cases of Meniere's disease (Linsay, 1942; Yazawa and Kitahara, 1981). However, fluctuating hearing loss and/or episodic vertiginous attack is inexplicable based only on retention hydrops, which is expected to develop slowly and gradually. In addition to the passive process of fluid retention, some active process that promotes the over-production of endolymph is required. The clinical observations of high p-VP levels in Meniere's disease (Takeda et al., 1995; Aoki et al., 2005) seem to provide a key for the solution of the mechanism underlying the overaccumulation of endolymph in Meniere's disease. Since an elevation in p-VP was not observed in hydrops animals with surgical obliteration of the endolymphatic duct (Kitano et al., 1994), high p-VP levels are thought to result not from the formation of EH, but rather to result in the development of EH.

Recently, AQP2 protein was confirmed to be expressed in the stria vascularis and endolymphatic sac, which are thought to be the main site of the secretion and/or absorption of endolymph (Fukushima et al., 2005). Molecular biological studies revealed that AQP2 mRNA expression in the cochlea as well as in the endolymphatic sac was upregulated by systemic application of VP (Sawada et al., 2002), and downregulated by OPC application into the scala tympani (Takeda et al., 2003). Morphologically, the endolymphatic space was increased by systemic application of VP (Takeda et al., 2000), and decreased by the application of OPC into the scala tympani (Takeda et al., 2003). These experimental results indicate that the water homeostasis of the inner ear fluid is regulated via the VP-AQP2 system. Namely, the VP-AQP2 system is thought to operate in such a way that its activation results in an influx of endolymph into the endolymphatic compartment, while its inhibition results in an efflux of endolymph from the endolymphatic compartment. Such clinical and basic lines of evidence strongly suggest that overactivity of the VP-AQP2 system is one of the etiological factors in Meniere's disease.

Treatment of this disease principally aims at a reduction in excess water retention in the endolymphatic compartment. Osmotic diuretic is one of the optional medicines. Since the mal-regulation of the VP-AQP2 system in the inner ear is thought to play an important role in the pathogenesis of Meniere's disease, application of an inhibitor of the VP-AQP system also seems to be a rational treatment strategy for Meniere's disease. In the present study, systemic and RW applications of OPC-31260, an inhibitor of the VP-AQP system, reduced the endolymphatic space in the same manner as the previously reported direct application to the inner ear (Takeda et al., 2003). The reduction in the endolymphatic space was more evident with RW application. Here, a question arises about the cumulative effect of xanthan gum used for sustained release of OPC. A possible dehydrating effect of xanthan gum, however, is ruled out, because RW application of xanthan gum did not exert any influence on the endolymphatic volume. The reduction in the endolymphatic space caused by RW application of OPC is thought to have been mainly caused by the aquaretic effect of OPC.

As to the OPC delivery via the RW, we have no exact knowledge about the pharmacokinetics in the inner ear. According to the report of Salt and Ma (2001), the OPC concentration in the inner ear is expected to be maintained with a basal to apical gradient for a period of days after the RW application. In the present study, however, the dehydration effects of OPC were not different among the 4 turns (the basal, 2nd, 3rd, and apical turns). Since the collapse was more marked in the basal turn in the previous study of direct infusion of OPC into the scala tympani of the basal turn (Takeda et al., 2003), RW application would be expected to provide a milder concentration gradient along the length of the cochlea.

It should be noted that systemic application produced no distinct volumetric change in the non-obliterated ear in spite of the significantly extended Reissner's membrane. These results are puzzling, because it is naturally expected that the bilateral ears would be affected in the same manner by systemic application. However, the high p-VP levels in animals with systemic application of OPC seem to give some explanation for the conflicting observations. Systemic application of OPC produces excessive urinary excretion, which causes dehydration and an increase in p-OSM, resulting in a rise in the p-VP level (Marples et al., 1998; Fleeman et al., 2000). This elevation of p-VP levels counteracts the pharmacological action of OPC. The blood concentrations of OPC and p-VP levels are expected to change with the passage of time. OPC exerts a dehydratic effect on the inner ear via the VP-AQP2 system in the inner ear, while high p-VP exerts a hydratic effect on the inner ear. These two effects are suspected to work in the inner ear in a competitive manner, resulting in the lack of a volumetric change in the non-obliterated ear, although a slight bulging and folding were seen in Reissner's membrane. Such offset effects might be one of the reasons why systemic application of OPC did not produce a remarkable reduction in the endolymphatic spaces in the obliterated ears.

The main site of action of OPC is thought to be in the stria vascularis and endolymphatic sac, especially the stria vascularis, in RW application. If OPC is toxic to the inner ear, the stria vascularis could be involved. However, RW application exerted no harmful influence on EP, and electron microscopic studies also revealed that the stria vascularis was not injured. Another possible ototoxic effect of RW application is thought to be hair cell damage due to osmotic changes of the inner ear fluid. RW application of OPC might cause dehydration of the inner ear fluid, resulting in an increase in the osmolarity of the inner ear fluid. Hypertonic inner ear fluid is suspected to shrink the hair cells. However, our TEM study revealed that the ultrastructural appearance was normal both in the stereocilia and in the hair cells. These results indicated that the ototoxicity of OPC-31260, even if applied via the RW, was negligible.

The results of the present study suggest that RW application of the gelatinform of OPC-31260 mixed with xanthan

gum seems to be the most useful drug delivery method. Meanwhile, systemic application of OPC has some drawbacks for clinical use. The dosage that can be used is limited due to the diuretic effect of OPC. Systemic application of OPC also requires controlling the increase in p-VP due to the systemic dehydration. If the p-VP level could be controlled to within the normal range, for example, by adequate hydration, systemic application of OPC could be one of the treatment options in cases of bilateral involvement. OPC-31260, whether applied systemically or locally, is a promising drug for the treatment of Meniere's disease.

## Acknowledgements

This study was supported by a Research Grant for Intractable Disease (Vestibular Disorders) from the Ministry of Health, Labour and Welfare, Japan (2005–2006) and a Grant-in Aid for Scientific Research [C(2) 17591792] provided by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

## References

Agre, P., Sasaki, S., Chrispeels, M.J., 1993. Aquaporins: a family of water channel proteins. Am. J. Physiol. Renal. Physiol. 265, F461.

Aoki, M., Ando, K., Yokota, Y., Mizuta, K., Ito, Y., 2005. The association of antidiuretic hormone with an attack of Meniere's disease. Clin. Otolaryngol. 30, 521–525.

Beitz, E., Kumagami, H., Krippeit-Drews, P., Ruppersberg, J.P., Schultz, J.E., 1999. Expression pattern of aquaporin water channels in the inner ear of the rat. The molecular basis for a water regulation system in the endolymphatic sac. Hear. Res. 132, 76–84.

Fleeman, L.M., Irewin, P.J., Phillips, P.A., West, J., 2000. Effects of an oral vasopressin receptor antagonist (OPC-31260) in a dog with syndrome of inappropriate secretion of antidiuretic hormone. Aust. Vet. J. 78, 825–830.

Fukushima, K., Takeda, T., Kakigi, A., Takeda, S., Nishioka, R., Azuma, H., Taguchi, T., 2005. Effects of lithium on endolymph homeostasis and experimentally-induced endolymphatic hydrops. ORL J. Otorhinolaryngol. Relat. Spec. 67, 282–288.

Huang, D., Chen, P., Chen, S., Nagura, M., Lim, D.J., Lin, X., 2002. Expression patterns of aquaporins in the inner ear: evidence for concerted actions of multiple types of aquaporins to facilitate water transport in the cochlea. Hear. Res. 165, 85–95.

Kitano, H., Takeda, T., Pulec, JL., Saadat, D., Uchida, K., 1994. The relationship between vasopressin and endolymphatic hydrops in the guinea pig. Ear Nose Throat J. 73, 824–828.

Kitano, H., Suzuki, M., Kitanishi, T., Yazawa, Y., Kitajima, K., Takeda, T., Kimura, H., Tooyama, I., 1999. Regulation of inner ear fluid in the rat by vasopressin. Neuroreport 10, 1205–1207.

Kumagami, H., Loewenheim, H., Beitz, E., Wild, K., Schwartz, H., Yamashita, K., Schultz, J., Paysan, J., Zenner, H.P., Ruppersberg, J.P., 1998. The effect of anti-diuretic hormone on the endolymphatic sac of the inner ear. Pflugers Arch. 436, 970–975.

Linsay, J.R., 1942. Labyrinthine hydrops and Meniere's disease. Arch. Otolaryngol. 35, 853–867.

Marcus, D.C., Rokugo, M., Ge, X., Thalmann, R., 1983. Response of cochlear potentials to presumed alterations of ionic conductance: endolymphatic perfusion of barium, valinomycin and nystatin. Hear. Res. 12, 17–30.

Marples, D., Christensen, B.M., Frøkiaer, J., Knepper, M.A., Nielsen, S., 1998. Dehydration reverses vasopressin antagonist-induced diuresis and aquaporin-2 downregulation in rats. Am. J. Physiol. Renal. Physiol. 275, F400–F409.

Merves, M., Bobbitt, B., Parker, K., Kishore, BK., Choo, D., 2000. Developmental expression of aquaporin 2 in the mouse inner ear. Laryngoscope 110, 1925–1930.

Mhatre, A.N., Steibach, S., Hribar, K., Shamsul Hoque, A.T.M., Lalwani, A.K., 1999. Identification of aquaporin 5 (AQP5) within the cochlea: cDNA cloning and in site localization. Biochem. Biophys. Res. Commun. 264, 157–162.

Minami, Y., Shimada, S., Miyahara, H., Matsunaga, T., Toyama, M., 1998. Selective expression of mercurial-insensitive water channel (AQP-4) gene in Hensen and Claudius cells in the rat cochlea. Acta Otolaryngol. (Stockh.) Suppl. 533, 19–21.

Nielsen, S., Kwon, T.H., Christensen, B.M., Promeneur, D., Frokiaer, J., Marples, S., 1999. Physiology and pathophysiology of renal aquaporins. J. Am. Soc. Nephrol. 10, 647–663.

Salt, A.N., Ma, Y., 2001. Quantification of solute entry into cochlear perilymph through the round window membrane. Hear. Res. 154, 88–97.

Sawada, S., Takeda, T., Kitano, H., Takeuchi, S., Kakigi, A., Azuma, M., 2002. Aquaporin-2 regulation by vasopressin in the rat inner ear. Neuroreport 13, 1127–1129.

Shambaugh, G.E., Clemis, J.D., Arenberg, I.K., 1969. Endolymphatic duct and sac in Meniere's disease. 1. Surgical and histopathologic observations. Arch. Otolaryngol. 89, 816–825.

Stankovic, K.M., Adams, J.C., Brown, D., 1995. Immunolocalization of aquaporin CHIP in the guinea pig inner ear. Am. J. Physiol. 269, C1450–C1456.

Sterkers, O., Ferrary, E., Amiel, C., 1988. Production of inner ear fluids. Physiol. Rev. 68, 1083–1128.

Takeda, T., Kakigi, A., Saito, H., 1995. Antidiuretic hormone (ADH) and endolymphatic hydrops. Acta Otolaryngol. (Stockh.) 519 (Suppl.), 219–222.

Takeda, T., Takeda, S., Kitano, H., Okada, S., Kakigi, A., 2000. Endolymphatic hydrops induced by chronic administration of vasopressin. Hear. Res. 140, 1–6.

Takeda, T., Sawada, S., Takeda, S., Kitano, H., Suzuki, M., Kakigi, A., Takeuchi, S., 2003. The effects of $V_2$-antagonist (OPC-31260) on endolymphatic hydrops. Hear. Res. 182, 9–18.

Takeda, T., Sawada, S., Kakigi, A., Saito, H., 1997. Computed radiographic measurement of the dimensions of the vesibular aqueduct in Meniere's disease. Acta Otolaryngol. (Stockh) 528 (Suppl.), 80–84.

Takumi, Y., Nagelhus, E.A., Eidet, J., Matsubara, A., Usami, S., Shinkawa, H., Nielsen, S., Ottersen, O.P., 1998. Select types of supporting cell in the inner ear express aquaporin-4 water channel protein. Eur. J. Neurosci. 10, 3584–3595.

Yamamura, Y., Ogawa, H., Yamashita, H., Chihara, T., Miyamoto, H., Nakamura, S., Onogawa, T., Yamashita, T., Hosokawa, T., Mori, T., Tominaga, M., Yabuchi, Y., 1992. Characterization of a novel aquaretic agent, OPC-31260, as an orally effective, nonpeptide vasopressin $V_2$ receptor antagonist. Br. J. Pharmacol. 105, 787–791.

Yazawa, Y., Kitahara, M., 1981. Electron microscopic studies of the endolymphatic sac in Meniere's disease. ORL J. Otorhinolaryngol. Relat. Spec. 43, 121–130.

Original Paper

ORL

ORL 2006;68:217–220
DOI: 10.1159/000093299

Received: October 21, 2005
Accepted after revision: March 17, 2006
Published online: May 12, 2006

# Antidiuretic Hormone and Osmolality in Isosorbide Therapy and Glycerol Test

Akinobu Kakigi Taizo Takeda Shoichi Sawada Daizo Taguchi

Department of Otolaryngology, Kochi Medical School, Kochi, Japan

**Key Words**
Ménière's disease · Antidiuretic hormone · Plasma osmolality · Osmotic diuretics · Isosorbide · Glycerol test

**Abstract**
Changes in plasma antidiuretic hormone (p-ADH) and plasma osmolality (p-Osm) levels were studied in 63 patients with Ménière's disease before and after isosorbide administration and a glycerol test. Increments in both p-ADH and p-Osm levels were observed after isosorbide administration and the glycerol test. The p-ADH secretion appeared to be secondarily stimulated by an increase in the p-Osm level. This p-ADH level increase affects cochlear fluid homeostasis. Based on these results, we must consider both the p-Osm and p-ADH levels when treating patients with Ménière's disease by osmotic diuretics.

Copyright © 2006 S. Karger AG, Basel

## Introduction

Endolymphatic hydrops is the most consistent finding in Ménière's disease. As we reported previously, this endolymphatic homeostasis disorder appeared to be strongly related to antidiuretic hormone (ADH), i.e. the plasma ADH (p-ADH) level is increased in patients with Ménière's disease [1], chronic administration of ADH induces endolymphatic hydrops in guinea pigs [2], and ADH antagonist reduces endolymphatic hydrops in guinea pigs [3]. Concerning treatment in patients with Ménière's disease, osmotic diuretics are widely used [4–9]. Previously, we reported that hearing improvement, though in a limited number of cases, can be expected with isosorbide [10]. The mechanism underlying the efficacy of osmotic diuretics treatment is thought to be as follows: the change in endolymph volume is dependent upon the osmotic pressure gradient between the perilymph and the endolymph [11]. As long as the osmotic pressure of the perilymph is greater than that of the endolymph, endolymphatic hydrops decreases, and consequently, the hearing loss caused by endolymphatic hydrops improves. To increase the osmotic pressure of the perilymph, the plasma osmolality (p-Osm) must be greater than that of the perilymph. However, the increase in p-Osm in the osmotic diuretics treatment features the following problems. Consistent with the finding that the p-ADH level is regulated by the p-Osm level [12], osmotic diuretics treatment may worsen endolymphatic hydrops via a p-ADH level increase. Actually, we occasionally find that osmotic diuretics do not effect the hearing loss caused by endolymphatic hydrops, i.e. they are associated with a nega-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2006 S. Karger AG, Basel
0301–1569/06/0685–0217$23.50/0

Accessible online at:
www.karger.com/orl

Akinobu Kakigi, MD, PhD
Department of Otolaryngology
Kochi Medical School
Nankoku, Kochi 783-8505 (Japan)
Tel. +81 88 880 2393, Fax +81 88 880 2395, E-Mail kakigia@med.kochi-u.ac.jp

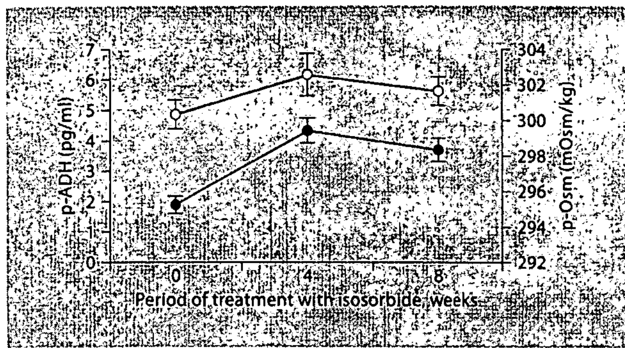


**Fig. 1.** Changes in p-ADH and p-Osm after therapy with isosorbide. The p-ADH level showed a statistical increase after 4 weeks of isosorbide administration compared with preisosorbide administration (paired t test, $p < 0.05$). The p-Osm level showed a statistical increase at 4 and 8 weeks after isosorbide administration, as compared with that prior to isosorbide administration (paired t test, $p < 0.001$). Open circles indicate the mean value of the plasma ADH level. Closed circles indicate the mean value of the p-Osm level. Error bars indicate the standard error of the means.

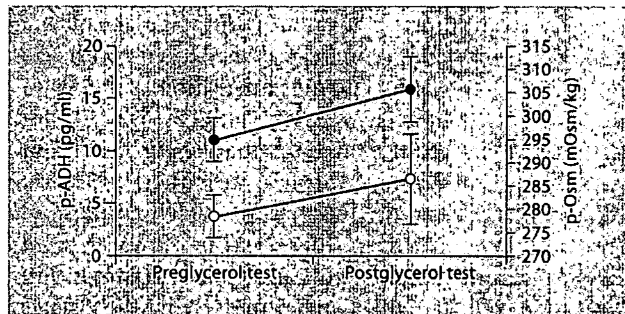


**Fig. 2.** Changes in p-ADH and p-Osm after the glycerol test. The p-ADH and p-Osm levels after glycerol administration showed a statistical increase ($p < 0.005$ and $p < 0.001$, respectively, paired t test). Open circles indicate the mean value of the plasma ADH level. Closed circles indicate the mean value of the p-Osm level. Error bars indicate the standard error of the means.

tive result on the glycerol test and a lack of effect of isosorbide treatment. Isosorbide, an osmotic diuretic, is widely used as a therapeutic agent for Ménière's disease in Japan [6, 13–15]. To gain further knowledge of the effects of osmotic diuretics, we investigated changes in p-ADH and p-Osm levels associated with isosorbide therapy and the glycerol test.

## Materials and Methods

The subjects included in the study were 63 patients with Ménière's disease. Of these 63 patients, 43 definitely represented Ménière's disease and 20 had cochlear Ménière's disease. Ménière's disease was diagnosed according to the criteria proposed by The Vestibular Disorder (Ménière's disease) Research Committee of Japan [16]. Each patient who had not been treated before was given 70% isosorbide orally. The dose administered was 40 ml three times a day for 2 weeks, then 30 ml for 2 weeks, and subsequently, 25 ml for 4 weeks. In all of the patients, p-ADH and p-Osm levels were determined just before and 4 and 8 weeks after isosorbide administration. With the patient seated, venous blood samples were collected between 10 and 12 a.m. For the glycerol test, 50% glycerol solution was administered orally at a dose of 1.3 ml/kg body weight. All patients had fasted for at least 3 h before the test. Pure-tone audiometry was performed immediately before and 3 h after glycerol ingestion. A threshold improvement exceeding 10 dB at two octaves was regarded as positive and a threshold improvement of 5–10 dB at three adjacent octaves was regarded as false positive. In 20 of 63 patients, p-ADH and p-Osm levels were determined just before and 3 h after glycerol ingestion. With the same procedures as in the isosorbide administration study, venous blood samples were collected. All patients gave informed consent before participation.

p-ADH was measured with a radioimmunoassay using a reversed-phase C18 silica column. This technique allows us to completely eliminate interference from nonspecific substances. The upper limit of the normal range of the p-ADH level was defined to be 3.5 pg/ml, on the basis of data for 105 normal subjects.

## Results

Figure 1 shows mean values of p-ADH and p-Osm just before (week 0) and 4 and 8 weeks after isosorbide administration. Both p-ADH and p-Osm levels were increased 4 weeks after isosorbide administration and then slightly decreased. The mean values and standard deviations of p-ADH levels 0, 4 and 8 weeks after isosorbide administration were 4.9 ± 3.8, 6.2 ± 5.4 and 5.6 ± 3.6 pg/ml, respectively. The p-ADH level showed a statistical increase with 4 weeks of isosorbide administration compared with the p-ADH level of preisosorbide administration (paired t test, $p < 0.05$). The mean values and standard deviations of p-Osm levels 0, 4 and 8 weeks after isosorbide administration were 295.2 ± 3.7, 299.4 ± 5.2 and 298.4 ± 5.1 pg/ml, respectively. The p-Osm level showed a statistical increase with 4 and 8 weeks of isosorbide administration compared with that of preisosorbide administration (paired t test, $p < 0.001$). The p-Osm level 4 weeks after isosorbide administration increased to 101.4% of that of preisosorbide administration. The p-Osm level 8 weeks after isosorbide administration de-

creased to 99.7% of that 4 weeks after isosorbide administration.

Figure 2 shows mean values of p-ADH and p-Osm just before and after glycerol administration. The mean values and standard deviations of the p-ADH level before and after glycerol administration were 3.8 ± 2.0 and 7.4 ± 4.3 pg/ml. There was a significant difference between the p-ADH level before and after glycerol administration (paired t test, $p < 0.005$). The mean values and standard deviations of the p-Osm level before and after glycerol administration were 295 ± 4.6 and 306 ± 6.9 mOsm/kg. There was a significant difference between the p-Osm level before and after glycerol administration (paired t test, $p < 0.001$). The post-glycerol test p-Osm level was 103.7% of the preglycerol test level.

## Discussion

Since Verney [17] reported the factors determining ADH release, it has been known that the p-Osm level determines the p-ADH level [12, 18]. The most common factor to increasing ADH release may be dehydration induced by restriction of water intake. Intravenous and intraperitoneal administrations of ionic, e.g. sodium chloride, hypertonic water increase p-Osm, and thus, the p-ADH level increases as well. There is some complexity in the fashion in which ADH secretion is stimulated by nonionic hypertonic water administration. The hypertonicity resulting from materials that are less permeable to the plasma membrane, e.g. sucrose and mannitol, stimulates ADH secretion. On the other hand, the hypertonicity resulting from materials that are easily permeable to the plasma membrane, e.g. urea and glucose, does not stimulate ADH secretion. For example, glucose hypertonicity decreases the plasma sodium level, and thus, ADH secretion is thought to be inhibited. Administration of hypertonic glycerol water increases p-Osm [19–21]. In this present study, results were the same as in previous reports. The average p-Osm level increased by 11 mOsm/kg (3.7%) after the glycerol test. In general, a 1% increase in p-Osm stimulates ADH secretion as a 1-pg/ml increase. In this study, a 3.7-pg/ml ADH increase was expected theoretically. Actually, the average p-ADH level increased by 2.6 pg/ml, which is close to the theoretical value based on the p-Osm increase. Concerning isosorbide, administration of hypertonic isosorbide water increased p-Osm. The average p-Osm level 4 weeks after isosorbide administration increased by 4.2 mOsm/kg (1.4%) compared with the p-Osm level just before administration. As mentioned above, a 1.4-pg/ml ADH increase was expected theoretically. Actually, the average p-ADH level increased by 1.3 pg/ml, which is close to the theoretical value based on the p-Osm increase. The result suggests that isosorbide is less permeable to the plasma membrane and that hypertonic isosorbide water stimulates ADH secretion. Moreover, the secondary ADH level increase may worsen endolymphatic hydrops. Based on this result, we must consider the p-Osm and p-ADH levels when treating patients with Ménière's disease with osmotic diuretics.

## References


1 Takeda T, Kakigi A, Saito H: Antidiuretic hormone (ADH) and endolymphatic hydrops. Acta Otolaryngol Suppl 1995;519:219–222.

2 Takeda T, Takeda S, Kitano H, Okada T, Kakigi A: Endolymphatic hydrops induced by chronic administration of vasopressin. Hear Res 2000;140:1–6.

3 Takeda T, Sawada S, Takeda S, Kitano H, Suzuki M, Kakigi A, Takeuchi S: The effects of V2 antagonist (OPC-31260) on endolymphatic hydrops. Hear Res 2003;182:9–18.

4 De Vincentiis I, Celestino D, Iannetti G: New pathogenic and therapeutic conceptions in Ménière's disease. Minerva Otorhinolaryngol 1972;22:223–228.

5 Duvall AJ 3rd, Santi PA, Hukee MJ: Cochlear fluid balance. A clinical/research overview. Ann Otol Rhinol Laryngol 1980;89:335–341.

6 Kitahara M, Takeda T, Yazawa Y, Matsubara H, Kitano H: Treatment of Ménière's disease with isosorbide. ORL J Otorhinolaryngol Relat Spec 1982;44:232–238.

7 Juhn SK, Prado S, Pearce J: Osmolality changes in perilymph after systemic administration of glycerin. Arch Otolaryngol 1976;102:683–685.

8 Kitahara M, Takeda T, Yazawa Y, Matsubara H, Kitano H: Experimental study on Ménière's disease. Otolaryngol Head Neck Surg 1982;90: 470–481.

9 Takeda T, Takeuchi S, Saito H: Effect of glycerol on pressure differences between perilymph and endolymph. Acta Otolaryngol (Stockh) 1990;110:68–72.

10 Kakigi A, Takeda T, Saito H, Kataoka H: Effect of isosorbide on hearing loss due to endolymphatic hydrops. Acta Otolaryngol Suppl 1995;519:223–226.

11 Kakigi A, Takeda S, Takeda T, Sawada S, Azuma H, Higashiyama K, Yamakawa K, Takeuchi S: Time course of dehydrating effects of isosorbide on experimentally induced endolymphatic hydrops in guinea pigs. ORL J Otorhinolaryngol Relat Spec 2004;66:291–296.

12 Robertson GL, Shelton RL, Athar S: The osmoregulation of vasopressin. Kidney Int 1976; 10:25–37.

13 Yamazaki T, Imoto T, Hayashi N, Watanabe S, Kozaki H, Abe T: Meniersche Krankheit und Isosorbid. Arch Otorhinolaryngol 1982; 234:97–104.

14 Kitano H, Kitahara M: The treatment of Ménière's disease with isosorbide; in Kitahara M (ed): Ménière's Disease. Tokyo, Springer-Verlag, 1990, pp 165–171.

15 Nozawa I, Nakayama H, Hashimoto K, Imamura S, Hisamatu K, Murakami Y: Efficacy of long-term administration of isosorbide for Ménière's disease. ORL J Otorhinolaryngol Relat Spec 1995;57:135–140.

16 Watanabe I: Ménière's disease. With special emphasis on epidemiology, diagnosis and prognosis. ORL 1980;42:20–45.

17 Verney EB: The antidiuretic hormone and the factors which determine its release. Proc Roy Soc 1947;135:25–106.

18 Schrier RW, Berl T, Anderson RJ: Osmotic and nonosmotic control of vasopressin release. Am J Physiol 1979;236:F321–F332.

19 Juhn SK, Prado S, Pearce J: Osmolality changes in perilymph after systemic administration of glycerin. Arch Otolaryngol 1976;102:683–685.

20 Coats AC, Alford BR: Ménière's disease and the summating potential. 3. Effect of glycerol administration. Arch Otolaryngol 1981;107:469–473.

21 Cohen J, Morizono T, Long C: The effect of glycerol on cochlear function and ionic concentration. Arch Otorhinolaryngol 1985;241:285–293.

*The Laryngoscope*
Lippincott Williams & Wilkins, Inc.
© 2007 The American Laryngological,
Rhinological and Otological Society, Inc.

# Expressions of Aquaporin-2, Vasopressin Type 2 Receptor, Transient Receptor Potential Channel Vanilloid (TRPV)1, and TRPV4 in the Human Endolymphatic Sac

Daizo Taguchi, MD; Taizo Takeda, MD; Akinobu Kakigi, MD; Masaya Takumida, MD;
Rie Nishioka, MD; Hiroya Kitano, MD

***Objective:*** **To localize aquaporin (AQP)2, vasopressin type 2 receptor ($V_2$-R), and transient receptor potential channel vanilloid subfamily 1, 4 (TRPV1, TRPV4) in the human endolymphatic sac (ES).** ***Methods:*** **Three samples of human ES were sampled during the removal of vestibular schwannoma by way of the translabyrinthine approach. The samples were immediately fixed in 4% paraformaldehyde and embedded in OCT compound; immunohistochemistry was performed with AQP2, $V_2$-R, TRPV1, and TRPV4 polyclonal antibodies.** ***Results:*** **AQP2, $V_2$-R, TRPV1, and TRPV4 proteins were detected in the epithelial layer of the ES but were not observed in connective tissue around the ES. TRPV1 was also expressed in blood vascular endothelial cells of the connective tissue of ES.** ***Conclusions:*** **AQP2, $V_2$-R, and TRPV4 were expressed in the luminal epithelium of human ES. The same characteristic distribution of water and ion channels is seen in the kidney, where a significant amount of fluid is filtrated and resorbed. ES probably plays an active role in the homeostasis of the endolymph.** ***Key Words:*** **Aquaporin, vasopressin, transient receptor potential channel vanilloid, inner ear.**

***Laryngoscope*, 117:695–698, 2007**

From the Department of Otolaryngology (D.T., T.T., A.K., R.N.), Kochi Medical School, Nankoku, Kochi, Japan; the Department of Otolaryngology (M.T.), Hiroshima University, Faculty of Medicine, Hiroshima, Hiroshima, Japan; and the Division of Otolaryngology (H.K.), Head and Neck Surgery, Tottori University, Faculty of Medicine, Yonago, Tottori, Japan.

Editor's Note: This Manuscript was accepted for publication December 15, 2006.

Presented at the 43rd Inner Ear Biology, Montpellier, France, September 16–20, 2006.

This study was supported by a Health and Labor Science Research Grant in Japan (Research on Measures for Intractable Disease [Vestibular Disorders]) and a Grant-in-Aid for Scientific Research C(2) (17591792) provided by the Ministry of Education, Science and Culture.

Send correspondence to Dr. Daizo Taguchi, Department of Otolaryngology, Kochi Medical School, Kohasu, Oko-cho Nankoku, Kochi, 783-8505 Japan. E-mail: jm-taguchid@kochi-u.ac.jp

DOI: 10.1097/mlg.0b013e318031c802

## INTRODUCTION

The homeostasis of inner ear fluid is essential for maintaining inner ear function. Because water channels as well as ion channels have been elucidated as essential for fluid homeostasis in a living body, water channels are expected to exist in the inner ear. Indeed, proteins or mRNAs of many types of aquaporins (AQP) are reported to be expressed in the inner ear of experimental animals.[1–3] It is noteworthy that proteins or mRNAs of AQP2 and vasopressin type 2 receptor ($V_2$-R) are expressed in the cochlea and endolymphatic sac (ES), where they are thought to be the main sites of absorption and secretion of the endolymph. Vasopressin (VP) is regulated by osmosis, and therefore water homeostasis of the inner ear fluid may be under the control of VP-mediated systemic osmoregulation. In addition, transient receptor potential vanilloid ion channels (TRPV)1 and 4 were recently confirmed to be histochemically identified in the same area.[4,5] TRPV4, initially named vanilloid receptor-related osmotically activated channel, might function as an osmosensor and hydrostatic pressure sensor in vertebrates as well invertebrates.[6,7] TRPV4, expressed in the stria vascularis and ES, is thought to play an important role in osmotic equilibrium and hydrostatic pressure regulation in the endolymph.

As described above, AQP2 and TRPV4 are reported to be expressed in the rodent inner ear and also in the kidney, where water homeostasis is under VP-AQP2 regulation. Water flux by way of the AQP2 water channel is driven by osmotic pressure,[8] and TRPV4 is gated by osmotic and hydrostatic pressure; therefore, AQP2 and TRPV4 are likely to act in close relation with each other by way of VP-regulated osmosis. If this supposition is true in all vertebrates, AQP2, $V_2$-R, and TRPV4 should be coherently expressed in the human inner ear. In this study, the expressions of AQP2, $V_2$-R, TRPV1, and TRPV4 were investigated using tissue slices of human ES collected when an acoustic neurinoma was removed by way of the translabyrinthine approach.

## MATERIALS AND METHODS

### *Tissue Sampling and Preparations*

Three samples of human ES were sampled during the removal of vestibular schwannoma by way of the translabyrinthine approach. The samples were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 2 hours, soaked in 15% sucrose in PBS at 4°C overnight, and then embedded in Tissue Tec OCT Compound (Sakura Finetechnical Co, Ltd., Tokyo, Japan). Slices of 7 $\mu$m thickness were cut with a cryostat (HM 505E, MICROM) and collected on slides. In accordance with Kochi Medical School ethics committee approval, all subjects were informed of the risks and purposes of the study before their written consent was obtained, and tissue sampling was approved by all patients before the operation.

### *Histochemistry*

The sections were preincubated for 1 hour in a solution containing 10% normal goat serum. The specimens were then exposed overnight at 4°C to rabbit antibodies specific for rat AQP2 (Cat. AQP21-A, Lot 561011A5, Alpha Diagnostic, San Antonio, TX), diluted 1:100, rat $V_2$-R (Cat. AVPV21-A, Lot 341314A2, Alpha Diagnostic), diluted 1:100, human TRPV1 (Cat. ALX-210-417, Lot L17951, Alexis Biochemicals, San Diego, CA), diluted 1:25, and rat TRPV4 (Alomone Labs, Ltd., Jerusalem, Israel), diluted 1:200. The homologies of rat AQP2, $V_2$-R, and TRPV4 with humans are 93%, 90%, and 84% identical residues, respectively. The sections were incubated for 6 hours at room temperature in Alexa 546 goat anti-rabbit IgG (A-11035, Molecular Probes, Inc., Eugene, OR), diluted 1: 100. Sections were washed thoroughly with PBS containing TWEEN 20 (Sigma-Aldrich Co, Ltd., Tokyo, Japan) after each antibody incubation. Sections incubated with Alexa 546 fluorescent antibodies were observed using a confocal laser scanning microscope system (LSM 410, Zeiss, Jena, Germany). The fluorescence of Alexa 546 was observed using an LSM 410 microscope equipped with a helium-neon laser (543 nm), a dicroicreflector (FT543), and an emission filter (FT570). Control sections were incubated with PBS instead of the primary antibodies; the results of these sections were invariably negative. Some sections were stained with hematoxylin-eosin (H&E) and observed under a light microscope for morphologic study.

### *Pixel Counts*

After each experiment, fluorescence intensities were measured and analyzed from stored digital image sequences using IP

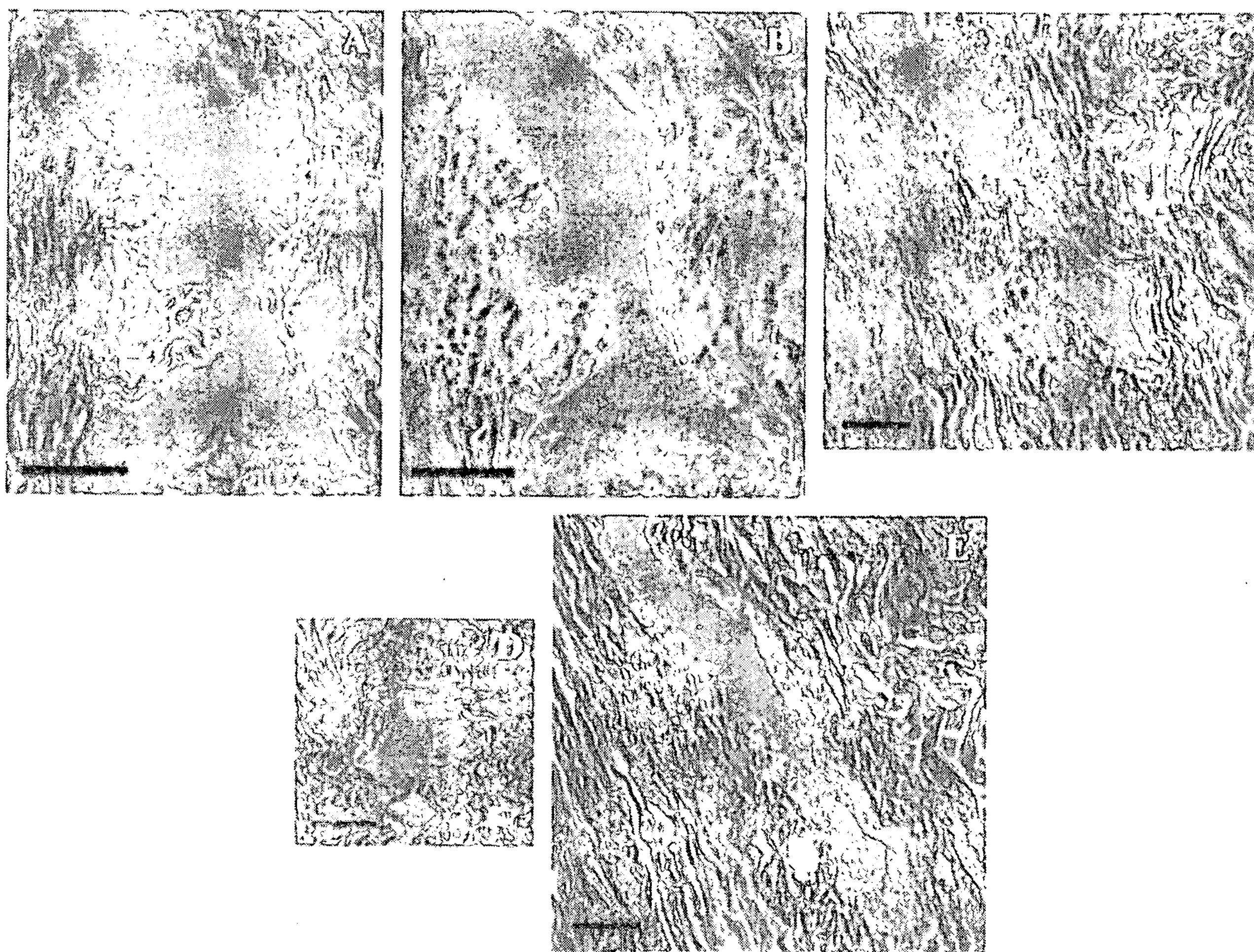


Fig. 1. Immunostaining of aquaporin (AQP)2, vasopressin type 2 receptor ($V_2$-R), transient receptor potential vanilloid (TRPV)1, and TRPV4 in rugose portion of human endolymphatic sac (ES). Both AQP2 (A) and $V_2$-R (B) proteins are expressed in epithelial layer of ES. No immunoreactivity was observed in connective tissue of ES. Scale bar = 50 $\mu$m. Immunohistochemistry studies revealed that TRPV1 was expressed in epithelial cells and blood vascular endothelial cells of connective tissue in ES (C and D). TRPV4 was expressed in epithelial cells of ES (E) but was not observed in the connective tissue of ES. Scale bar = 50 $\mu$m.

TABLE I.
Fluorescence Intensity of Aquaporin-2 (AQP2), Vasopressin Type 2 Receptor ($V_2$-R), and Transient Receptor Potential Channel Vanilloid (TRPV)1 and TRPV4 in Human Endolymphatic Sac and Blood Vessel.

| | AQP2(ES), 29.9 ± 8.4 | $V_2$-R(ES), 61.9 ± 12.7 | TRPV1(ES), 13.0 ± 3.6 | TRPV1(V), 21.0 ± 10.2 | TRPV4(ES), 14.7 ± 3.8 |
|---|---|---|---|---|---|
| AQP2(ES) | — | $P < .01$ | $P < .01$ | $P < .01$ | $P < .01$ |
| V2-R(ES) | | — | $P < .01$ | $P < .01$ | $P < .01$ |
| TRPV1(ES) | | | — | $P < .01$ | NS |
| TRPV1(V) | | | | — | $P < .01$ |

Fluorescence intensity of control specimen was 1.0 ± 0.4 in endolymphatic sac (ES) and 0.8 ± 0.2 in blood vessel (V).
Significance levels were assessed according to one-way analysis of variance.
NS = not significant.

Lab Spectrum software (version 3.0; Signal Analytics Corp., Rockville, MD). For statistical analysis, 20 epithelial cells were randomly selected from each specimen, and the fluorescence intensity (0–255) of each cell was measured at each time point. Means and standard errors were calculated from the fluorescence intensity values of the 20 cells. These data were analyzed by one-way analysis of variance (ANOVA).

## RESULTS

Immunoreactivity to antibodies of AQP2, $V_2$-R, TRPV1, and TRPV4 was present in all three samples. Meanwhile, immunoreactivity was not observed in sections incubated with PBS instead of the primary antibodies (not shown). Representative photographs of immunohistochemical staining of AQP2, $V_2$-R, TRPV1, and TRPV4 are presented in Figure 1. AQP2, $V_2$-R, TRPV1, and TRPV4 proteins were expressed in the epithelial layer of the ES but were not observed in connective tissue around the ES (Fig. 1A, B, C, and E). TRPV1 was also expressed in blood vascular endothelial cells of the connective tissue of ES (Fig. 1D). Table I shows the results of pixel counts. Pixel counting analysis of fluorescence intensity revealed that the fluorescence intensity of specimens stained with TRPV1 was significantly higher in blood vascular endothelial cells than in the epithelial layer of ES ($P < .01$, one-way ANOVA). Immunoreactive sites were observed using H&E-stained specimens to confirm the morphologic characteristic of the epithelial structure of ES and blood vessels (Fig. 2).

## DISCUSSION

Systemic osmotic pressure is maintained by VP-mediated feedback regulation of body fluid. As is well known, VP mainly regulates urinary volume by binding to $V_2$-R in the collecting duct of the kidney. $V_2$-R is reported to exist in the inner ear of rodents, and AQP2, a VP-mediated water channel protein, is also expressed.[9] Water homeostasis of the inner ear fluid was confirmed to be regulated using the VP-AQP2 system.[10,11]

In this study, AQP2, $V_2$-R, TRPV1, and TRPV4 were expressed in human ES. In particular, AQP2, $V_2$-R, and TRPV4 were expressed in the epithelial layer of ES. The same distribution pattern was also observed in the kidney from the cortex through the inner medulla,[12,13] where a large amount of water and solute is filtrated and reabsorbed. The water channel composed of AQP2 is mediated by VP, and its driving force is the osmotic gradient, whereas TRPV4 is activated by hypotonic stress. These two channels are expected to be closely interconnected by way of osmosis. Although the possible role of AQP, $V_2$-R, and TRPV4 in kidney function is still poorly understood, the expressions of AQP2, $V_2$-R, and TRPV4 in the ES epithelium suggest the active transport of water and solute similar to that in the kidney.

TRPV1 protein could be localized to sensory and autonomous ganglia cells and also to urothelial cells lining the pyelon, ureter, and bladder. In the rodent inner ear, TRPV1 was detected in hair cells and supporting cells of the organ of Corti and in both vestibular and spiral ganglion cells.[5] In human ES, TRPV1 was faintly expressed in epithelial cells, concordant with data from guinea pigs,[5] and also faintly expressed in the vessel layer. A recent study indicated the possible mechanisms of TRPV1 to autoregulate vascular myogenic tone in response to mechanical stimuli in blood vessels. TRPV1 expressed in the blood vascular layer of ES likely plays a role in the autoregulation of blood circulation in ES.

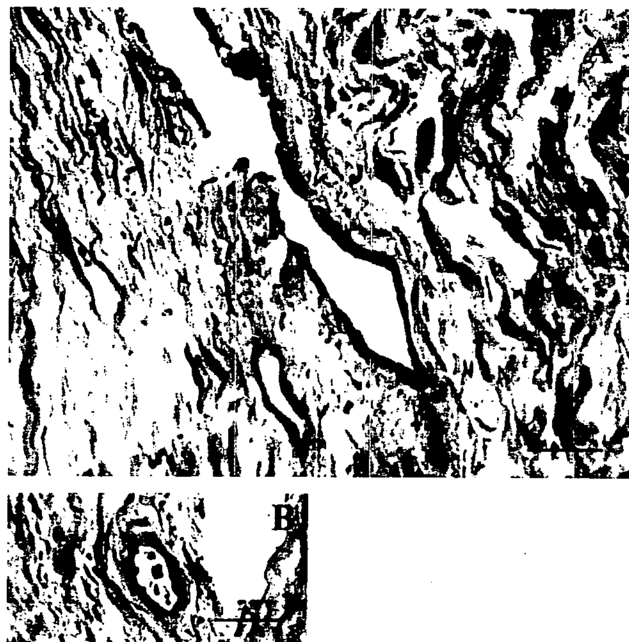


Fig. 2. Hematoxylin-eosin staining in rugose portion of human ES. (A) Cuboidal epithelial cells of endolymphatic sac (ES) and underlying connective tissue. Scale bar = 50 μm. (B) Blood vascular squamous endothelial cells of connective tissue of ES and underlying connective tissue. Scale bar = 50 μm. Morphologic characteristic of epithelial structure of ES and vessels was confirmed.

It is reported that patients with acoustic neuromas have abnormally high perilymph protein levels.[14] This increase in protein level will likely disrupt normal osmotic pressure and homeostasis between endolymph and perilymph. Therefore, the distinct expressions of AQP2, V2-R, TRPV1, and TRPV4 proteins in the present study might represent the up-regulation of these proteins in response to abnormal endolymphatic homeostasis caused by elevated perilymph (and possibly endolymph) protein levels caused by the presence of acoustic neuromas.

In the longitudinal theory of endolymph flow/absorption, mal-homeostasis of water and solute in ES is thought to cause the development of endolymphatic hydrops. This study revealed that a VP-mediated water channel and TRPV4 ion channel exist in the endothelial cells of ES. There is currently not sufficient knowledge as to how these channels participate in homeostasis of inner ear fluid in ES; however, there can be little doubt that intimate interaction between these channels is essential to maintain normal homeostasis of the inner ear fluid. Further studies might open a new avenue to better understand inner ear fluid homeostasis and the pathogenesis of diseases caused by its disturbance.

## BIBLIOGRAPHY


1. Sawada S, Takeda T, Kitano H, et al. Aquaporin-1 (AQP1) is expressed in the stria vascularis of rat cochlea. *Hear Res* 2003;181:15–19.
2. Sawada S, Takeda T, Kitano H, et al. Aquaporin-2 regulation by vasopressin in the rat inner ear. *Neuroreport* 2002;13: 1127–1129.
3. Fukushima K, Takeda T, Kakigi A, et al. Effects of lithium on endolymph homeostasis and experimentally-induced endolymphatic hydrops. *ORL J Otorhinolaryngol Relat Spec* 2005;67:282–288.
4. Liedtke W. TRPV4 as osmosensor: a transgenic approach. *Pflugers Arch* 2005;451:176–180.
5. Takumida M, Kubo N, Ohtani M, et al. Transient receptor potentials channels in the inner ear: presence of transient receptor potential channel subfamily 1 and 4 in the guinea pig inner ear. *Acta Otolaryngol* 2005;125:929–934.
6. Mutai H, Heller S. Vertebrate and invertebrate TRPV-like mechanoreceptors. *Cell Calcium* 2003;33:471–478.
7. Liedtke W, Choe Y, Marti-Renom MA, et al. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* 2000;103: 525–535.
8. Agre P. The aquaporin water channels. *Proc Am Thorac Soc* 2006;3:5–13.
9. Kitano H, Takeda T, Suzuki M, et al. Vasopressin and oxytocin receptor mRNAs are expressed in the rat inner ear. *Neuroreport* 1997;8:2289–2292.
10. Takeda T, Takeda S, Kitano H, et al. Endolymphatic hydrops induced by chronic administration of vasopressin. *Hear Res* 2000;140:1–6.
11. Takeda T, Sawada S, Takeda S, et al. The effects of $V_2$-antagonist (OPC-31260) on endolymphatic hydrops. *Hear Res* 2003;182:9–18.
12. Nielsen S, Frøklær J, Marples D, et al. Aquaporins in the kidney: from molecules to medicine. *Physiol Rev* 2002;82: 205–244.
13. Cohen DM. TRPV4 and the mammalian kidney. *Pfluger Arch Eur J Physiol* 2005;451:168–175.
14. Silverstein H, Schuknecht HF. Biochemical studies of inner ear fluid in man. Changes in otosclerosis, Ménière's disease, and acoustic neuroma. *Arch Otolaryngol* 1966;84: 395–402.

Downloaded By: [Kouchi Medical College] At: 01:19 12 February 2008

*Acta Oto-Laryngologica*
2008, 1–5, iFirst article

informa
healthcare

ORIGINAL ARTICLE

# Effects of gadolinium injected into the middle ear on the stria vascularis

AKINOBU KAKIGI[1], MASAHIKO NISHIMURA[1], TAIZO TAKEDA[1], TERUHIKO OKADA[2], YORIKO MURATA[3] & YASUHIRO OGAWA[3]

*Departments of [1]Otolaryngology, [2]Department of Anatomy and [3]Department of Radiology, Kochi Medical School, Kochi, Japan*

**Abstract**
*Conclusion.* The concentration of gadolinium (Gd) used clinically showed no remarkable effects on the stria vascularis; however, a higher concentration had adverse effects. The concentration of Gd must be borne in mind when injecting Gd into the tympanic cavity. *Objective.* Endolymphatic hydrops has been visualized using high resolution MRI with the intratympanic administration of Gd in patients with Meniere's disease. We attempted to investigate the effects of Gd on the stria vascularis. *Materials and methods.* Gd hydrate diluted eightfold with saline or non-diluted Gd or saline was injected into the tympanic cavity of guinea pigs. To investigate the effects of Gd on the stria vascularis, we measured endocochlear DC potential (EP) and observed the stria vascularis using transmission electron microscopy. *Results.* Intratympanic injections of Gd hydrate diluted eightfold with saline (1/8 Gd) and saline did not cause apparent changes in the EP. Moreover, the amplitude of the EP decreased significantly 60 min after non-diluted Gd was injected. Transmission electron micrographs of the stria vascularis revealed no significant morphological difference between the ears injected with 1/8 Gd and those injected with saline. There was significant morphological change in the ear injected with non-diluted Gd. The intercellular spaces were markedly enlarged.

**Keywords:** *TRPV4, endocochlear DC potential, stria vascularis, endolymphatic hydrops, MRI, gadolinium, Meniere's disease*

## Introduction

As a pathological feature of Meniere's disease is endolymphatic hydrops, a definite diagnosis had been made by examining temporal bone histopathologic specimens in autopsy cases. Nakashima, et al. [1] recently reported that endolymphatic hydrops in patients with Meniere's disease could be visualized using a 3 T magnetic resonance imaging (MRI) unit. They injected gadolinium (Gd) hydrate diluted eightfold with saline intratympanically through the tympanic membrane. No adverse effects of the injection were observed, and there was no change in tinnitus. In a study with guinea pigs, auditory brainstem response (ABR) measurements showed no significant shifts in threshold after the application of Gd to the middle ear, indicating that gadolinium is non-toxic to the guinea pig cochlea [2]. However, Gd is known to be a blocker of transient receptor potential channel vanilloid subfamily 4 (TRPV4) [3,4]. Moreover, TRPV4 was found in the inner ear, i.e. hair cells and supporting cells of the organ of Corti, in marginal cells of the stria vascularis, spiral ganglion cells, sensory cells, transitional cells, dark cells in the vestibular end organs, vestibular ganglion cells, and epithelial cells of the endolymphatic sac. Notably, TRPV4 expression is marked in the region of the stria vascularis [5]. Based on these reports, we were interested in whether Gd influences the inner ear, especially the stria vascularis. The purpose of this study was to investigate the effects of Gd hydrate on the stria vascularis, both physiologically and morphologically, using endocochlear DC potential (EP) measurements and electron microscopy.

Correspondence: Akinobu Kakigi, MD, PhD, Department of Otolaryngology, Kochi Medical School, Nankoku, Kochi 783-8505, Japan. Tel: +81 88 880 2393. Fax: +81 88 880 2395. E-mail: kakigia@kochi-u.ac.jp

*(Received 11 October 2007; accepted 23 October 2007)*

ISSN 0001-6489 print/ISSN 1651-2551 online © 2008 Taylor & Francis
DOI: 10.1080/00016480701769776

Downloaded By: [Kouchi Medical College] At: 01:19 12 February 2008

## Materials and methods

### *Intratympanic Gd injection and EP measurement*

Thirteen healthy female guinea pigs (Hartley) weighing 260–400 g were used. The animals were divided into a Gd group ($n = 5$), a 1/8 Gd group ($n = 5$), and a control group ($n = 3$). In the Gd group, the middle ear bulla was injected with 1.0 ml of non-diluted Gd hydrate (Omniscan, Daiich Pharmaceutical Co. Ltd, Tokyo, Japan). In the 1/8 Gd group, the middle ear bulla was injected with 1.0 ml of Gd hydrate diluted eightfold with saline (1:7 v/v), which was decided based on the procedures described by Nakashima et al. [1]. In the control group, the middle ear bulla was injected with 1.0 ml of saline. Gd hydrate, 1/8 Gd hydrate, and saline were kept at 38°C.

Guinea pigs were anesthetized with ketamine (30 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.) and artificially ventilated with room air. The right cochlea was exposed by the ventral approach. EP levels were recorded from the scala media of the second cochlear turn through the lateral wall of the cochlear duct using a glass microelectrode (tip diameter approximately 0.5 μm) filled with 150 mM KCl and connected to an electrometer (FD223, WPI, Sarasota, FL, USA). An Ag-AgCl wire placed in the neck muscle served as a reference. The recording system was zeroed when the pipette tip was placed on the spiral ligament before insertion into the scala media. Voltage drifts were within ±2 mV when measured with the pipette tip pulled back to the spiral ligament after the experiment had been completed. Data were obtained from animals whose EP was stable at normal levels (>80 mV) for 6 min before experimental trials. The EP was monitored for 1 h after the Gd hydrate, 1/8 Gd hydrate, or saline was injected.

### *Data presentation and statistics*

Data are presented as the mean ± SE. Data were compared with a paired Student's *t* test, and changes were regarded as significant when $p < 0.05$. All the changes and differences mentioned in the text are statistically significant.

### *TEM study*

After termination of the EP recording, the cochlea was fixed by perilymphatic perfusion via the round window of a fixative containing 1.0% paraformaldehyde, 1.5% glutaraldehyde, and 1% $OsO_4$ in 0.07 M phosphate buffer (pH 7.2) with 3% sucrose for 15 min. Thereafter, the cochlea was quickly ablated, and the stria vascularis of the basal turn was harvested from extirpated cochlea in the same fixative. We selected the basal turn stria vascularis to avoid the effect of fenestration. The stria vascularis was immersed in the same fixative for an additional 60 min in the cold. After fixation, the tissues were washed in 0.1 M phosphate buffer (pH 7.2), dehydrated in a graded ethanol series, and embedded in Spurr's resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, and observed using a Hitachi H700-H electron microscope at 75 kV.

### *Animal care*

The care and use of animals in this study were approved by the Kochi Medical School Animal Care and Use Committee.

## Results

### *EP measurement*

The typical pattern of the EP measurement in the 1/8 Gd group is presented in Figure 1A. The intratympanic injection of Gd hydrate diluted eightfold with saline (1/8 Gd) did not cause apparent changes in the EP for 60 min. The amplitude of the EP was 87.6 ± 2.97 mV and 88.8 ± 3.06 mV before and after the injection, respectively. There was no significant change. The typical pattern of the EP change in the Gd group is presented in Figure 2A. In the experiment with non-diluted Gd, the amplitude decreased from 88.0 ± 1.29 mV to 81.9 ± 1.14 mV 60 min after the injection. This difference was significant ($p = 3.41042 \times 10^{-5}$). The typical pattern of the EP measurement in the control group was the same as in the 1/8 Gd group (not shown). The intratympanic injection of saline did not cause apparent changes in the EP for 60 min. The amplitude of the EP was 87.0 ± 1.68 mV and 86.9 ± 1.36 mV before and after the injection, respectively. There was no significant change.

### *TEM study*

Figure 1B, C shows transmission electron micrographs of the stria vascularis in the ear injected with 1/8 Gd. A number of autophagosomes were seen in the marginal cells. However, vacuolar degeneration was not detected. The intercellular spaces were tight. No blebs were seen at the apical membrane. In the stria vascularis, there was no significant morphological difference between the ears injected with 1/8 Gd and those left untreated. Figure 2B, C shows transmission electron micrographs of the stria vascularis in the ear injected with non-diluted Gd. Protrusions were seen at the apical membrane of the

Downloaded By: [Kouchi Medical College] At: 01:19 12 February 2008

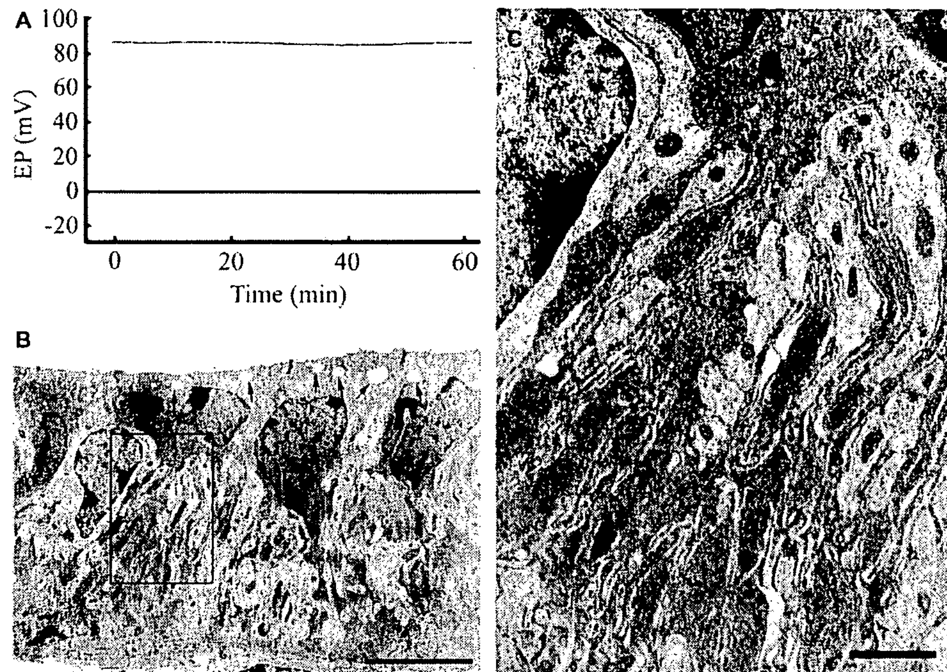


Figure 1. Effect of 1/8 Gd on the EP and morphology of the stria vascularis. (A) EP recording during injection of 1/8 Gd. After the termination of the recording, the strial tissue was chemically fixed by perilymphatic perfusion of the fixative. (B) Electron micrograph. Arrow, autophagosome. Scale bar = 5 μm. (C) Magnification of the rectangular area in B. The intrastrial space was not enlarged. Scale bar = 1 μm.

marginal cells. The intercellular spaces were enlarged. The intermediate cell's volume appeared to be decreased. In the stria vascularis, there was significant morphological difference between the ears injected with non-diluted Gd and the untreated ears. The stria vascularis in the ears injected with

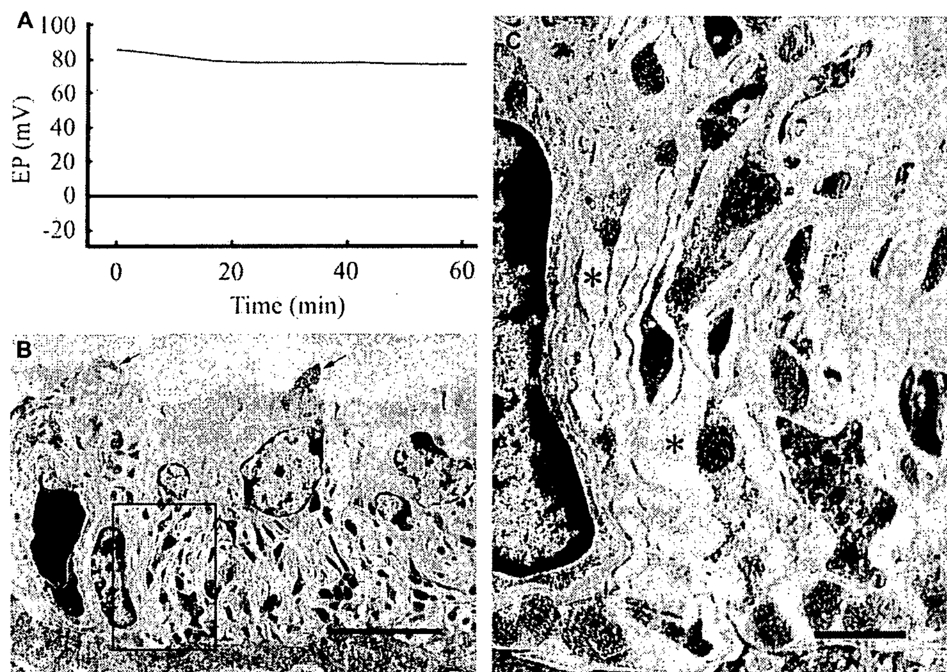


Figure 2. Effect of non-diluted Gd on the EP and morphology of the stria vascularis. (A) EP recording during injection of non-diluted Gd. After the termination of the recording, the strial tissue was chemically fixed by perilymphatic perfusion of the fixative. (B) Electron micrograph. Note the protrusion of the apical membrane of marginal cells to the endolymphatic side (arrow). Scale bar = 5 μm. (C) Magnification of the rectangular area in B. The intrastrial space was enlarged (asterisks). Scale bar = 1 μm.

Downloaded By: [Kouchi Medical College] At: 01:19 12 February 2008

saline had no significant morphological difference from those left untreated (not shown).

## Discussion

Recently, endolymphatic hydrops in the human cochlea has been visualized using high resolution MRI combined with the injection of Gd into the middle ear [1]. Gd is known to be a blocker of TRPV4, which is present in the inner ear [3,4]. Toxicity of Gd was excluded experimentally by ABR measurements [2]. This experimental study applied only 25–200 μl of Gd on a round window membrane. In a human MRI study, however, the tympanic cavity was filled with Gd for 60 min [1]. In this study, we tried to prove the validity of the human MRI study procedures.

In the present study, there was no change in EP 60 min after the injection of 1/8 Gd and no morphological change in the stria vascularis. These results support the validity of the human MRI study. In contrast, the EP decreased and the intrastrial space was enlarged 60 min after the non-diluted Gd was injected. These results indicate that the higher concentration of Gd has ototoxic effects. Gd was described as an inhibitor of TRPV4 and cation-selective stretch-activated ion channels (SACs) [6–8]. TRPV4 expression is marked in the region of the stria vascularis [5]; however, the expression of SACs has not been reported yet. Within the stria vascularis, TRPV4 was detected in marginal cells [9,10], nonsensory cells that maintain the ionic composition of endolymph [11] and the EP [12].

The ability of cells to regulate their volume if exposed to an anisotonic environment is a fundamental physiological function. Under hypotonic conditions, the influx of water along its osmotic gradient leads to cell swelling. In most vertebrate cells, and even some unicellular organisms, swelling is followed by a process called regulatory volume decrease (RVD), which enables the cell to regain its former volume even though the cell remains in a hypotonic environment. Loss of ions, mainly $K^+$ and $Cl^-$, followed by loss of water provides the mechanism of volume reduction [13]. A possible participant in RVD is the nonselective cation channel TRPV4 that has been shown to react to hypotonic stimuli with conductance of $Ca^{2+}$ [9]. In the stria vascularis, there seems to be an anisotonic environment because there was thought to be water flux through it and the endolymph is hyperosmotic to perilymph by 11–40 mOsmol/kg $H_2O$ [14–16]. In this study, non-diluted Gd seemed to block TRPV4 in the stria vascularis and water flux may have stopped at the level of the marginal cell. This may cause the protrusion of the marginal cell and the enlargement of the intrastrial space. Concerning the intermediate cell, its volume appeared to be decreased. In the intermediate cell, the existence of aquaporin1 (AQP1), which facilitates the osmotic movement of water molecules, has been reported [17]. The efflux of water from the intermediate cells to the intrastrial space may occur. These results may indicate that the intrastrial space is hyperosmotic to the intermediate cells.

TRPV4 was also detected in cochlear outer hair cells (OHCs) and inner hair cells (IHCs) and in vestibular hair cells [5,9]. It has been reported [18,19] that hypo-osmotic challenge induces rapid contraction of vestibular and cochlear isolated hair cells. These changes were reversible upon returning cells to iso-osmotic conditions. The TRPV4 channel may therefore act as an osmotic sensor in the fluid homeostasis of these cells [9]. TRPV4 is also considered as a $Ca^{2+}$-permeable channel, and it has been recently proposed to be an osmo- and mechano-sensitive channel [7,9]. A recent study demonstrated the functional expression of TRPV4 to be involved in the hypotonic stimulation-induced $Ca^{2+}$ influx in OHCs of the mouse cochlea [20]. These authors suggested that TRPV4 may function as an osmo- and mechano-sensory receptor in OHCs [20]. They also suggested that TRPV4 may play an important role in the active function of OHCs in cochlear micromechanics. The function of TRPV4 in the inner ear sensory cells remains unclear, although it has been suggested that Gd should induce adverse effects on the hearing and balance.

Gd blocks SACs and thereby inhibits a variety of physiologic and pathophysiologic processes. Actually, non-diluted Gd decreased the amplitude of the EP in this study. Gd sensitivity has become a simple and widely used method for detecting the involvement of SACs and, conversely, Gd insensitivity has been used to infer that processes are not dependent on SACs. The limitations of this approach are not adequately appreciated; however, Gd remains a useful tool for studying SACs, but appropriate care must be taken in experimental design and interpretation to avoid both false negative and false positive conclusions [6]. Based on reports, SACs may exist in the stria vascularis and its inhibition may decrease the EP's amplitude.

We report here the effects of Gd on the stria vascularis. The concentration used clinically showed no remarkable effect; however, a higher concentration of Gd had adverse effects on the stria vascularis. Visualizing the endolymphatic hydrops provides many advantages clinically, for example, when making a diagnosis and selecting treatments.

Downloaded By: [Kouchi Medical College] At: 01:19 12 February 2008



## Conclusions

(1) The EP showed no change and a decrease 60 min after the injection of 1/8 Gd and non-diluted Gd into the tympanic cavity, respectively. A higher concentration of Gd decreases the EP. (2) Morphologically, the stria vascularis showed no change and an enlargement of the intrastrial space 60 min after the injection of 1/8 Gd and non-diluted Gd into the tympanic cavity, respectively. A higher concentration of Gd affects the marginal cells of the stria vascularis. (3) The concentration of Gd used clinically showed no remarkable effect; however, a higher concentration had adverse effects on the stria vascularis. These results seem to be caused by the blocking effect of Gd on TRPV4 and SACs. However, the concentration of Gd should be borne in mind when injecting Gd into the tympanic cavity.

## References


[1] Nakashima T, Naganawa S, Sugiura M, Teranishi M, Sone M, Hayashi H, et al. Visualization of endolymphatic hydrops in patients with Meniere's disease. Laryngoscope 2007;117: 415–20.

[2] Duan M, Bjelke B, Fridberger A, Counter SA, Klason T, Skjönsberg A, et al. Imaging of the guinea pig cochlea following round window gadolinium application. Neuroreport 2004;15:1927–30.

[3] Andrade YN, Fernandes J, Vázquez E, Fernández-Fernández JM, Arniges M, Sánchez TM, et al. TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. J Cell Biol 2005;168:869–74.

[4] Becker D, Blase C, Bereiter-Hahn J, Jendrach M, Villalón M, Valverde MA. TRPV4 exhibits a functional role in cell-volume regulation. J Cell Sci 2005;118:2435–40.

[5] Takumida M, Kubo N, Ohtani M, Suzuka Y, Anniko M. Transient receptor potential channels in the inner ear: presence of transient receptor potential channel subfamily 1 and 4 in the guinea pig inner ear. Acta Otolaryngol (Stockh) 2005;125:929–34.

[6] Caldwell RA, Clemo HF, Baumgarten CM. Using gadolinium to identify stretch-activated channels: technical considerations. Am J Physiol 1998;275:C619–21.

[7] Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. Nat Cell Biol 2000;2:695–702.

[8] Patapoutian A, Peier AM, Story GM, Viswanath V. ThermoTRP channels and beyond: mechanisms of temperature sensation. Nat Rev Neurosci 2003;4:529–39.

[9] Liedtke W, Choe Y, Martí-Renom MA, Bell AM, Denis CS, Sali A, et al. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. Cell 2000;103:525–35.

[10] Dai C, Steyger PS, Lee H, Caterina MJ, Nuttall AL. Expression of OTRPC4 in the rat inner ear. Abstr Assoc Res Otolaryngol 2002;25:607.

[11] Wangemann P. $K^+$ cycling and the endocochlear potential. Hear Res 2002;165:1–9.

[12] Takeuchi S, Ando M, Kakigi A. Mechanism generating endocochlear potential: role played by intermediate cells in stria vascularis. Biophys J 2000;79:2572–82.

[13] Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, Morishima S. Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). J Physiol 2001;532:3–16.

[14] Konishi T, Hamrick PE, Mori H. Water permeability of the endolymph-perilymph barrier in the guinea pig cochlea. Hear Res 1984;15:51–8.

[15] Sterkers O, Ferrary E, Amiel C. Inter- and intracompartmental osmotic gradients within the rat cochlea. Am J Physiol 1984;247:F602–6.

[16] Sziklai I, Ferrary E, Horner KC, Sterkers O, Amiel C. Time-related alteration of endolymph composition in an experimental model of endolymphatic hydrops. Laryngoscope 1992;102:431–8.

[17] Sawada S, Takeda T, Kitano H, Takeuchi S, Okada T, Ando M, et al. Aquaporin-1 (AQP1) is expressed in the stria vascularis of rat cochlea. Hear Res 2003;181:15–9.

[18] Dulon D, Aran JM, Schacht J. Osmotically induced motility of outer hair cells: implications for Meniere's disease. Arch Otorhinolaryngol 1987;244:104–7.

[19] Lapeyre PN, Cazals Y. Guinea pig vestibular type I hair cells can show reversible shortening. J Vestib Res 1990;91:241–50.

[20] Shen J, Harada N, Kubo N, Liu B, Mizuno A, Suzuki M, et al. Functional expression of transient receptor potential vanilloid 4 in the mouse cochlea. Neuroreport 2006;17: 135–9.

Downloaded By: [Kouchi Medical College] At: 01:12 12 February 2008

*Acta Oto-Laryngologica*
2008, 1–9, iFirst article

informa
healthcare

ORIGINAL ARTICLE

# Expression and immunolocalization of aquaporin-6 (Aqp6) in the rat inner ear

DAIZO TAGUCHI[1], TAIZO TAKEDA[1], AKINOBU KAKIGI[1], TERUHIKO OKADA[2], RIE NISHIOKA[1] & HIROYA KITANO[3]

[1]*Department of Otolaryngology and* [2]*Department of Anatomy and Cell Biology, Kochi Medical School, Kochi and* [3]*Division of Otolaryngology, Head and Neck Surgery, Tottori University Faculty of Medicine, Tottori, Japan*

**Abstract**

*Conclusion.* Since aquaporin-6 (Aqp6) protein was located in the membrane of intracellular vesicles of the stria vascularis, endolymphatic sac, and vestibule, Aqp6 might be involved in some distinct physiological function of acid–base metabolism and water balance in endolymphatic fluid homeostasis. However, its lack of expression on the plasma membrane indicates that Aqp6 does not have a direct role in water flux via the plasma membrane. *Objective.* To evaluate the expression and immunolocalization of Aqp6 in the rat inner ear. *Materials and methods.* Wistar rats were used. Aqp6 mRNA expression in the rat inner ear was investigated in the vestibulum as well as in the cochlea and endolymphatic sac using the reverse transcription-polymerase chain reaction (RT-PCR) method, and detailed immunolocalization of Aqp6 in the rat inner ear was investigated using immunohistochemical methods including immunofluorescence microscopy and immunoelectron microscopy. *Results.* We obtained novel data showing that not just Aqp6 mRNA but also Aqp6 protein is expressed in the cochlea, endolymphatic sac, and vestibule. Immunoelectron microscopic studies revealed that the immunolabelled gold was diffusely seen in the intracellular area of the stria vascularis, endolymphatic sac, and vestibule, but never in the plasma membranes.

**Keywords:** *Endolymph, water transport, polymerase chain reaction, immunohistochemistry, immunoelectron microscopy*

## Introduction

The homeostasis of inner ear fluid is essential for maintaining inner ear function. Since water channels as well as ion channels have been demonstrated to play an important role in the fluid homeostasis of the living body, water channels are expected to exist in the inner ear. Indeed, proteins or mRNAs of many types of aquaporins (Aqps), structural proteins of water channels, are reported to be expressed in the inner ear of experimental animals: Aqp1 [1], Aqp2 [2–8], Aqp3 [4,5], Aqp4 [4,5], Aqp5 [4,7], Aqp6 [9], Aqp7 [4,5], Aqp8 [4,5], and Aqp9 [5]. Most of these Aqps are thought to mediate water homeostasis in the inner ear. In particular, the participation of Aqp2 in water homeostasis in the endolymphatic compartment is apparent from the experimental findings that endolymph volume is mediated by vasopressin and vasopressin type 2 ($V_2$) antagonist [10,11]. However, Aqp6 is unlike other members of the Aqp family in terms of distribution and function. Aqp6 resides exclusively in the membranes of intracellular vesicles of acid-secreting intercalated cells in the renal collecting duct, where it co-localizes with $H^+$-ATPase [12], and is thought to participate in distinct physiological functions in the kidney: glomerular filtration, tubular endocytosis, and acid–base metabolism [13]. Although the systemic distribution of Aqp6 has not been extensively examined, Aqp6 mRNA is reported to be expressed in the rat inner ear [9], and the immunolocalization of Aqp6 protein in the human cochlea and vestible has been reported [14]. However, the immunolocalization of Aqp6 protein in the inner ear of experimental animals has not been reported.

Correspondence: Daizo Taguchi, MD, Department of Otolaryngology, Kochi Medical School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan. Tel: +81 88 880 2393. Fax: +81 88 880 2395. E-mail: taguchident@aol.com

*(Received 28 September 2007; accepted 22 October 2007)*

ISSN 0001-6489 print/ISSN 1651-2551 online © 2008 Taylor & Francis
DOI: 10.1080/00016480701765691

Downloaded By: [Kouchi Medical College] At: 01:12 12 February 2008

In this study, the localization of Aqp6 was investigated in the rat inner ear via reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical methods including immunofluorescence microscopic and immunoelectron microscopic studies.

## Materials and methods

### *Subjects*

Twenty healthy female Wistar rats (200–280 g) were used. The animal protocols of this study were approved by the Animal Care Committee of Kochi Medical School.

### *RT-PCR*

Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneally) and perfused with phosphate-buffered saline (PBS, 10 mM phosphate buffer salts, 137 mM NaCl, 2.7 mM KCl, pH 7.4) from the left ventricle to remove blood. Tissue specimens were then dissected under a

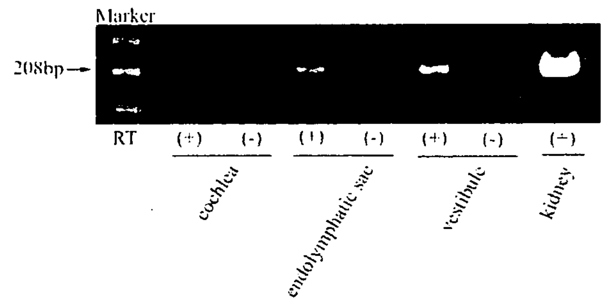


Figure 1. RT-PCR analysis of Aqp6 expression in the rat inner ear. RT-PCR products were electrophoresed on 3% agarose gels. RT (+), templates with reverse transcription; RT (−), templates with no reverse transcription as negative controls. The bands of all templates (cochlea, endolymphatic sac, vestibule, and kidney) with reverse transcription showed the expected size of 208 bp. Total RNA concentrations (ng/μl) of the templates were 16.6 (cochlea), 24.8 (endolymphatic sac), 27.6 (vestibule), and 203 (kidney). The sequence of all RT-PCR products was exactly the same as the known sequence of rat Aqp6 cDNA.

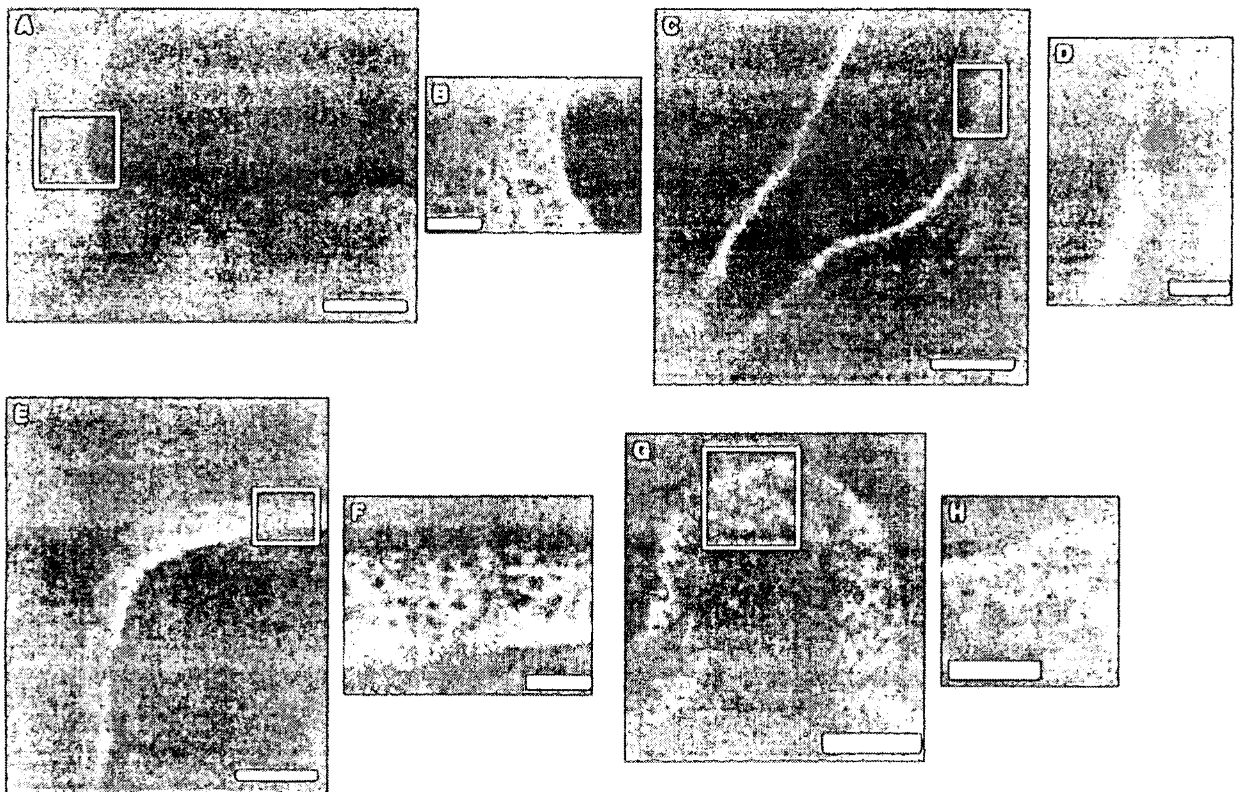

Figure 2. Immunostaining of the rat inner ear for Aqp6 detected with Alexa 546-conjugated secondary antibody (in green). (A) Low-power view of the cochlea. Scale bar, 100 μm. The stria vascularis was stained in the cochlea and the organ of Corti was not so clearly stained. (B) Higher-magnification view of the boxed area in (A). Scale bar, 25 μm. Immunoreactivity was diffusely observed in the cytoplasmic compartment of all three types of cells of the stria vascularis. (C) Low-magnification view of the endolymphatic sac. Scale bar, 100 μm. The epithelial layer of the endolymphatic sac was well stained. (D) Higher-magnification view of the boxed area in (C). Scale bar, 25 μm. (E) Low-magnification view of the saccule. Scale bar, 100 μm. The sensory epithelium of the saccule was stained. (F) Higher-magnification view of the boxed area in (E). Scale bar, 25 μm. Immunoreactivity was diffusely observed in the cytoplasm of sensory epithelium. (G) Low-magnification view of the cupula. Scale bar, 50 μm. The sensory epithelium of the cupula was stained. (H) Higher-magnification view of the boxed area in (G). Scale bar, 25 μm. Immunoreactivity was diffusely observed in the cytoplasm of sensory epithelium.

Downloaded By: [Kouchi Medical College] At: 01:12 12 February 2008



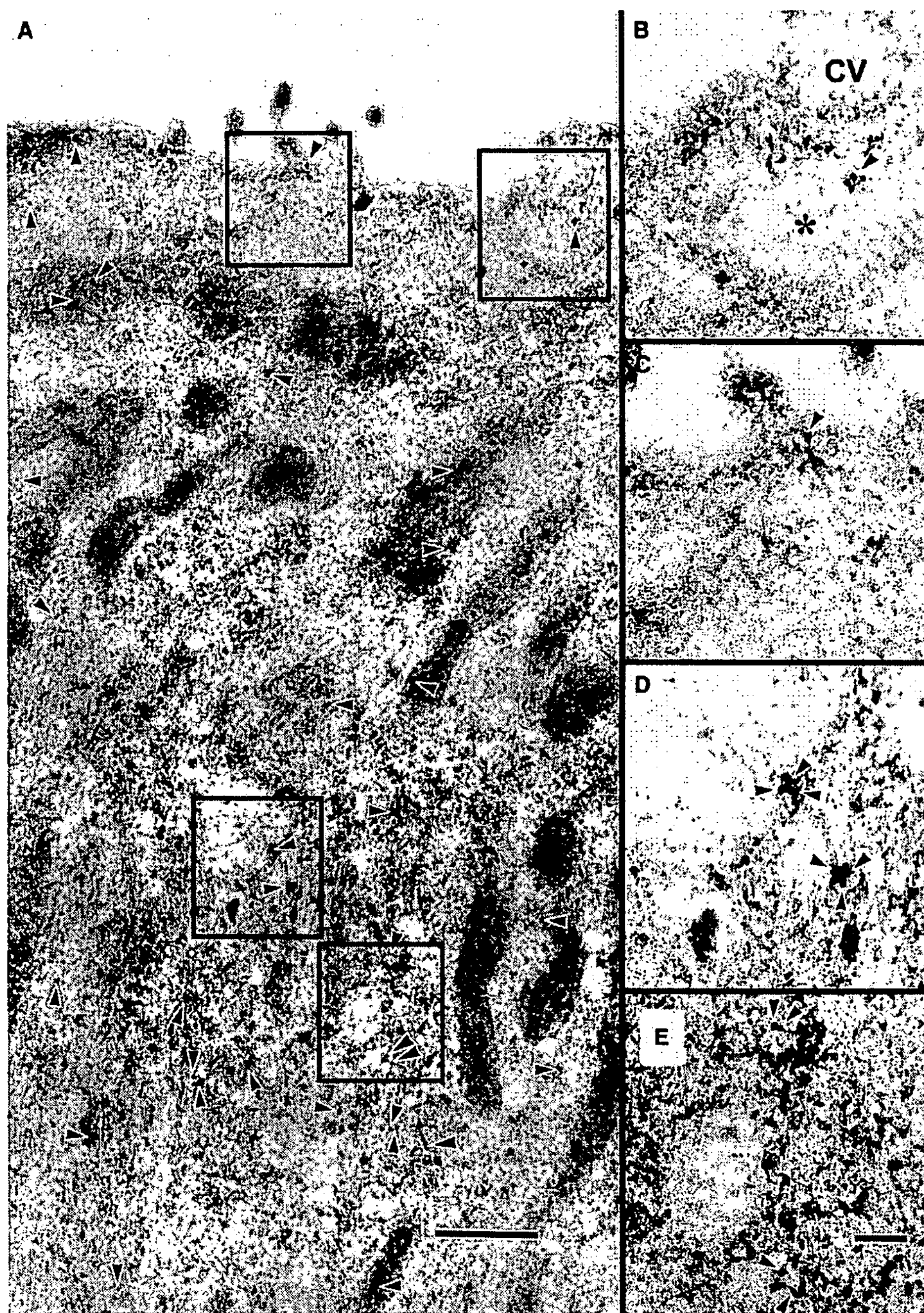


Figure 3. Immunoelectron microscopy of Aqp6 in the apical part of the marginal cells. Immunogold Aqp6 labelings (arrowheads) were observed at the small vesicular or tubular structures (A), as seen in B, C, D, E (higher magnification view of area enclosed by rectangles in A). CV, coated vesicle; asterisk, intracellular vesicle. Scale bar, 0.5 μm in A, 0.1 μm in B, C, D, E.

microscope. mRNA was prepared from three kinds of inner ear tissues (cochlea, endolymphatic sac, and vestibule) and from the kidney, for use as a positive control. Two inner ears were used to obtain mRNA for the cochlea, endolymphatic sac, and vestibule. The endolymphatic sac specimen was carefully separated from the vestibular aqueduct, although it was difficult to completely remove connective tissue from around the endolymphatic sac. Total RNA was extracted from each tissue using an RNeasy Mini Kit (Qiagen, Hilden, Germany). To eliminate residual genomic DNA that might produce a false positive amplification signal, we performed on-column DNase digestion using an RNase-free DNase kit (Qiagen). cDNA was synthesized from total RNA by reverse transcription (SuperScriptII, Invitrogen,

Downloaded By: [Kouchi Medical College] At: 01:12 12 February 2008

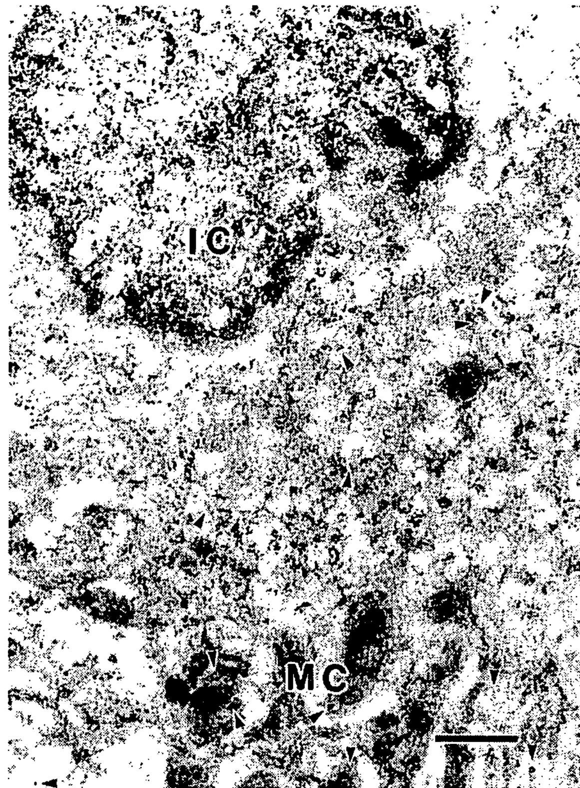


Figure 4. Immunoelectron microscopy of Aqp6 in the intermediate cell. Immunogold Aqp6 labelings of intracellular structures (arrowheads) were few. MC, marginal cell; IC, intermediate cell. Scale bar, 0.5 μm.

USA). PCR was performed using HotStar Taq (Qiagen). The primers used for detection of specific cDNA were 5′-AGCAGACCCGAAGCGACCAG-3′ (sense) and 5′-TGGCCAGGTTGAACGTGATG-3′ (antisense) for rat Aqp6.

PCR was carried out using a thermal cycler (TP3000, Takara, Otsu, Shiga, Japan) with an initial denaturing period of 15 min at 95°C, followed by 35 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and a final extension period of 10 min at 72°C. PCR amplified products (10 μl) were electrophoresed on 3% agarose gels and stained with ethidium bromide. The PCR products were sequenced directly using an ABI PRISM 310 with a BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA).

*Immunohistochemistry*

Animals were anesthetized by the same method described above and perfused from the left ventricle with PBS, followed by a fixative (pH 7.4) containing 4% paraformaldehyde in PBS. Temporal bones were dissected and immersed in the fixative overnight, and then decalcified in 0.12 M ethylenediamine tetraacetic acid in PBS (pH 7.4) for 7 days. They were soaked in 15% sucrose in PBS at 4°C overnight, and then embedded in Tissue Tec OCT Compound (Sakura Finetechnical, Tokyo, Japan). They were cut with a cryostat (HM 505E, Microm) to 7 μm thickness and collected on slides. Sections were washed extensively with PBS containing Triton X-100 for permeabilization. The sections were preincubated for 1 h in a solution containing 10% normal goat serum. The specimens were then exposed overnight at 4°C to rabbit antibody specific for Aqp6 (#AB9024, Chemicon, Temecula, CA, USA), diluted 1:100. The sections were incubated for 6 h at room temperature with Alexa 546 goat anti-rabbit IgG (A-11035, Molecular Probes, Oregon, USA) diluted 1:100. Sections were washed extensively with PBS containing Tween 20 (Sigma-Aldrich, Tokyo, Japan) after each antibody incubation. The sections incubated with Alexa 546 fluorescent antibodies were observed using a confocal laser scanning microscope system (LSM 410, Zeiss, Jena, Germany). Alexa 546 fluorescence was observed using an LSM 410 microscope equipped with a helium-neon laser (543 nm), a dichroic reflector (FT543), and an emission filter (FT570). Control sections were incubated with PBS instead of the primary antibody; the results obtained with these sections were invariably negative.

*Immunoelectron microscopy*

Tissues were dehydrated in a graded series of ethanol and embedded in LR White embedding resin (Electron Microscopy Sciences, Hatfield, PA, USA). Ultra-thin sections were cut with a Reichert ultamicrotome E (Reichert-Jung, Depew, NY, USA), mounted on nickel grids and processed for immunogold cytochemistry. The sections were washed three times in a 10-fold dilution of Block Ace (Dainippon Sumitomo Pharma, Tokyo, Japan) containing 0.1% Tween 20 (Sigma-Aldrich). Nonspecific binding was blocked with a fourfold dilution of Block Ace. Then sections were incubated with a 25-fold dilution of anti-Aqp6 antibody (#AB9024, Chemicon) in a 10-fold dilution of Block Ace containing 0.1% sodium azide for 8 h at room temperature. After incubation with the primary antibody, the sections were washed three times in PBS (0.15 M saline buffered at pH 7.2 with 0.5% BSA and 0.05% Tween 20), and incubated with a 10-fold dilution of protein A labeled with 20 nm colloidal gold and a 10-fold dilution of anti-rabbit IgG labeled with 10 nm colloidal gold (Sigma-Aldrich) in PBS for 6 h at room temperature. Finally, the sections were counterstained with uranyl acetate and lead citrate and examined with a Hitachi

Downloaded By: [Kouchi Medical College] At: 01:12 12 February 2008

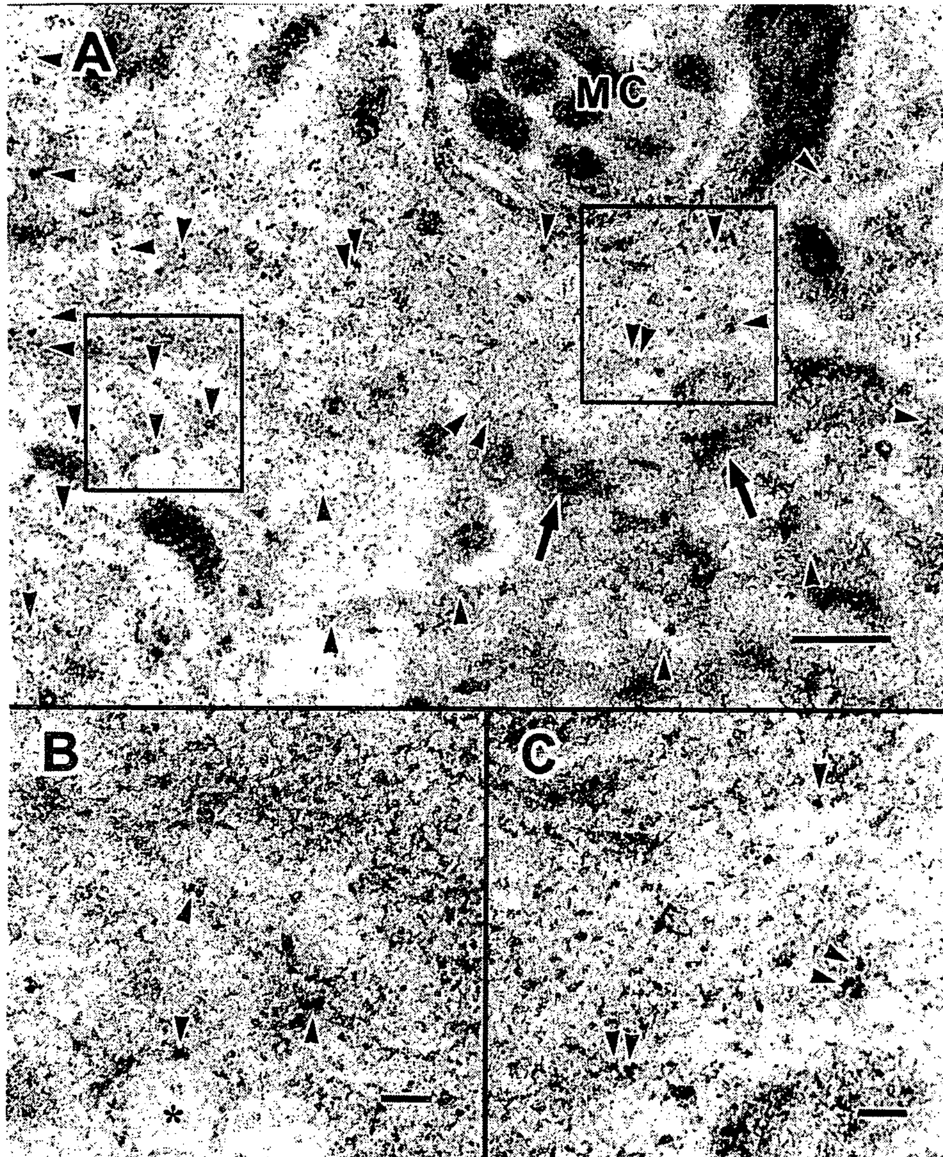


Figure 5. Immunoelectron microscopy of Aqp6 in the basal cells. Many immunogold Aqp6 labelings (arrowheads) were observed at the small vesicular structures (A), as seen in the higher magnification views (B, C) (area enclosed by rectangles in A). MC, marginal cell; arrows, desmosomes; asterisk, intracellular vesicle. Scale bar, 0.5 μm in A, 0.1 μm in B and C.

7100 electron microscope. Control sections were incubated with PBS instead of the primary antibodies; the results obtained with these sections were invariably negative.

## Results

RT-PCR of micro-dissected tissues revealed that Aqp6 mRNA was clearly expressed in the cochlea, endolymphatic sac, and vestibule (Figure 1). The RT-PCR products were the expected size of 208 bp and the nucleotide sequence of the bands agreed exactly with the known sequence of rat Aqp6 (GenBank accession no. AF083879). In immunohistochemical studies, Aqp6 immunoreactivity was observed in the cochlea, endolymphatic sac, and vestibule (Figure 2). In the cochlea, Aqp6 was strongly expressed in all types of cells of the stria vascularis (basal, intermediate, and marginal cells) but not so clearly expressed in the organ of Corti (Figure 2A). Aqp6 was also expressed in the epithelial layer of the endolymphatic sac (Figure 2C). In the vestibule, immunoreactivity was extensively observed in the sensory epithelium of the saccule (Figure 2E), utricle (data not shown), and cupula (Figure 2G). High-magnification micrographs show that immunoreactivity was diffusely observed in the cytoplasmic compartment of these