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Cystic fibrosis transmembrane conductance regulator in the endolymphatic sac of the rat

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ABSTRACT

Objective: Na^+ and Cl^- are dominant ions in the endolymphatic fluid in the endolymphatic sac and are important for volume regulation in the endolymphatic sac. An epithelial sodium channel (ENaC) and other Na^+ transporters have been identified in the endolymphatic sac epithelia, and they are involved in the regulation of endolymph. Although the presence of Cl^- channels in the endolymphatic sac epithelia has been speculated, no Cl^- channels have been identified. In this study, we confirmed the expression of cystic fibrosis transmembrane conductance regulator (CFTR) in the endolymphatic sac by reverse transcriptase polymerase chain reaction (RT-PCR) and by immunohistochemical staining.
Methods: Pure mRNA from endolymphatic sac epithelia was prepared using laser capture microdissection (LCM) and examined using RT-PCR. Localization of CFTR and ENaC in the endolymphatic sac was examined using immunohistochemistry.
Results: mRNA of the CFTR was expressed in the endolymphatic sac. Immunohistochemical analysis showed the expression of the CFTR on apical side of the endolymphatic sac epithelia and co-localization with the ENaC.
Conclusion: RT-PCR and immunohistochemistry were used to identify the expression of CFTR in the endolymphatic sac epithelia, which gives us a clue for understanding Cl^- transport in the endolymphatic sac. These results suggest a pathway for Cl^-, possibly through interaction with the ENaC, which may regulate the endolymph in the endolymphatic sac.

1. Introduction

The endolymphatic sac (ES) is a part of the inner ear. It consists of the endolymph-filled membranous labyrinth, which contains the cochlea, vestibular organs, and semicircular canals. The ES epithelium is believed to absorb endolymphatic fluid, and dysfunction of ES may result in excess accumulation of endolymphatic fluid (i.e., endolymphatic hydrops) [1]. In the endolymphatic fluid in the ES, Na^+ and Cl^- are dominant ions, and both are thought to be important for volume regulation in the ES [2]. Several Na^+ transporters are identified in the ES. Na^+, K^+-ATPase at the basolateral membrane of ES epithelial cells provides the driving force for Na^+ absorption [3]. The Na^+ flow is generally coupled with Cl^- flow to neutralize the charge movement, thereby guaranteeing ionic neutrality [4]. However, no chloride channels have been identified.

Cystic fibrosis transmembrane conductance regulator (CFTR) is a plasma membrane cAMP-regulated Cl^- channel that is responsible for transepithelial salt and fluid transport [5,6]. The CFTR also acts as a regulator by exerting modulatory influences over a variety of other ion channels and transporters such as the epithelial sodium channel (ENaC) [7–10]. ENaC was identified in the ES of human and guinea pigs [11,12]. This ENaC is localized at the apical membrane and constitutes the main pathway for Na^+ absorption in various tissues, including the ES, kidneys, and the airways [12–14].

The aim of this study was to examine the expression of CFTR in ES epithelia, which may play roles in the regulation of endolymph in the ES.

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 study was

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

For immunohistochemical analysis, the rats were deeply anesthetized and perfused via the left ventricle with a fixative solution (4% paraformaldehyde in phosphate-buffered saline [PBS]) for approximately 5 min. Then, the rats were decapitated. Their temporal bones were removed, and the ES on both sides, including the surrounding bone tissues, was dissected carefully under a stereomicroscope. The colon and kidney were also collected. The samples were fixed in 4% paraformaldehyde in PBS for 6 h at 4 °C. The temporal bones that contained the ES were then decalcified in 0.12 M ethylenediaminetetraacetic acid (EDTA; pH 6.5) at 4 °C for approximately 14 days. The decalcified temporal bones and other organs were embedded in an optimal cutting temperature (OCT) tissue compound (Sakura Fintechnical, Tokyo, Japan). Sections (7–10 μm thick) were cut on a cryostat at −20 °C and mounted on Matsunami Adhesive Slide (MAS)-coated glass slides (Matsunami Glass, Osaka, Japan).

For laser capture microdissection (LCM), anesthetized rats were exsanguinated via the left ventricle with 70% ethanol/RNase-free water and decapitated. The ES within the temporal bones was collected from both sides. The pancreas was also collected and immediately immersed into liquid nitrogen for use in RT-PCR. The temporal bones that contained the ES were 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 (Life Technologies, CA, USA) for approximately 7 days at 4 °C. The samples were embedded in OCT tissue compound, frozen in liquid nitrogen, and stored at −80 °C.

2.2. LCM

LCM was performed as previously described with minor modifications [15]. Briefly, the entire ES in the temporal bone was cut into slices (10–12-μm thick) using a cryostat at −20 °C and mounted on MAS-coated glass slides. The sections were re-fixed and then dehydrated in a stepwise manner for 1 min each in 70%, 90%, and 100% ethanol/RNase-free water. This was followed by 5-min incubation in xylene before the sections were air-dried. LCM was performed using Applied Biosystems Arcturus Capsure HS LCM caps (Life Technologies, CA, USA), and ES epithelia (from the distal portion to the proximal portion) were selectively collected from the sections. RNA was isolated from the LCM samples using an Applied Biosystems Arcturus PicoPure RNA isolation kit (Life Technologies, CA, USA) in accordance with the manufacturer's protocol. Dissected ES epithelia from both sides were combined to obtain 1 RNA sample (LCM-ES).

2.3. RT-PCR and sequencing

RT-PCR for LCM-ES was performed as previously reported elsewhere [16]. Briefly, RNA that was isolated from the LCM samples was reverse-transcribed into cDNA by incubation with a random primer and Moloney murine leukemia virus reverse transcriptase (Takara Bio, Otsu, Japan). The cDNA fragments were amplified by 30 cycles of PCR (94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min) with Ex-Taq DNA polymerase, dNTPs (Takara Bio, Otsu, Japan), and specific primer pairs for the CFTR: sense, 5′-GACTACATGGAACACATACCTTCG-3′; and antisense, 5′-ATAGCAAGCAAAGTGTCGGCTACTC-3′ [17]. The predicted size of the PCR product was 258 bp. The PCR products were separated by electrophoresis on 1.0% agarose gels and visualized by ethidium bromide staining. The housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a control with the following primers: sense, 5′-GGTGATGCTGGTGCTGAGT-3′; and antisense, 5′-CAGTCTTCTGAGTGGCATTG-3′. The predicted size of the PCR product was 301 bp. cDNA from the pancreas was used as a positive control. RT-PCR without reverse transcriptase was performed as a negative control. PCR template that had been replaced with pure water was used for the negative control.

2.4. Immunohistochemical analysis

Immunostaining was performed as described in our previous study with minor modifications [18]. The sections were washed with distilled water and PBS that contained 0.2% Tween 20, and were covered with a blocking reagent (Dako Japan, Tokyo, Japan) for 30 min. A specific primary antibody against CFTR, goat anti-CFTR (N-20) polyclonal antibody (diluted at 1:50 in PBS; Santa Cruz Biotechnology, Inc, CA, USA), and that against ENaC, rabbit anti-ENaC gamma polyclonal antibody (1:50; Abcam Japan, Tokyo, Japan), were applied to the sections, and the sections were then incubated overnight at 4 °C. After repeated washes with PBS that contained 0.2% Tween 20, the sections were incubated with the secondary antibody Alexa Fluor-546 donkey anti-goat IgG or Alexa Fluor-488 donkey anti-rabbit IgG (diluted at 1:200 in PBS; respectively; Life Technologies, CA, USA) for 2 h at room temperature. After repeated washes with PBS that contained 0.2% Tween 20, 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain (diluted at 1:5000 in distilled water; Life Technologies, CA, USA) was applied for 2 min. The sections were then rinsed several times in distilled water, mounted with 50% glycerol on a slide, and then observed under an Olympus BX51 light microscope (Olympus, Tokyo, Japan). The intermediate portion of the ES was used for the analysis. The positive control experiments were performed using the colon for the CFTR and the kidney for ENaC antibodies. Negative controls were treated similarly but by omitting the primary antibodies. To confirm the accuracy and reproducibility of this procedure, 15 rats (30 ESs) were used.

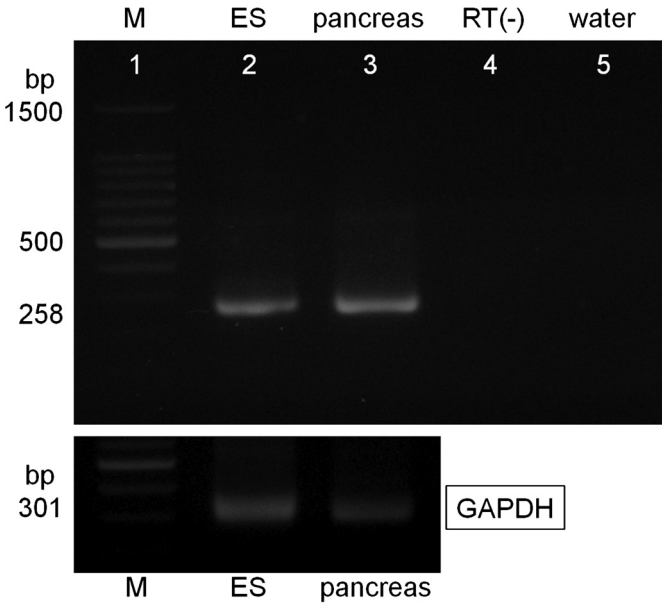

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR)-amplified products. The band for cystic fibrosis transmembrane conductance regulator (CFTR) from the endolymphatic sac (ES) was detected at the expected size (258 bp; Lane 2). The band for CFTR from the pancreas is shown as a positive control (Lane 3). Reverse transcriptase (RT)-PCR without reverse transcriptase (Lane 4) and PCR template replaced with pure water (Lane 5) were used for negative controls. Bands for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected at the expected sizes (301 bp) for mRNA isolated from the ES and pancreas (lower picture). M: size marker.

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