant in the IE, EP2 moderately, and EP3 weakly, while EP1 was scarcely present at all. This indicates that EP4 (and probably EP2) plays an important role in the action of PGE1 in the inner ear. It has been shown that activation of the EP2 or EP4 receptors stimulates cAMP, generally affording protection of nervous tissue from an insult. In contrast, activation of EP1 receptors causes an increase in intracellular tissue calcium and generally exacerbates any detrimental insult. Activation of EP3 receptors is associated mainly with reduced cAMP metabolism and reportedly is both beneficial for and detrimental to cell survival [8].

In the inner ear, EP4 mRNA expression in the mouse cochlea was demonstrated earlier. Immunohistochemical study also revealed that EP4 expression occurred in stria vascularis, spiral ligament, spiral ganglion, supporting cells, and in OHCs and IHCs [3]. The present investigation has confirmed these findings. EP4 was abundant in the cochlea, vestibular end organs, spiral and vestibular ganglia, and ES. Concerning the functional role of EP4, recent studies suggest the potential of EP4 agonists for neuroprotection. An *in vitro* study using primary culture of cortical neurons revealed that EP4 agonists protect neurons from amyloid β-peptide toxicity via the cAMP-PKA route, which is also involved in mechanisms for promoting auditory neuron survival [9]. One EP4 agonist, ONO-AE1-329, has been reported to attenuate brain injury from glutamate excitotoxicity *in vivo* [10]. Excitotoxicity mediated by glutamate receptors plays an important role in mechanisms of cochlear damage induced by noise trauma and ischemic injury [11]. In the inner ear, protective effects of ONO-AE1-329 against noise trauma have been confirmed. Topical EP4 agonist treatment of the cochlea significantly limited the increase in auditory brainstem response (ABR) thresholds and OHC loss [3].

There are several explanations for the mechanisms underlying otoprotection by EP4 agonists. One possible mechanism is direct action of EP4 agonists on cochlear hair cells (HCs). Stimulation of EP4 in HCs may raise intracellular cAMP levels, thus activating the PKA pathway, and promoting HC survival. Regulation of growth factors (GFs) such as vascular endothelial GF (VEGF) and hepatocytic GF (HGF) in the cochlea by EP4 agonists is also included among otoprotective mechanisms. Earlier reports have shown that PGE2 stimulates VEGF expression via EP4 receptors [12]. The up-regulation of VEGF in the inner ear helps the recovery of auditory function following acoustic trauma, with potentially important clinical and therapeutic implications [13]. HGF gene transfer to the subarachnoid space using virus vectors can prevent or ameliorate hearing impairment, and local HGF protein application to the cochlea attenuates noise-induced cochlear damage [14]. Such

mechanisms might be involved in the EP4 agonist-induced protection of auditory HCs from injury caused by exposure to noise [3].

The present study revealed moderate expression of EP2 in the inner ear as well. This suggests a possible otoprotective function in the inner ear. Actually, EP2 agonist, butaprost, also demonstrated a neuroprotective effect in rat retinal cultures. Generation of reactive oxygen species and apoptosis were attenuated by EP2 agonists [8]. It has been shown that treatment of immortalized human microglia and CCF-STTG1 astrocytes with either PGE2 or the EP2 selective agonist butaprost stimulates the release of brain-derived neurotrophic factor (BDNF). Both cell lines express mRNA for EP2, whereas transcripts for the other subtypes are not detected. Pharmacological studies using PGE2 and modulators of cyclic AMP signaling implicate this pathway in PGE2-stimulated BDNF release. These results indicate that EP2 activation induces BDNF secretion by stimulating cyclic AMP-dependent signaling. Endogenous PGE2 could contribute to either neurotoxicity or neuroprotection in the injured brain by inducing BDNF release from microglial cells and astrocytes [15]. This mechanism also applies in the inner ear. Several studies have demonstrated the beneficial effects of BDNF on the survival of compromised auditory and vestibular neurons [16]. Thus, the induced BDNF secretion by EP2 seems to contribute to the otoprotection.

The present study revealed marked expression of IP in the inner ear. Recent studies on prostanoids have shown that some PRs are expressed in rat dorsal root ganglion (DRG) neurons. These findings suggest that PRs are involved in the excitation mechanism of DRG neurons. In PCR experiments, IP was found in L6 and S1 rat DRG neurons. The functional role of IP receptor agonists was examined by measuring cAMP accumulation and substance P (SP) release in primary cultured DRG neurons. Pretreatment of DRG neurons with IP agonist, iloprost, sensitized the DRG neurons and hence potentiated the lys-bradykinin-induced SP release. The increase of SP release by lys-bradykinin plus IP agonists was proportional to cAMP accumulation. The effect of iloprost, the most potent agonist inducing cAMP accumulation and SP release, is mediated by IP receptor. Moreover, capsaicin-, ATP- and KCl-induced SP release was also enhanced by iloprost, although it did not affect intracellular Ca^{2+} and membrane depolarization induced by these chemical stimuli. These results strongly indicate that IP plays an important role in the sensitization of rat sensory neurons [17]. It is therefore suggested that IP receptors may play an important role in the sensory cell transduction in the inner ear as well.

The present study revealed that FP prostanoid receptors are abundantly distributed in the mouse inner ear, including ES, as was noted earlier in guinea pigs and humans [2]. This finding suggests that PGF2α seems essential in the inner ear [2]. Clinically, it has been reported that a selective FP prostanoid receptor agonist, latanoplast, alleviated vertigo/dysequilibrium and improved hearing in Meniere's disease [18]. In the kidney, FP receptor stimulation inhibited vasopressin-stimulated osmotic water permeability [19]. In the inner ear, vasopressin was detected in the inner ear fluid transporting cells [20], those expressing FP. These findings indicate an important role for FP in regulating inner ear fluid homeostasis.

Recently, localization and expression of COX1 and COX2 enzymes in the cochlea have been demonstrated [2]. The present study revealed the expression of COX1 in mouse inner ear, while COX2 was generally not observed. It is questionable whether the COX2 enzyme is at all constitutively expressed in the inner ear. Ziegler et al. [4] reported that COX2 was found in all cell types in the inner ear, in the guinea pig cochlea, while Stjernschantz et al. [2] demonstrated that the COX2 enzyme appeared to be lacking in guinea pig and human cochleae. Nevertheless, their localization in various cell types in the cochlea, irrespective of the vascular architecture, suggests an additional function beyond taking part in regulating cochlear blood flow [2].

Acknowledgments

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ORIGINAL ARTICLE

Localization of aquaporins in the mouse vestibular end organs

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Abstract

Conclusion: We found that aquaporins (AQPs) in the fluid transporting cells, such as vestibular dark cells and endolymphatic sac epithelial cells, seem to be of importance in fluid transport in the inner ear, while those in the sensory and ganglion cells may play a functional role in sensory cell transduction. *Objective:* Expression of AQPs (0–12) was analyzed in normal mouse vestibular end organs. *Methods:* CBA/J mice were used in this study. Localization of AQPs 0–12 in the vestibular end organs and endolymphatic sac was investigated by immunohistochemistry. *Results:* The AQPs were found abundantly distributed in many structures in the vestibular end organs, i.e. vestibular sensory and supporting cells, vestibular dark cells, vestibular ganglion cells, and the endolymphatic sac.

Keywords: *Endolymphatic sac, immunohistochemistry, fluid transport, sensory cell transduction*

Introduction

Aquaporins (AQPs) are essential in mediating bidirectional water transport through membranes by reducing the energy needed for water permeation. At present, 13 members of the AQP family (AQPs 0–12) are known to be ubiquitously distributed in mammalian tissues including the nervous system, kidney, gastrointestinal tract, skin, respiratory tract, eye, and inner ear [1–3]. These channels have been highly conserved throughout evolution and the family is currently divided according to sequence homology and permeability into: aquaporins, aquaglyceroporins, and unorthodox AQPs. The AQPs subgroup, comprising AQPs 0, 1, 2, 4, and 5, is considered to be permeable mainly to water having a high flow rate. AQP1 is also permeable to volatile solutes such as CO_2. Water diffusion via AQPs is inhibited by mercury, except for AQP4, which is mercury insensitive. The second subgroup, aquaglyceroporins, comprises AQPs 3, 7, 9, and 10. These channels are permeable to water, glycerol, and urea. AQP9 was also called 'neutral channel.' Indeed, the presence of AQP9 in Xenopus oocytes or proteoliposomes facilitated diffusion of not only water, but also polyols (glycerol, mannitol, and sorbitol), purines (adenine), pyrimidines (uracil and the chemotherapeutic agent 5-fluorouracil), and monocarboxylates (lactate, β-hydroxybutyrate). However, the osmotic water coefficient for AQP9 is lower than in a pure water channel such as AQP4. In addition to AQPs and aquaglyceroporins, a third subfamily of related proteins was discovered and recently designated unorthodox AQPs. They include the mammalian AQPs 6, 8, 11, and 12. Few papers have been published on these, possibly because their functions have not yet been clearly demonstrated and they appear to be localized inside the cells [1,2].

In the inner ear, expression of proteins and/or mRNAs of AQPs has recently been reported. AQPs 0–9 were identified within the inner ear and their expression was confirmed in certain cochlear tissues [3–5]. AQPs 1–4 and 6–9 were identified in the

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endolymphatic sac (ES) by means of RT-PCR and immunohistochemistry [5,6]. However, no study concerning the expression of all types of AQPs in the vestibular end organs has yet been carried out. The detailed localization and functional significance of AQPs in the vestibular end organs await clarification. The present study was devised to determine the detailed localization of all types of AQPs (AQPs 0–12) in the vestibular end organs and ES, using immunohistochemistry to ascertain their role in water homeostasis.

Material and methods

We used six healthy, otomicroscopically normal, 8-week-old CBA/J mice with body weights in the range 20–25 g and a normal Preyer reflex. The care and use of the animals was approved by the Animal Experimentation Committee, Hiroshima University School of Medicine (permit no. A10-53), all in accordance with the Guide to Animal Experimentation, Hiroshima University and the guidelines of the Committee on Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine. The animals were deeply anesthetized with pentobarbital and fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4. The temporal bones were excised and immersed in the same fixative for a further 1 h. They were decalcified with 0.1 M buffered Na-EDTA for 14 days. The specimens were frozen in OCT mounting medium (Sakura Finetechnical Co. Ltd, Tokyo, Japan), serially sectioned on a cryostat at 4 μm, and mounted on glass slides. After pretreatment with blocking serum, the specimens were incubated with a rabbit polyclonal antibody to AQP0 (Acris Antibodies Gmbh, Herford, Germany) at a dilution of 1:100; a rabbit polyclonal antibody to AQP1 or 3 (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:100; a rabbit polyclonal antibody to AQP2 (Novus Biologicals, CO, USA) at a dilution of 1:100; AQPs 4, 5, 6, 8, and 9 (Santa Cruz Biotechnology) at a dilution of 1:50; a rabbit polyclonal antibody to AQPs 7, 10, and 12 (Novus Biologicals) at a dilution of 1:300; or with a rabbit polyclonal antibody to AQP11 (Novus Biologicals) at a dilution of 1:20 in 0.3% Triton X-100 containing phosphate-buffered saline (PBS) at 4°C for 48 h. The specimens were then washed in PBS and incubated for 1 h with Alexa Fluor 488 goat anti-rabbit secondary antibodies (1:500; Molecular Probes, OR, USA). The sections were washed and cover-slipped with DakoCytomatia Fluorescent Mounting Medium (DakoCytomatia, CA, USA). The specimens were viewed in a Nikon fluorescence microscope (Eclipse E600) equipped with an appropriate filter set. Fluorescence analog images were obtained with an intensified digital color charge-coupled device camera (C4742-95; Hamamatsu Photonics) and stored as digital images using IP Lab Spectrum software (version 3.0; Signal Analytics Corporation). Control sections were incubated with PBS instead of the primary antibodies; the results in these sections were invariably negative.

Results

Vestibular sensory epithelium

In the vestibular end organs, immunoreactivity to AQP0 was identified in the sensory cells of the utricle, saccule, and crista ampullaris. The nerve fibers in the subepithelial tissue displayed immunofluorescence to AQP0. Immunofluorescence to AQP1 was observed in sensory cells and subepithelial nerve fibers, and to AQP2 in the sensory cells, especially in the cuticular plate, whereas supporting cells were not fluorescent. In the subepithelial tissue, positively stained nerve fibers were commonly observed. Immunoreactivity to AQP3 was observed in the sensory cells, and to AQP4 was identified in the basal part of the sensory epithelium, including the basement membrane (BM) of the utricle and crista ampullaris. In the saccule, immunoreactivity to AQP4 was detected in the supporting cells, and to AQP5 in the sensory cells of the utricle, saccule, and crista ampullaris. The nerve fibers in the subepithelial tissue showed moderate immunofluorescence to AQP5. Immunoreactivity to AQP6 was distributed in the subapical vestibular supporting cells and BM. Immunofluorescence to AQP7 was observed in supporting cells and in nerve fibers of subepithelial tissue. Immunofluorescence to AQP8 was observed in the supporting cells of the crista ampullaris, saccule, and utricle. Intense immunofluorescence to AQP9 was observed in the sensory cells, and moderately in nerve fibers of the subepithelial tissue. Immunofluorescence to AQP10 was observed in vestibular sensory cells (VSCs), moderately so in nerve fibers of the subepithelial tissue; to AQP11 in the cuticular plates of VSCs, with weak fluorescence in the nerve fibers; and to AQP12 in both vestibular sensory and supporting cells, with moderate fluorescence in nerve fibers of the subepithelial tissue (Figures 1 and 2).

Vestibular ganglion

In the vestibular ganglion cells (VGCs), immunoreactivity to AQP0 was observed in those cells showing weaker staining in the nerve fibers; to AQP1 in VGCs and nerve fibers; to AQP2 in VGCs; and to AQP3 in VGCs and nerve fibers. Weak immunoreactivity to

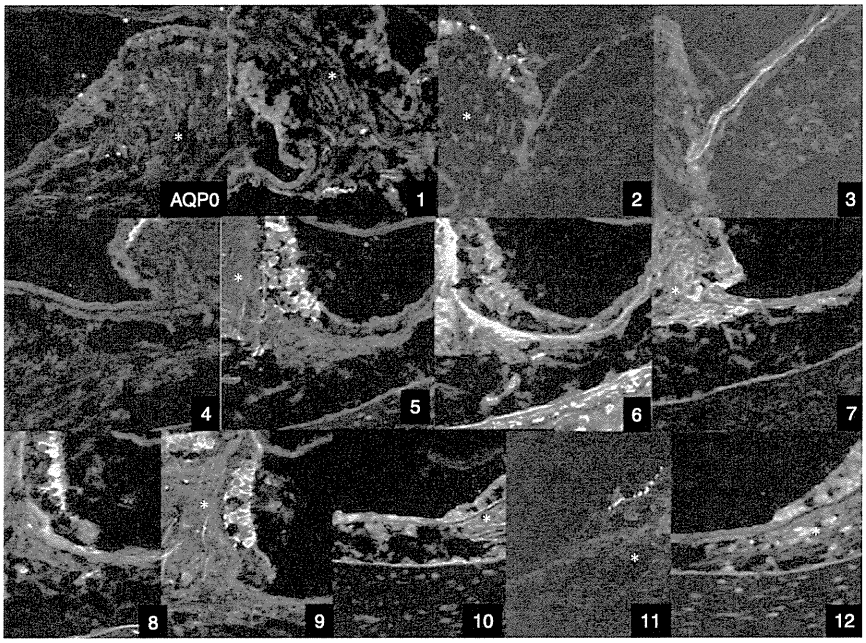

Figure 1. In the crista ampullaris, immunoreactivity to aquaporins (AQPs) 0–12 is evident. The sensory cells show immunoreactivity to AQPs 0–3, 5, 9–12, the supporting cells to AQPs 4, 6–8, and the subepithelial nerve fibers to AQPs 0, 1, 2, 5, 7, 9–12 (asterisks).

AQP4 was evident in ganglion cell bodies and nuclei, and to AQP5 in ganglion cell bodies, with moderate reactivity in the nerve fibers. Immunoreactivity to AQP6 was observed in ganglion cells, with more intense labeling in the nerve fibers. For AQP7, reactivity was intense in VGCs and nerve fibers. Immunoreactivity to AQPs 8 and 9 was evident in VGCs and nerve fibers, but to AQP10 in VGCs only. Immunoreactivity to AQP11 was detected in both VGCs and nerve fibers (Figure 3).

Vestibular dark cells

Vestibular dark cells (VDCs) displayed immunoreactivity to all types of AQPs (AQPs 0–12). In particular it was noted that AQP1 was expressed in the apical and basolateral parts of the cells. AQP2 was observed in the basolateral part, AQP3 in the basal part, and AQP4 in both apical and basolateral plasma membranes. AQP6 was present in cytoplasm and basal parts of the cells. AQPs 7, 8, and 9 were expressed mainly apically in VDCs, while AQPs 0, 5, 10, 11, and 12 were expressed in VDC cytoplasm (Figure 4).

Endolymphatic sac

In the ES, immunoreactivity to AQP0 was observed in the epithelial cells, and to AQP1 in the BM, whereas epithelial cells showed little or no immunoreactivity. Immunoreactivity to AQP2 was evident in the epithelial cells, and to AQP3 mainly in the basolateral part. AQP4 immunoreactivity was observed in the basolateral plasma membrane, whereas no AQP5 immunoreactivity was seen at all in the ES. Marked AQP6 immunoreactivity was noted in the BM, with weaker labeling in the epithelial cells. AQP7 immunoreactivity was observed in epithelial cells, mainly in the apical and lateral parts. AQP8 immunoreactivity was evident mainly in the BM, while the epithelial cells showed weak immunofluorescence. Intense immunoreactivity to AQP9 was observed in the apical plasma membrane of the epithelial cells and in the BM. Immunoreactivity to AQP10 was observed in the epithelial cells, especially in the light cells, while reactivity to AQPs 11 and 12 was noted in the cytoplasm of epithelial cells (Figures 5 and 6).

Discussion

The presence of AQPs in the cochlea and ES has been thoroughly investigated at both light and electron microscopic level, while there are fewer studies concerning AQPs in the vestibular end organs. Expression of AQP2 was reported in the mouse saccule, utricle, and crista ampullares. AQP6 was observed in the vestibular end organs of both rat and mouse. In

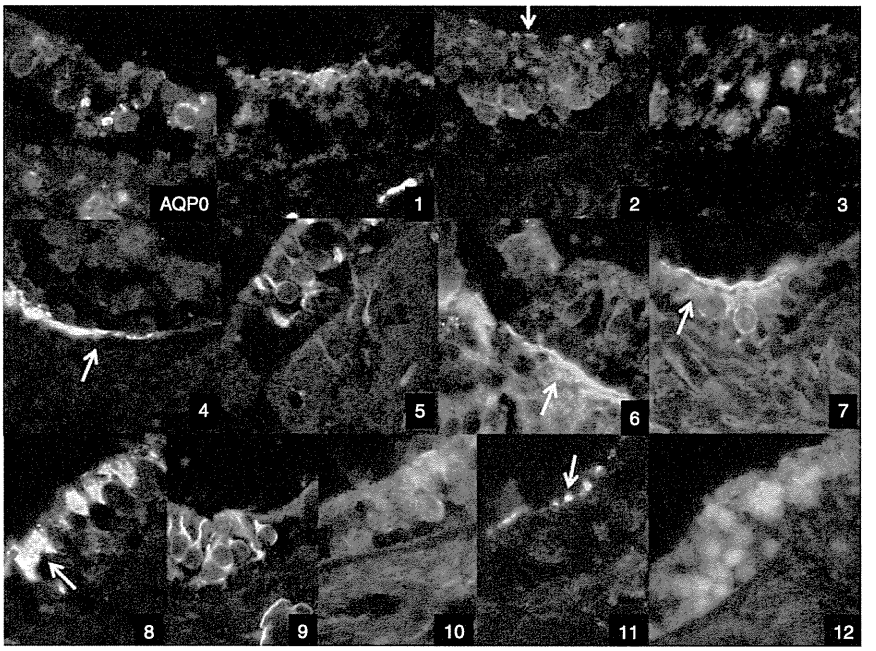

Figure 2. Immunoreactivity to aquaporins (AQPs) 0–3, 5, 9–12 is evident in sensory cells; to AQPs 2 and 11 in the cuticular plates of the vestibular sensory cells (arrows); to AQP4 in the basement membrane (arrow). Immunoreactivity to AQP6 is distributed in the subapical vestibular supporting cells and basement membrane (arrow); to AQPs 7 and 8 in the supporting cells (arrows); and to AQP12 in both vestibular sensory and supporting cells.

humans, AQPs 2, 4, and 9 were identified in the saccule and AQPs 1, 4, and 6 in the utricle [3,5,7]. Expression of other AQPs in the vestibular end organs still remains obscure.

We first consider the VSCs. The present study revealed that AQP0 was expressed in VSCs and subepithelial nerve fibers of the utricle, saccule, and crista ampullares. In the eye, AQPs 0, 1, and 9 are

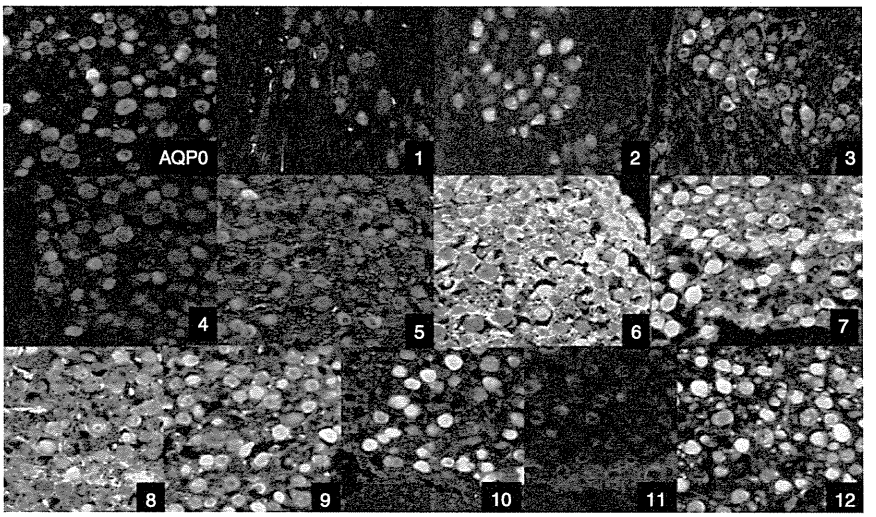

Figure 3. In vestibular ganglion cells (VGCs), immunofluorescence to aquaporins (AQPs) 0, 1, 3, 5–9, and 12 is observed in the ganglion cells and nerve fibers, and to AQPs 2, 4, 10, and 11 in the ganglion cells.

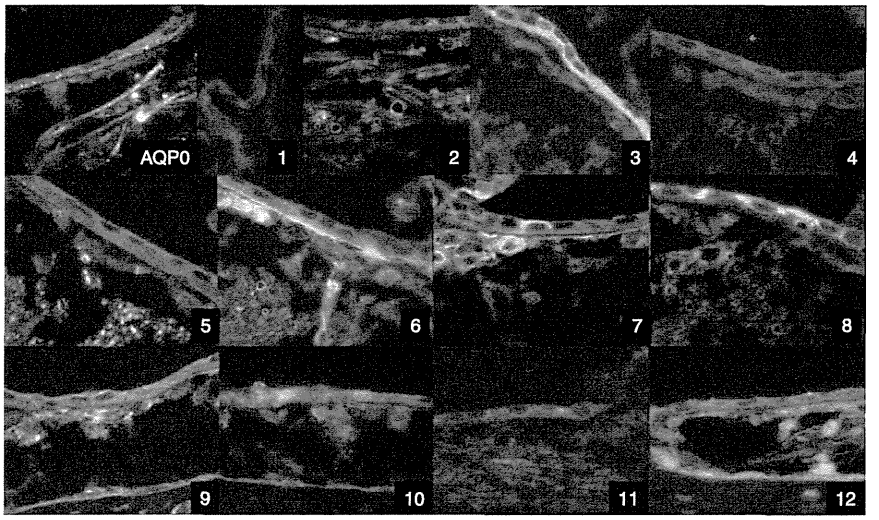

Figure 4. Vestibular dark cells (VDCs) are immunoreactive to all aquaporin (AQP) types. AQP1 is localized in the apical and basolateral parts of the cell; AQP2 in the basolateral part, and AQP3 in the basal part; AQP4 in the apical and basolateral plasma membranes; AQP6 in the cytoplasm and basal parts of the cell; AQPs 7, 8, and 9 in the apical part of the cell; and AQPs 0, 5, 10–12 in the cytoplasm.

reportedly expressed specifically in a subpopulation of neurons. AQP0 was predominantly observed in the bipolar cells of rat retina and in the retinal nerve fibers of diabetic rat retina. It is concluded that AQP0 is involved in regulating the activity of retinal second order neurons [8]. A similar function of AQP0, which regulates the activity of VSCs, can therefore be suggested.

Immunofluorescence to AQP1 was identified in VSCs and subepithelial nerve fibers. In the nervous system, AQPs 1, 2, 4, and 9 have been found. Generally, they are involved in water transport in nerve tissue, whereas recent data suggest the involvement of AQPs also in neurotransmission [2,9]. Concerning the functional role of AQP1 in the vestibuli, it has been reported that, after repeated stimulus by rotation, the alleviation of motion sickness symptoms in mice was, at least in part, due to increased AQP1 expression in the inner ear. AQP1 in the inner ear was suggested to be one important factor causing motion

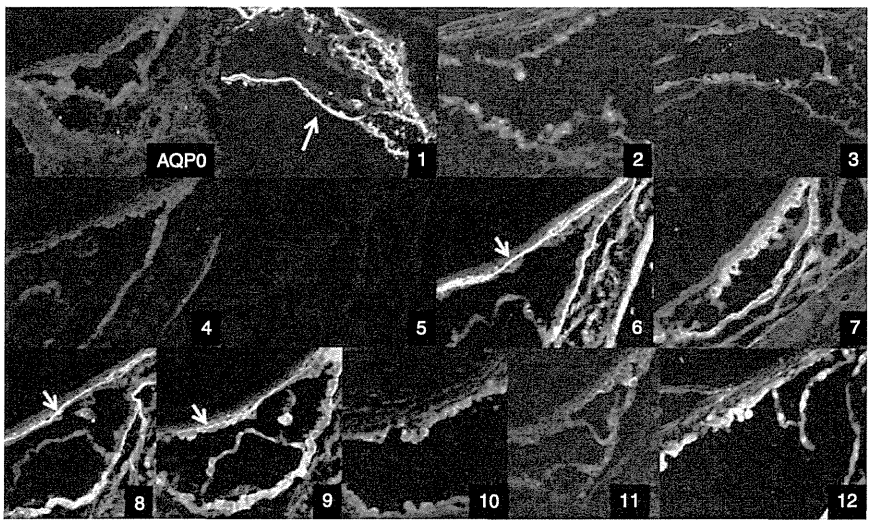

Figure 5. In the endolymphatic sac (ES), immunoreactivity to aquaporins (AQPs) 0–4 and AQPs 6–12 is evident, but AQP5 is not. The basement membrane shows immunoreactivity to AQPs 1, 6, 8, and 9 (arrows).

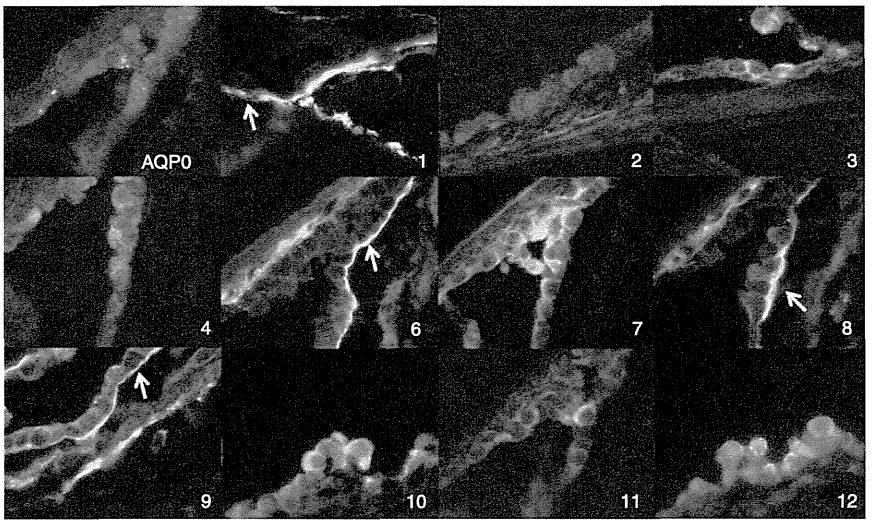

Figure 6. Immunoreactivity to aquaporin (AQP) 0 is observed in epithelial cells; AQP1 in the basement membrane (arrow); AQP2 in epithelial cells; AQP3 mainly in the basolateral part of the cell; AQP4 in the basolateral plasma membrane; AQP6 in the basement membrane (arrow) with weaker labeling in the epithelial cells; AQP7 mainly in the apical and lateral part of the cell; AQP8 mainly in the basement membrane (arrow); AQP9 in the apical plasma membrane and the basement membrane (arrow); AQP10 in the epithelial cells, especially in the light cells; and AQPs 11 and 12 in the cytoplasm.

sickness [10], which might mean that AQP1 in VSCs is related to this role.

Expression of AQP2 in VSCs, especially in the cuticular plate, suggests a mechanosensory role for AQP2 as a component of sensory cells in the vestibular end organs, since it has been reported that TRPV4 is present in the vestibular sensory and ganglion cells where it appears to function as an osmoreceptor, or is part of an osmoreceptor complex, controlling vasopressin release [5].

The present study revealed expression of AQPs 3 and 5 in VSCs. AQPs 3 and 5 are reportedly present in astrocytes and in neurons in the brain. Although a possible relationship between AQP5 and intracranial edema following ischemic injury has been suggested, the functional significance of AQPs 3 and 5 in the brain remains obscure [2]. Similarly, the functional significance of AQPs 3 and 5 in VSCs also awaits clarification.

In the human saccule, AQP9 was localized in the basolateral part of the sensory epithelium, as shown by co-staining with laminin 1 [7]. On the other hand, Huang et al. [11] detected AQP9 mainly at the surface of the sensory epithelium of mouse saccule, indicating a species difference [7]. The present study revealed strong immunofluorescence to AQP9 in mouse VSCs, being moderate in the nerve fibers of the subepithelial space. AQP9 is an aquaglyceroporin that is permeable to a wide variety of non-charged solutes such as lactate and glycerol. The functional significance of AQP9 as a lactate-glycerol channel in the central nervous system (CNS) has been attracting increasing attention, as both lactate and glycerol can serve as fuel for neurons, indicating that they are involved in energy metabolism [2]. Water homeostasis is critical for maintaining physiological neuronal activity in the CNS, including the retina and optic nerve, as water transit is coupled to ionic currents that are the basis for neuronal excitability. These results confirm that AQPs 5 and 9 are also active within the vestibular sensory transduction system.

The present study also revealed immunoreactivity to AQP10 in VSCs, AQP11 in the cuticular plates of VSCs, and AQP12 in both VSCs and supporting cells. The functional significance of AQPs 10, 11, and 12 in VSCs still remains unknown.

We turn now to vestibular supporting cells and the BM. Earlier studies have revealed that AQP4 is present in the vestibular supporting cells of the rat [12,13]. AQP4 was also identified in the basal portion of supporting cells in the human utricular sensory epithelium [14] as well as in the basolateral part of the human saccular sensory epithelium. This basolateral location of AQP4 was confirmed by co-expression with laminin 1, a BM protein [7]. The present findings confirm the expression of AQP4 in the mouse vestibular supporting cells, as well as in the BM. Concerning the functional role of AQP4 in vestibular supporting cells, it seems likely that AQP4 (at least in part) regulates ionic and water homeostasis, similar to

astroglial cells in the brain, and counteracts local increases in K^+ ion concentration in supporting cells. Vasopressin has been shown to activate water permeability of AQP4 in renal epithelial cells via vasopressin type 2 receptors (V2Rs) linked to cAMP production, and cAMP has been shown to increase AQP4 production in astrocytes. AQP4 also participates in regulating water homeostasis in the brain by transporting water at the blood–brain and ependymal–cerebrospinal barriers. A similar role has been suggested in the vestibular supporting cells and the BM, as regards water transport between blood, endolymph, and perilymph compartments [7].

Previous studies have shown that AQP6 is expressed in human, rat, and mouse inner ear. The protein localization is still controversial, however, possibly due to species differences, has been observed for other AQPs, whereas in vestibular epithelia, immunoreactivity to AQP6 has been localized to the subapical vestibular supporting cell cytoplasm in the human, mouse, and rat inner ear [13,15]. Immunoelectron microscopic studies revealed that the immunogold AQP6 labeling count in the supporting cells exceeded than that in the sensory cells in the utricle and saccule, but no difference in labeling was observed between utricle and saccule [15]. Our data agree with those previous findings, showing strong AQP6 expression mainly in the vestibular supporting cells of the saccule, utricle, and crista ampullaris. To our knowledge, no AQP6 knockout mouse is available, and the role of AQP6 in the inner ear is still obscure. In the cochlea, AQP6 may be involved in acid–base homeostasis, as there is a constant passive flux of protons from the endolymph [12,15]. Light and electron microscopic examination of vestibular end organs obtained from patients with intractable Meniere's disease demonstrated increased expression of AQP6, which seems to reflect an altered acid–base balance in the inner ear. Other studies have shown that AQP6 is markedly increased in rats rendered chronically alkalotic with bicarbonate, also indicating a change in acid–base balance. This too suggests the involvement of AQP6 in acid–base homeostasis in the vestibular organs [14].

In mouse vestibular end organs, AQP7 immunoreactivity was observed in supporting cells of both the saccule and utricle [10]. The present study confirmed those previous findings. The functional significance of AQP7 in the vestibular supporting cells is obscure. In the cochlea, AQP7 levels decreased in response to vasopressin [16]. It is currently believed that K^+ in the endolymph is recycled by means of supporting cells of the organ of Corti. Along the recycling pathways various AQPs are expressed in cells. This pathway may provide the necessary water permeability regulated by AQPs in order to compensate for osmotic changes as a result of K^+ flow. Reduced expression of AQP7 mRNA in the cochlea caused by vasopressin seems to limit water permeability in the K^+ recycling pathway of the organ of Corti, thereby blocking the endolymph circulation. A similar mechanism is also suggested in the vestibular supporting cells.

The present study revealed immunoreactivity to AQP8 in the supporting cells of the crista ampullaris, saccule, and utricle. AQP8 expression, initially described in the testis, pancreas, placenta, and liver, was also observed in the spinal cord, especially in the ependymal cells lining the central canal and faint staining of cells surrounding the canal, suggesting slight AQP8 expression in astrocytes too. These expression patterns of AQP8 suggest that this protein participates together with AQP4 and AQP9 in the water transport process [9]. The functional significance of AQP8 in the vestibular supporting cells is obscure, but a similar role for water transport may be suggested.

In the vestibular ganglion, ganglion cells showed immunoreactivity to AQPs 0–12 and the nerve fibers to AQPs 0, 1, 3, 5–9, and 12. In the nervous system, only AQPs 1, 2, 4, and 9 have been found. Generally, they are involved in water transit in nerve tissue; nevertheless, recent data suggest the involvement of AQPs also in neurotransmission [2]. Recently, expression of AQPs 1 and 2 in the trigeminal ganglia of mice, using an animal model of perioral acute inflammatory pain, has been evaluated by means of immunohistochemistry and immunoblotting. The change in AQP2 expression showed increased and intracellular redistribution of AQP2, mainly in small neurons and Schwann cells; otherwise, the AQP1 expression remained unaltered. On the whole, these data do support the hypothesis that AQP2 is involved in pain transmission in the peripheral nervous system [17]. Although the functional significance of AQP2 in VSCs and VGCs remains unclear, it has been suggested that AQP2 is involved in neurotransmission in the vestibular end organs [5].

AQP9 is enriched in certain neuronal populations and is unique in its ability to act as a lactate-glycerol channel supplying neurons with alternative fuel under ischemic conditions [2]. In retinal ganglion cells and in retina AQP0 was detected by Western blotting, RT-PCR, and immunohistochemistry. Retinal ganglion cells subjected to hypotonic stress increased their cell volume that was blocked by a known inhibitor of AQP9 (phloretin, 40 μM). Retinal ganglion cells subjected to hypoxia showed an increase in AQP9 expression when judged by Western blotting and RT-PCR. Similarly, hypotonic shock

(50%) increased AQP9 expression, when determined by RT-PCR. AQP9 is involved in energy balance as a glycerol-lactate channel and also appears to regulate cell volume in retinal ganglion neurons. This water channel thus plays a key role in retinal ganglion pathology [18]. A similar function of AQP9 may also apply to VGCs.

In the present study we observed expression of AQPs 0–12 in VDCs, whose functional significance was attributed to endolymph secretion and the resorption of otoconia. In stria vascularis, RT-PCR study revealed expression of AQPs 1–5 and 7–9 in rat cochlea. By means of immunohistochemistry, AQPs 0, 1, 2, 3, 6, 7, and 9 were reportedly expressed and suggested to play an important role in the endolymph secretion. AQP1 was expressed in type III fibrocytes and found diffusely in stria vascularis. AQPs 2, 3, and V2R were located in strial basal cells, while vasopressin-AQP2-mediated water transit work actively in basal cells [4]. AQPs 1, 2, 3, and V2R have also reportedly been expressed in VDCs [5], indicating that similar vasopressin-AQP2-mediated water transit works actively in VDCs.

AQP6 is abundant in marginal cells as well as in intermediate and basal cells. The AQP6 water channel is a potential candidate of water and ion co-transport. Among the many members of the water channel family, AQP6 is the only one known to function also as an ion channel, allowing cation and anion permeation with marked specificity for nitrate [4]. AQP6 was found to be diffusely expressed in the cytoplasm, indicating that the primary role of AQP6 is cell regulation rather than transepithelial fluid flux [14]. A similar role has been also indicated in the VDCs.

Concerning the functional role of AQPs 7 and 9, it was suggested that they participate in the transport of water from extracellular spaces into the scala media in the cochlea. AQPs 7 and 9 are expressed in the apical region of marginal cells [4]. Recent reports have shown that AQPs are present in cytoplasmic vesicles in human parotid acinar cells and mouse liver, and also in intracellular vesicles in rat kidney – transport vesicles involved in endocytosis and exocytosis. Application of vasopressin elicits increased vesicle formation in marginal cells. These results suggest that the AQP7 observed on the vesicles immediately beneath the apical membrane is related to exocytosis as a water transit route under vasopressin regulation [4]. AQP7 is permeable not only to water but also to glycerol and so-called aquaglyceroporins (AQPs 3, 7, and 9) [1]. Water flux through the apical membrane is attributed to AQP9 water channels [4]. The present study revealed both AQPs 7 and 9 in the apical part of dark cells. Morphological similarities between dark cells and strial marginal cells, with increased surface area and microvilli located at the apical and basal sides, indicate involvement in fluid transport. Together with the similarities of the subcellular localization of AQPs 7 and 9 between stria marginal cells and VDCs, the latter are also related to exocytosis as a water transit route under vasopressin regulation.

The functional significance of other AQPs (AQPs 4, 5, 8, 10, 11, and 12) is still obscure. The relationship to endolymph secretion or other functions of VDCs, such as transport of potassium ions, absorption of otoconia, etc., may be suggested, but needs further investigation.

In a previous study of the mouse ES, mRNAs and/or proteins of AQPs 1, 3, 4, 7, and 9 were expressed, mRNA of AQP5 weakly so, and mRNAs of AQPs 2 and 8 not at all [11]. In the rat ES, AQPs 1–4 and 6–9 were detected [6]. The present study revealed expression of AQPs 0–4 and 6–12 in mouse ES epithelial cells as well as AQPs 1, 6, 8, and 9 in ES BM, whereas AQP5 was not detected.

Expression of AQPs 1–4 and 6–8 was observed in the kidney, from the cortex through the inner medulla [19], where large quantities of water and solute are filtered and reabsorbed. As ES absorbs endolymph, its function resembles that of the kidney. In the kidney, AQP1 is present in both apical and basolateral plasma membranes, in proximal tubules and descending thin limbs, consistent with the role of AQP1 in the transit of water through both cell surfaces. AQP2 is present in apical plasma membrane and apical vesicles in the collecting duct of principal cells and, less abundantly, in connecting tubules. AQPs 3 and 4 are present in the collecting duct of principal cells where they are located in the basolateral plasma membranes and represent potential exit pathways from the cell for water entering via AQP2. AQP6 is present in cortical and outer and inner medullary collecting ducts of intercalated cells but not in plasma membranes [19]. AQP7 occurs in the brush border of proximal tubule cells and is involved in proximal tubule water reabsorption [19]. AQP8 is present intracellularly in low abundance in proximal tubules and collecting duct principal cells [19]. In the ES, the present study revealed that AQP2 in the apical plasma membrane, AQPs 3 and 4 in the basolateral plasma membrane, and AQP6 in cytoplasm and BM, are identical to the kidney. AQP7 was present in the apical and lateral intercellular spaces and AQP8 in the cytoplasm and BM. These findings suggest that at least AQPs 2, 3, 4, and 6 and probably AQPs 7 and 8 participate in the absorption of endolymph in the same manner as in the kidney, while AQP1 was located in the BM, which suggests a function different from that in the kidney.

Concerning the function of the ES, based on the hypotheses of endolymph absorptive function and the localization of AQPs and V2R, water flux occurs through the ES epithelium from the apical to the basolateral plasma membrane. The entry for water flux seems to be AQP2 and its exit AQPs 3 and 4 [6].

In the ES, water can move through both surfaces of the epithelium, depending on the osmotic gradient. In the kidney, AQP6 appears to be an exclusively intracellular water and ion channel [19], and its role the same in the ES [6]

AQP7 diminished in response to vasopressin. In the ES, endolymph fluid is disposed of by active pinocytosis against an osmotic gradient. And in the ES epithelium, the internalized hyperosmolar fluid is removed by ion secretion through the basolateral membrane and concurrent water flow. The reduced expression of AQP7 mRNA in the ES caused by vasopressin probably reduces water permeability in the basolateral membrane, in turn resulting in decreased absorption of endolymph [16].

AQP9 is expressed in liver hepatocytes, and in Leydig cells in the testis [20], but not in the kidney [19]. In the liver, AQP9 is expressed on microvilli facing the circulation and the characteristic ability of AQP9 to permeate a broad spectrum of neutral solutes supports the view that it plays a pivotal role in the rapid transit of a variety of metabolites through the cell membrane, with minimal osmotic perturbation [20]. In the testis, AQP9 is expressed on the plasma membrane of Leydig cells in the peritubular interstices. The transit of water and solute through the membrane is not well understood in these cells, although the localization of AQP9 on the membrane of Leydig cells surrounding the interstitial vessels suggests its involvement in copious water transit between the cells and blood vessels or interstitial space [20]. We found expression of AQP9 in the apical plasma membrane of ES epithelial cells and in the BM. A similar AQP distribution pattern in the ES and liver or testis confirms that AQP9 in the ES plays an important role in a variety of metabolites.

AQP10 is thought to be involved in water and glycerol permeability and is expressed in the small intestine. A similar function is suggested also in the ES. AQP11 is expressed in kidney, testis, liver, brain, intestine, heart, and adipose tissue, but its function is still unknown. AQP12 appears to be expressed specifically in pancreatic acinar cells, and as well as AQPs 0 and 11, it has not been characterized functionally. The precise roles of AQPs 0, 8, 10, 11, and 12 in the ES are also still poorly understood [1,2].

The present study revealed AQPs 1, 6, 8, and 9 in the BM, which is known to be of importance for the dynamic properties of ES water transport. It seems that AQPs 1, 6, 8, and 9 in the BM are closely related to water and ion transport through the BM.

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