PERITONEAL MEMBRANE SOLUTE TRANSPORTER EXPRESSION AND FUNCTION IN HEALTH, CHRONIC KIDNEY DISEASE AND PERITONEAL DIALYSIS

Ph.D. thesis

Eszter Lévai, MD

Károly Rácz Conservative Medicine Division
Semmelweis University

Supervisor: Prof. Attila J. Szabó MD, D.Sc,
Prof. Claus P. Schmitt MD., DHC
Official reviewers: Andrea Berkes MD, PhD
Ákos G. Pethő MD, PhD
Head of the Complex Examination Committee:
András Szabó, MD, D.Sc.
Members of Complex Examination Committee:
Tamás Szabó, MD, PhD
András Tislér, MD, PhD

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1. Introduction

Chronic kidney disease (CKD) is increasingly common worldwide, leads to major cardiovascular complications, a reduced life-expectancy and places a heavy burden on healthcare systems.

In end-stage kidney disease, the last stage of CKD, initiation of kidney replacement therapy (KRT) is needed. Due to the limited availability of kidney transplantation (the optimal alternative regarding superior survival rate) and the increasing number of CKD patients, the reliance on dialysis as a life-maintaining therapy is growing significantly.

Peritoneal dialysis (PD) and hemodialysis (HD) are both suitable for bridging- and long-term therapies. In pediatrics, PD is a preferred chronic dialysis mode, since it provides a wide flexibility for school-aged children, less hospital visits and thus a better quality of life.

The peritoneum works as an endogenous semipermeable dialysis membrane and is being used as a peritoneal dialyser for more than 50 years. There are ultrastructural changes in the peritoneal membrane, that already start at CKD, and accelerate amid the supraphysiological glucose-, GDP- and uremic toxin concentrations of PD, and effectivity of toxin-, salt- and water removal declines. (1, 2)

In 1993, Rippe and his colleagues described peritoneal transport with a mathematical “3-pore model”. It hypothesizes three different sizes of pores for the exchange of molecules, a) transcellular/ultrasmall pore, primarily responsible for water transport (aquaporin-1 - AQP1), b) small pore, responsible for small solute and ion transport, and c) the large pore, subject of macromolecule transport. Only the molecular counterparts of the transcellular pore, AQP-1 is identified to date. (3) The molecular mechanisms of the remaining water and of solute removal is still uncertain.

Peritoneal permeability is characterized by transcellular and paracellular transport. Considering the peritoneum as a leaky membrane, allowing for rapid diffusion of water and small solutes, paracellular pathways have a major role in underlying transport mechanisms. (4-6) Tight junctions (TJ) are the key elements of mostly permselective paracellular transport in endothelial and epithelial barriers, and also indirectly effect transcellular transport properties.
This thesis focuses on the peritoneum as a dialysis membrane, the underlying molecular mechanisms of peritoneal solute transport and CKD and PD induced expression and functional alterations. As a proof of concept, a first experimental pharmacological intervention reversing PD induced changes is presented. This is of particular interest in view of the increasing affected population and time spent on PD per individual.

2. Objectives

During the time of my PhD studies, the aim was to investigate yet little described components of peritoneal membrane transport, i.e. tight junction molecules, transporters and channels during the course of CKD and PD treatment. The following questions have been addressed.

1. Expression of key molecules of peritoneal trans-and paracellular transport in health, CKD and during PD in mesothelium and endothelium in the peritoneal membrane
2. Correlation of peritoneal transcellular and paracellular protein expression and peritoneal membrane function during PD
3. Establishment of an experimental workflow for studying barrier integrity and permeability within the same polarized cell monolayer of interest in the context of PD, mesothelial and endothelial mono-cell layer barriers.

4. Reversing the PD-fluid induced disintegration of the endothelial membrane by pharmacological means (with AlaGln)
5. The effect of high GDP load in PD on endothelial cell junction and cytoskeleton disruption in vasculopathy

3. Methods

In the work of this dissertation we used a multimodal approach, based on histological studies, cell culture experiments (from human umbilical vein endothelial cells, human umbilical arteriolar endothelial cells and human primary mesothelial cells – HUVEC, HUAEC and HPMCs) and transendothelial resistance experiments tied together with fluorescent dextrane transport measurements and also immunostaining. We used partly and automated imaging and automatedimage analysis, and uniquely tied it with Single Molecule Localization Microscopy (SMLM) method with the help of physicist colleagues from the Kirchoff Institute in Heidelberg. Additionally Western Blotting was performed for protein quantification.

For further studies an experimental workflow for studying barrier integrity in monolayers was established. This workflow enables the investigator to uniquely describe the paracellular permeability changes from a functional, temporal and spatial aspect.

All immunohistochemistry studies listed in this dissertation were performed from tissues collected within the International Peritoneal Biobank (IPPB, registered at www.clinicaltrials.gov—NCT01893710). The studies using human samples were performed according to the Declaration of Helsinki which sets ethical principles regarding human experimentation. The studies have been approved by all local institutional review boards.

4. Results

4.1. Human peritoneal tight junction, transporter and channel expression in health and kidney failure, and associated solute transport

4.1.1. Tight junction protein abundance in children with normal kidney function, children with CKD5 and on PD)

Abundance of sealing claudins (CLDN1, CLDN3, CLND5), pore-forming claudins (CLDN2, CLDN4, CLDN15) and ZO-1, OCL and TriC were measured in the mesothelial and in the arteriolar areas. CKD5 patients showed a higher abundance of peritoneal mesothelial CLND1 and arteriolar CLDN2 and -3 than in controls. PD patient samples had the highest mesothelial and arteriolar CLDN1 and mesothelial CLDN2 abundance, while mesothelial and arteriolar CLDN3 were the lowest.

4.1.2 Transcellular sodium channel (ENaC) and sodium and phosphate transporter proteins (SGLT1 and PiT1) in healthy individuals, CKD5 and in PD

Mesothelial ENaC, mesothelial and arteriolar SGLT1 and PiT1 abundance was unchanged over CKD5 and PD treatment. Arteriolar ENaC abundance was lowest in PD, compared to controls.

In patients with CKD5, peritoneal mesothelial CLDN1 and arteriolar CLDN2 and -3 abundances were higher than in controls. Peritoneum from patients on PD had the highest mesothelial and arteriolar CLDN1 and mesothelial CLDN2 abundance, while mesothelial and arteriolar CLDN3 and arteriolar ENaC were lowest.

4.1.3 Lower arteriolar CLDN5/CLDN1 abundance ratio in peritoneal dialysis, compared to control and CKD5 indicates an impaired cellular barrier function in PD

Change of the ratio of arteriolar CLDN5 and CLDN1 sealing proteins due to peritoneal dialysis, indicating an impaired

sealing function of the endothelium and sample stainings from all investigated groups (healthy, CKD, PD).

4.1.4. Correlation of arteriolar CLDN2 and mesothelial CLDN4, CLDN15 and PiT1 abundance with D/P_{Crea} and D/D_0 glucose obtained from peritoneal equilibration test data in a subcohort of patients at the start of PD treatment (CKD5) and on chronic PDs

D/P_{Crea} and D/D_0 Glucose significantly correlated with CLDN2 in the arteriolar area, CLDN15 in the mesothelial area and D/D_0 Glucose with CLDN4 (with D/P_{Crea} p=0.06, r=0.50) in mesothelium and D/P_{Crea} with PiT1 (with D/D_0 glucose p=0.19, r=-0.41).

In a multivariable analysis of arteriolar CLDN2 abundance, submesothelial vessel density (quantified by CD31 positivity) and age, solely arteriolar CLDN2 predicted D/P_{Crea} and D/D_0 glucose ratios (p = 0.086/0.036).

4.2. Experimental workflow to study protein expression and transport function in a single, polarized cell monolayer

We have established an experimental workflow for human umbilical vein endothelial cells (HUVECs), that were cultured on Transwell filters to ensure polarization and performed transendothelial electrical resistance (TER) and FITC dextrane (10 kDa) flux measurements in paralell. In addition to these functional measurements, the same monolayers underwent immunolabeling (ZO-1 and CLDN5), and automated immunofluorescent imaging covering large monolayer areas. Finally, the same monolayers were used for single molecule localization microscopy (SMLM), allowing for ZO-1 and CLDN5 spatial clustering analysis on the nanoscale.

Dipeptide Alanyl-Glutamin (AlaGln) was used to modulate the properties of the endothelial monolayers, SMLM showed the non-random, higher degree of clustering of CLDN5 cell-cell junction areas (7, 8).

4.3. Alanyl-Glutamine restores endothelial ZO-1 organization after disruption by a conventional peritoneal dialysis fluid

Alanyl-glutamine (AlaGln) in cell medium dose-dependently increased TER in human umbilical vein endothelial cells (HUVEC) (ANOVA $p < 0.001$). Endothelial ZO-1 abundance is decreased and CLDN5 increased with 8 mM AlaGln ($p < 0.05$). Supplementation of 8 mM alanyl-glutamine (AlaGln) to CPDF and LPDF increased electrical resistance of HUVEC after 1 and 5 h. 10 kDa and 70 kDa dextran transport was reduced with CPDF only (p<0,05). Reduction of ZO-1 and CLDN5 protein abundance in CPDF treated HUVEC was restored with AlaGln supplementation(p=0,07/0.05).

Single molecule localization microscopy was used for quantification and clustering of single ZO-1 molecules at the junction areas in HUVEC, cultured on Transwell filters for proper polarization. Alanyl-glutamine (AlaGln) reduced the number of fluorophore signals in the membrane of endothelial cell incubated with medium and increased it with LPDF incubation. ZO-1 molecule clustering was changed in a characteristic way when AlaGln was added.

4.4. GDP induce arteriolar tight junction disintegration

3,4-dideoxyglucosone-3-ene (3,4-DGE) disrupts membrane ZO-1 (zonula occludens-1) and increases endothelial monolayer permeability. Human umbilical arterial endothelial cells (HUAEC) were incubated with 3,4-DGE at different concentrations in high–glucose degradation products (GDP) PD fluids for 24 h.

Total endothelial ZO-1 was unchanged, cell membrane ZO-1 was disintegrated.

In Transwells, increasing doses of 3,4-DGE progressively disrupted membrane ZO-1 molecule organization. Functional studies demonstrated a dose-dependent decline in transendothelial electrical resistance and increased permeability

for 4 kDa dextran with 5 to 20 µmol/L 3,4-DGE exposure Endothelial ZO-1 (zonula occludens-1) clustering was modified by high–glucose degradation products (H-GDP) PD, and this distinct difference was also made visible by Single Molecule Localization Microscopy.

5. Conclusions

This dissertation outlines the novel importance of paracellular transport proteins as molecular counterparts of peritoneal membrane function. This work has been done through a multimodal approach.

By proving consistent detectability and describing the changes of distinct sealing, pore-forming and scaffolding TJ proteins (CLDN1-5, and CLDN15, ZO-1, OCL, TriC) and a few transcellular transporters and channels (PiT1, SGLT1, ENaC), the probable role of these molecules in peritoneal membrane changes during PD was pointed out. Description of mesothelial abundances and changes prepared further functional studies of our group to emphasize the role of the mesothelium as a barrier in peritoneal solute transport. (9)

Finding associations of target TJ molecules and peritoneal membrane transport functional data highlights CLDN2, CLDN4, CLDN15 and PiT1 as possible target molecules for future interventions to enhance PD function.

To standardize and enhance studying barrier integrity, permeability and TJ composition, localization and clustering in a single monolayer, a new experimental workflow was established. Thus, maximizing the retrievable coherent information content from a single monolayer, making it possible for future investigators to provide a triad of functional, temporal and spatial description of paracellular permeability changes, with particular regard to effects on the TJs.

Opening up a window to PD fluid additive research AlaGln was tested and proven to seal the epithelial barrier. Dialysis fluids

affect TJ organization by disruption, which can be counteracted by AlaGln through increasing transepithelial resistance and decreasing small and middle molecule transport. Increased CLDN5 and ZO-1 abundance was present with AlaGln supplementationd to CPDF and AlaGln also improved the clustering of ZO-1 in the endothelial membrane. This example of targeting the molecular basis of peritoneal transport is a key to improve PD efficacy and patient outcome.

To address further complications of PD treatment and therefore provide a new interface of interventions to molecular counterparts of the endothelial barrier, a link between GDP load and systemic vasculopathy was established. GDP induces endothelial cell junction and cytoskeleton disruption, next to the induction of apoptosis. This finding is crucial for prevention of long-term cardiovascular complications in PD patients, especially children on chronic PD.

6. Bibliography of the candidate's publications

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