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The mRNA of claudins is expressed in the endolymphatic sac epithelia

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ABSTRACT

Objective: Claudins are a family of membrane proteins which localize to tight junctions (TJs). Recent studies have shown that claudins can form pores for ions in the TJs and regulate the permeability of epithelial paracellular ion transport. The endolymphatic sac (ES) is a part of the inner ear, absorbing the endolymphatic fluid. ES dysfunction may result in endolymphatic hydrops. In this study, we focused on the paracellular transport and examined claudin mRNA expression in the ES epithelia.
Materials and methods: Total RNA was isolated from whole ES epithelia of rats by laser capture microdissection. RT-PCR was used to evaluate the expression of claudins. The expression of each claudin mRNA in the epithelial cells of rat ES was confirmed by *in situ* hybridization.
Results: RT-PCR indicated the expression of cldn2, cldn4, cldn6, cldn7, cldn9, cldn11, cldn12, and cldn14. The expression of these claudin mRNAs in the epithelial cells of rat ES was confirmed by *in situ* hybridization.
Conclusion: We demonstrated mRNA expression of multiple claudins in the rat ES epithelia. These results in the ES epithelia were consistent with a role of claudins in paracellular ion transport.

1. Introduction

Tight junctions (TJs) are sets of intercellular contacts at the apical end of the lateral cell membrane, and seal the paracellular pathway across epithelia. Tight junctions between endolymphatic sac (ES) epithelial cells have been reported in an electron microscopy study [1]. Freeze-fracture electron microscopy of tight junctions has shown intramembranous networks of strands (TJ strands), mainly composed of claudins [2]. The claudin family constitutes at least 24 members, each claudin expresses as complex patterns in specific organs. TJs basically work as barriers in epithelia, however, recent studies have demonstrated that some claudins in TJs can form pores for ions with size- and/or charge-selectivity. In the kidney, a representative absorptive organ, each nephron segment expresses multiple claudins, which can be consistent with different functions on the resorption system of the individual segment [3]. The characteristic claudin distribution in the kidney indicates that claudins in renal tubules can form cation or anion pores and contribute to paracellular transport [4]. Several reports have also shown the expression and distribution of claudins in the cochlea and vestibule [5]. However, the presence of claudins in the ES epithelia has not been investigated.

The ES is a part of the inner ear and consists of the endolymph-filled membranous labyrinth that contains the cochlea, vestibular organs, and semicircular canals. The ES epithelia is believed to absorb the endolymphatic fluid and its dysfunction may induce excess of the endolymphatic fluid; endolymphatic hydrops [6]. In the endolymphatic fluid in the ES, Na^+ and Cl^- are dominant ions different from in the cochlea, and both ions are thought to be important for volume regulation in the ES [7–9]. Several transporters and channels have been identified in the ES epithelia. Na^+,K^+-ATPase in the ES epithelia has the sufficient capability to maintain volume of endolymph from the viewpoint of Na^+ and water reabsorption [9]. On the other hand, whole-cell patch clamp studies have shown no evidence of Cl^- conductance in ES epithelial cells [10]. The study using ionic lanthanum as a tracer has shown the morphological evidence of both intercellular and paracellular pathway in the epithelia of the ES and endolymphatic duct [11]. Takumida et al. has also indicated a hypothetical mechanism of water and ion transport in the ES epithelium based on the ultrastructual observation of widened lateral intercellular spaces, suggesting the paracellular ion transport pathway [12]. However, there are no reports of the paracellular transport in the ES by molecular biological or physiological approaches. In the present study, we focused on the mRNA expressions of the claudins which may participate in the resorption system through paracellular pathway in the ES epithelia.

2. Materials and methods

2.1. Animals and tissue preparation

Four-week-old female Sprague–Dawley rats were purchased from Charles River JAPAN (Yokohama, Japan). This research was

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approved by the Animal Care and Use Committees of Kagawa University.

For *in situ* hybridization, 20 rats were deeply anesthetized by diethyl ether and then perfused via the left ventricle with a fixative solution (4% paraformaldehyde in phosphate buffered saline: PBS, treated with RNase-free water) for approximately 5 min, and then decapitated. The temporal bones were removed and the ES on bilateral sides, including the surrounding bone tissues, was dissected carefully under stereomicroscopy. Kidneys were also dissected. The ES and kidneys were fixed in 4% paraformaldehyde in PBS for 6 h at 4 °C and the ES was decalcified in 0.12 M EDTA (pH 6.5) at 4 °C for about 14 days. Decalcified ES and kidneys were embedded in OCT tissue compound (Sakura Fintechnical, Tokyo, Japan). Sections (7–10 μm thick) were cut on a cryostat at −20 °C and mounted on MAS-coated glass slides (MATSUNAMI GLASS, Osaka, Japan).

For laser capture microdissection (LCM), 20 anesthetized rats were exsanguinated via the left ventricle with 70% ethanol/RNase-free water and decapitated. ES on bilateral sides in temporal bone were separated and fixed in 70% ethanol/RNase-free water for 6 h at 4 °C, and decalcified in 0.12 M EDTA (pH 6.5) including RNAlater (Ambion Inc., TX, USA) for about 10 days at 4 °C. The samples were embedded in OCT tissue compound, frozen in liquid nitrogen, and stored at −80 °C.

2.2. Laser capture microdissection

LCM was performed as previously described with minor modifications [13]. Briefly, the entire ES was cut into slices (10–15 μm thick) using a cryostat at −20 °C, re-fixed and stepwise dehydrated for 1 min each in 70%, 90%, and 100% ethanol/RNase-free water, followed by 5 min incubations in xylene before being air-dried. LCM was performed using Arcturus Pixcell II (MDS Analytical Technologies, CA, USA) and whole ES epithelia were collected. RNA was isolated from LCM samples with the PicoPure RNA isolation kit (Ambion Inc.) in accordance with the manufacturer's protocol. Bilateral dissected ES were combined to obtain one RNA sample (LCM-ES).

2.3. RT-PCR and sequencing

RT-PCR from LCM-ES was performed as previously reported [13]. Briefly, RNA isolated from LCM-ES was reverse-transcribed into cDNA by incubation with a random primer and MMLV (Molony murine leukemia virus) reverse transcriptase (TAKARA BIO, Otsu, Japan). cDNA fragments were amplified by 30 cycles of PCR (94 °C for 60 s, 60 °C for 60 s, and 72 °C for 1 min) using a TaKaRa PCR thermal cycler MP (TAKARA BIO) with Ex Taq DNA polymerase, dNTPs (TAKARA BIO), and specific primer pairs as shown in Table 1 [14]. GenBank ACCESSION No. of each claudin is as follows, cld1: NM_031699, cld2: NM_001106846, cld3:NM_031700, cld4: NM_001012022, cld6: NM_001102364, cld7: NM_031702, cld8: NM_001037774, cld9: NM_001011889, cld10: NM_001106058, cld11: NM_053457, cld12: NM_001100813, cld14: NM_001013429, cld16: NM_131905, cld18: NM_001014096, cld19: NM_001008514, GAPDH: NM_017008. PCR products were separated by electrophoresis on 1.0% agarose gels and visualized by ethidium bromide staining. The specificity of the primers was confirmed using positive controls and a negative control. As positive controls, cDNA obtained from the kidney was used for claudin-1, -2, -3, -4, -6, -7, -8, -9,-10, -11, -14, -16, -19 and cDNA obtained from cochlea for claudin-12, and from the lung was used for claudin-18. cDNA obtained from the blood was used as the negative control.

2.4. Hybridization probes

Total RNA was isolated by RNeasy Mini kit (QIAGEN, Tokyo, Japan) from the ES and the kidney according to the manufacturer's protocol and was reverse-transcribed into cDNA as described above. cDNA was amplified with same specific primers as used for cDNA derived from LCM-ES and ligated into the pCR II-TOPO-vector (Invitrogen, Tokyo, Japan) using Ligation-high (TOYOBO, Osaka, Japan). Plasmid DNAs with inserts were purified and the nucleotide sequences were determined using a ABI PRISM 377 DNA Sequencer (Applied Biosystem, Osaka, Japan) using Big Dye terminator ver 3.1 (Applied Biosystem).

2.5. In situ hybridization

Dried cryosections of the intermediate portion of the ES and kidney were washed with PBS and 1.5% Triton-X in PBS for 15 min. The slides were incubated with 1 μg/ml proteinase K (Merck, Tokyo, Japan) at 37 °C for 10 min, postfixed in 4% paraformaldehyde, and acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride. After prehybridization for 1 h at 62 °C, sections were hybridized to sense or antisense riboprobes for 16 h at 62 °C and washed. The slides were incubated with 10 μg/ml RNaseA (MoBiTec, Gottingen, Germany) for 30 min at 37 °C. After quenching of nonspecific antibody binding sites with blocking medium, alkaline- phosphatase-conjugated anti-digoxigenin antibody (Roche diagnostics Japan) was applied to each slide for 1 h at 37 °C. The color was developed using NBT/BCIP overnight at room

Table 1
Oligonucleotide sequences used in the RT-PCR experiments.

Gene	Primer (forward)	Primer (reverse)	Product length (bp)
Rattus norvegicus			
Cldn1	5′-AGGTCTGGCGACATTAGTGG-3′	5′-TGGTGTTGGGTAAGAGGTTG-3′	202
Cldn2	5′-TCTGGATGGAGTGTGCGAC-3′	5′-AGTGGCAAGAGGCTGGGC-3′	467
Cldn3	5′-GCACCCACCAAGATCCTCTA-3′	5′-AGGCTGTCTGTCCTCTTCCA-3′	246
Cldn4	5′-TGGATGAACTGCGTGGTGC-3′	5′-CCCTACGACTGAGAGAAGC-3′	153
Cldn6	5′-GGAGGGGCTATGGATGTC-3′	5′-GCAGATGGGAATGAGGGT-3′	271
Cldn7	5′-ACTACTGGGCTTTTCAATGGC-3′	5′-CACCGAGTCGTACATTTTGC-3′	190
Cldn8	5′-GCTGGAATCATCTTCTTCAT-3′	5′-CATCCACCAGCGGGTTGTAG-3′	100
Cldn9	5′-CTTCATTGGCAACAGCATCG-3′	5′-CCTTGGCACCTTCGTCCTC-3′	242
Cldn10	5′-CATATTGTCAGGTCTGTGTTC-3′	5′-TGGGTGTTTTGTTGTTGTC-3′	200
Cldn11	5′-ATTGGCATCATCGTCACAAC-3′	5′-ATGTCCACCAGGGGCTTG-3′	158
Cldn12	5′-CCTTCAAGTCTTCGGTGCC-3′	5′-CAGGAGGATGGGAGTACAG-3′	312
Cldn14	5′-CTGTACCTGGGCTTCATC-3′	5′-CACACATAGTCATTCAACCTG-3′	230
Cldn16	5′-ATCTTCTTCAGTACGCTGCC-3′	5′-CGATGAGTAATACGGTCCC-3′	372
Cldn18	5′-CCGTTCAGACCAGGTACAC-3′	5′-CTCCAGGCTTATAGGCAAC-3′	180
Cldn19	5′-TGCTGAAGGACCCATCTG-3′	5′-TGTGCTTGCTGTGAGAACTG-3′	129
GAPDH	5′-GGTGATGCTGGTGCTGAGT-3′	5′-CAGTCTTCTGAGTGGCATTG-3′	301

Cldn, claudin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.