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Claudin-1 Leads to Strong Formation of Tight Junction in Cultured Mouse Lung Microvascular Endothelial Cells

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Abstract

We aimed to examine paracellular barrier function in cultured mouse lung microvascular endothelial cells (LMECs). The transcellular resistance of LMEC monolayers yielded an electrical resistance of approximately 19 Ω × cm^2 at days 6 - 7 in culture when the cells reached confluence, and paracellular permeable clearance of sodium fluorescein was the lowest on day 6 in culture, suggesting the formation of tight junctions (TJs) in cultured LMECs. Moreover, the expression of TJ-associated proteins, occludin, claudin-1 and -4 and zonula occludents 1 (ZO-1) was detected in LMECs at day 6 in culture. However, mRNAs of occludin, claudin-1 and -4 and ZO-1 were already expressed on day 1 after culture, and large variations were absent in the mRNA levels of occludin, claudin-4 and ZO-1 between days 1 and 7 in culture, when the level of each mRNA on day 1 in culture was used as a basal level. However, the claudin-1 mRNA level gradually increased up to approximately 7-fold on day 7 in culture over the basal level. These results indicate that the drastic increase in the mRNA expression level of claudin-1 leads to the strong formation of TJs.

Keywords

Mouse Lung Microvascular Endothelial Cells, Paracellular Permeability, Tight Junction, Occludin, Claudins, Zonula Occludents

1. Introduction

The lung is a heavily vascularized organ and the gas-blood barrier in the lung consists of single layers of

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epithelial and endothelial cells [1]. Endothelial cells also comprise nearly 50% of the total cellular population of the lungs [2], and the microvascular endothelial cells have unique and efficient protective systems controlling the passage of materials. The transport of macromolecules across the endothelium may be dependent on the paracellular and transcellular routes [3] [4]. A regulated paracellular barrier to the movement of water, solutes and immune cells between both epithelial and endothelial cells is created by tight junctions (TJs) [5]. In these cells, TJs are directly involved in barrier and fence functions by sealing them to generate the primary barrier against the diffusion of solutes via the paracellular pathway and by acting as a boundary between the apical and basolateral plasma membrane domains to create and maintain cell polarity [5] [6]. The molecular architecture of TJ exhibits a complex arrangement of interacting cytoplasmic adaptor proteins (e.g. zonula occludents [ZO-1, ZO-2 and ZO-3] as well as 7H6, AF6, vinculin and cingulin), which mediate the cytoskeletal tethering and cell-cell partnering of the transmembrane linker protein (e.g. occludin, claudins and junctional adhesion molecules 1, 2 and 3) [7]. TJ may be closely associated with the actin cytoskeleton in terms of both structure and function [8] [9].

In the present study, we examined whether cultured mouse LMECs form TJs. In addition, the expression of occludin, claudins and ZO-1 proteins and genes was observed in cultured LMECs in relation to paracellular barrier function.

2. Experimental

2.1. Materials

Gentamicin sulphate, amphotericin B and sodium fluorescein (Na-FS) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium: Nutrient mixture F-12 (D-MEM/F-12), heparin, piperazine-N'-(2-ethane-sulfonic acid) (HEPES), Hank's balanced salt solution (HBSS), dispase, epidermal growth factor (EGF), foetal bovine serum (FBS) and donor horse serum (HS) were obtained from Gibco BRL, Life Technologies (Rockville, MD). Percoll was purchased from Pharmacia (Uppsala, Sweden). Collagenase P was obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of reagent grade and were obtained commercially.

2.2. Isolation and Culture of Mouse LMECs

Male C57 BL/6 mice (3 weeks old) purchased from Japan SLC (Hamamatsu, Japan) were housed at a constant temperature (23˚C ± 1˚C) and humidity (55% ± 5%) with automatically controlled lighting (07:00-19:00). Mouse LMECs were isolated as described previously (Magee *et al*. 1994; Sakurai *et al*. 2002). In total, 20 mice were euthanized by decapitation. The lungs were removed and placed in a beaker containing M199 solution with 0.005% antibiotic solution. The visceral pleura were first stripped from each lobe, and the outer 3 - 5 mm of the peripheral lung tissue were dissected free of the remaining tissue. The pooled pieces of lung periphery were finely minced and washed with M199 and the fragments collected on a 40-µm nylon mesh. With constant gentle mechanical agitation, the tissue was digested with 0.6% collagenase in M199 at 37˚C for 20 min and then incubated with 2.1% dispase in M199 at 37˚C for 30 min. The suspension was mixed in M199 with 5% FBS. After centrifugation at 600 ×*g* for 10 min, the resulting tissue pellet was resuspended in M199 and filtered through a 100-µm mesh. The microvessels were collected by centrifugation at 600 ×*g* for 10 min and resuspended in M199. The suspension was layered on a Percoll gradient formed by the centrifugation of 50% Percoll at 26,000 ×*g* at 4˚C for 60 min and then centrifuged at 600 ×*g* for 10 min. After Percoll gradient centrifugation, three layers were observed. The endothelial cells aggregates formed a band around the middle third of the gradient, and the entire middle layer was collected from gradients. The cells were resuspended in M199 and collected by centrifugation at 600 ×*g* for 10 min. The cell suspensions were seeded onto collagen-coated 225 cm^2 tissue culture flasks (Iwaki Glass, Funabashi, Japan). The cells were allowed to attach and grow to monolayers at 37˚C in a humidified atmosphere of 5% CO_2/95% air. The culture medium (D-MEM/F-12 containing 14 mM sodium bicarbonate, 20 ng/mL of EGF, 50 µg/mL of gentamycin-amphotericin B solution, 10 U/ mL of heparin, 5% FBS and 5% HS) was changed every 3 days. Subculture was performed when the cells reached confluence after approximately 6 - 8 days. Cells were trypsinized at a ratio of 1:3 after reaching confluence using 0.025% trypsin in HBSS containing 0.02% EDTA. The primary cultured LMECs were stored at −80˚C in a culture medium con-

taining 20% horse serum and 10% DMSO until use.

2.3. Transendothelial Electrical Resistance and Paracellular Permeability Measurements

LMECs were grown on Transwells (polycarbonate membrane; pore size, 12 μm; diameter, 12 mm; surface area, 1 cm^2; Costar, Cambridge, MA, USA) in 12-well cluster dishes (Costar). Prior to seeding, the polycarbonate filter was coated with rat tail collagen under UV light and then with human fibronectin. Primary cultured LMECs were seeded at 2.0×10^5 cells/cm^2 on Transwells and cultured at 37˚C under 95% air and 5% CO_2. The time course of transendothelial electrical resistance (TEER) and cell monolayer integrity changes was examined.

TEER, which reflects the flux of mainly sodium ions through cell layers in culture conditions, was measured using a Millicell ESR metre (Millipore Corp., Bedford, MA, USA). TEER of coated but cell-free filters was subtracted from the measured TEER values of the models shown as $\Omega \times cm^2$.

Na-FS was used as a hydrophilic marker for cell monolayer integrity. Cell culture inserts were transferred to 12-well plates containing 1.5 mL incubation buffer (136 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10 mM NaH_2PO_4, 25 mM glucose and 10 mM Hepes, pH 7.4) in the basolateral compartments. In the apical compartments, the culture medium was replaced by 0.5 mL incubation buffer containing 250 ng/mL Na-FS. At the conclusion of the incubation times (15, 30, 45 and 60 min), 100-μL aliquots were sampled from the apical and basolateral compartments, respectively, and replaced with the same volume of incubation buffer. The concentrations of Na-FS in the apical and basolateral compartments were determined using a fluorescence multiwell plate reader (excitation: 485 nm, emission: 535 nm). Flux across a cell-free insert was also measured. The permeability of the monolayer was expressed as a clearance (μL/min/cm^2), calculated as the apical to basolateral flux of the marker molecule divided by the concentration of the marker molecule in the apical compartment.

2.4. Western Blotting

Secondary cultured LMECs cultured for 6 days were washed with ice-cold phosphate buffered saline [PBS(−)]. LMECs were then harvested and the pellet was lysed in RIPA buffer (Nacalai tesque, Kyoto, Japan). The lysates were incubated for 15 min on ice prior to sonication and then centrifuged at 10,000 ×*g* for 20 min at 4˚C. Protein content was determined by BCA protein assay (Thermo Scientific, Rockford, USA). Equivalent protein aliquots (5 μg) were separated by 10% SDS-PAGE and transferred electrophoretically to an Immobilon-P transfer membrane (Millipore, Billerica, MA, USA). The membrane was blocked with Blocking One (Nacalai tesque, Kyoto, Japan) and then incubated with primary antibodies against occludin (Rabbit polyclonal IgG at a dilution of 1:1000, Abcam, Cambridge, United Kingdom), claudin-1 and claudin-2 (Rabbit polyclonal IgG at dilution of 1:1000, Abcam, Cambridge, UK) and ZO-1 (Rabbit polyclonal IgG at a dilution of 1:1500, Abcam, Cambridge, United Kingdom). The primary antibodies used in this experiment have specificity in human, rat and mouse, respectively. The blots were incubated with HRP-conjugated anti-rabbit antibody (GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England) and detected with ECL Plus Western blotting detection reagents (GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England).

2.5. Real-Time RT-PCR

The total RNA was isolated using the Real Time ready Cell Lysis Kit (Roche Applied Science, Rotkreuz, Switzerland). Reverse transcription (RT) was performed with Transcriptor Universal cDNA Master and PCR was performed with the FastStart Universal Probe Master Rox on a Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Primers for occludin, claudin-1, -4 and ZO-1 were designed with the online Universal ProbeLibrary System. The mRNA levels were calculated by normalizing to the housekeeping gene beta actin using the ΔCt method.

2.6. Statistics

The data are expressed as mean ± SEM for n experiments. A comparison among the groups was conducted using analysis of variance (ANOVA) and Dunnett's *post hoc* multiple comparison test. The differences were considered statistically significant at $p < 0.05$ (two-tailed).

3. Results

3.1. Tightness of Cultured Mouse LMEC Monolayer

The isolated cells reached confluence after 6 - 8 days in culture. The level of TEER in LMECs monolayer on day 6 in culture increased up to 19.0 ± 2.9 Ω × cm^2 (**Figure 1(a)**). The paracellular permeability of the LMEC monolayer measured by the water soluble small marker Na-FS was inversely the lowest on day 6 in culture (**Figure 1(b)**).

3.2. Expression of Occludin, Claudin and ZO-1 Proteins in Mouse LMECs

The occludin, claudin-1, -4 and ZO-1 bands detected on day 6 in culture were 59, 30, 22 and 139 kDa, respectively, in mouse LMECs (**Figure 2**).

3.3. mRNA Expression of Occludin, Claudin and ZO-1 Genes

The temporal mRNA expression of occludin, claudin and ZO-1 genes in mouse LMECs is shown (**Figure 3**).

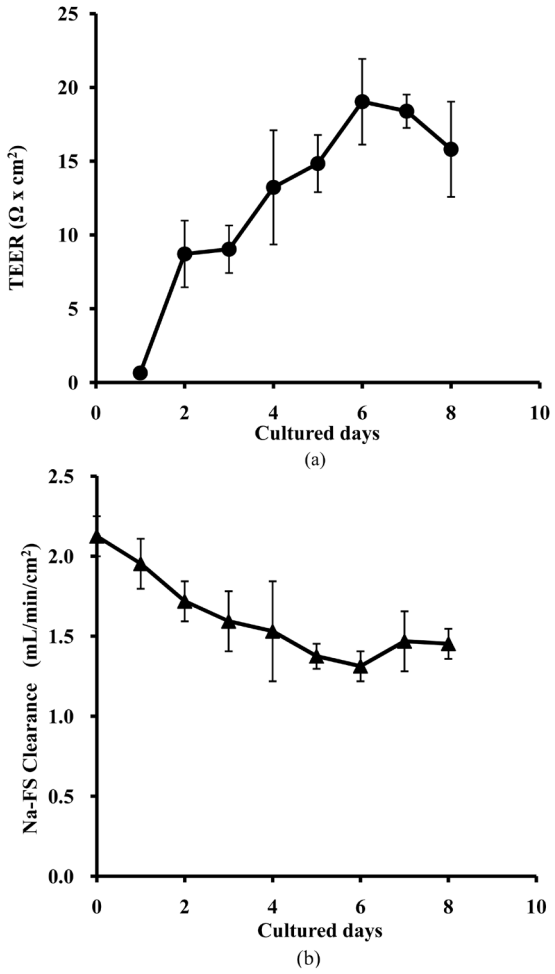

Figure 1. (a) The induction of transendothelial electrical resistance (TEER) in mouse LMECs. Values of TEER are expressed as the mean ± SE of five experiments. (b) Transendothelial permeability (Na-FS clearance) changes for paracellular permeability marker Na-FS in mouse LMECs monolayer. Each value is expressed as the mean ± SE of five experiments.

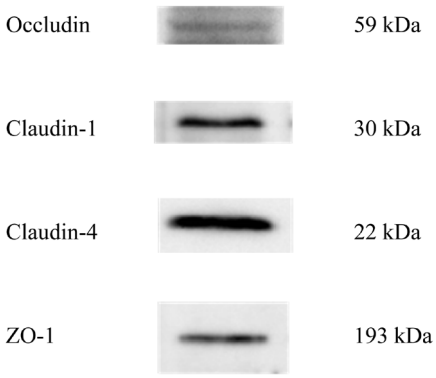

Figure 2. Detection of TJ-associated proteins by western blotting on day 6 in culture in mouse LMECs.

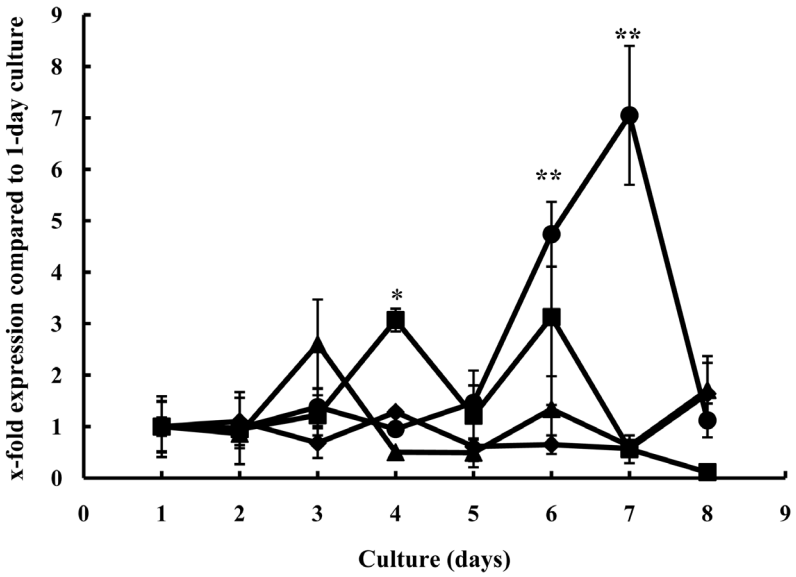

Figure 3. Quantification of occludin (▲), claudin-1 (●) and -4 (■) and ZO-1 (♦) mRNAs by real-time PCR in cultured mouse LMECs. The level of each mRNA on day 1 in culture was used as a basal level to compare relative levels at the other culture times. $^{*}p < 0.05$ and $^{**}p < 0.01$ for comparison with each basal level. Each value is expressed as the mean ± SE of five experiments.

The mRNA of occludin, claudin-1 and -4 and ZO-1 were already expressed on day 1 after culture, and the large variation was absent in the mRNA levels of occludin, claudin-4 and ZO-1 between days 1 and 7 of culture, when the level of each mRNA on day 1 of culture was used as a basal level. However, the claudin-1 mRNA levels gradually increased up to approximately 7-fold over the basal level on day 7 in culture.

4. Discussion

We studied paracellular barrier functions in cultured mouse LMECs. TEER in mouse LMECs monolayer on day 6 after inoculation increased up to approximately 19.0 ± 2.9 Ω × cm^2. This value was similar to that of cultured wild-type rat lung endothelial cells [10], but was approximately 20% lower than that of cultured rat brain endothelial cells in the confluent condition [11] [12]. On the other hand, the paracellular permeability of mouse LMEC monolayers by the water soluble small marker fluorescein is the lowest on day 6 after inoculation. Most evidence indicates that the endothelial barrier function in the various organs is regulated in large part by the state of intracellular junctions between endothelial cells [13], suggesting that the cultured mouse microvascular endothelial cells in the lung also create the barrier.

This barrier function consists of TJs and adherens junctions. Adherens junctions consist of integral membrane proteins: E-cadherin, α-catenin, p120-catenin and α-catenin [14]. TJs consist of occludin, the claudin family of proteins, junctional adhesion molecules and ZO-1, -2 and -3 proteins [15]. Occludin is a transmembrane molecule localized at TJs [16] [17]. Initial studies of the epithelial system demonstrated that this molecule is important in the regulation of not only paracellular permeability but also the fence function of TJs [18]. Occludin is abundant in endothelial cells, such as those of brain capillaries, and is important for the functions of TJs in these cells [19]. On the other hand, the claudins represent a family of more than 20 proteins, 20 - 24 kDa in size, characterized by four transmembrane domains, two extracellular loops and two cytoplasmic tails. Their ability to regulate TJs is evidenced by the sensitive response to cell conductance and permeability to charged molecules affected by changes in the claudin expression or by specific claudin mutations [20] [21]. Claudins can also regulate cell permeability via size selectivity, as evidenced by increased permeability of the blood-brain barrier to small molecules (<800 Da) in mice deficient in claudin-5 [22]. Moreover, several peripheral membrane proteins, such as ZO-1 [23], ZO-2 [24], ZO-3 [18], cingulin [25], 7H6 antigen [26] and symplekin [27] were shown to be concentrated at the cytoplasmic surface of TJs. Freshly isolated cerebral endothelial cell culture aggregates and 7-day-old cerebral endothelial cell culture in primary culture express ZO-1 protein [11]. In this study, the expression of TJ-associated proteins occludin, claudin-1, -4 and ZO-1 was observed in LMECs on day 6 in culture when paracellular permeability was the lowest. On the other hand, although a large variation was absent in the mRNA levels of occludin, claudin-4 and ZO-1 between days 1 and 7 in culture, the claudin-1 mRNA level gradually increased up to approximately 7-fold over the basal level on day 7 in culture. This drastic increase in the mRNA expression level of claudin-1 corresponded inversely with the paracellular permeability of monolayer, suggesting that the mRNA expression of claudin-1 directly led to the strong formation of TJs.

The barrier function of pulmonary endothelial or epithelial cells plays an important role in maintaining alveolar membrane permeability and preventing the destruction of alveolar endothelial or epithelial cell junctions, which could lead to acute lung injury and respiratory distress syndromes [28] [29]. Therefore, our future experiments on the physiological and pathological roles of TJ-associated proteins using cultured mouse lung microvascular endothelial cells are warranted.

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