Nocturnal Hemodialysis Is Associated with Restoration of Early-Outgrowth Endothelial Progenitor-Like Cell Function

Darren A. Yuen,*† Michael A. Kuliszewski,* Christine Liao,* Dmitriy Rudenko,* Howard Leong-Poi,*† and Christopher T. Chan†‡

Summary

Background and objectives Angiogenesis is a key response to tissue ischemia that may be impaired by uremia. Although early-outgrowth endothelial progenitor-like cells promote angiogenesis in the setting of normal renal function, cells from uremic patients are dysfunctional. When compared with conventional hemodialysis, it was hypothesized that nocturnal hemodialysis would improve the in vivo angiogenic activity of these cells in a well described model of ischemic vascular disease.

Design, setting, participants, and measurements Early-outgrowth endothelial progenitor-like cells were cultured from healthy controls (n = 5) and age- and gender-matched conventional hemodialysis (12 h/wk, n = 10) and nocturnal hemodialysis (30 to 50 h/wk, n = 9) patients. Cells (5 × 10⁵) or saline were injected into the ischemic hindlimb of athymic nude rats 1 day after left common iliac artery ligation.

Results Although conventional dialysis cell injection had no effect versus saline, nocturnal hemodialysis and healthy control cell injection significantly improved ischemic hindlimb perfusion and capillary density. Nocturnal hemodialysis cell injection was also associated with significant increases in endogenous angiopoietin 1 expression in the ischemic hindlimb compared with saline and conventional dialysis cell injection.

Conclusions In contrast to a conventional dialytic regimen, nocturnal hemodialysis is associated with a significantly improved ability of early-outgrowth endothelial progenitor-like cells to promote angiogenesis and thus restore perfusion in a model of ischemic vascular disease.


Introduction

Cardiovascular disease is one of the leading contributors to morbidity and mortality in ESRD patients (1). Current therapies have failed to reduce this vascular risk, with renin-angiotensin system blockade (2), BP control (3), and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibition (4) all failing to show benefits in clinical trials.

One of the major contributors to vascular disease in ESRD is endothelial injury because uremia is associated with dysfunction (5) and even loss (6) of the endothelium. Early-outgrowth endothelial progenitor-like cells (EPLCs), a novel bone-marrow-derived mononuclear cell population, promote endothelial repair and new endothelial growth (7) through the release of soluble factors that activate local angiogenic processes (8,9), maintaining healthy blood vessels and preserving tissue perfusion in the setting of ischemic injury (10,11). Importantly, preliminary data suggest that uremia induces early-outgrowth EPLC dysfunction, at least in vitro (12).

Nocturnal hemodialysis (NHD) greatly augments uremic toxin clearance compared with conventional thrice weekly hemodialysis (CHD) through a combination of increased dialysis duration and frequency. NHD is associated with better cardiovascular outcomes, including improved endothelial function (13), attenuated vascular calcification (14), reduced BP (15), and regression of left ventricular hypertrophy (16) when compared with its conventional counterpart. Intriguingly, conversion to NHD has also been associated with symptomatic and structural improvements in severe cases of ischemic vascular disease (17). Recently, we demonstrated that NHD is associated with improved circulating early-outgrowth EPLC number and in vitro function (18). Here we show for the first time that (1) early-outgrowth EPLCs derived from CHD patients are unable to restore perfusion in ischemic tissue in vivo, and (2) NHD is associated with a reversal of this early-outgrowth EPLC angiogenic defect when tested in an in vivo rodent model of ischemic vascular disease.

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1345
Study Population and Methods

Patient Enrollment

This protocol was approved by the Research Ethics Boards of the Toronto General Hospital, University Health Network, and St. Michael’s Hospital. Two groups of age- and gender-matched ESRD patients were studied: (1) CHD patients \( (n = 10) \), and (2) NHD patients \( (n = 9) \). A separate group of healthy individuals \( (n = 5) \) was also studied as controls. CHD and NHD patients had been on their respective dialysis modalities for a minimum duration of 6 months. None of the patients had any acute illness or symptomatic cardiovascular disease (including congestive heart failure and acute coronary syndrome). Written informed consent was obtained from each patient.

Clinical Data Collection

In both ESRD cohorts, clinical assessment (including weight, height, BP, and routine dialysis-related biochemical analyses) was performed. Seated BP in each patient after 5 minutes of rest was measured during a clinic visit. Blood samples were obtained at midweek and predialysis for CHD patients. To minimize circadian variation and to replicate steady-state CHD and NHD conditions, blood samples were drawn at the same time of day for NHD patients (a minimum of 4 hours after the end of a regular NHD session). Dialysis dose per treatment was estimated by equilibrated Kt/V (\( eKt/V \)) as described by Daugirdas and colleagues (19), where \( eKt/V = spKt/V - 0.693 \times spKt/V \times 10^-3 \), where \( spKt/V \) is the single-pool Kt/V, \( K \) is the delivered clearance, \( t \) is the dialysis time, and \( V \) is the urea distribution volume. Single-pool Kt/V was determined using the blood urea reduction ratio (\( URR \)). Cardiovascular medications were documented, including diuretics, \( \beta \)-blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor antagonists, calcium channel blockers, and HMG-CoA reductase inhibitors. The dose of erythropoiesis-stimulating agent prescribed was also documented because of its well established effects on early-outgrowth EPLC number and function (21).

Hemodialysis Prescriptions

NHD patients received hemodialysis at home for 6 to 8 hours, 5 to 6 nights per week. Vascular access was achieved through an internal jugular catheter (Udall Catheter, Cook Critical Care, Bloomington, IN) or an arteriovenous fistula. A dialysate flow rate of 350 ml/min, a blood flow rate of 200 to 300 ml/min, and Polyflux polyamide dialyzers (Gambro, Lund, Sweden or Baxter, McGaw Park, IL) were used for each treatment. CHD patients received hemodialysis for 4 hours three times per week via similar vascular access. A blood flow rate of 400 ml/min, a dialysate flow rate of 500 to 750 ml/min, and F80 polysulphone dialyzers (Fresenius Medical Care, Lexington, MA) were used for CHD delivery. Unfractionated heparin was used for anticoagulation on CHD and NHD.

Early-Outgrowth EPLC Isolation

Early-outgrowth EPLCs were isolated by enriched medium isolation as described previously (18,22). Briefly, peripheral venous blood was collected from ESRD patients and healthy control subjects. The mononuclear cell fraction was isolated by Ficoll–Paque density gradient (Becton Dickinson, Mississauga, Canada) centrifugation and washed 3 times with PBS (Sigma-Aldrich, Mississauga, Canada), and cells were plated at a density of 10⁶ mononuclear cells/cm² on fibronectin-coated culture slides (Becton Dickinson) in endothelial cell basal medium-2 (Lonza) supplemented with endothelial growth medium SingleQuots and 20% fetal bovine serum. Cells were trypsinized after 10 days of culture and washed with PBS. Cells \( (5 \times 10^5) \) were resuspended in 1 ml of PBS and then used for \textit{in vivo} injection.

Unilateral Chronic Hindlimb Ischemia Model, Tissue Preparation, and Histology

All animal studies were approved by the St. Michael’s Hospital Animal Ethics Committee in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no 85-23, revised 1996). Male athymic nude rats \( (6 \text{ to } 8 \text{ weeks}, \text{ Charles River, Montreal, Canada}) \) were obtained and maintained at the St. Michael’s Hospital Animal Research Vivarium in a temperature-controlled \( (22°C) \) room with \textit{ad libitum} access to commercial irradiated standard rat chow and autoclaved water. Rats underwent unilateral left-sided chronic hindlimb ischemia induction as described previously (23). Briefly, rats were anesthetized with intraperitoneal chloral hydrate (2% to 3%). Using aseptic technique, the left common iliac artery and small proximal branches were exposed and doubly ligated with 4-0 suture. The incision was closed in layers, and the animals were recovered. One day after left common iliac artery ligation, athymic nude rats were injected with serotonin \( (5 \times 10^5 \text{ early-outgrowth EPLCs at five intramuscular sites using a 1-cc syringe and 25-gauge needle in the ischemic hindlimb, as described previously (24), using aseptic technique and under sedation. \textit{In vivo} injection per rat was 1 cc (0.2 cc per site). At study end 27 days later, rats were sacrificed, and the right (nonischemic) and left (ischemic) hindlimb muscles were immediately isolated and immersion-fixed in 10% neutral-buffered formalin, embedded in cryostat matrix (Tissue-Tek, Sakura, Kobe, Japan), or flash frozen in liquid nitrogen. Formalin-fixed tissues were routinely processed, embedded in paraffin, and sectioned (5 \text{ mm} thickness). Endothelial cell density within formalin-fixed, paraffin-embedded sections of ischemic hindlimb tissue was assessed using staining with biotin-conjugated isolecitin GS-B4 (Invitrogen, Burlington, Canada, catalog number I21414), and the Vectastain ABC system (Vector Laboratories, Burlington, Canada) was used for detection of specific isolectin B4 staining as described previously (25). Omission of isolecitin B4 served as the negative control. Quantification of ten nonoverlapping \( 160 \times \) fields was performed for each animal in a blinded fashion with a grid technique.

RNA Extraction and Quantitative Real-Time PCR

Quantitative real-time PCR (RT-PCR) was performed using SYBR green. In brief, frozen rat hindlimb tissue, stored at \(-80°C\), was homogenized (Polytron, Kinematica GmbH, Littau, Switzerland), and total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY). Total RNA \( (4 \mu g) \) was treated with RQ1 DNase (Promega, Madison, WI) to remove genomic DNA. RNA was reverse transcribed into cDNA with 1 \mu\text{L} of random hexamers \( (2 \mu g/\mu l) \), 2.5 \mu l of 10 mmol/L dNTP.
mix, 0.5 μl RNase inhibitor (40 U/μl) (Roche, Indianapolis, IN), and 0.5 μl avian myeloblastosis virus reverse transcriptase (25 U/μl) (Roche). cDNA samples were stored at −20°C until further analysis. RT-PCR was performed on an ABI Prism 7900HT Fast PCR System (Applied Biosystems, Foster City, CA). Sequence-specific primers were designed to span exon-exon boundaries using Primer Express software version 1.5 (Applied Biosystems) and were obtained from Sigma-Aldrich. Primer sequences are provided in supplemental Table 1. Experiments were performed in triplicate, and data analysis was performed using the Applied Biosystems Comparative CT method.

All values were referenced to the mRNA transcript levels of the housekeeper gene Rpl13a (10) and are presented as ratios of mRNA levels between the ischemic (left) and nonischemic (right) limbs indexed to the value found for saline-treated animal controls.

Contrast-Enhanced Ultrasound Imaging

Contrast-enhanced ultrasound imaging of the proximal hindlimb adductor muscles was performed with gated pulse inversion imaging (HDI 5000, Philips Ultrasound, Andover, MA) at a mechanical index of 1.0 and a transmission frequency of 3.3 MHz (L7-4 transducer). Perfusion in the adductor muscles was assessed during continuous intravenous infusion of lipid microbubbles (1 × 10^7 min^-1) as described previously (23).

Statistical Analyses

All data are shown as mean ± SEM unless otherwise stated. Differences between groups were analyzed by one-way ANOVA with post hoc Fisher’s least significant difference or Mann–Whitney test. Bivariate correlation analysis was performed with Spearman’s ρ test. All statistics were performed using GraphPad Prism 4.00 for Windows.

### Table 1. Demographic and clinical characteristics

<table>
<thead>
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<th>Variable</th>
<th>Healthy Control</th>
<th>CHD</th>
<th>NHD</th>
</tr>
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<tr>
<td>n</td>
<td>5</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Age (years), mean ± SEM</td>
<td>35 ± 6</td>
<td>52 ± 4</td>
<td>49 ± 5</td>
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<td>Gender (male:female)</td>
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<tr>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>PCKD (1)</td>
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<tr>
<td>Reflux nephropathy (1)</td>
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</tr>
<tr>
<td>Diabetes mellitus (%)</td>
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</tr>
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<td>Cardiovascular medications (number of patients)</td>
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<tr>
<td>angiotensin converting-enzyme inhibitor</td>
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<td>diuretic</td>
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<td>1</td>
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<tr>
<td>HMG-CoA reductase inhibitor</td>
<td>NA</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

NA, not applicable; HTN, hypertension; PCKD, polycystic kidney disease; CHD, conventional thrice weekly hemodialysis; NHD, nocturnal hemodialysis; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA.

### Table 2. Clinical variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>CHD</th>
<th>NHD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>eKt/V (per session)</td>
<td>1.30 ± 0.07</td>
<td>2.18 ± 0.18</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Predialysis urea (mmol/L)</td>
<td>22.2 ± 2.4</td>
<td>11.3 ± 2.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Postdialysis urea (mmol/L)</td>
<td>7.0 ± 1.8</td>
<td>1.9 ± 0.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Predialysis creatinine (μmol/L)</td>
<td>699 ± 106</td>
<td>556 ± 121</td>
<td>0.18</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>135 ± 10</td>
<td>126 ± 13</td>
<td>0.39</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>82 ± 6</td>
<td>80 ± 10</td>
<td>0.78</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>72 ± 5</td>
<td>70 ± 8</td>
<td>0.67</td>
</tr>
<tr>
<td>Plasma hemoglobin (g/L)</td>
<td>116 ± 8</td>
<td>136 ± 9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Darbepoetin alpha dose (μg/wk)</td>
<td>31 ± 7</td>
<td>34 ± 13</td>
<td>0.84</td>
</tr>
<tr>
<td>Predialysis potassium (mmol/L)</td>
<td>5.0 ± 0.4</td>
<td>4.6 ± 0.4</td>
<td>0.24</td>
</tr>
<tr>
<td>Predialysis bicarbonate (mmol/L)</td>
<td>24.7 ± 2.1</td>
<td>26.1 ± 2.1</td>
<td>0.48</td>
</tr>
<tr>
<td>Predialysis calcium (mmol/L)</td>
<td>2.42 ± 0.06</td>
<td>2.42 ± 0.10</td>
<td>0.99</td>
</tr>
<tr>
<td>Predialysis phosphate (mmol/L)</td>
<td>1.72 ± 0.30</td>
<td>1.09 ± 0.19</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Parathyroid hormone (pmol/L)</td>
<td>33.5 ± 14.0</td>
<td>15.3 ± 11.2</td>
<td>0.15</td>
</tr>
</tbody>
</table>

eKt/V, equilibrated Kt/V.
Demographic and Clinical Characteristics

Five healthy control individuals, 10 prevalent CHD, and 9 prevalent NHD patients were enrolled. The NHD and CHD patients were age and gender matched. Relevant demographic data are presented in Table 1, and clinical data are presented in Table 2. All patients were nonsmokers. NHD was associated with an increased sessional dialysis dose (eKt/V, \( P < 0.05 \)) and reduced predialysis urea and phosphate (\( P < 0.05 \) for both). NHD was also associated with higher hemoglobin concentrations despite similar erythropoiesis-stimulating agent usage (\( P < 0.05 \)). Although systolic and diastolic BP were not significantly different between the CHD and NHD cohorts, CHD patients used a greater number of antihypertensives to achieve this level of control. HMG-CoA reductase usage was slightly higher in the CHD cohort.

NHD Is Associated with an Improved Ability of Early- Outgrowth EPLCs to Restore Microvascular Perfusion in Ischemic Tissue

The effect of dialysis modality on human early-outgrowth EPLC-mediated neovascularization ability was assessed in a rodent model of chronic unilateral ischemic vascular disease. One day after ligation of the left common iliac artery, athymic nude rats were injected at five intramuscular sites in the ischemic leg with saline or \( 5 \times 10^5 \) early-outgrowth EPLCs derived from ex vivo culture of patient-derived mononuclear cells. Twenty-eight days postligation, contrast-enhanced ultrasound demonstrated that injection of NHD patient–derived early-outgrowth EPLCs induced a robust increase in microvascular perfusion of the ischemic limb similar in magnitude to that achieved with healthy control-derived cell

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**Figure 1.** Microvascular perfusion of ischemic hindlimb tissue is improved 27 days after injection of early-outgrowth endothelial progenitor-like cells (EPLCs) from nocturnal hemodialysis (NHD) patients. (A) Representative contrast-enhanced ultrasound perfusion images of ischemic hindlimb muscle at increasing pulsing intervals from one animal in each of the four treatment groups at 4 weeks postligation. Contrast enhancement into ischemic skeletal muscle was greater and occurred faster in muscle treated with early-outgrowth EPLCs derived from healthy controls and NHD patients as compared with muscle treated with saline (negative control), whereas early-outgrowth EPLCs derived from conventional thrice weekly hemodialysis (CHD) patients had no beneficial effect when compared with saline. (B) Microvascular blood flow ratio. (C) Microvascular blood volume ratio. Results are presented as ratios of ischemic hindlimb to nonischemic hindlimb control. \( *P < 0.05 \) versus saline-treated animal. \( \dagger P < 0.05 \) versus healthy early-outgrowth EPLC-treated animal. \( \ddagger P < 0.05 \) versus NHD early-outgrowth EPLC-treated animal.
injection (Figure 1). In contrast, CHD patient–derived cell injection had no significant effect on microvascular perfusion when compared with saline-injected controls (Figure 1 and Supplemental Movies). Similar trends were seen in microvascular blood volume, an index of blood volume contained within the ischemic hindlimb microvasculature (Figure 1). Dialysis dose, as measured by eKt/V, correlated positively with microvascular blood flow ($r = 0.536$, $P < 0.05$) and volume ($r = 0.656$, $P < 0.01$) (Figure 2).

**NHD Is Associated with an Improved Ability of Early-Outgrowth EPLCs to Promote Neovascularization in Ischemic Tissue**

To assess the structural correlates of the observed changes in microvascular perfusion, sectioning of ischemic hindlimb tissue and lectin staining were performed to investigate differences in capillary density. Correlating with the improvement in microvascular perfusion and blood volume, NHD and healthy control early-outgrowth EPLC treatment were associated with significant and equivalent increases in capillary density when compared with saline-treated controls (Figure 3). In contrast, CHD cell treatment did not alter capillary density compared with saline-treated controls (Figure 3). As observed with microvascular perfusion parameters, dialysis dose correlated positively with capillary density ($r = 0.713$, $P < 0.01$) (Figure 4).

**Injected NHD Patient-Derived Early-Outgrowth EPLCs Promote the Expression of Endogenous Angiopoietin 1**

To assess the molecular mechanisms underlying NHD early-outgrowth EPLC-mediated improvements in capillary density and microvascular perfusion, the expression of key angiogenic factors was interrogated by quantitative RT-PCR 4 weeks after cell therapy. Although endogenous expression of the vessel-stabilizing factor angiopoietin 1 (Ang1) in the ischemic hindlimb of CHD early-outgrowth EPLC-treated animals was no different from saline-treated controls, rat Ang1 mRNA levels were markedly upregulated in NHD cell-treated ischemic hindlimb tissue (saline: $1.00 \pm 0.36$ arbitrary units [AU], CHD: $0.86 \pm 0.51$ AU, NHD: $5.48 \pm 2.68$ AU, $P < 0.05$). In contrast, expression of the early angiogenic factors vascular endothelial growth factor (VEGF-A), angiopoietin 2 (Ang2), and stromal-cell-derived factor-1α (SDF-1α) was not significantly increased above saline-treated controls in the CHD or NHD early-outgrowth EPLC-treated ischemic hindlimbs (supplemental Table 2).

**Discussion**

Vascular disease is common in ESRD patients and is a substantial contributor to the poor outcomes seen in uremia (26,27). Importantly, no evidence-based therapies exist to treat vascular disease in ESRD, with most studies reporting a poor prognosis regardless of treatment choice (4,28,29). In the study presented here, we show that (1) CHD is associated with a marked inability of early-outgrowth EPLCs to restore blood flow and microvasculature in an *in vivo* model of ischemic vascular disease, and (2) NHD is associated with significant restoration of early-outgrowth EPLC function in this model.

Multiple lines of evidence support the concept that early-outgrowth EPLCs participate in endothelial repair. Preclinical proof-of-concept studies have demonstrated that early-outgrowth EPLCs promote neovascularization in ischemic tissue (30–32) and inhibit capillary rarefaction in chronic kidney disease (10). Clinical studies have demonstrated that human early-outgrowth EPLC administration induces significant proangiogenic benefits on top of standard-of-care therapy in the peripheral vascular disease (33) and postmyocardial infarction (11,34) settings. Strong epidemiologic links have also been made between circulating early-outgrowth EPLC number and cardiovascular risk (35,36).

Endothelial dysfunction plays a key role in the pathogenesis of ESRD-associated vascular complications (37). Interestingly, the ESRD milieu appears to adversely affect mechanisms of endothelial repair critical in maintaining

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**Figure 2.** Correlation between dialysis dose and microvascular perfusion. Correlation analysis between dialysis dose as measured by eKt/V and (A) microvascular blood flow ratio and (B) microvascular blood volume ratio.
endothelial homeostasis in the face of injury. Previous reports have demonstrated that early-outgrowth EPLCs of predialysis chronic kidney disease (38) and dialysis (12) patients demonstrate dysfunction in vitro. These reports tested the ability of early-outgrowth EPLCs to migrate along an in vitro VEGF-A chemotactic gradient and to incorporate into “vascular networks” with human umbilical vein endothelial cells in culture (12,39). As our understanding of early-outgrowth EPLC biology has evolved, it has become clear that the proangiogenic functions of these cells are complex, and, in contrast to true endothelial progenitor cells, are likely not due to transdifferentiation into progeny endothelial cells and incorporation into existing vasculature (40). Instead, early-outgrowth EPLCs play an

Figure 3. | Capillary density. Ischemic hindlimb sections were stained with isolectin B4 4 weeks postligation to identify endothelial cells. (A through D) Representative ischemic hindlimb muscle images. Original magnification: ×160. (A) Saline-treated animal. (B) Healthy early-outgrowth EPLC-treated animal. (C) CHD early-outgrowth EPLC-treated animal. (D) NHD early-outgrowth EPLC-treated animal. (E) Capillary density (number of capillaries per 100× field) in the ischemic hindlimb. *P < 0.05 versus saline-treated animal. †P < 0.05 versus healthy early-outgrowth EPLC-treated animal. ‡P < 0.05 versus NHD early-outgrowth EPLC-treated animal.
important supportive role in the neovascularization response, likely recruiting local angiogenic mechanisms (9,41,42) and promoting tissue protection (10). Recognizing that the exact mechanisms by which early-outgrowth EPLCs mediate their proangiogenic effects are still incompletely understood, we chose instead in this translational study to examine the effects of hemodialytic modality on the function of these cells using a model of ischemic vascular disease that integrates these mechanisms in vivo. In doing so, we demonstrate for the first time that early-outgrowth EPLCs derived from CHD patients demonstrate a marked defect in their ability to restore perfusion and microvasculature in ischemic leg tissue, whereas augmenting uremic clearance with NHD can return the in vivo angiogenic capacity of these cells to near-normal levels.

An interesting observation from our study is the demonstration that injected human early-outgrowth EPLCs lead to the endogenous production of Ang1 in ischemic hindlimb muscle. In line with the complexity of the angiogenic process, multiple signaling molecules play stage-specific roles in coordinating new blood vessel growth (43). Ischemic hindlimb muscle was collected 28 days postligation, at a time when the levels of factors such as VEGF-A, Ischemic hindlimb muscle was collected 28 days postligation, at a time when the levels of factors such as VEGF-A, Ang2, and SDF-1α that are activated early in angiogenesis have returned back to baseline (23). In contrast, Ang1 expression is often maintained for weeks after ischemia onset (23). Ang1 plays a critical role in the maturation of newly formed vascular networks in postnatal angiogenesis through the recruitment of pericytes (44) and has been shown to promote neovascularization in the setting of ischemia (45). Because exogenously administered early-outgrowth EPLCs have been shown to regulate host angiogenesis (41), the finding that NHD patient–derived cells, but not CHD patient–derived cells, upregulated endogenous Ang1 expression and ischemic neovascularization raises important mechanistic questions beyond the scope of the study presented here. Because NHD could alter many aspects of early-outgrowth EPLC biology, future studies are needed to clarify the relevance of this finding.

In an effort to assess the effect of hemodialysis regimen on early-outgrowth EPLC angiogenicity in vivo, the study presented here compared early-outgrowth EPLCs derived from age-, gender-, and comorbidity-matched CHD and NHD patients with those derived from healthy controls. Although it is possible that unmeasured variables might explain some of the differences observed in early-outgrowth EPLC angiogenicity, CHD and NHD patients were equally matched for known variables affecting early-outgrowth EPLC function, including age, smoking status, and diabetes prevalence. Although CHD and NHD patients did differ in antihypertensive usage, this is a known improvement associated with NHD (13,15) and may reflect improvements in endothelial function (13). Importantly, although early-outgrowth EPLCs from CHD patients had similar effects as saline injection, cells derived from NHD patients exerted angiogenic activity similar to cells derived from younger healthy controls, suggesting dramatic differences in function that seem unlikely to be fully explained by nondialytic factors.

In conclusion, this study is the first to document that NHD is associated with significant improvements in the in vivo angiogenic activity of peripheral-blood-derived early-outgrowth EPLCs using an immunodeficient rodent model of ischemic vascular disease. The study presented here suggests that augmenting dialysis dose might restore the ability of early-outgrowth EPLCs to mediate vascular repair. Future studies are urgently needed to test the effects of NHD on early-outgrowth EPLC angiogenicity in the setting of human vascular disease.

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Disclosures
None.

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14. O’Hare AM, Feinglass J, Sidaway AN, Bacchetti P, Rodrige


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H.L. and C.T.C. contributed equally to this manuscript.

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Supplemental information for this article is available online at www.cjasn.org.
**Supplemental Table 1: Primer sequences used to assess endogenous angiogenic gene expression in rat hindlimb muscle.**

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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Ang1 (forward)</td>
<td>GAG CAT AAA ATC CTA GAA ATG GAG GG</td>
</tr>
<tr>
<td>Ang1 (reverse)</td>
<td>TGC AGA ACA CTG TTG TTG CTG GTA GC</td>
</tr>
<tr>
<td>Ang2 (forward)</td>
<td>TTT GTC TCC CAG CTG ACC AGT GG</td>
</tr>
<tr>
<td>Ang2 (reverse)</td>
<td>GAC AGG TAG AAG TGC TCA TAC AG</td>
</tr>
<tr>
<td>VEGF-A (forward)</td>
<td>CCC TGG CTT TAC TGC TGT ACC T</td>
</tr>
<tr>
<td>VEGF-A (reverse)</td>
<td>TCC ATG AAC TTC ACC ACT TGA TG</td>
</tr>
<tr>
<td>SDF-1α (forward)</td>
<td>GCT CTG CAT CAGT GAC GGT AAG</td>
</tr>
<tr>
<td>SDF-1α (reverse)</td>
<td>TGG CGA CAT GGC TCT CAA A</td>
</tr>
<tr>
<td>Rpl13a (forward)</td>
<td>GAT GAA CAC CAA CCC GTC TC</td>
</tr>
<tr>
<td>Rpl13a (reverse)</td>
<td>CAC CAT CCG CTT TTT CTT GT</td>
</tr>
</tbody>
</table>

**Abbreviations:** Ang1: angiopoietin 1, Ang2: angiopoietin 2, VEGF-A: vascular endothelial growth factor-A, SDF-1α: stromal cell derived factor-1α.
**Supplemental Table 2**: Ratios of angiogenic factor expression levels in ischemic to nonischemic hindlimb muscle 28 days post-ischemia.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>CHD</th>
<th>NHD</th>
<th>One-way ANOVA p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang1 (AU)</td>
<td>1.00 ± 0.36</td>
<td>0.86 ± 0.51</td>
<td>5.48 ± 2.68 *</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Ang2 (AU)</td>
<td>1.00 ± 0.75</td>
<td>1.04 ± 0.64</td>
<td>1.16 ± 0.66</td>
<td>0.99</td>
</tr>
<tr>
<td>VEGF-A (AU)</td>
<td>1.00 ± 0.20</td>
<td>0.45 ± 0.08 †</td>
<td>1.11 ± 0.18</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>SDF-1α (AU)</td>
<td>1.00 ± 0.19</td>
<td>0.76 ± 0.27</td>
<td>1.08 ± 0.20</td>
<td>0.62</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. saline- and CHD early outgrowth EPLC-treated animals. † p < 0.05 vs. saline and NHD animals. All values are referenced to mRNA transcript levels of the housekeeper gene Rpl13a, and represent ratios of mRNA levels between the ischemic (left) and nonischemic (right) limbs. All values are indexed to saline-treated animals.

**Abbreviations**: Ang1: angiopoietin 1, Ang2: angiopoietin 2, VEGF-A: vascular endothelial growth factor-A, SDF-1α: stromal cell derived factor-1α, AU: arbitrary units, CHD: conventional hemodialysis, NHD: nocturnal hemodialysis.
Supplemental Movie 1: Saline-treated ischemic hindlimb muscle. Representative 20 second time lapse contrast enhancement video of ischemic hindlimb muscle treated with saline injection. Video was obtained 28 days post-iliac artery ligation.

Supplemental Movie 2: Healthy control early outgrowth EPLC-treated ischemic hindlimb muscle. Representative 20 second time lapse contrast enhancement video of ischemic hindlimb muscle injected with \(5 \times 10^5\) early outgrowth EPLCs derived from a healthy control individual. Video was obtained 28 days post-iliac artery ligation.

Supplemental Movie 3: CHD early outgrowth EPLC-treated ischemic hindlimb muscle. Representative 20 second time lapse contrast enhancement video of ischemic hindlimb muscle injected with \(5 \times 10^5\) early outgrowth EPLCs derived from a CHD patient. Video was obtained 28 days post-iliac artery ligation.

Supplemental Movie 4: NHD early outgrowth EPLC-treated ischemic hindlimb muscle. Representative 20 second time lapse contrast enhancement video of ischemic hindlimb muscle injected with \(5 \times 10^5\) early outgrowth EPLCs derived from a NHD patient. Video was obtained 28 days post-iliac artery ligation.