The endothelial surface layer: a new target of research in kidney failure and peritoneal dialysis

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Chapter 3

The Endothelial Surface Layer after Successful Kidney Transplantation

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Abstract

Background
Dialysis patients have alterations of the endothelial surface layer (ESL) and increased plasma levels of glycocalyx constituents. It is not known whether renal transplantation leads to restoration of the endothelial glycocalyx. Here, we investigated the state of ESL in stable renal transplant recipients (RTR), compared to healthy individuals (HI).

Methods
Investigations were performed in 15 stable RTR and 19 HI. Plasma levels of the glycocalyx constituents hyaluronic acid (HA) and syndecan-1, and hyaluronidase activity were measured by ELISA. The ESL was assessed by sidestream darkfield imaging of the sublingual microvasculature and the perfused boundary region (PBR), which reflects the erythrocyte permeable part of the ESL, was measured.

Results
The estimated glomerular filtration rate (eGFR) was 47 (39-67) ml/min/1.73m² in RTR and 98 (92.5-104) ml/min/1.73m² in HI (p<0.001). Plasma HA levels were similar in RTR and HI: 25.3 ± 11 ng/ml versus 20.5 ± 11 ng/ml, p=0.25; syndecan-1 concentration and hyaluronidase activity were slightly higher in patients compared to HI: 30.8 ± 15 versus 20.4 ± 12 ng/ml, and 25.8 (24.3-27.9) U/ml versus 23.6 (21.1-26.6) U/ml, p=0.06 for both. PBR was thicker in patients compared to HI (3.3 ± 0.4 vs 2.9 ± 0.5 µm, p=0.005). In addition, correlations were present between PBR and both eGFR (p=0.004, r=-0.51) and syndecan-1 (p=0.005, Spearman’s rho=0.49).

Conclusions
After successful renal transplantation no significant changes in plasma levels of HA, syndecan-1 and hyaluronidase activity were found as compared to HI. However, the thicker perfused boundary region suggests that alterations of the ESL may still be present in these patients and their magnitude is dependent on the graft function.
Introduction

Amongst the different renal replacement therapies, successful renal transplantation confers the highest survival benefit. However, mortality caused by cardiovascular disease remains increased in renal transplant recipients (RTR) as compared to healthy individuals (HI). This suggests the presence of endothelial dysfunction, which may be due to the preceding renal failure, but also to persistent cardiovascular disease and/or effects of the immunosuppressive treatment.

Essential for the vascular homeostasis is an unaltered endothelial glycocalyx (EG). This negatively charged, highly hydrated mesh of proteoglycans (PG), glycoproteins and glycosaminoglycans (GAGs) covers the vascular endothelium, and together with associated plasma molecules forms the endothelial surface layer (ESL). Under physiological conditions, the endothelial glycocalyx has several well defined functions aimed at preserving the integrity of the vessel wall. Thus, it is involved in the regulation of vascular permeability, lipid homeostasis, mechanotransduction of shear stress, redox regulation, leucocyte adhesion and (anti) coagulant responses. Several data indicate that disruption of the glycocalyx results in increased vascular vulnerability and accelerates atherosclerosis.

Recently, our group and others have shown that patients with end stage renal failure (ESRD) on dialysis have alterations of the ESL and high concentrations of glycocalyx constituents in plasma. Also, relationships were found between levels of circulating glycocalyx components and markers of endothelial activation and dysfunction. Here, we questioned whether successful renal transplantation is associated with restoration of the endothelial glycocalyx, contributing to the improved risk profile of this patient group. The measurements of plasma levels of glycocalyx components represent a useful tool for the assessment of ESL in humans. Hyaluronic acid (HA), the only non-sulfated GAG within the endothelial glycocalyx, is essential for the maintenance of vascular integrity. It is involved in the regulation of vascular permeability, mechanotransduction of fluid shear stress, leucocyte adhesion. Syndecan-1 is a transmembrane heparan sulfate (HS) PG, which binds various ligands via its GAGs side chains, and subsequently modulates inflammation, angiogenesis, microbial attachment and entry, matrix remodeling, and carcinogenesis. In this study we investigated the state of the ESL in stable renal transplant recipients and compared the data to those of healthy individuals. HA, syndecan-1 and the activity of the regulating enzyme hyaluronidase, were measured in plasma. In addition, using sidestream darkfield (SDF) imaging of the sublingual microcirculation, we measured the perfused boundary region (PBR), which reflects the erythrocyte permeable part of the ESL.
Materials and methods

Study design and subjects
We performed a cross-sectional study and included 15 transplant recipients of a first renal allograft, with age between 18 and 70 years, who were treated with prednisolone in combination with cyclosporine. In addition, we studied 19 age and sex-matched healthy individuals. All patients received their kidney transplant between 2001 and 2008. Their immunosuppressive regimen included quadruple therapy (basiliximab, prednisolone, mycophenolate sodium and cyclosporin) in the first 6 months after transplantation, followed by double therapy with prednisolone and cyclosporine. All RTR had a stable renal allograft function (estimated glomerular filtration rate >30 ml/min/1.73m²), and no change in the immunosuppressive regimen in the previous 6 months. Exclusion criteria were: smoking, history of diabetes mellitus, any recent acute inflammatory episode, the use of angiotensin converting enzyme inhibitors or angiotensin receptor blockers on the day of the measurements, use of anti-oxidants. The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the Committee of Medical Ethics of the Academic Medical Center, University of Amsterdam. Written informed consent was obtained from all participants.

Laboratory measurements
Blood was drawn done after an overnight fast. Blood pressure, heart rate and body weight were also measured. All routine laboratory measurements were performed in the central laboratory of the Academic Medical Center on a Hitachi P-800 (Roche Diagnostics, Germany), with reagents provided by the same company. Glucose, creatinine, alanine aminotransferase, and urea were measured by enzymatic methods. The glycated hemoglobin (HbA1c) was measured in patients by HPLC, and C-reactive protein (CRP) by an immunoturbidimetric assay. Total cholesterol was measured by colorimetry and LDL cholesterol was calculated with the Friedewald formula. Estimated glomerular filtration rate (eGFR) was calculated using the abbreviated MDRD formula: GFR = 175 x (Pcr ÷ 88.4)-1.154 x age-0.203 (female: multiply result by 0.742, black: multiply result by 1.210).

Circulating glycocalyx constituents and regulating enzyme
Quantitative total plasma hyaluronic acid levels were measured by enzyme-linked immunosorbent assay (ELISA) (Corgenix Inc., Broomfield, Colorado, USA), as was syndecan-1 (Diaclone, Gen-Probe Inc., California, USA). Hyaluronidase activity was determined using an assay previously described, it could not be determined in nine of the healthy individuals because of unavailability of blood samples.

Sidestream Darkfield (SDF) imaging of the microcirculation
Intravital microscopic imaging of the sublingual microvasculature was performed all study participants using a SDF MicroScan videomicroscope (MicroVision Medical
Inc., Wallingford, PA, USA). An extensive description of the method has been given previously. In short, movies of the sublingual microvasculature are recorded, and the red blood cell column width (RBCW) is measured in blood vessels with a diameter up to 50 µm. Images were collected with a 5x objective with a 0.2 NA providing a 325-fold magnification on screen and were sized 720x576 pixels. The frame rate was 23/s. Video sequences of 40 consecutive frames of 950x700 µm sublingual tissue surface area were recorded using Streampix software (Norpix Inc. Montreal, Canada) in at least 10 different areas. All visible vessels are automatically identified and measurements lines are placed every 10 µm, perpendicular to the vessel direction. At each measurement sites intensity profiles were obtained and RBCW was measured. A maximum of 3000 measurements were obtained per individual. By measuring the dynamic of RBCW near the vessel wall, the perfused boundary region (PBR) is calculated, which reflects the erythrocyte permeable part of the ESL (GlycoCheck B.V.).

Statistical analysis
Results are expressed as median and interquartile range or mean and standard deviation, depending on the distribution of the data. Between-group differences were analysed using the unpaired t-test, Mann Whitney U test, or \( \chi^2 \)-test as appropriate. Bivariate correlations between different variables were assessed using Spearman of Pearson correlation test. For statistical analyses, we used SPSS 20.0 (Chicago, IL, USA).

Results
Patients
Two patients were excluded from the analysis because of the presence of liver disease, which may interfere with the breakdown of the glycocalyx constituents.

Clinical characteristics
The baseline characteristics of the study participants are summarized in Table 1. As expected, RTR had a lower eGFR, higher systolic and diastolic blood pressure, and a different lipid profile compared with healthy individuals.

Circulating glycocalyx constituents
Hyaluronic acid levels were similar in RTR and HI: 25.3 ± 10.7 and 20.5 ± 11.3 ng/ml, p=0.25, as shown in Figure 1. Both syndecan-1 and hyaluronidase activity were moderately increased in RTR, but the difference did not reach statistical significance (syndecan-1: 30.7 ± 15.1 versus 20.4 ± 12 ng/ml, p=0.06, and hyaluronidase activity: 25.8 (24.3-27.9) versus 23.6 (21.1-26.6) U/ml in HI, p=0.06).
Imaging of the microcirculation
PBR was significantly increased in patients compared to controls (3.3 ± 0.4 versus 2.9 ± 0.5 µm, p=0.005).

Relationships between glycocalyx parameters and other clinical and biochemical parameters
Relationships were present between the perfused boundary region and both eGFR (p=0.004, r=-0.51) and syndecan-1 (p=0.005, Spearman’s rho=0.49). Neither syndecan-1 nor HA was associated with renal function. A positive correlation was present between HA levels and age (p=0.004, Spearman’s rho=0.50). We found no other relationships between the parameters for ESL and other biochemical or clinical parameters, including the blood pressure, lipid profile, glucose, glycated haemoglobin, time on dialysis or time since transplantation (data not shown).

Table 1. Baseline characteristics of renal transplant recipients and healthy individuals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Renal transplant recipients (n=13)</th>
<th>Healthy individuals (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>48.2 ±10.5</td>
<td>50.0 ± 9.9</td>
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<tr>
<td>Male gender n (%)</td>
<td>8 (61.5)</td>
<td>11 (57.9)</td>
</tr>
<tr>
<td>Time since transplantation (mo)</td>
<td>29 (23.5-83)</td>
<td>-</td>
</tr>
<tr>
<td>Time on dialysis (mo)</td>
<td>29.9± 26.4</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.4 ± 4.8</td>
<td>24.5 ± 3.6</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>142 ± 19</td>
<td>125 ± 16*</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>88 ± 11</td>
<td>76 ± 9*</td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73m²)</td>
<td>47 (39-67)</td>
<td>98 (92.5-104)*</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>5.2 (4.8-5.6)</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.0 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>ALAT (U/l)</td>
<td>28 (20-51)</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>4.8 ± 0.7</td>
<td>5.5 ± 0.8*</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>2.4 ± 0.5</td>
<td>3.3 ± 0.8*</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.7 (0.6-2.0)</td>
<td>1.0 (1.0-1.2)</td>
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<tr>
<td>Statins (%)</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Antihypertensives (%)</td>
<td>75</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation) or median (inter-quartile range) depending on the distribution. ALAT: alanine aminotransferase; BMI: body mass index; BP: blood pressure; TC: total cholesterol; eGFR estimated by the abbreviated MDRD. *p<0.05 Mann Whitney U test or t- test depending on the distribution of the data.
Measurements of circulating glycocalyx constituents in stable RTR with moderately reduced kidney function, showed normal or slightly increased plasma levels of HA, syndecan-1 and hyaluronidase activity compared to HI. Analysis of the ESL by SDF imaging of the sublingual microvasculature identified a significantly thicker perfused boundary region in patients compared to controls.

The direct assessment of the endothelial glycocalyx in vivo is challenging and hence plasma concentrations of its constituents have been used to assess the state of EG in humans. Although not specific for the endothelial cells, high plasma levels of HA and syndecan-1 have been associated with alterations of the endothelial glycocalyx in various conditions, e.g., kidney failure, ischemia-reperfusion, diabetes mellitus (DM), sepsis.14-16,20-23

Discussion

Figure 1. Hyaluronan, syndecan-1 and hyaluronidase activity have similar levels in renal transplant recipients (RTR) and healthy individuals (HI). The perfused boundary region is significantly increased in RTR compared to HI. Mean with SEM are shown.
Also, robust correlations of their plasma concentrations with markers of endothelial dysfunction have been reported in kidney disease.\textsuperscript{16,24} HA is present in the extracellular matrix but is also an essential constituent of the EG, where it is critical for the vascular homeostasis. Renal excretion is of minor importance for the clearance of hyaluronan from circulation, and therefore, of no significance in patients with good renal function.\textsuperscript{25} Hyaluronidases but also inflammatory cytokines and reactive oxygen species are involved in the degradation of HA.\textsuperscript{17} Within the EG, hyaluronidase binds HA but also other GAGs, like HS and chondroitin sulfate (CS). Therefore, the plasma hyaluronidase activity is indicative for its glycocalyx degrading capacity, and increased values have been found in conditions associated with alterations of ESL in patients with type 1 and type 2 DM, and uremia.\textsuperscript{14,25-27} Syndecan-1 is a proteoglycan present primarily on epithelial and plasma cells, but also expressed by endothelial cells.\textsuperscript{7} It has attachment sites for both HS and CS,\textsuperscript{28} binds various chemokines, growth factors, cytokines, extracellular matrix components, and thus functions primarily as co-receptor.\textsuperscript{29} The syndecan-1 ectodomain is shed from the cell surface via various processes, including proteolytic or oxidative mechanisms stimulated by inflammation.\textsuperscript{18} In kidney disease, plasma levels of syndecan-1 are elevated in ESRD and chronic kidney disease stage 4, but normalize in RTR with good or reduced graft function.\textsuperscript{14-16} Also, high levels were found in microalbuminuric type 1 DM patients but not in matched patients with normoalbuminuria, suggesting that this proteoglycan may be important in the pathogenesis of diabetic nephropathy.\textsuperscript{30} A recent study excluded a role for renal accumulation in the high syndecan-1 levels reported in kidney disease,\textsuperscript{16} suggesting the importance of increased shedding in this setting. Although an association between renal function and plasma levels of syndecan-1 has been previously reported,\textsuperscript{15,16} we did not confirm this finding in our group of patients, possibly due to the limited number of patients.

The measurement of the erythrocyte permeable region of the ESL by non-invasive imaging of the sublingual microvasculature showed a thicker PBR in RTR compared to HI. This reflects increased accessibility to RBCs and is suggestive of a disrupted endothelial surface layer. Our data seems to be at odds with a study that reported similar thickness of PBR in RTR and HI.\textsuperscript{15} This discrepancy can be explained by the different renal function in the two patient groups, as our transplant recipients had lower eGFR compared to the other study. Importantly, in both studies, a thicker PBR was associated with a low eGFR, suggesting the important effect of reduced renal function on the ESL. Interestingly, in our study higher syndecan-1 levels were associated with a thicker PBR. The RBC excluding properties of the ESL are determined by the complex organization and the interactions between its components. To date little is known about the exact contribution of each component to the mechanical stability of this structure.
A limitation of our study is the cross-sectional design which does not allow for follow-up data. In addition, the direct effects of the immunosuppressive medication on the ESL were not addressed. Both calcineurin inhibitors and glucocorticoids have been associated with the development of hypertension, dyslipidemia and diabetes, and have been shown to promote the development of atherosclerosis. Taking into account the effects on the endothelium, we cannot rule out an effect of immunosuppression on the EG.

Although renal transplantation cannot erase the associated cardiovascular burden, it does solve the uremia related complications, leading to less oxidative stress and less inflammation. Whether this leads to restoration of the endothelial glycocalyx and reduced vascular vulnerability which may contribute to the improved prognosis of these patients, is not known. Here we show that successful renal transplantation is associated with normalization of plasma level of glycocalyx constituents and regulating enzyme, which is indicative of a normal turnover within this structure. However, disruption of the endothelial surface layer is still present and its magnitude is determined by the graft function.

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