Effect of claudins 6 and 9 on paracellular permeability in MDCK II cells

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Sas D, Hu M, Moe OW, Baum M. Effect of claudins 6 and 9 on paracellular permeability in MDCK II cells. Am J Physiol Regul Integr Comp Physiol 295: R1713–R1719, 2008. First published September 10, 2008; doi:10.1152/ajpregu.90596.2008.—The neonatal proximal tubule has a lower permeability to chloride, higher resistance, and higher relative sodium-to-chloride permeability (PNa/Pcl) than the adult tubule, which may be due to maturational changes in the tight junction. Claudins are tight-junction proteins between epithelial cells that determine paracellular permeability characteristics of epithelia. We have previously described the presence of two claudin isoforms, claudins 6 and 9, in the neonatal proximal tubule and subsequent reduction of these claudins during postnatal maturation. The question is whether changes in claudin expression are related to changes in functional characteristics in the neonatal tubule. We transfected claudins 6 and 9 into Madin-Darby canine kidney II (MDCK II) cells and performed electrophysiological studies to determine the resultant changes in physiological characteristics of the cells. Expression of claudins 6 and 9 resulted in an increased transepithelial resistance, decreased chloride permeability, and decreased PNa/Pcl and PiHCO3/Pcl. These findings constitute the first characterization of the permeability characteristics of claudins 6 and 9 in a cell model and may explain why the neonatal proximal tubule has lower permeability to chloride and higher resistance than the adult proximal tubule.

METHODS

Cell culture and transfection. MDCK II cells, a leaky variant of the Madin-Darby Canine Kidney cell line, were cultured in a 37°C, 95% air-5% CO2 atmosphere in high-glucose (450 mg/dl) DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. The low resistance of MDCK II cells compared with MDCK type I cells is due to the expression of claudin 2 in MDCK II cells (17). Cells were grown on Snapwell Transwell-Clear polyester filters (12 mm, 1 cm2 pore size; Corning Costar, Acton, MA). Upon reaching 80% confluence, claudins 6 and 9 and empty vector (controls) were transfected into MDCK II cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per standard protocol. Claudin expression was observed to be optimal (both in abundance and localization) after 72 h of transfection, which is when experiments were performed.

Plasmid constructs. Full-length mouse claudins 6 and 9 were amplified using the following primers. Claudin 6: forward, 5′-GCAAGCTTCCACCATGCGCTTACTGGTTGCTTCAG-3′; reverse, 5′-GCCGATCTCCCACAATAATCTCTGTCG-3′; claudin 9: forward, 5′-GCAAGCTTCCACCATGCGCTTACTGGTTGCTTCAG-3′; reverse, 5′-GCCGATCTCCCACAATAATCTCTGTCG-3′.

The resulting product was ligated, inserted into the plasmid vector p3XFLAG-CMV-10, and the sequence verified. Mouse claudin 6 (NCBI accession no. BC050138) and claudin 9 (NCBI accession no. BC058186) were purchased from American Type Culture Collection (Manassas, VA) and Open Biosystems (Huntsville, AL), respectively.

Immunoblot. Cells were scraped and harvested in ice-cold RIPA (50 mM Tris·HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS) containing fresh mini-EDTA-free protease inhibitor (1 tablet/10 ml; Roche Applied Science, Indianapolis, IN). After vigorous agitation, the lysate was centrifuged at 14,000 g, 4°C for 15 min, the supernatant was collected, and the protein content determined by the Bradford method (12). Sixty micrograms of each sample was then diluted in 5× loading buffer (5 mM Tris·HCl [pH 6.8], 10% glycerol, 1% β-mercaptoethanol, 10%
SDS, 1% bromophenol blue) and heated in an 85°C water bath for 5 min. Samples were fractionated by 15% SDS-polyacrylamide gel and electrophoretically transferred to PVDF membranes. Membranes were washed, blocked with 5% nonfat milk, and 0.05% Tween 20 solution for 1 h, washed with PBS-0.1% Tween 20, and membranes were then probed overnight at 4°C with primary antibody at 1:250 dilution [claudin 6 (polycyclonal) (Santa Cruz Biotechnology, Santa Cruz, CA); claudin 9 (polyclonal) (Oribigen, San Diego, CA)]. These antibodies produced one band of the appropriate molecular weight, and specificity of these antibodies was confirmed in transfection studies (see Figs. 1–2). The membranes were then washed with PBS-0.1% Tween 20 solution (6 × 20 min each) before labeling with either anti-mouse or anti-rabbit horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed as above and developed using Western Lighting Chemiluminescence Reagent (PerkinElmer LAS, Boston, MA) per protocol. Membranes were then stripped for 30 min at 50°C in buffer containing 100 mM 2-β-ME, 2% SDS, and 62.5 mM Tris-HCl pH 6.7 with occasional agitation, and probed again with the appropriate claudin antibody [claudin 1–3 (polycyclonal), claudin 4 (monocyclonal) (Zymed, San Francisco, CA)] or β-actin (serving as a loading control; Sigma-Aldrich, St. Louis, MO), followed by washing, incubation with secondary antibody, and development as above.

**Immunocytochemistry.** To visualize the localization of the claudins, immunostaining of both empty vector-transfected and claudin 6 and 9-transfected MDCK II cells was performed. Cells were grown to confluence on Snapwell Transwell-Clear polyester filters (12-mm, 1-cm² growth area, 0.4-μm pore size; Corning Costar, Acton, MA). After electrophysiological and permeability experiments were performed, cells on the filters were washed with room temperature (1× PBS), then fixed with 4% paraformaldehyde for 10–20 min at 4°C. Cells were then washed with 1× PBS 4–6 times for 20 min each at 4°C. Cells were permeabilized with 0.1% Triton X-100 for 3 min and washed three times at room temperature with 1× PBS and blocked for 1 h with 1.5% BSA/10% goat serum at room temperature. Cells were then incubated overnight with claudin 6 or 9 antibody (1:100 dilution in 1.5% BSA/5% goat serum) at 4°C, washed as above, and incubated at room temperature with secondary antibody (Texas Red; Molecular Probes, Eugene, OR) at 1:800 dilution in 1.5% BSA/5% goat serum for 1 h. The cells were mounted on slides, viewed with a fluorescent confocal microscope (LSM 510; Carl Zeiss, Thornwood, NY), and images were recorded.

**Electrophysiological studies.** Empty vector-, claudin 6-, and claudin 9-transfected cells were grown to confluence on the aforementioned filters for 72 h after transfection. Studies were performed using an Ussing chamber with Ag/AgCl voltage and current electrodes bridged with 3M KCl agar, and computer-controlled voltage/current clamp (Physiologic Instruments, San Diego, CA). Chambers were bubbled with 95% air-5% CO2. The solutions were warmed to 37°C, and pH was checked before and after experiments to ensure pH of 7.35–7.40. Blank filters were used at the start of each experiment in the modified Ussing chamber to calibrate the instrument and software per manufacturers’ instructions. Once calibration was complete, filters with the confluent cells were placed in the chamber, first with solution A (Table 1) on both the apical and basolateral sides. TER was measured by passing a known current (5–50 μA) across the cell monolayer and measuring the change in voltage.

**Typical experiments to determine the dilution potential across the cell monolayers for each of the three transfected cell types (empty vector- and claudin 6- and 9-transfected) proceeded as follows (Fig. 4).** With the filter in the chamber and both the apical and basolateral chambers filled with equal volumes of solution A, voltage was recorded for 2 min. Next, the basal solution was replaced with solution D, and the voltage was recorded for 2 min. The basal solution was then replaced with solution A for 2 min, and the voltage was allowed to return to baseline. Next, the apical solution was replaced with solution D for 2 min to observe whether a voltage deflection of equal magnitude, but opposite sign, occurred. The apical solution was replaced with solution A and again returned to baseline for 2 min. This pattern was repeated for each of the different dilutional solutions. The order in which the different solutions were used was rotated to eliminate artifact from the duration of experiment. The transepithelial potential differences are designated as the apical side with respect to the basolateral or reference side.

The ratio of permeabilities, PNa/PCl and PNa/PCHO/PCl were calculated from the transepithelial potential difference values recorded during imposition of an ion concentration gradient across cell monolayers using the following format of the Goldman-Hodgkin-Katz equation

\[ E = 61 \text{ mV log} \left( \frac{P_{Na}}{P_{Cl}(Na^+) + (Cl^-)} \right) + \frac{P_{CHO}}{P_{Cl}(HCO_3^-)} \]

where A is the apical solution A and B is the basal solution.

To characterize chloride permeability, claudin- or empty vector-transfected cells were grown on filters to confluence and placed in the Ussing chamber as described above. While at 4°C, the apical side was bathed in chloride-containing solution (in mmol: 104 NaCl, 25 NaHCO3, 4 NaHPO4, 7.5 Na acetate, 5 KCl, 1 MgSO4, 1 CaCl2, 5 alanine, 5 glucose, 5 HEPES) and the basal side in a chloride-free solution (in mmol: 115 Na gluconate, 25 NaHCO3, 2.5 K2HPO4, 1 Mg gluconate, 2.5 Ca gluconate, 5 alanine, 5 glucose, 5 HEPES) (Table 2). Samples from the basolateral solution were taken at time 0 and 1 h. Chloride concentration was measured by potentiometry (Vitros 250; Ortho-Clinical Diagnostics, Rochester NY). Chloride permeability (PcI) was calculated using the following equation:

\[ \text{P}_{Cl} = \frac{J_{Cl}}{\left| \Delta [Cl_{apical}] - [Cl_{basal}] \right|} \]

where JCl is net flux and [Cl] is the chloride concentration on either the apical or basolateral side. JCl was calculated as ΔCl/time × area, where ΔCl is the difference in chloride from time 0 to 1 h (33).

**Statistical analysis.** ANOVA and determination of statistical significance of difference between groups (using the Student-Newman-Keuls method) were performed using SigmaStat 3.5 (Systat Software, San Jose, CA).

**RESULTS**

**Expression and localization of transfected claudins.** The expression level of transfected claudins 6 and 9 was detected by immunoblot. Expression of other endogenously expressed claudins was not affected by expression of claudin 6 or 9 (Fig. 1). As shown in Fig. 2, transfected claudins 6 and 9 localized to the tight junction.

**Measurement of TER.** MDCK II cells transfected with claudin 6 or 9 had a higher TER than control MDCK II cells transfected with empty vector (Fig. 3). Mean TER in control cells was 80 ± 3.9 Ohms/cm² (n = 8), which is consistent with findings in previous studies (20, 39). TER in claudins 6 and 9 transfected cells was 268 ± 8.0 Ohms/cm² (n = 8) and 188 ± 8.1 Ohms/cm² (n = 8), respectively (P < 0.05).

| Table 1. Solution amounts for electrophysiology experiments |
|---------------|---|---|---|---|
|              | A | B | C | D |
| NaCl         | 120 | 60 | 120 | 30 |
| Mannitol     | 0  | 120 | 0  | 180 |
| NaHCO3       | 24 | 24 | 24 | 24 |
| HEPES        | 10 | 10 | 10 | 10 |
| KCl          | 5  | 5  | 5  | 5  |
| CaCl2        | 1.2 | 1.2 | 1.2 | 1.2 |
| MgSO4        | 1  | 1  | 1  | 1  |

Values are in mM.
Applying a 2:1 NaCl concentration gradient across the MDCK II cell monolayer by changing the basal solution to solution B induced an average transepithelial potential difference of 6.9 ± 0.7 mV in empty vector-transfected cells, which is consistent with findings in previous studies (25, 35, 36). The transepithelial potential difference was blunted in claudins 6- and 9-transfected cells at 2.0 ± 1.0 mV and 4.8 ± 1.2 mV, respectively (P < 0.05; data not shown). Increasing that gradient to 4:1 induced an average transepithelial potential difference of 11.4 ± 1.0 mV in empty vector-transfected cells, 3.3 ± 0.8 mV in claudin 6-transfected cells, and 8.8 ± 0.8 mV in claudin 9-transfected cells (P < 0.05; data not shown). As shown in Fig. 4, reversing the concentration gradient in each experiment by replacing the apical (rather than basal) solution induced a transepithelial potential difference of equal magnitude and opposite sign. Applying a 10:1 HCO₃⁻ concentration gradient across the cell monolayer resulted in an average transepithelial potential difference of 4.2 ± 1.0 mV in empty vector-transfected cells, 2.6 ± 0.7 mV in claudin 6-transfected cells, and 3.1 ± 1.2 mV in claudin 9-transfected cells (P < 0.05 for transfected cells compared with control).

The Goldman-Hodgkin-Katz equation was used to calculate P_{Na/Cl} and P_{HCO_3/Cl}. Applying a 2:1 NaCl concentration gradient across the MDCK II cell monolayer by changing the basal solution to solution B induced an average transepithelial potential difference of −6.9 ± 0.7 mV in empty vector-transfected cells, which is consistent with findings in previous studies (25, 35, 36). The transepithelial potential difference was blunted in claudins 6- and 9-transfected cells at −2.0 ± 1.0 mV and −4.8 ± 1.2 mV, respectively (P < 0.05; data not shown). Increasing that gradient to 4:1 induced an average transepithelial potential difference of −11.4 ± 1.0 mV in empty vector-transfected cells, −3.3 ± 0.8 mV in claudin 6-transfected cells, and −8.8 ± 0.8 mV in claudin 9-transfected cells (P < 0.05; data not shown). As shown in Fig. 4, reversing the concentration gradient in each experiment by replacing the apical (rather than basal) solution induced a transepithelial potential difference of equal magnitude and opposite sign. Applying a 10:1 HCO₃⁻ concentration gradient across the cell monolayer resulted in an average transepithelial potential difference of 4.2 ± 1.0 mV in empty vector-transfected cells, 2.6 ± 0.7 mV in claudin 6-transfected cells, and 3.1 ± 1.2 mV in claudin 9-transfected cells (P < 0.05 for transfected cells compared with control).

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Chloride permeability. A chloride concentration gradient was applied across confluent cell monolayers on filters with 111 mmol chloride solution on the apical side and chloride-free solution on the basal side. These experiments were performed at 4°C to inhibit active transport. Samples were taken from the basal side at time 0 and 1 h and the chloride permeability was calculated as described in METHODS. (Shorter time periods did not give reproducible results.) Both claudins 6- and 9-transfected cells had lower chloride permeability compared with controls (control: 14.9 ± 1.6 mmole/M·h·cm²; claudin 6: −1.4 ± 2.1 micromole/M·h·cm²; claudin 9: 7.4 ± 1.7 micromole/M·h·cm²; P < 0.05) (Fig. 7). These results indicate that the MDCK II cells transfected with claudins 6 and 9 are less chloride-permeable than control cells.

![Fig. 1. Effect of claudins 6 or 9 transfection on expression of endogenous claudins in Madin-Darby canine kidney II (MDCK II) cells.](http://ajpregu.physiology.org/)
DISCUSSION

Previous studies have shown that different claudins can affect ion-specific permeability in cell monolayers (2, 35, 37, 39). We sought to characterize the effect of claudins 6 and 9 on paracellular permeability. Our results indicate that expression of claudins 6 and 9 result in decreased chloride permeability, increased TER, and decreased $P_{Na}/P_{Cl}$ and $P_{HCO3}/P_{Cl}$.

We have previously shown that there are postnatal developmental changes in a number of properties of the proximal tubule paracellular pathway. The neonatal rabbit and rat proximal straight tubule had a higher TER and lower chloride permeability than that of the adult segment (11, 23). In the rabbit proximal straight tubule, the $P_{Na}/P_{Cl}$ was significantly higher in the neonate compared with the adult (23). In the rat, the $P_{Na}/P_{Cl}$ was comparable in adult and neonatal proximal straight tubules (23), but the $P_{Na}/P_{Cl}$ was again higher in the neonatal rat and mouse proximal convoluted tubule than that in the adult (1, 11). Our laboratory has also shown that claudins 6 and 9 are expressed in the neonatal mouse, but that expression is reduced as the mouse matures (1). Expanding on this, our hypothesis was that claudins 6 and 9 are responsible for the developmental changes in the properties of the paracellular pathway. Our finding of a significant decrease in chloride permeability and increase in TER is consistent with our hypothesis; however, our finding of a lower $P_{Na}/P_{Cl}$ in cells expressing claudins 6 and 9 differs from the findings in the neonatal rabbit, mouse, and rat (1, 10, 11, 23, 31). Thus, these data show that, while there was a decrease chloride permeability with expression of claudins 6 and 9, the decrease in sodium permeability is even greater.

Our data show that transfection of claudins 6 and 9 result in a significant change in TER and chloride permeability. These results are consistent with findings that the claudin isoforms expressed in the tight junction affect the TER and permeability properties of the epithelia. For example, the high cation permeability of the thick ascending limb is due to the presence of claudin 16 (32). In MDCKII cells, claudin 2 results in a decrease in resistance in MDCK cells compared with MDCKI cells that do not express claudin 2 (17). In addition, MDCKII cells are permeable to cations and transfection of claudin 8 in
MDCKII cells reduces claudin 2 expression and decreases the cation permeability (3, 39). There are a number of potential explanations for the fact that expression of claudins 6 and 9 did not result in the expected increase in $P_{Na}/P_{Cl}$. It is possible that the difference between relative $P_{Na}/P_{Cl}$ in our cell model compared with perfused neonatal rabbit, mouse, and rat tubules is due to some other characteristics in the epithelia aside from claudins 6 and 9. While it has been shown that the charge generated by the amino acid sequence of the first extracellular domain determines the ion selectivity in claudins in in vitro studies (14, 15, 36–38), it is likely that tight junction regulation is more complex in vivo and may be regulated by more than just the charge on the first extracellular loop of each claudin (4). The amino acid sequence in the predicted first extracellular loop of claudins 6 and 9 has both acidic and basic residues, making the dominant charge of the domain unclear. In fact, the greatest degree of heterogeneity between claudin isoforms is found not in the amino acid sequence of the extracellular loops, but rather in the amino acid sequence in the intracellular COOH-terminus (4). This suggests a role for intracellular signaling and regulation, indicating that the ultimate effect of claudins on the tight junction may be multifactorial and that there may be a developmental change in regulation that affects the expression or properties of the claudins. In addition, the transfection of claudins 6 and 9 likely results in a greater expression than would naturally occur in neonatal proximal tubule cells in vivo. While we do show that claudin 1-4 protein expression does not change, other claudin proteins may have

Fig. 3. Transepithelial resistance (TER) in MDCK II cells transfected with claudin 6 or 9. TER was measured across cell monolayers in a modified Ussing chamber. Results expressed as TER in $\Omega \cdot \text{cm}^2$ ($n = 8$ for each sample). Bars and error bars represent means ± SE. *$P < 0.05$.

Fig. 4. Representative voltage tracing showing transepithelial potential differences across a MDCK II cell monolayer using asymmetric solutions with a bracketed method. NaCl and HCO$_3^-$ concentrations were changed in apical or basal chambers bathing MDCK II cell monolayers. Black solid line represents empty vector-transfected cells; dashed line represents claudin 6-transfected cells. NaCl and HCO$_3^-$ concentrations for each segment are indicated at the top of the figure.

Fig. 5. Sodium permeability-to-chloride permeability ratio ($P_{Na}/P_{Cl}$) in MDCK II cells transfected with claudin 6 or 9. The relative ionic permeability was calculated using the Goldman-Hodgkin-Katz equation based on the transepithelial potential difference across each cell monolayer with each replacement solution ($n = 8$ for each sample). Bars and error bars represent means ± SE. *$P < 0.05$.
been affected with transfection resulting in the unexpected change in the P_{Na}/P_{Cl}.

Our previous findings showed that there was a developmental change in claudins 6 and 9 mRNA and protein abundance (1). By examining claudins 1-16, we found there was no difference in neonatal and adult proximal tubule mRNA expression for claudins 1, 2, 10a, and 12. The other claudin isoforms were not detected in the neonatal or adult segment. However, our previous study does not rule out the possibility that there are differences in protein abundance in these claudins at the tight junction or the presence of another claudin that is affected during development that may be responsible for the higher P_{Na}/P_{Cl} in the neonate compared with the adult proximal tubule. Nonetheless, the current studies do not support our hypothesis that the expression of either claudin 6 or 9 by themselves result in all the changes in the paracellular pathway seen during postnatal development.

While there is a consistent decrease in proximal tubule P_{Na}/P_{Cl} in different species comparing neonates to adults (6, 10, 11, 23), the changes that occur in P_{HCO3}/P_{Cl} during postnatal development are not as consistent (10, 11, 23). In the rabbit, proximal straight tubule P_{HCO3}/P_{Cl} is less in the adult than in the neonate, while in the rat proximal straight and convoluted tubule the P_{HCO3}/P_{Cl} is comparable in neonates and adults. In the mouse proximal convoluted tubule there is a maturation increase in the P_{HCO3}/P_{Cl} and a developmental decrease in claudins 6 and 9 expression (1). The lower P_{HCO3}/P_{Cl} in MDCK cells expressing claudins 6 and 9 are consistent with the lower P_{HCO3}/P_{Cl} in the neonatal mouse. The mechanism by which this developmental change in claudin isoforms occurs has not been elucidated. We know that the normal developmental surge in glucocorticoids and thyroid hormone is responsible for other maturational changes in tubular transport mechanisms in the kidney (8, 9, 13, 18, 19, 30). It may be that these also lead to the decrease in abundance of claudins 6 and 9 in the proximal tubule during maturation. In summary, these experiments help characterize claudins 6 and 9 and their role in tight junction permeability, as well as provide a possible mechanism for decreased chloride permeability in the neonatal proximal tubule. The importance of their effect on regulation of ion permeability in vivo, the mechanism by which the claudin isoform change occurs, as well as their role in the pathogenesis of disease, requires further study.

**Perspectives and Significance**

The neonatal kidney has a lower glomerular filtration rate and thus a lower requirement for reclamation of filtered solutes. Most previous studies have demonstrated that there is a maturational increase in active solute transport and a corresponding increase in the transporters expressed in the adult kidney. However, it is now clear that there are developmental transporter protein isoforms that are more highly expressed in the neonate than the adult, such as the apical membrane sodium phosphate cotransporter, NaPi-2c, and the sodium hydrogen exchanger, NHE8 (1, 28). Recently, it has been shown that there are developmental changes in claudin isoforms, and this study shows that the claudins expressed in neonates can affect paracellular permeability (1). Clearly, it is time to reexamine postnatal development in view of these findings as there are likely other developmentally regulated transporters and paracellular proteins. The factors that cause the developmental changes in transporter and paracellular protein expression will be an interesting area of future investigation.

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