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Assessment of increased glomerular permeability associated with recurrent focal segmental glomerulosclerosis using an in-vitro model of the glomerular filtration barrier

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Abstract

The presence of circulating permeability factors (cPFs) has been hypothesized to be associated with recurrence of focal segmental glomerulosclerosis (rFSGS) in renal allografts. The available methods to detect cPFs are complex, not easily repeatable and inappropriate to represent the anatomical characteristics of the three-layer glomerular filtration barrier (GFB). Here we describe a novel method which measures the permeability to bovine serum albumin (BSA) through a three-layer device (3LD). The 3 layers comprise: 1. conditionally immortalized human podocytes (HCiPodo), 2. collagen type IV coated porous membrane and 3. human glomerular endothelial cells (HCiGEnC). Using this method, we found that sera from all rFSGS patients increased albumin permeability, while sera from non recurrent (nrFSGS) and genetic (gFSGS) forms of FSGS did not. The mechanisms underlying the increase of albumin permeability are probably due to endothelial cell damage as an initial event, which was demonstrated by the decrease of Platelet endothelial cell adhesion molecule (PECAM-1 or CD31), while the podocytes' expressions of synaptopodin and podocin were normal. Furthermore, we also found that the plasmapheretic treatment (PPT) eliminated the effect of increasing BSA permeability in sera from rFSGS patients. These preliminary data suggest that our in vitro GFB model could not only be useful in predicting the recurrence of FSGS after renal transplantation (RTx), but also be a valuable in vitro model to study podocyte and endothelial cell biology.

Keywords: FSGS, permeability factors, filtration membrane, albumin permeability, co-culture, podocyte

Introduction

Primary focal segmental glomerulosclerosis (pFSGS) is a glomerular disease of unknown etiology, often characterized by heavy proteinuria, which in more than 50% of cases does (do) not response to the available therapies and progresses toward end stage kidney failure [1-2]. It has long been hypothesized that the presence of circulating permeability factors (cPFs) which damage the glomerular filtration barrier (GFB) could contribute to the pathogenesis of pFSGS [3].This hypothesis was based on the frequent recurrence of FSGS after renal transplantation (RTx, 30-50%), the reported proteinuria resolution when an FSGS kidney is transplanted in an unaffected recipient, the appearance of proteinuria in the newborn of a mother affected by FSGS, the regression/reduction of proteinuria after plasmapheresis (PP) in some affected patients [4-12].

The traditional method to determine the circulating permeability factors was based on the measurement of albumin reflection coefficient with isolated rat glomeruli [13], but this method is very complex and requires animal sacrifice. Pegoraro et al proposed a simpler method using cultured rat glomerular epithelial cells grown on the Millicell filters, which allow sampling of apical and basolateral media. Through measuring the ^{125}I-labeled human serum albumin across the epithelial cell monolayer, they tested the circulating permeability activity of sera from idiopathic nephrotic syndrome patients [14]. In a recent paper, Kachurina et al described a novel method based on cultured mice podocytes challenged with sera from FSGS patients, using immunofluorescence microscopy followed by computerized image-processing analysis [15]. More recently, Srivastava et al developed a reporter-based assay, using transfected podocyte cells for the study of activated genes after exposure to the plasma from recurrent FSGS (rFSGS) patients [16]. However the main limitation of all these models, except the first one [13], is that the assays are based on only one epithelial monolayer, which is far from the in-vivo physiological structure of GFB which is a three-layer structure with glomerular endothelial cells, podocytes and the basement membrane in between.

Here we described a method of co-culture of conditionally immortalized human podocytes and glomerular endothelial cells on the opposite of a porous membrane, which does not rely on special instruments nor on the synthesis of devoted biomaterials, so it may be widely adopted by the scientific community and is more easily performed than the original method based on isolated rat glomeruli [13]. Moreover this device not only permits us to study separately the events occurring in podocytes and/or endothelial cells, but also to detected the albumin permeability activity of sera from patients through a simple colorimetric analysis of BSA across the three-layer device (3LD).

Materials and Methods

Patient samples

Seven recurrent FSGS (rFSGS), 10 non recurrent FSGS (nrFSGS), and 5 genetic forms of FSGS (gFSGS) sera from adult and pediatric patients (Table 1), who signed an informed consent [M. 02. F (A)], were collected in Adult and Pediatric Nephrology, Dialysis and Renal Transplant Units (Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico- Milan). 8 healthy blood donor sera were collected in the Blood Transfusion Center of the same hospital. rFSGS was diagnosed when renal transplanted (RTx) patients with a histological confirmed diagnosis of FSGS in their native kidneys (5 patients) or with a history of steroid resistant nephrotic syndrome (SRNS) as the cause of native kidney failure (2 patients), developed significant proteinuria (> 3 g/day) and/or had histologic finding of FSGS at the renal graft biopsy within the first year after RTx. All the seven rFSGS had been submitted to PP treatment. In each rFSGS patient, pre- and post-PP sera were collected before and immediately after PP treatment.

In 6 of rFSGS cases, the blood sample was collected at the first or second PP therapy, performed immediately after the FSGS recurrence (within first 2 months), while in one patient (subject number 2), transplanted in 2011 and affected by an early FSGS recurrence, who required chronic PP therapy, the blood collection was performed after 6 years of RTx. PPs were performed using albumin in most patients (subjects 1, 2, 4, 5, 6), while plasma was utilized in patients 3 and 7.

Serum samples were processed under sterile laminar flow hood (Heraeus, Hanau, Germany) and divided into small volume aliquots to avoid multiple freeze/thaw cycles and stored at -80°C. Before use, sera were thawed on ice immediately before experiment and diluted to 2% with cell culture medium.

Cell cultures

Conditionally immortalized human glomerular endothelial cells (HCiGEnC) and conditionally immortalized human podocytes (HCiPodo) (both from University of Bristol, Bristol, UK) were cultured as previously described [17, 18]. Briefly, for propagation, cells were grown at 33°C and 5% CO_2 in endothelial growth medium 2-microvascular (EGM2-MV, Lonza, Walkersville, MD USA) containing 5% fetal calf serum (FCS) and growth factors as supplied, excepting vascular endothelial growth factor (VEGF) or in RPMI-1640 medium containing 10% Fetal bovine serum (FBS), 5 μg/ml transferrin, 5 ng/ml sodium selenite, 0.12 U/ml insulin, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Sigma Aldrich, Milan, Italy). HCiGEnC and HCiPodo were utilized within 35 and 20 passage respectively.

In-vitro model of glomerular filtration barrier (GFB) and assessment of permeability

The in-vitro model of GFB is assembled according to a modified previously described methodology [19]. Briefly, using the Millicell hanging cell culture inserts with Polyethylene Terephthalate (PET) microporous (1 μm diameter) membrane (Millipore, Milan, Italy) coated on both sides with collagen type IV (Sigma), 75,000 endothelial cells are seeded on the lower side of the membrane and allowed to adhere to it for 4 h; then the endothelial cells are cultured in EGM2-MV medium containing 5 ng/ml of VEGF (Sigma) at 37°C and, after 3 days, 65,000 podocytes are seeded on the upper side of the membrane and cultured by their own medium. Podocytes and endothelial cells are co-cultured in their respective medium for an additional 5 days before experiment. To assess albumin permeability, after carefully washing 3LD with PBS, RPMI-1640 only and RPMI-1640 plus 40 mg/ml BSA are added in the upper (podocyte) and lower (endothelial) compartment respectively. After 2 hours incubation, the concentration of BSA is assessed in the upper compartment and taken as the basal level (BSAb). Thereafter, both compartments are emptied, washed again, and the lower compartment is filled with the experimental solution (i.e.: adriamycin at concentration of 0.8, 1.4 and 2 μM; or 2% serum to be tested, added to the medium), while the upper compartment with podocytes' medium. The 3LD is then incubated for 24 hours with adriamycin or 48 hours

with patient's serum. At the end of the incubation, the two compartments are emptied again, washed and BSA permeability assay is repeated as before (BSAexp). The changes in BSA permeability are expressed as: (BSAexp-BSAb)/BSAb %. BSA has been measured by spectrometry using the DC protein assay kit (Bio-Rad, Milano, Italy). Each patient serum and each ADR concentration were tested in 3 different devices contemporarily and the results are expressed as mean ± SD. Healthy control experiments were obtained by incubating the device with sera from 8 healthy blood donors in triplicate. (Fig.1 A)

Immunofluorescence

After removing the membrane from the insert using a sharp scalpel and placing it onto a microscope slide, cells to be studied (pocodytes or endothelial cells) were washed and fixed with 10% neutral buffered formalin for 10 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS. Sequentially, the cells are incubated overnight at 4 °C with the primary antibody (rabbit anti-CD31, 1:100, Abcam, Cambridge, UK; rabbit anti-synaptopodin, 1:50 and rabbit anti-podocin, 1:50, Sigma), followed by the addition of the appropriate fluorescent-labelled secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, 1:200 Invitrogen, S. Giuliano Milanese, Italy) and 4',6-diamidin-2-fenilindolo (DAPI, 1:1000, Sigma). The specificity of the primary antibody was demonstrated by the lack of staining after substituting the primary antibody with proper control immunoglobulin (rabbit IgG isotype control, Zymed, California, USA (Supplementary Fig.1). Images were acquired by AxioObserver microscope and recorded by AxioVision software 4.8 (all from Zeiss, Milan, Italy). The parameters for image acquisition were first established with cells exposed to healthy sera and then were applied to all samples treated with patients' sera. The percentage of the membrane surface covered by cells and mean fluorescence intensity (FI) from 10 images of each sample were analysed by Image J software. The results were expressed as percentage of FI of healthy controls.

Statistical analysis

Data are presented as mean ± SD. At least three replicates were performed for each sample. Two-tail Student's t test was used for analysis of data when two groups of data were compared. ANOVA test was applied when comparing more than 2 groups of data. P values <0.05 were considered significant.

Results

Assembly of the in-vitro model of GFB

Based on our previously published method [19], we successfully substituted the immortalized mouse podocytes and mouse capillary endothelial cells with the conditionally immortalized human podocytes and human glomerular endothelial cells. As shown by Fig. 1 B, immunofluorescence staining of cells grown on the membrane with anti-CD31, a specific endothelial cell marker (left) and anti-podocin, a specific podocyte marker (right) demonstrated the homogeneous formation of the cell monolayer on the two different sides of the membrane (average area covered by endothelial cells and podocytes 91±4.9% and 89.9±1.9 %, respectively) (Fig. 1B).

Validation of the albumin permeability assay

For validation of the albumin permeability assay, we used adriamycin to induce cell damage, which in turn must result in increasing the BSA passage across the three-layer structure. In physiological conditions (medium alone), BSA permeability was 1.3 ± 4.1%. Incubation with adriamycin at concentration of 0.8, 1.4 and 2 µM for 24h induced a statistically significant and dose dependent increase of BSA permeability as compared with cells incubated with medium alone (18.95 ± 2.05%, *p<0.05; 31.5±1.98%, **p<0.01; 41.75±5.59%, **p<0.01 respectively) (Fig.1C). Immunofluorescence Staining showed that treatment with ADR induced both endothelial cells and podocytes damage(s). As seen in supplementary Fig.2, there was a remarkable decrease of CD31 expression on endothelial cells, while podocytes presented not only the decrease of podocin and synaptopodin immunostaining intensity but also the changes of their distribution (podocin: from cytoplasmic and membrane expression to weak cytoplasmic expression; synaptopodin: from cytoplasmic, along acting filaments and cell processes expression to cortical, near to the cell membrane expression.).

Effects of sera from patients with diverse forms of FSGS on the GFB

To check if the in-vitro GFB model can be used to detect the circulating permeability factors, we added in the endothelial cell compartment the sera from 7 patients with early post-transplant rFSGS, 10 from nrFSGS, 5 from pediatric patients with gFSGS and 8 from healthy blood donors (HBD). As shown in Fig.2A, all rFSGS samples increased significantly the BSA permeability (27 % ± 5.27), while the nrFSGS, gFSGS and HBD samples did not (-2.1 % ± 6.2; 3.0 % ± 3.5; 0.4% ± 5.2 respectively). The differences

between the effects on BSA permeability of rFSGS vs nrFSGS, gFSGS and HBD sera were statistically significant (**P < 0.01).

To verify the reproducibility of BSA permeability assay, serum from 3 patients with rFSGS was used in three independent different experiments at two weeks distance of each other, each experiment was performed in triplicate at the same time. as shown in Fig.2B, the variation among experiments was not statistically significant (Sample1: 40.3 ± 7.2%; 34.7± 6.5%; 32.2 ± 0.9%; ANOVA P = 0.39. Sample 2: 23.6±6.3; 23.8±1.1; 20.1±3.3; ANOVA P=0.69. Sample3: 20.6±1.5; 17.9±2.2; 20.8±1.1; ANOVA P=0.22).

Effects of pre- and post- plasmapheresis sera on the GFB

To verify if PP can change the GFB permeability to albumin, we compared the effects of sera collected pre- and post PP from the 7 rFSGS patients. As shown in Fig.2C, incubation with pre-PP sera induced a consistent increase in BSA permeability, but this effect was no longer evident with sera collected immediately after PP treatment. (26.1 vs -9.5; 23.6 vs -9.4; 32.3 vs -6.7; 20.6 vs -6.0; 28.7 vs -0.63; 22 vs 1.5; 22.6 vs 1.7). The differences between pre- and post PP sera were statistically significant (*P < 0.05; ** P < 0.01).

Immunofluorescence staining

Then we assessed if the changes in BSA permeability induced by rFSGS sera were associated with any change in the expression of some molecules specific for either podocytes or endothelial cells. Fig.3A showed that sera from the majority of patients with rFSGS (5 out of 7) induced an evident decrease of Platelet endothelial cell adhesion molecule (PECAM-1 or CD31) expression on endothelial cells, while sera from nrFSGS and gFSGS did not; the normalized mean fluorescence intensity (% of healthy sera) was: rFSGS, 47.7% ± 34.6; nrFSGS, 100% ± 9.7; gFSGS, 98.1% ± 12.5 respectively) (Fig.3B). Staining with anti-synaptopodin presented the expression along acting filaments and cell processes (arrows) (Fig.4 A-D, I) and anti-podocin showed a filamentous cytoplasmic, cell surface and cell processes(e)? distribution (arrows) (Fig.4 E-H, J). Incubation on endothelial cells? with sera from different patients, which is presumably the initial event occurring in vivo, didn't change the intensity or the distribution of these molecules.

Discussion

We successfully assembled a system of HCiPodo and HCiGEnC co-culture which mimics the in-vivo GFB structure (Fig.1A, B), whose permeability to BSA was highly increased in a dose-dependent way by adding adriamycin (Fig.1C), a chemotherapeutic nephro-toxic drug already utilized for inducing chronic proteinuric nephropathy in rodents [20, 21].

Although some clinical aspects might predict recurrence of FSGS after RTx [2], a reliable and repeatable laboratory methodology could be useful in the clinical practice. Testing the sera drawn from 7 patients with rapid rFSGS after RTx (from 5 to 150 days), we found a significant increase of BSA permeability in all these patients (Fig.2A). The genetic or secondary forms of FSGS are not expected to recur at all (genetic) or need a long time before recurrence (metabolic) after RTx [4]. In fact, we were not able to find any evident change in BSA permeability with sera collected from those patients, some of whom received a RTx, but did not recur, suggesting a high specificity of the test for the rFSGS (Fig.2A).

The repeatability of our method was confirmed by the overlapping results obtained by three independent experiments performed in the same serum sample (Fig. 2B).

PP has been reported to have beneficial effects in some patients with rFSGS [5, 8, 9]. In fact, sera collected before PP in 7 rFSGS patients induced a consistent increase in BSA permeability; these effects were no longer evident when 3LD was challenged with sera collected immediately after PP (Fig. 2C). These results reinforce the suggestion of the presence of cPFs in most patients with FSGS who recurred after RTx [4-12].

Although it has long been hypothesized that rFSGS is the consequence of podocyte damage due to various pathogenic CPFs [22, 23, 24], the initial events occurring when the glomerular endothelial cells come in contact with the hypothesized cPFs are still unknown. Fig.3 showed that 48 hours incubation on endothelial cells with the majority of rFSGS patients sera (5 of 7) induced a noticeable decrease of CD31, while at the same time point the podocytes still presented a normal expression of synaptopodin (Fig.4 A-D, I) and podocin (Fig.4 E-H, J). CD31 is a cellular adhesion and signalling receptor that is highly expressed at endothelial cell–cell junctions and have implications in the maintenance of vascular barrier integrity [25].

More than 15 years ago, Ferrero and colleagues reported that PECAM-1-specific monoclonal antibody augments transit of 125I-labelled albumin across endothelial cell junctions, both in cultured cells and in mice [26]. Our results suggest that the hypothesized cPFs might trigger the pathological events first by damaging the endothelial cells, followed by an increase of GFB permeability and consequently a podocyte damage. It is worth underlining that not all sera from our rFSGS patients (2 of 7) induced a decrease of CD31 expression, implying that the possible mechanism(s) may well be characterized by different pathogenic pathways. So, the purpose for future research could be that of better understanding and defining these possible different pathways.

In conclusion, these preliminary data suggest that the assessment of BSA permeability by our in vitro 3LD could be a valuable method for predicting the recurrence of FSGS after RTx. Moreover this device could be a worthwhile instrument to study separately the biology of podocytes and endothelial cells and their cross-talking during physiological and pathological processes.

Acknowledgments

This study was funded by Fondazione IRCCS Ca' Granda Current Research, Associazione Bambino Nefropatico and Fondazione La Nuova Speranza.

Compliance with ethical standards

Conflict of interest All the authors declared no competing interests. Dr Li and Dr Rastaldi are the inventors of the 3D co-culture method (Patent application PCT/IB2010/003298, 12/2010, filed by Fondazione IRCCS Ospedale Maggiore Policlinico di Milano; Italian Patent December 28, 2012, No. 0001397084; European Patent August 10, 2016, No. EP2513295).

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study. The informed consent of involved patients had been previously approved by the EC of Fondazione IRCCS Ca' Granda Ospedale Maggiore – Policlnico of Milan (ITA) [M. 02. F (A)]

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Legends to Figures

Fig. 1. Co-culture assembly and BSA permeability after adriamycin (ADR) treatment. A) Schematic drawing of the preparation of the in vitro GFB and BSA permeability assay. HCiGEnC and HCiPodo were grown on the opposite of the PET membrane coated both side with Collagen type IV. The three-layer structure divided the system into a lower (endothelial) and upper (podocyte) compartment. For BSA permeability assay, the lower compartment was filled with 40 mg/ml of BSA in cell culture medium while the upper compartment with medium alone. **B)** Immunofluorescence staining with anti-CD31 (left, green), anti-podocin (right, green) and 4', 6-diamidin-2-fenilindolo (DAPI, blue) on cells grown on the membrane. Scale bar: 20 µm. **C)** Incubation on endothelial cell compartment with ADR at different concentration for 24h induced a dose-dependent increase of BSA permeability. * $P < 0.05$, **$p<0.01$. Each bar represents the mean ± SD of triplicate experiments.

Fig. 2. Effect of sera incubation on BSA permeability. A) Effect of sera from patients with diverse forms of FSGS on BSA permeability. Incubation on lower (endothelial) compartment with 2% of sera from recurrent (n = 7), non recurrent (n = 10), genetic (n = 5) FSGS and healthy blood donors (n = 8) for 48h. BSA permeability was consistently increased in all the 7 rFSGS patients, without any relevant increase in the other cases. Tx-rFSGS, renal transplantation with recurrent FSGS; Tx-nrFSGS, renal transplantation without recurrent FSGS; nTx-gFSGS, non renal transplantation with genetic FSGS; HBD, healthy blood donor. **B)** Reproducibility. Variation of BSA permeability among three independent experiments with serum from each of 3 rFSGS patients. The results, expressed as percentages of basal condition, demonstrate no statistically significant difference. E1, E2, E3: experiment 1, 2, 3. **C)** Effect of pre- and post-plasmapheresis sera on BSA permeability. Incubation of sera collected immediately before and after PP showed the complete disappearance of the effect of pre-PP sera on albumin permeability by PP. All results were expressed as percentages of basal condition. Each bar represents the mean ± SD of triplicate experiments. * $P < 0.05$, ** $P< 0.01$

Fig. 3. Immunofluorescence staining on endothelial cells after sera incubation. A) Representative images of CD31 expression. After 48 hours incubation of endothelial cell compartment with 2% sera from

recurrent (n = 7), non recurrent (n = 5), and genetic (n =5) FSGS patients, CD31 expression was considerably decreased in 5 out of 7 rFSGS patients, while there were no evident change in all the others. **B)** Semi quantitative analysis of the mean fluorescence intensity (FI) showed that sera from majority of rFSGS patients decreased the mean FI, while sera from nrFSGS and gFSGS did not. The results were expressed as percentages of FI of healthy control. Scale bar: 50 µm. ** P< 0.01

Fig. 4. Immunofluorescence staining on podocytes after sera incubation. Representative images of expression of synaptopodin (**A**-**D**) and podocin (**E**-**H**). After 48 hours incubation of the endothelial cell compartment with 2% sera from recurrent (n = 7), non recurrent (n = 5), genetic (n = 5) FSGS patients, the intensity as well as the distribution of synaptopodin and podocin were not changed. Semi quantitative analysis of FI of synaptopodin (**I**) and podocin (**J**) showed that there were no significant variations among the different groups. Scale bar: 50 µm