Antibodies to kidney endothelial cells contribute to a “leaky” glomerular barrier in patients with chronic kidney diseases

Nidia Maritza Hernandez,1 Anna Casselbrant,2 Meghnad Joshi,1 Bengt R. Johansson,3 and Suchitra Sumitran-Holgersson1

Departments of 1Transplantation Surgery, 2Gastrointestinal Research, and 3Medical Chemistry and Cell Biology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

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Hernandez NM, Casselbrant A, Joshi M, Johansson BR, Sumitran-Holgersson S. Antibodies to kidney endothelial cells contribute to a “leaky” glomerular barrier in patients with chronic kidney diseases. Am J Physiol Renal Physiol 302:F884–F894, 2012. First published December 21, 2011; doi:10.1152/ajprenal.00250.2011.—Anti-endothelial cell antibodies (AECA) have been reported to cause endothelial dysfunction, but their clinical importance for tissue-specific endothelial cells is not clear. We hypothesized that AECA reactive with human kidney endothelial cells (HKEC) may cause renal endothelial dysfunction in patients with chronic kidney diseases. We report that a higher fraction (56%) of end-stage renal disease (ESRD) patients than healthy controls (5%) have AECA reactive against kidney endothelial cells (P <0.001). The presence of antibodies was associated with female gender (P < 0.001), systolic hypertension (P < 0.01), and elevated TNF-α (P < 0.05). These antibodies markedly decrease expression of both adherens and tight junction proteins VE-cadherin, claudin-1, and zona occludens-1 and provoked a rapid increase in cytosolic free Ca2+ and rearrangement of actin filaments in HKEC compared with controls. This was followed by an enhancement in protein flux and phosphorylation of VE-cadherin, events associated with augmented endothelial cell permeability. Additionally, kidney biopsies from ESRD patients with AECA but not controls demonstrated a marked decrease in adherens and tight junctions in glomerular endothelium, confirming our in vitro data. In summary, our data demonstrate a causal link between AECA and their capacity to induce alterations in glomerular vascular permeability.

CHRONIC KIDNEY DISEASE (CKD) afflicts a very high number of individuals and can be identified by the presence of abnormal quantities of albumin in the urine or a low glomerular filtration rate (GFR) (9). Microvascular irregularity of the blood-urine interface within kidney glomeruli, leading to microalbuminuria, may reflect the renal sign of global endothelial dysfunction and has been associated with an elevated risk of serious cardiovascular events in these patients (25, 33). Despite this, the exact pathophysiological mechanisms leading to vascular irregularity within the kidney glomeruli are not well understood.

The renal vasculature regulates permeability and modulates vasomotor, inflammatory, and hemostatic responses. Impairment of these vital endothelial cell (EC) functions can contribute to augmented capillary permeability, increased levels of albumin in the urine, reduced renal perfusion, and, subsequently, a decrease in GFR. Increased endothelial permeability in some models of renal ischemic and oxidative injury has been associated with the redistribution of endothelial tight junction (TJ) proteins and the disassociation and internalization of vascular endothelial (VE)-cadherin from endothelial adherens junctions (AJ) (43). Morphological alterations of EC after ischemia are observed with functional consequences characterized by loss of normal endothelial function and/or barrier (7, 28, 31, 43). Thus glomerular endothelial dysfunction might play a pivotal role in the derangement of glomerular permeability. Understanding the underlying molecular mechanisms and structural changes leading to a “leaky” glomerular “barrier” is therefore of importance.

TJ serve the major functional purpose of providing a “fence” within the membrane, by regulating paracellular permeability and maintaining cell polarity. Many TJ protein components have been defined with complex composition and function, e.g., occludins, claudins, and junctional adhesion molecules and intracellular proteins, such as zona occludens (ZO-1) (18, 30). On the other hand, AJ play an important role in contact inhibition of EC growth, paracellular permeability to circulating leukocytes, and solutes (3, 50). EC express a specific AJ called VE-cadherin (48). Disruption of endothelial AJ in vivo has been demonstrated to induce gaps between EC and increase in endothelial permeability, as well as enhanced leukocyte infiltration reflecting compromise of the semiselective EC barrier that controls the movement of fluids, macromolecules, and leukocytes between vascular compartments and the interstitium (15, 45, 47). These findings underscore the importance of EC-cell junctions in maintaining the integrity of the endothelial permeability barrier.

Antibodies that react with the surface of vascular EC (anti-endothelial cell antibodies; AECA) are found in a variety of diseases associated with vascular injury, including systemic sclerosis, systemic lupus erythematosus (SLE), Takayasu’s arteritis, Wegener’s granulomatosis, Behcet’s syndrome, and transplant arteriosclerosis (4, 37). A number of mechanisms have been proposed whereby AECA binding to EC may exert pathogenic effects, including the induction of EC inflammatory activation and thrombogenicity, the stimulation of leukocyte free radical production and cellular cytotoxicity, and the induction of EC apoptosis (6, 20, 34, 35). Investigations of these mechanisms have traditionally used EC isolated from easily accessible tissues such as large vessels of the human umbilical cord (human umbilical vein endothelial cells; HUVEC). Today, with more recent knowledge in the field of EC heterogeneity, it is clear that there are many fundamental differences between different organs, within the vascular loop of a given organ as well as between neighboring EC of a single blood
vessel (1, 11, 13, 23, 38). Therefore, the endothelium of various vascular beds is phenotypically and functionally heterogeneous, and the use of HUVEC as targets may not always be clinically relevant. Existing data provide indirect support for a pathogenic role for AECA in lupus nephritis and uremic patients on hemodialysis. AECA were associated with histological evidence of active renal injury and serological evidence of EC dysfunction (24, 49). However, factors affecting dysfunction of the glomerular endothelium are poorly understood, and the exact molecular mechanisms leading to the loss of the renal endothelium filtration barrier integrity remain unclear.

In this pilot study, we investigated whether AECA in end-stage renal disease (ESRD) patients reactive with tissue-specific EC such as human kidney endothelial cells (HKEC) cause glomerular endothelial dysfunction and impair kidney vascular permeability. Since TJ and AJ control permeability, we examined the functional effects of these antibodies on TJ and AJ expressed in HKEC.

**MATERIALS AND METHODS**

**Patients**

This cohort of patients represents part of an ongoing prospective cohort study, which has been described before (41) (Table 1). Informed consent was obtained from each individual. A total of 45 ESRD patients (22 men), aged 52 ± 12 yr, were included, enrolled at a time point close to the start of renal replacement therapy with a median GFR 5.7 (range 1.9–13.7 ml/min). The exclusion criteria were an age below 18 or above 70 yr, clinical signs of acute infection, a time point close to the start of renal replacement therapy with a

**Table 1. Clinical characteristics of ESRD patients**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AECA Negative (n = 20)</th>
<th>AECA Positive (n = 11)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>52 ± 11</td>
<td>51 ± 7</td>
<td>NS</td>
</tr>
<tr>
<td>Men, %</td>
<td>80</td>
<td>36</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25 ± 3</td>
<td>23 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>30</td>
<td>36</td>
<td>28</td>
</tr>
<tr>
<td>Cardiovascular disease, %</td>
<td>30</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>6.2 (3.4–13.7)</td>
<td>5.7 (1.9–10.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum albumin, g/l</td>
<td>33 ± 5</td>
<td>32 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>hs-CRP, mg/l</td>
<td>2.9 (0.3–31.0)</td>
<td>5.3 (0.2–218.0)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6, pg/l</td>
<td>6.1 (0.8–24.0)</td>
<td>11 (1.1–43.0)</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α, pg/l</td>
<td>10.5 (6.1–17.3)</td>
<td>13.3 (5.2–22.0)</td>
<td>0.05</td>
</tr>
<tr>
<td>IL-10, pg/l</td>
<td>1.2 (0.9–9.7)</td>
<td>1.7 (0.9–131)</td>
<td>NS</td>
</tr>
<tr>
<td>ICAM-1, ng/ml</td>
<td>229 (161–557)</td>
<td>223 (120–398)</td>
<td>NS</td>
</tr>
<tr>
<td>VCAM-1, ng/ml</td>
<td>1455 (885–2051)</td>
<td>1355 (891–1835)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum creatinine, μmol/l</td>
<td>849 ± 264</td>
<td>639 ± 191</td>
<td>0.08</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>1.9 ± 1.1</td>
<td>1.6 ± 0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>150 ± 17</td>
<td>134 ± 15</td>
<td>0.005</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>86 ± 11</td>
<td>79 ± 13</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Values are means ± SD. ESRD, end-stage renal disease; AECA, anti-endothelial cell antibodies; BMI, body mass index; GFR, glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; NS, not significant. The group of patients with activity are divided according to low (≤1:50) or high (≥1:100) anti-kidney endothelial cell antibody titers.

**Blood Samples and Biochemical Analyses**

Morning blood samples were taken after an overnight fast for generation of plasma and serum and were stored at −70°C pending analyses, if not analyzed immediately. Blood pressure was recorded on the same occasion.

**Isolation and Cultivation of HKEC**

HKEC were freshly isolated from one kidney donor (intended but not used for transplantation as no suitable recipient was found) and cultivated as described earlier (17). In short, kidney tissue was manually dissected and minced (into small cubes) and enzymatically digested with dispase (1.6 U/ml) overnight at 4°C. Thereafter, the individual tissue pieces were manually disaggregated with a flat instrument to release the EC. The decanted cell suspensions were centrifuged at 5,000 g 4°C for 10 min, and the organ-specific EC were isolated on a density gradient of 35% Percoll (Sigma, St. Louis, MO) at 5,000 g 4°C for 10 min. The top band of the gradients was collected, and the cells were washed in PBS, seeded on gelatin-coated tissue culture flasks, and grown at 37°C in a humidified atmosphere of 5% CO2 in air. In cultures where fibroblast contamination was observed, the EC were handpicked by adding a few drops of trypsin on the EC colony. The detached cells were then picked by a fine pipette and transferred to a different culture dish for further culture.

Morning blood samples were taken after an overnight fast for generation of plasma and serum and were stored at −70°C pending analyses, if not analyzed immediately. Blood pressure was recorded on the same occasion. GFR, corrected for body surface area, was estimated as the mean of urea and creatinine clearance from 24-h urinary samples. Serum concentrations of VCAM-1, ICAM-1, IL-6, and TNF-α were measured by using commercially available ELISA assays (R&D Systems, Minneapolis, MN). Serum cholesterol and triglyceride levels were analyzed by means of standard enzymatic procedures (Roche Diagnostics, Mannheim, Germany). Serum albumin (bronnecrulep method), high sensitivity C-reactive protein (hsCRP), HbA1C, and urinary creatinine and urea were determined by routine procedures at the Department of Clinical Chemistry, Karolinska University Hospital Huddinge.

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Cell monolayers were passaged at confluence using trypsin-EDTA. HKEC were routinely cultured on 0.5% gelatin-coated culture flasks in MCDB 131, which was further supplemented with EGM-2 single andquot; (Clonetics, Stockholm, Sweden). The addition of 50 ng/ml VEGF was made to maintain fenestrae in HKEC. HUVEC were used as control cells (Clonetics). Capillary formation in Matrigel and morphological characterization of HKEC were performed by light microscopy. Scanning electron microscopy was used to detect fenestrae in the EC.

**Screening of Patient Sera for AECA by Flow Cytometry**

In all experiments, one set of cell samples remained untreated, while another set was stimulated for 16 h with recombinant human IFN-γ (200 ng/ml) and TNF-α (20 ng/ml). For the flow cytometric assay, unstimulated/stimulated HKEC/HUVEC were used for the screening of AECA in sera of 45 ESRD patients and 20 healthy controls. Sera from healthy nontransfused blood group AB men known not to have any antibodies served as negative controls. A pool of sera from patients who had formed alloantibodies as a result of multiple blood transfusions or organ transplantations was used as a positive control. For the assay, 5 × 10⁵ cells were incubated with 50 μl of patient serum for 1 h at room temperature (rt), and then washed twice with PBS. Ten microliters of (4 μg/ml) diluted fluoresceinated F(ab′)₂ fragments of goat anti-human IgG (Fc specific) and anti-human IgM (μ chain specific) antibodies were added and incubated at 4°C on ice for 30 min. The cells were washed, resuspended in 200 μl of PBS, and immediately before analysis 4 μl of propidium iodide (PI; 50 μg/ml) was added to detect dead cells. Cells were then analyzed on a Becton Dickinson flow cytometer (FACSorter, Becton Dickinson). Fluorescence signals from 10,000 cells were counted, and the percentage of FITC-positive cells was recorded. A shift in the mean fluorescence of 20 channels in the test sample compared with negative control was considered as positive, determined as described before (29). All sera giving positive reactions were further diluted 1:50, 1:100, and 1:500 to determine titers of AECA.

**Preparation of IgG Fractions From Sera of AECA-Positive and AECA-Negative Patients**

Sera from ESRD patients with AECA reactive against HKEC and patients without AECA were pooled separately. Total IgG fractions were isolated using goat anti-human IgG (Fc-chain specific) agarose beads 127 (Sigma-Aldrich Sweden, Stockholm, Sweden) according to the standard protocol, and IgG concentration was determined by the beads 127 (Sigma-Aldrich Sweden, Stockholm, Sweden) according to the manufacturer’s recommendations. After about 10 s of recording, 50 μl of IgG from ESRD patients with or without AECA or normal IgG were added to the cells in final concentrations of 1.0, 0.5, 0.3, and 0.1 mg/ml. The total recording time was 200 s. For analysis of F-actin formation, the ability of IgG to mobilize calcium flux, which is a primary feature of cell activity, was tested as described earlier (29, 44) using HKEC and HUVEC. The cells were incubated with 5 μM fura 2-AM (Calbiochem, La Jolla, CA) and 0.3 mg/ml pluronic F-127 (Sigma-Aldrich Sweden) in HBSS (Ca and Mg included, Invitrogen, Stockholm, Sweden) with 10 mM HEPES for 30 min at 37°C and 5% CO₂. The results were recorded as the ratio of fluorescence between 340 and 380 nm, calibrated and calculated with commercially available software (MiraCal, Life Science Resources) according to the manufacturer’s recommendations. After about 10 s of recording, 50 μl of IgG from ESRD patients with or without AECA or normal IgG were added to the cells in final concentrations of 1.0, 0.5, 0.3, and 0.1 mg/ml. The total recording time was 200 s. For analysis of F-actin formation in HKEC in response to AECA IgG activation, confluent HKEC monolayers grown on gelatin-coated coverslips were incubated for 15 min at 37°C with either purified IgG from ESRD patients with or without AECA or from controls in medium identical to the procedures described above. The monolayers were fixed in 3.7% formaldehyde in PBS for 10 min at rt, washed twice, and permeabilized with 0.2% Triton X-100 in PBS (1 min at 4°C). The cells were

**Measurement of EC Barrier Function**

HKEC were cultured as described previously. HKEC in the third passage were detached by a brief (2 min) trypsin-EDTA treatment (0.25% trypsin/0.01% EDTA) and replated onto detachable cell culture inserts (Snapwells, 12-mm diameter, 0.4-μm pore size Corning, Costar, Cambridge, MA). To promote cellular differentiation and enhance attachment of EC, the filters were pretreated with 50 μl 0.2% gelatin and air-dried. EC were seeded at a density of 2 × 10⁵ cells/filter and incubated in culture medium at 37°C in a humidified atmosphere of 5% CO₂ in air. The EC were grown to confluent monolayers, as assessed by daily microscopic observation and were then treated overnight with CKD-AECA or normal IgG for subsequent Ussing chamber experiments.

**Ussing chamber experiments.** EC membranes were detached from the inserts and mounted in the chambers using Krebs buffer solution (118.07 mM NaCl, 4.69 mM KCl, 2.52 mM CaCl₂, 1.16 mM MgSO₄, 1.01 mM NaH₂PO₄, 25 mM NaHCO₃, and 11.10 mM glucose), maintained at 37°C continuously oxygenated with 95% O₂-5% CO₂, and stirred by gas flow. FITC-dextran (Mw 4,000, Mw 70,000, and Mw 150,000, Sigma) was added to the lumen of the half-chamber to a final concentration of 0.15 mg/ml, respectively. Epithelial electrical resistance was assessed by use of the Ussing pulse method, a method having the advantage of estimating specifically epithelial electrical resistance and which is described in detail elsewhere (11a). In the present setup, data sampling and pulse inductions were computer controlled using specially constructed hardware and software developed in LabView (National Instruments, Austin, TX). Serosal samples of 0.2 ml were then collected at 5, 10, 20, 40, 60, 90, and 120 min after the start and analyzed. The luminal-to-serosal permeability of the probes was measured by fluorescence at wavelengths of 480 and 535 nm for excitation and emission, respectively.

**Determination of EC Intracellular Ca²⁺ Concentration and F-Actin Formation**

The ability of IgG to mobilize calcium flux, which is a primary feature of cell activity, was tested as described earlier (29, 44) using HKEC and HUVEC. The cells were incubated with 5 μM fura 2-AM (CalBiochem, La Jolla, CA) and 0.3 mg/ml pluronic F-127 (Sigma-Aldrich Sweden) in HBSS (Ca and Mg included, Invitrogen, Stockholm, Sweden) with 10 mM HEPES for 30 min at 37°C and 5% CO₂. The results were recorded as the ratio of fluorescence between 340 and 380 nm, calibrated and calculated with commercially available software (MiraCal, Life Science Resources) according to the manufacturer’s recommendations. After about 10 s of recording, 50 μl of IgG from ESRD patients with or without AECA or normal IgG were added to the cells in final concentrations of 1.0, 0.5, 0.3, and 0.1 mg/ml. The total recording time was 200 s. For analysis of F-actin formation in HKEC in response to AECA IgG activation, confluent HKEC monolayers grown on gelatin-coated coverslips were incubated for 15 min at 37°C with either purified IgG from ESRD patients with or without AECA or from controls in medium identical to the procedures described above. The monolayers were fixed in 3.7% formaldehyde in PBS for 10 min at rt, washed twice, and permeabilized with 0.2% Triton X-100 in PBS (1 min at 4°C). The cells were

**Immunocytochemistry for Endothelial Adherens and TJ Proteins**

Tissue culture plates with a 0.2% gelatin coating were used to grow 3 × 10⁵ HKEC. Cells were allowed to attach (24 h) before staining. For immunocytochemistry, cells were washed twice with PBS and incubated with either AECA-positive IgG fractions from ESRD patients or controls. The primary monoclonal antibodies used were anti-human claudin-1, ZO-1, and occludin-1 (Zymed Laboratories, San Francisco, CA). The secondary antibodies used were CY3-conjugated goat anti-mouse antibodies (diluted 1:500 in PBS, Jackson ImmunoResearch) and Alexa Fluor 488 donkey anti-mouse (diluted 1:100, Invitrogen). After incubation for 1 h at 4°C on ice, the cells were washed twice with PBS, nuclei staining with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) was performed, and specimens were mounted and dried for microscopic analysis under a confocal fluorescence microscope (Olympus).

**Binding of Purified AECA-Positive IgG to HKEC by Flow Cytometry**

Flow cytometric analysis as described earlier was performed to demonstrate that purified IgG fractions retained the capacity to bind HKEC. Similarly, HUVEC, human aortic EC (HAEC), and human lung EC (HLEC) primary cells were used to examine whether IgG was directed against other EC. Stained cells were analyzed by flow cytometry. Using initial experiments, a concentration of 3 mg/ml of ESRD IgG was estimated as optimal for activation of HKEC and was used for all subsequent analysis.
washed twice and stained for actin filaments with FITC-conjugated phalloidin (Sigma-Aldrich Sweden) for 20 min at 37°C. After three additional washes, the HKEC were viewed in a fluorescence microscope.

Tyrosine Phosphorylation of VE-Cadherin

Tyrosine phosphorylation of VE-cadherin is known to be associated with increased EC permeability. We therefore investigated whether antibodies to HKEC cause phosphorylation of VE-cadherin. For this purpose, we performed two different analyses: 1) immunocytochemistry as described above and 2) Western blotting. HKEC were stimulated with either 3 mg/ml normal IgG or IgG from AECA-positive or AECA-negative patients for 30 min, 1 h, and 16 h. One set of cells was left unstimulated. Recombinant human VEGF (100 ng/ml)-stimulated cells were used as a positive control (19). Cell lysates prepared as described previously (42) were immunoblotted with anti-phospho VE-cadherin antibodies (dilution 1:200; Invitrogen) using standard SDS-PAGE and Western blot analysis.

Kidney Biopsy Staining

Kidney biopsies were obtained from six ESRD patients with AECA, three without AECA, and four normal kidney samples from transplanted donors. Kidney specimens were fixed in 4% neutral-buffered formaldehyde at 4°C for 24 h and then dehydrated in an alcohol series, treated with xylene, and embedded in paraffin. Four-micrometer sections were cut and placed on microscope slides. After deparaffinization, immunostaining was performed by the biotin-peroxidase complex method. Antigen retrieval was done using 10 mmol citrate buffer (pH 6.0) for occludin and claudin-1 in a pressure cooker at 100°C for 20 min followed by 20-min cooling time. ZO-1 and VE-cadherin were done using proteinase K (20 mmol/ml, Mbiotech, Seoul, Korea) for 15 min. Endogenous peroxidase activity was quenched by 1% H2O2, and nonspecific binding was blocked with 5% horse serum. The slides were incubated overnight at 4°C with anti-human ZO-1 (1:200), occludin (1:200), claudin 1 (1:100), (Invitrogen, Carlsbad CA), and VE-cadherin (1:50; BD Biosciences Pharmingen, San Jose CA) and then washed three times with PBS for 10 min.

Table 2. Phenotypic characteristics of endothelial cells isolated from human kidney and umbilical cord vein

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>CD 141</th>
<th>CD 142*</th>
<th>CD 144</th>
<th>Acetyl-ated LDL</th>
<th>Ulex Europaus</th>
<th>CD 106*</th>
<th>CD 62E*</th>
<th>CD31</th>
<th>vWF*</th>
<th>CD105</th>
<th>Fibroblast</th>
<th>α-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HKEC</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

HUVEC, human umbilical cord vein endothelial cells; HKEC, human kidney endothelial cells; vWF, von Willebrand factor, *Expressed only activated endothelial cells.

Fig. 1. A: human kidney endothelial cells (HKEC) exhibited typical endothelial cell morphology when grown in endothelial cell-selective medium; magnification ×40. B and C: the presence of fenestrae using scanning electron microscopy. D: HKEC formed capillary-like structures in Matrigel; magnification ×40. E: flow cytometric analysis demonstrating the strong binding of IgG isolated fractions from anti-endothelial cell antibodies (AECA)-positive end-stage renal disease (ESRD) patients from (0.5 mg/ml; filled histogram) to HKEC, but not to human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells (HAEC), or human lung microvascular endothelial cells (HLEC). Normal IgG (0.5 mg/ml; grey lines) and AECA-negative IgG (0.5 mg/ml; grey dotted lines) did not bind to any of the cells tested. A secondary antibody was used as negative control (black lines).
Biotinylated horse anti-rabbit/mouse secondary antibody (ImmPRESS REAGENT KIT anti-rabbit/mouse Ig, Vector Laboratories, Burlingame CA) was used in a 40-min incubation step at rt. After washing, detection was carried out using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen and finally counterstained with Gill’s hematoxylin (Vector Laboratories) for 20 s to stain the nuclei of the cells. Negative and positive controls were included to detect possible nonspecific reactions.

**Statistical Analysis**

All values are expressed as means ± SD unless otherwise indicated. A P value <0.05 was considered to be statistically significant. Differences among four groups were analyzed by ANOVA using a Kruskal-Wallis test. The statistical analysis was performed using SAS software (version 9.1.3, SAS, Cary, NC). Relative changes in electrical resistance as well as cumulative permeability of probes were performed by Student’s t-test for paired and unpaired values when appropriate, using SPSS 19.0 software (SPSS, Chicago, IL).

**RESULTS**

**Characterization of HKEC**

Using flow cytometry, phenotypic characterization of HKEC showed that the cells expressed all the endothelial markers tested (Table 2). In addition, light microscopy, demonstrated endothelial morphology and maintained contact inhibition for about eight passages, after which the cells ceased to divide. We used freshly isolated HKEC in passages 3–4 (Fig. 1A) for all studies. Their glomerular origin was confirmed by the presence of fenestrae using scanning electron microscopy (Fig. 1, B and C), and in Matrigel these cells formed capillary-like structures, indicating their endothelial origin (Fig. 1D).

**A High Number of ESRD Patients Have AECA Against HKEC**

A significantly higher proportion of ESRD patients had AECA that bound to both unstimulated and stimulated HKEC

<table>
<thead>
<tr>
<th>Patients/Subjects</th>
<th>HUVEC</th>
<th>HKEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>Stimulated</td>
</tr>
<tr>
<td>ESRD (n = 45)</td>
<td>2 (4%)</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Controls (n = 20)</td>
<td>1 (5%)</td>
<td>2 (10%)</td>
</tr>
</tbody>
</table>

Table 3. **Number and percentage of ESRD patients and healthy controls with antibodies against the tested endothelial cells**

Fig. 2. Immunocytochemical analysis demonstrated that normal IgG and AECA-negative IgG-treated HKEC from ESRD patients showed strong expression of zonula occludens (ZO)-1 and claudin-1 (green and red staining, arrows), while stimulation with AECA-positive IgG markedly decreased expression of the same molecules. Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI; blue) was done, and staining with secondary antibodies alone served as a negative control. Magnification ×40.
(25/45, 56%) compared with normal healthy controls (1/20, 5%) \((P < 0.0001)\) (Table 3). Furthermore, 2/45 ESRD patients had antibodies that bound HUVEC compared with 1/20 in controls \((P = \text{not significant})\). In addition, purified IgG isolated fractions from ESRD patients with AECA were tested for their sustained capacity to bind HKEC (Fig. 1E) and were found to bind strongly to HKEC. The same IgG fraction did not bind HUVEC, HLEC, or HAEC (Fig. 1E).

**IgG Fractions From ESRD Patients With AECA Decrease Expression of TJ Proteins on HKEC**

Immunocytochemical staining of cultured untreated HKEC with antibodies to TJ proteins showed strong expression on these cells. We found abundant expression of the TJ proteins claudin-1 and ZO-1 but not occludin-1. However, treatment of HKEC with AECA-positive IgG fractions from ESRD patients markedly decreased expression of these molecules. On the other hand, IgG fractions from AECA-negative ESRD patients or normal subjects did not have any effect on the expression of TJ on HKEC (Fig. 2). Immunocytochemical staining of cultured untreated HUVEC with antibodies to TJ proteins showed strong expression of occludin and VE-cadherin and weak expression of claudin-1 but no ZO-1. Expression of these proteins was not affected by treatment with AECA-positive IgG.

**AECA-Positive IgG From ESRD Patients Elicits an Increase in HKEC Monolayer Permeability**

Next, we studied the capacity of AECA-positive IgG fractions from ESRD patients to cause increased macromolecular flux across the HKEC monolayer, which was estimated through measuring the diffusion of different FITC-dextrans over a time lapse of 2 h and calculated as the percentage of epithelial resistance. We found that activation of the HKEC by AECA-positive IgG from ESRD patients elicited augmented dextran flux significantly higher than for EC treated with normal IgG (Fig. 3A). The smaller probe (Mw 4,000, \(P = 0.003\)) permeated more readily than the larger one (Mw 150,000, \(P = 0.001\)), indicating that the permeability of FITC-dextran was markedly increased in IgG-treated HKEC compared with controls but unchanged in HUVEC (data not shown). Similarly, when we compared the change in electrical resistance of both treated HUVEC and HKEC monolayers (baseline resistance 38 \pm 2.2 in HKEC and 33 \pm 5.5 \(\Omega\) cm² in HUVEC), after 20 min resistance in AECA-positive IgG-treated HKEC started to significantly decline by 30% \((P = 0.012\) but not HUVEC, suggesting to us that these IgG fractions are strongly reactive on the binding proteins of these cells (Fig. 3B).

**AECA-Positive IgG From ESRD Patients Increases Cystosolic Free Ca\(^{2+}\) and F-Actin Content in HKEC**

Purified IgG from AECA-positive patients elicited a dose-dependent Ca\(^{2+}\) response in HKEC that peaked after 5–10 s, lasted for nearly 60 s, and resulted in a Ca\(^{2+}\) change of 250 nM (maximal plateau value, calculated from baseline value before stimuli challenge) (Fig. 4A). No Ca\(^{2+}\) change was detected upon stimulation with normal IgG (Fig. 4A) while negligible Ca\(^{2+}\) change was observed with IgG fractions from AECA-negative patients. The same effects were not observed with HUVEC (Fig. 4A). In further experiments, HKEC monolayers were incubated with AECA-positive IgG from ESRD patients and normal subjects and then stained for actin filaments with FITC-conjugated phalloidin. Laser-scanning confocal microscopy revealed that untreated HKEC exhibited few stress fibers (Fig. 4B). On the other hand, HKEC incubated with AECA-positive IgG from patients but not normal subjects demonstrated a marked increase in the number and density of F-actin stress fibers (Fig. 4B). In addition, no effect of IgG from ESRD patients without AECA was observed.

**AECA-Positive IgG From ESRD Patients Decreases Expression and Causes Tyrosine Phosphorylation of VE-Cadherin**

Immunocytochemical staining of cultured untreated or normal IgG-treated HKEC with antibodies to endothelial-specific adherens protein VE-cadherin showed strong expression on these cells (Fig. 5A). However, treatment of HKEC with AECA-positive IgG but not AECA-negative IgG from ESRD patients markedly decreased expression of this molecule (Fig. 5A). Furthermore, we found that normal IgG and AECA-negative IgG fractions from ESRD patients did not cause...
phosphorylation of VE-cadherin (Fig. 5B). In contrast, activation of HKEC by AECA-positive IgG from ESRD patients resulted in a 10-fold increase in tyrosine-phosphorylated VE-cadherin (Fig. 5, C and D). This effect occurred within 1 h and lasted through 16 h of activation.

ESRD Patients with AECA-Positive IgG Have Markedly Decreased Expression of Claudin-1 and VE-Cadherin

Strong expression of occludin, ZO-1, claudin-1, and VE-cadherin was found in normal kidney sections (n = 4) (Fig. 6A). Occludin and ZO-1 were found to be localized to tubular endothelial and glomerular epithelial and endothelial cells, while claudin-1 was expressed mainly in the glomerulus and VE-cadherin was expressed in the peritubular and tubular capillaries and around vessel areas in normal kidneys (Fig. 6B). In kidney biopsy sections from ESRD patients with AECA-positive IgG (n = 6), expression of claudin-1 and VE-cadherin in glomeruli was markedly decreased or absent. In AECA-negative patients (n = 3), the expression of claudin-1 was decreased but VE-cadherin was not altered.

Clinical Correlations of AECA Reactive with HKEC

All clinical characteristics of ESRD patients are presented in Table 3. The AECA-positive group (n = 25) was further divided into patients with low-titered AECA (1:50, n = 11) or high-titered AECA (1:100, n = 14), respectively. We found that the prevalence of female ESRD patients was significantly higher in patients with AECA. Moreover, both systolic (P < 0.01) and diastolic (P < 0.05) blood pressure were significantly higher in patients with higher AECA titers. The slightly lower S-creatinine levels found in patients with the presence of AECA may be attributed to the high prevalence of women in this group of patients. There were no differences in age or the prevalence of CVD and diabetes mellitus between patients with and without AECA. Similarly, no difference in the median hsCRP, VCAM-1, ICAM-1, or IL-6 level was found between the different AECA groups. However, median TNF-α levels tended to be higher in patients with low and high AECA activity, respectively. Thus, when low- and high-titered AECA patients were grouped together (n = 25), median TNF-α was significantly higher in patients with AECA (13.0 vs. 10.5 pg/l; P < 0.05).
Recent studies provided evidence that endothelial cell injury plays an important role in the pathophysiology of renal barrier dysfunction (1, 13, 38). The present study identifies a novel factor that may contribute to the endothelial dysfunction. Herein, we provide evidence for a causal connection between AECA and kidney EC and their capacity to induce alterations in EC barrier function in ESRD patients. We further explored the mechanisms by which these antibodies may cause EC permeability. We demonstrated that upon binding these antibodies promptly triggered activation of HKEC as detected by a rapid calcium flux response within seconds. Moreover, it increased HKEC monolayer permeability by affecting TJ and AJ, consistent with immunohistochemical staining for these proteins in the glomerulus of ESRD patients with AECA. The marked loss of TJ proteins in biopsies of ESRD patients with AECA further implicates an important role for AECA in causing endothelial damage. It is important to mention that the same effects were not observed with HUVEC. A main difference could be the specificity of the antibodies reacting with HKEC compared with HUVEC, HLEC, or HAEC. As demonstrated, only 4% of all ESRD patients had anti-HUVEC compared with 56% with anti-HKEC antibodies. Obviously, the tissue specificity of AECA seems to play an important role in the pathogenic progress. This indicates the importance of using the relevant target cells for studying the effects of AECA in various diseases and disorders. Thus antibodies to HKEC activate and increase kidney endothelial permeability, which may lead to a leaky glomerular barrier. However, it is important to mention that in the present study we used kidney EC from a single donor. The present results will need to be verified in further studies, including kidney EC from several donors.

Fig. 5. A: Immunocytochemical analysis demonstrated that normal IgG and AECA-negative IgG-stimulated HKEC from ESRD patients showed strong expression of VE-cadherin (green staining, arrows), while stimulation with AECA-positive IgG markedly decreased expression of this molecule (top). Bottom: nuclear staining of same cells with DAPI (blue). Magnification ×40. B: HKEC were either preincubated with 3 mg/ml normal IgG (lane 1) or IgG from AECA-negative (lane 2), or AECA-positive ESRD patients (lane 3) or 100 ng/ml rhVEGF (positive control, lane 4). VE-cadherin was immunoprecipitated (IP) from cell lysates and immunoblotted (IB) with anti-phospho VE-cadherin antibodies. Middle: tyrosine-phosphorylated VE-cadherin. Bottom: there was no difference in total immunoprecipitated VE-cadherin from the same immunoblots after stripping and reprobing with anti-VE-cadherin antibody. C and D: IgG from AECA-positive ESRD patients phosphorylation of VE-cadherin beginning at ~30 min and persisted for 16 h after stimulation with AECA IgG. Phosphorylation of VE-cadherin was quantitated by densitometric analysis using Imager (Quantity one, version 1.2). Results are means ± SD (n = 3). *P < 0.05, **P < 0.001 compared with nontreated cells.

**DISCUSSION**

Recent studies provided evidence that endothelial cell injury plays an important role in the pathophysiology of renal barrier dysfunction (1, 13, 38). The present study identifies a novel factor that may contribute to the endothelial dysfunction. Herein, we provide evidence for a causal connection between AECA and kidney EC and their capacity to induce alterations in EC barrier function in ESRD patients. We further explored the mechanisms by which these antibodies may cause EC permeability. We demonstrated that upon binding these antibodies promptly triggered activation of HKEC as detected by a rapid calcium flux response within seconds. Moreover, it increased HKEC monolayer permeability by affecting TJ and AJ, consistent with immunohistochemical staining for these proteins in the glomerulus of ESRD patients with AECA. The marked loss of TJ proteins in biopsies of ESRD patients with AECA further implicates an important role for AECA in causing endothelial damage. It is important to mention that the same effects were not observed with HUVEC. A main difference could be the specificity of the antibodies reacting with HKEC compared with HUVEC, HLEC, or HAEC. As demonstrated, only 4% of all ESRD patients had anti-HUVEC compared with 56% with anti-HKEC antibodies. Obviously, the tissue specificity of AECA seems to play an important role in the pathogenic progress. This indicates the importance of using the relevant target cells for studying the effects of AECA in various diseases and disorders. Thus antibodies to HKEC activate and increase kidney endothelial permeability, which may lead to a leaky glomerular barrier. However, it is important to mention that in the present study we used kidney EC from a single donor. The present results will need to be verified in further studies, including kidney EC from several donors.

Cytokines and ischemic injury have been associated with endothelial dysfunction, increased permeability, and intercellular gap formation (1, 6, 10, 16). In vitro conditions, studies have shown how the permeability of kidney and umbilical epithelial cells/EC may be induced or impeded transcellularly through the formation of caveolae and fenestrations (12). In the present study, we provide evidence that AECA are another important factor that may affect the endothelial permeability barrier. HKEC activation through AECA binding to its antigen(s) initiated an active response, as evidenced by rapid mobilization of cytosolic free Ca\(^{2+}\) and redistribution of actin filaments, leading to structural rearrangement of the HKEC.
cytoskeleton and impaired barrier function. This increase in EC permeability has been shown to be associated with calcium-dependent conformational changes in the EC cytoskeleton, leading to cell contraction and intercellular gap formation (8, 39, 44). Reorganization of actin filaments is essential for these cell shape changes, and formation of F-actin may thus be an important determinant of increased HKEC permeability (39). We demonstrated that stimulation of HKEC through AECA activation resulted in loss of peripheral actin bands and increased stress fiber density.

A considerable body of evidence now supports a role for tyrosine phosphorylation in the regulation of vascular permeability (5, 51). The remarkable cell specificity of VE-cadherin prompted us to further investigate the role of VE-cadherin in renal vascular permeability. In this study, we showed that AECA activation of HKEC induces phosphorylation of VE-cadherin. This is in line with recent observations where the tyrosine phosphorylation of VE-cadherin is an important regulatory pathway associated with TNF-induced EC barrier dysfunction (26, 32). Importantly, in kidney biopsies of normal controls, very strong expression of VE-cadherin was observed in the glomerular endothelium, and tubular and peritubular capillaries; however, a marked decrease in expression was observed in ESRD patients with AECA. This implies a role for AECA in adversely altering the expression of important AJ involved in maintaining vascular permeability.

Claudins, a family of TJ transmembrane proteins, appear to be the main structural component. In mouse and rabbit experimental models, recent studies demonstrate that the expression of specific claudins can alter transepithelial electrical resistance and charge selectivity (14, 21, 46). In the present study, we found that AECA markedly decreased the expression of claudin-1 and ZO-1 on HKEC, implying a loss of kidney endothelial integrity and polarity. Once again, our in vivo staining of kidney biopsies from ESRD patients with AECA showed reduced expression of these two main structural components of TJ compared with normal controls, implying a role for AECA in the loss of kidney endothelial integrity and polarity.

A pathogenic role for AECA has been thoroughly described in recently reviewed literature (2). Accordingly, EC-binding antibodies have been shown to activate cultured EC, as manifested by the expression of adhesion molecules (27). Perry et al. (36) found that AECA levels were associated with histological evidence of active renal damage on biopsy specimens, while George et al. (24) reported that the presence of AECA could contribute to future EC dysfunction with a resultant prothrombotic tendency. It was not the intention of the present study to define the antigens with which AECA react but rather to further strengthen the functional and clinical relevance of tissue-specific AECA in diseases with vascular injury. Moreover, ESRD patients represent a group with severe disease in general, and one could argue to find increased levels of antibodies to HKEC as an epiphenomenon. Nevertheless, our data demonstrate a causal link between HKEC-reactive antibodies and their capacity to induce alterations in glomerular vascular permeability. Thus the findings that these antibodies possess functional properties reflect more than an epiphenomenon. These results shed light in understanding some of the molecular mechanisms and structural changes leading to a leaky glomerular barrier. Our results justify the need to investigate the presence of these antibodies in patients with early renal disease and the use of appropriate and relevant target EC for the detection and signaling pathways of AECA in various diseases.

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REFERENCES


