Serum Endocan Correlated With Stage of Chronic Kidney Disease and Deterioration in Renal Transplant Recipients


ABSTRACT
Earlier detection and intervention for chronic renal allograft injury (CRAI) remain major challenges for transplantation physicians. Endocan plays a key role in the regulation of cell adhesion, inflammatory disorders, and tumor progression. We conducted this cross-sectional study of 97 renal transplant (RT) recipients with mean RT duration of 7.0 ± 5.7 years to determine whether Endocan could be a diagnostic and prognostic marker. The patients’ mean age was 43.6 ± 13.2 years, and 55.7% (54/97) were male. Higher Endocan levels were found in more advanced chronic kidney disease (CKD) stages in a dose-dependent manner. Interestingly, the Endocan ≥643.19 pg/mL group had higher creatinine (Cr; 1.2 ± 0.4 vs 1.6 ± 1.1 mg/dL; P = .029) and lower estimated glomerular filtration rate (eGFR; 67.8 ± 23.8 mL/min vs 54.4 ± 22.0; P = .006) than the Endocan <643.19 pg/mL group after 3 months of follow-up, respectively. Linear regression analysis found tumor necrosis factor (TNF)-α correlated well with Endocan. To elucidate the response of endothelium activation, we stimulated human umbilical vein endothelial cells (HUVECs) with TNF-α in vitro, and found the levels of Endocan (P = .022) and transforming growth factor (TGF)-β1 (P = .034) increased with time, but interleukin (IL)-10 decreased (P = .013). In summary, Endocan may reflect the degree of endothelial cell injury in renal allografts, and showed a trend of elevation in late-stage CKD. An in vitro study demonstrated TNF-α-activated HUVECs secreted high levels of Endocan and TGF-β1, which could lead to a better understanding of the role of endothelium in immune balance. In conclusion, Endocan may have potential as a useful long-term indicator of CRAI in RT recipients, but further study is needed to verify our findings.

EVEN WITH improved immunosuppressive agents and early graft survival, graft function still deteriorates due to immunologic and nonimmunologic causes in renal transplant (RT) recipients [1]. With the advances in potent immunosuppressive agents in recent years, acute graft rejection has become significantly less common, and 1-year patient and graft survival rates are now more than 90%; however, late graft loss due to chronic rejection and recurrent glomerulonephritis (GN) are major causes of end-stage renal disease (ESRD) [2–4]. Earlier detection and intervention for chronic renal allograft injury (CRAI) remain major challenges for transplantation physicians.

Endothelial cells are sites of early contact between donor and recipient cells, and are first recognized by the immune system after organ transplantation [5]. The persistent injury results in excessive turnover of graft vascular endothelial cells in renal allografts, so long-term RT inflammation and endothelial activation may play a relevant role in inflammation, organ fibrosis, and graft dysfunction [6]. Endocan is a proteoglycan expressed by endothelial cells in from the Division of Nephrology (K.-H.S., C.-H. Cheng., M.-J.W., T.-M.Y., Y.-W.C., S.-T.H., C.-H. Chen.), Department of Internal Medicine, Taichung Veterans General Hospital; Department of Internal Medicine, Chiayi Branch, Taichung Veterans General Hospital (C.-H. Chen.) the School of Medicine (C.-H. Chen.), China Medical University; the Department of Life Science (Y.-H.S., C.-P.H., C.-H. Chen.), Tunghai University; the Life Science Research Center (C.-P.H.), Tunghai University; and the Department of Medicine (K.-H.S., C.-H. Cheng., M.-J.W.), Chung Shan Medical University Hospital, Taichung, Taiwan.

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lungs and kidney and can be detected in human blood [7]. Endocan plays a key role in the regulation of cell adhesion and inflammatory disorders. Endothelial dysfunction after renal transplantation was associated with renal graft loss [8]. The aims of this pilot study were to investigate whether circulating Endocan increased in RT recipients with graft dysfunction and to determine its relationships with clinical status and prognosis. We also investigated the kinetics of Endocan secretion by endothelial cells in vitro.

MATERIALS AND METHODS

Patients

The study population consisted of 97 kidney transplant recipients treated for at least 3 months between February 1987 and June 2011 in our center. The study was approved by the institutional review board and ethics committee (no. CE13062). Most patients were treated with triple-therapy immunosuppression in a maintenance dose that consisted of prednisolone in 85 patients, either cyclosporine in 19 patients or tacrolimus in 75 patients, and either mycophenolate mofetil in 37 patients or mycophenolate sodium in 29 patients. Ten patients were also treated with sirolimus. Estimated glomerular filtration rate (eGFR) was calculated using the modified version of the Modification of Diet in Renal Disease equation (MDRD). Allograft function was assessed according to the stage of chronic kidney disease (CKD) classification as defined by the National Kidney Foundation’s Kidney Disease Outcomes Quality Initiative (KDOQI) because all patients with a kidney transplant were considered either to have CKD or to be at increased risk of CKD. Because the dynamic status of renal function might change in transplant recipients, we observed successive change of eGFR from baseline within 3 months. We defined CKD progression as more than 5% suppression of the change in eGFR ($\Delta$eGFR) from the baseline in 3 months. Endocan (Lunginnov s.a.s., Lille, France) [9], transforming growth factor (TGF)-β, and interleukin (IL)-10 (R&D Systems, Minneapolis, Minn, United States) concentrations in patients’ sera were analyzed using sandwich enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s instructions.

Human Primary Cultured Endothelial Cell Cultures

Human primary cultured endothelial cells (HUVECs) were derived from the vein of umbilical cords as previously described [10]. Third-passage HUVECs were cultured on fibronectin-coated 24-well culture plates (Corning, Corning, NY, USA) in M199 medium (Invitrogen, Carlsbad, Calif, United States) containing 20% fetal bovine serum, endothelial cell growth supplement (Millipore, Billerica, Mass, United States), and 2 mmol/L L-glutamine (Gibco, Grand Island, NY, United States). At confluency, the cells were washed once and incubated in M199 medium containing 2% fetal calf serum and 2 mmol/L L-glutamine for 18 hours and then washed

Fig 1. (A) The serum Endocan levels detected using ELISA showed a trend of progressive elevation in CKD staging of RT recipients. (B) The CKD progression (change in eGFR; $\Delta$eGFR > 5%) group had higher Endocan level than that of the group with stable CKD status by Kruskal-Wallis H test. (C) The cut-off value of serum Endocan level was 643.19 pg/mL, as determined using the receiver operating characteristics (ROC) curve.
once again with the same medium just before stimulation. Final concentrations of tumor necrosis factor (TNF-α) at 10 ng/mL (PEPROTECH, Rocky Hill, NJ, United States) were added in each well. The cell supernatants were centrifuged and stored at -20°C until quantitation of Endocan using ELISA.

Statistical Analysis

Statistical analysis was performed with SPSS for Windows, version 16.0 (SPSS, Chicago, IL, USA). Data are expressed as percentage and mean ± standard deviation. Demographic and outcome differences were compared using Pearson chi-square test, Fisher exact test, the Mann-Whitney U test, and independent sample t test as appropriate. Linear regression analysis was used to assess the relationship between serum Endocan levels and cytokines. \( P < .05 \) was considered statistically significant.

RESULTS

Serum Endocan Level Associated With CKD Stage in RT Recipients

We stratified the RT recipients by eGFR into CKD stages 1–2 (n = 48), stage 3 (n = 44), and stages 4–5 (n = 5). The serum Endocan levels showed a trend of elevation in the late-stage CKD (\( P = .077 \); Fig 1A). Most of the transplant recipients maintained stable graft function (n = 57), but patients with progression of CKD (n = 40) had higher serum Endocan levels than those without (966.3 ± 718.2 vs 593.8 ± 520.5 pg/mL; \( P = .004 \); Fig 1B). Thus, the cut-off value of Endocan level was 643.19 pg/mL, as determined using receiver operating characteristics (ROC) curve (specificity, 73.8; sensitivity, 65.7; Fig 1C). There were no differences in age of transplantation, duration of follow-up, gender, pre-existing diseases, mode of dialysis, initial Cr, and GFR between the 2 Endocan groups (Table 1). Interestingly, the group with higher serum Endocan (≥643.19 pg/mL) had higher creatinine (Cr; 1.2 ± 0.4 vs 1.6 ± 1.1 mg/dL; \( P = .029 \)) and lower eGFR (67.8 ± 23.8 mL/min vs 54.4 ± 22.0; \( P = .006 \)) than those of the lower serum Endocan group (<643.19 pg/mL) after 3 months of follow-up, respectively. CKD progression was significantly greater in the higher serum Endocan group (62%; \( P = .001 \)). Linear regression analysis was performed to assess the relationship between serum Endocan levels and cytokines and the results showed that TNF-α had a linear correlation with serum Endocan levels (correlation coefficient \( r = 0.286; P = .002 \)), but not with IL-10 and TGF-β (data not shown).

TNF-α Induces Sustained Release of Endocan by HUVECs

The proinflammatory cytokine TNF-α might play an important role in post-transplantation alloimmune reaction and could also enhance the expression of Endocan in RT recipients. We mimicked the regulation of Endocan secretion in HUVECs culture stimulated with graded dosage of TNF-α (0, 0.1, 1, 10, and 100 ng/mL) for 3 days, and found that Endocan increased in a dose-dependent manner; the optimal stimulatory dosage for induction of Endocan expression was 10 ng/mL (Fig 2A). There was a significant increase in Endocan production in the presence of TNF-α in primary cultured HUVECs from 6 to 72 hours (\( P = .022 \); Fig 2B). Meanwhile, the TGF-β1 levels in the supernatant of culture increased (\( P = .034 \); Fig 2C) in the first 48 hours, and decreased thereafter. In contrast, IL-10 production progressively decreased over time (\( P = .013 \); Fig 2D).

DISCUSSION

Maintenance of vascular endothelial integrity is important for the health of an organ, and involves keeping a balance between endothelial turnover and repair. Endothelial repair is augmented by growth factors, and progenitor cells are attracted to an injured organ to protect and prevent ongoing interstitial damage. In a graft kidney, the persistent posttransplantation alloimmune reaction between recipient leukocytes and endothelial cells may lead to inflammation and damage, and thus continued injury results in excessive turnover of graft vascular endothelial cells, leading to recruitment of both leukocytes and endothelial cell progenitors, which facilitates the overlapping processes of inflammation and angiogenesis [11]. In a recent report, Endocan appeared to reflect the degree of endothelial cell injury in renal allografts, and was postulated to have the potential to serve as a highly sensitive and specific marker for acute rejection after renal transplantation [5]. In our study, we found a correlation between serum Endocan level and CKD stage of a graft kidney, and a high level of circulating Endocan was associated with progression of graft renal function in 3 months. These

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Table 1. Comparison of Patients’ Clinical Characteristics Between Higher and Lower Serum Endocan Groups

<table>
<thead>
<tr>
<th>Endocan ≥643.19</th>
<th>Endocan &lt;643.19</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N = 58)</td>
<td>(N = 39)</td>
<td></td>
</tr>
<tr>
<td>Age of transplantation (y)</td>
<td>49.7 ± 11.6</td>
<td>52.0 ± 11.36</td>
</tr>
<tr>
<td>Duration of transplantation (y)</td>
<td>7.4 ± 5.8</td>
<td>8.3 ± 5.6</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>35 (60)</td>
<td>19 (49)</td>
</tr>
<tr>
<td>Original disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>8 (14)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>CGN</td>
<td>23 (40)</td>
<td>17 (43)</td>
</tr>
<tr>
<td>CTIN</td>
<td>5 (9)</td>
<td>4 (10)</td>
</tr>
<tr>
<td>Unknown</td>
<td>16 (31)</td>
<td>12 (31)</td>
</tr>
<tr>
<td>Mode of dialysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAPD</td>
<td>20 (35)</td>
<td>9 (23)</td>
</tr>
<tr>
<td>HD</td>
<td>34 (59)</td>
<td>24 (62)</td>
</tr>
<tr>
<td>No dialysis</td>
<td>4 (8)</td>
<td>6 (15)</td>
</tr>
<tr>
<td>Initial creatinine (mg/dL)</td>
<td>1.2 ± 0.4</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>Creatinine at 3 mo (mg/dL)</td>
<td>1.2 ± 0.4</td>
<td>1.6 ± 1.1</td>
</tr>
<tr>
<td>Initial GFR (mL/min)</td>
<td>65.4 ± 20.3</td>
<td>57.1 ± 21.0</td>
</tr>
<tr>
<td>GFR at 3 mo (mL/min)</td>
<td>67.8 ± 23.8</td>
<td>54.4 ± 22.0</td>
</tr>
<tr>
<td>CKD progression (ΔGFR&gt;5%)</td>
<td>16 (28)</td>
<td>24 (62)</td>
</tr>
<tr>
<td>No. HLA AB mismatches</td>
<td>1.5 ± 1.2</td>
<td>1.6 ± 1.3</td>
</tr>
<tr>
<td>No. HLA DR mismatches</td>
<td>0.9 ± 0.6</td>
<td>1.2 ± 0.7</td>
</tr>
</tbody>
</table>

Abbreviations: CGN, chronic glomerulonephritis; CTIN, chronic tubulointerstitial nephritis; CAPD, continuous ambulatory peritoneal dialysis; HD, hemodialysis.

*Pearson chi-square.
†Fisher exact test.
‡Independent samples t test.
preliminary data warrant confirmation by kinetic studies of Endocan. However, our data demonstrated that higher serum Endocan (>643.19 pg/mL) had a significant deleterious impact on renal graft function in 3 months. Interestingly, we also found a statistically significant correlation between Endocan and TNF-α, a cytokine that is known to stimulate endothelial cell activation and injury. In healthy subjects, TNF-α is usually not present in the kidneys, but is stimulated by and detected in rejected allografts, diabetic nephropathy, and GN [12]. TNF-α is a known attractant for leukocytes, enhances expression of adhesion molecules on endothelial cells, and, therefore, may play an important role in renal inflammatory processes and allograft rejection.

To elucidate the response of endothelium activation, we stimulated HUVECs with TNF-α in vitro, and found Endocan and TGF-β1 levels increased over time, but IL-10 decreased. Activated endothelium enhanced expression of Endocan, which binds directly to the integrin lymphocyte function-associated antigen-1 (LFA-1) of lymphocytes and monocytes, and consistently inhibits the specific binding of soluble intercellular Adhesion Molecule 1 (ICAM-1) to LFA-1 on inflammatory cells [13]. Endocan may be implicated in the regulation of leukocyte extravasation at inflammatory sites because of the essential role of ICAM-1/LFA-1 interactions during firm adhesion of human lymphocytes and monocytes. In addition, Endocan might modulate the LFA-1/ICAM-1 costimulatory pathway on T cells and might orientate the Th1/Th2 balance of the immune response [13]. Th1 responses have been implicated in most forms of acute rejection and graft-versus-host disease, whereas Th2 responses have been variably associated with either protection or chronic rejection. In this study, we also demonstrated the TNF-α–activated HUVECs also secreted TGF-β1, which is a multifunctional cytokine with immunosuppressive and fibrogenic properties. This phenomenon may be explained by the recruitment of inflammatory cells by the damaged endothelium (eg, TNF-α–activated HUVECs) to the injury site through the Endocan effect; meanwhile, it may also secrete anti-inflammatory cytokines (eg, TGF-β1 and IL-10) to clear inflammation and guide the repair of damaged tissue, resulting in the resolution of symptoms. TNF, therefore, appears to act in a “yin and yang” manner in modulating immunity.

The immune balance in renal transplantation might be disrupted through persistent injury by alloantigen-stimulated inflammation, endothelial activation, and overwhelming tissue repair, and thus progressive organ fibrosis and graft dysfunction may inevitably cause CRAI. Earlier detection and intervention of CRAI remain key challenges for transplantation physicians. It is often too late to prevent disease progression or renal damage once changes in serum Cr (sCr) levels and proteinuria have been detected. Deterioration of renal function over time, determined through slope analysis, is a more accurate indicator of CRAI, and earlier identification of renal deterioration may prompt earlier changes in immunosuppressive therapies. Endocan has been demonstrated to be a highly sensitive and specific diagnosing marker for acute rejection after renal transplantation [5], and our study also indicated that Endocan might be a valuable predictor for CRAI.

There were several outliers in the distribution found in our study. It is crucial to distinguish between non-immunologic or toxic and immunologic-derived endothelial damage, but a limitation in this preliminary study was that
no comparisons with patients without graft biopsy or correlations of dynamic change of Endocan and sCr levels were conducted.

In summary, Endocan may reflect the degree of endothelial cell injury in renal allografts. It showed a trend of elevation in late-stage CKD, and serum Endocan >643.19 pg/mL was correlated with a greater proportion of CKD progression. The in vitro study demonstrated TNF-α-activated HUVECs secreted greater levels of Endocan and TGF-β1, which might lead to a better understanding of the role of endothelium in immune balance. In conclusion, Endocan appeared to show promise as a long-term indicator for CRAI in RT recipients, but further study is needed to verify our findings.

REFERENCES