THE EFFECT OF CHRONIC KIDNEY DISEASE

ON ENDOTHELIAL FUNCTION AND PROGENITOR CELLS

by

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ABSTRACT

Patients diagnosed with chronic kidney disease (CKD) are more likely to die of cardiovascular disease than progress to end stage renal disease. Evidence indicates that increased cardiovascular mortality in CKD patients may be related to compromised vascular integrity and ultimately endothelial dysfunction (ED) leading to poor tissue oxygen perfusion at not only renal but also cardiovascular tissues. Consequentially, progenitor-cell-based therapies have been suggested to preserve vascular continuity and induce vascularization, thereby preventing endothelial cell apoptosis, ED, and ultimately organ failure and cardiovascular events in CKD patients. The purpose of this investigation was to enumerate circulating progenitor cell (CPC) subpopulations of hematopoietic, endothelial, and myeloid lineage in CKD patients and compare them to apparently healthy controls. Additionally, a colony forming unit (CFU) assay was performed and endothelial-dependent dilation (EDD) was assessed using a non-invasive measurement of brachial artery reactivity known as flow-mediated dilation (FMD). We hypothesized that progenitor cell subpopulations would be reduced and CFUs as well as EDD would be impaired in the CKD subjects as compared to the healthy controls. Also, regression analysis was performed looking at the relationships between CPC subpopulations, CFUs, subject characteristics, and EDD measurements. Ten individuals with stages 3-5 CKD and ten apparently healthy controls were recruited, blood was drawn and brachial artery FMD was performed. CPCs were then quantified using the cell surface markers CD34, KDR, and CD45 with flow cytometry, and peripheral blood mononuclear cells isolated from the venous

blood were cultured for the colony-forming assay. Renal function was significantly lower in the CKD patients as indicated by estimated glomerular filtration rate, elevated blood urea nitrogen, and increased serum creatinine levels. CKD patients also had significantly lower average percent changes in vessel diameter, and the hematopoietic progenitor subpopulations CD34+, CD34+/KDR+, CD34+/CD45-, and CD34+/KDR+/CD45- were reduced in this CKD sample. Furthermore, myeloid precursor populations CD45+ and CD45+/KDR+ were lower and colony forming ability was impaired in CKD patients with differences approaching statistical significance. Regression analysis indicated that the CPC subpopulations CD34+, CD34+/CD45-, and CD34+/KDR+/CD45-, in addition to EDD, were negatively associated with serum creatinine and blood urea nitrogen levels, whereas CD34+ and CD34+/KDR+/CD45- CPCs were also significantly positively correlated with percent change in vessel diameter. In conclusion, multiple subset populations of CPCs have been identified, shown to be reduced, and associated with EDD in the CKD population. Reductions in these progenitor cell subpopulations may be a potential mechanism by which vascular integrity is compromised and endothelial dysfunction results, increasing the risk of CVD and contributing to renal disease progression in CKD patients. Therefore, treatment strategies that target these specific subpopulations may be important in optimizing therapeutic effectiveness and ultimately improving renal and cardiovascular outcomes in this disease population.

Chapter 1

INTRODUCTION

Patients diagnosed with chronic kidney disease (CKD) are more likely to die than progress to end stage renal disease (ESRD; stage 5; GFR < 15 mL/min/1.73 m^2) (1). Evidence indicates that poor patient survival can be attributed to traditional and non-traditional cardiovascular risk factors paralleled with renal disease progression resulting in an accelerated development of cardiovascular disease (CVD) (19, 57). With CVD advancement, end-organ damage ensues, manifesting in such forms as myocardial infarction, cerebrovascular insults, and various ischemic diseases, which can now be considered the most significant causes of mortality and morbidity in the CKD population (9). Thus, establishing novel therapeutic strategies that target the underlying mechanisms of increased CVD risk in CKD is imperative.

Endothelial dysfunction (ED) is evident in CKD and suggested to play an important role in not only the development of CVD but also the persistence of renal insufficiency (50, 58). The endothelial monolayer lining the blood vessels helps maintain vascular homeostasis by mediating vascular tone, vessel wall inflammation, thromboresistance, and cellular adhesion through the release and regulation of various vasoactive substances (16). During healthy physiological conditions, the intact endothelium efficiently responds to physical and chemical changes within the vasculature and controls tissue oxygen perfusion without prolonged activation of host

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defense responses (18). Conversely, continual and/or repeated exposure to cardiovascular risk factors compromises vascular integrity resulting in ED, facilitating pro-atherosclerotic conditions, oxidative stress, and impaired tissue oxygen delivery to cardiovascular and renal tissues (9, 18). In this regard, progenitor-cell-based therapies have been suggested to preserve vascular continuity and induce vascularization, thereby preventing endothelial cell apoptosis, ED, and ultimately organ failure and cardiovascular events in CKD patients (9, 36).

Progenitor cells, both resident and circulating, have recently emerged as important mediators of vascular health with promising therapeutic potential. Re- and neovascularization are no longer thought to exclusively result from migration and proliferation of fully-differentiated resident endothelial cells, but rather different progenitor cell populations are thought to also facilitate these processes (12, 32, 68). Specifically, circulating progenitor cells (CPCs) have been proposed to migrate to sites of hypoxia and vascular injury and construct scaffolds for new capillary formation, release pro-angiogenic factors, and/or differentiate into mature endothelial cells, either replacing damaged cells or forming new capillaries (3, 39, 68, 77). In humans and animal models, these progenitor cells have been shown to improve blood flow, cardiac function, and left-ventricular function in myocardial infarction and critical limb ischemia (11, 20, 35, 56, 65). Further, progenitor cells are essential in normal cardiovascular functioning and are numerically and functionally impaired in various cardiovascular diseases, ESRD, and with CKD progression (13, 36, 47, 54, 70, 73, 76). Hence, progenitor cell insufficiencies in CKD could be potential mechanism

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by which vascular integrity is compromised and ED results, increasing the risk of CVD in CKD patients, and contributing to renal disease progression.

Among progenitor cell populations, Krenning et al, among others, have identified two in the peripheral blood involved in neovascularization with both distinct and overlapping functions including CD34+ and CD14+ cells, potentially of hematopoietic and myeloid lineage, respectively (5, 37, 53, 55, 68). CD14+ CPCs attach to the endothelium, transcellularly migrate, and tunnel through the endothelial basement membrane and extracellular matrix forming scaffolds for new capillary formation (2, 39, 45). CD34+ are postulated to then replace the CD14+ cells lining the tunnels while both CPC populations release various angiogenic factors creating a pro-angiogenic environment and stimulating proliferation of the mature endothelium in the surrounding tissue (3, 39). Studies using animal models support these hypotheses by showing CD14+ tunneling in the ischemic myocardium and CD14+/CD34+ induced neovascularization (2, 6, 45, 81).

CD14+ and CD34+ CPCs could also be involved in revascularization via paracrine signaling and/or incorporating into the endothelium replacing apoptotic endothelial cells (24, 68, 78). Both CPC populations secrete the angiogenic factors hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). Also, CD14+ CPCs have been shown to induce re-endothelialization *in-vivo* and differentiate into mature endothelial cells when cultured with HGF produced by CD34+ cells (6, 24, 38, 43, 81).

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CD34+ and CD14+ CPC have been identified in CKD patients (36). CD34+ cells were reduced in this disease population, while CKD progression had no significant effect on CD14+ cell numbers (36). However, subpopulations of CD34+ and myeloid CPCs in CKD where CVD risk is increased have not been identified. Additional cell surface markers indicative of progenitor cells including those of endothelial and myeloid lineage such as KDR (VEGF-Receptor II), and CD45, respectively, could be used to identify progenitor cell subpopulation deficiencies. This may be advantageous in progenitor-cell based therapy in order to mobilize and revitalize specific deficient progenitor cell subpopulations in CKD. Therefore, the purpose of this investigation was to enumerate CPC subpopulations in CKD patients and apparently healthy controls using the cell surface markers CD34, KDR, and CD45. Additionally, a colony forming unit (CFU) assay was performed and endothelial-dependent dilation (EDD) was assessed using a non-invasive measurement of brachial artery reactivity known as flow-mediated dilation (FMD). We hypothesized that progenitor cell subpopulations would be reduced and CFUs as well as EDD would be impaired in the CKD patients as compared to the healthy controls. Regression analysis was also performed to examine the relationships between CPC subpopulations, CFUs, subject characteristics, and EDD measurements.

Chapter 2

MATERIALS AND METHODS

Subjects, Screening, & Blood Samples

Ten Stage 3-5 CKD patients (53±6; 7 men and 3 women) and ten age and sex matched controls (48±4) were recruited for study. Exclusion criteria included history of coronary artery disease, myocardial infarction, and/or heart failure; untreated hypertension, current drug therapy for pulmonary, autoimmune, and/or HIV diseases; presence of hepatic disease, cancer, immunosuppressant therapy, antiretroviral therapy, and current tobacco use. Medications for hypertension, dyslipidemia (statins), and blood glucose regulation were continued during investigation. Estimated glomerular filtration rate (eGFR) was determined in all subjects using the Modification of Diet in Renal Disease (MDRD) equation based on serum creatine, age, gender, and race as recommended by the National Kidney Foundation (1, 41). CKD subjects' eGFRs were required to fall within the range of < 60 ml/min/1.73m^2 and healthy needed to be > 60. Written and verbal consent was administered to all subjects in accordance with the guidelines of the University of Delaware Human Subjects Review Board.

Subject screening was conducted after abstinence from food for at least 4 hours, alcohol and caffeine for 12 hours, and strenuous physical activity for 24 hours. All subjects completed a medical history questionnaire and underwent resting blood

pressure, heart rate, height, and weight measurements. Venous blood and urine samples were taken and sent to an approved commercial lab for assessment of liver enzymes, a lipid profile, renal function tests, hemoglobin, hematocrit, and blood glucose levels. From these data, patient eligibility was determined. Additional venous blood samples were taken either during the screening process or on a separate visit to be utilized for CPC enumeration and peripheral blood mononuclear cell (PBMNC) isolation.

CPC Flow Cytometry

Flow cytometry was used to quantify CPC subpopulations in the peripheral blood consistent with published practices and recommendations (21, 61). Each subject's venous blood sample collected was separated into five 150 µL aliquots to be processed and analyzed simultaneously: (1) received no antibody; (2) solely labeled with a fluorescein isothiocyanate (FITC) conjugated-CD34 antibody (BD Biosciences); (3) only labeled with a phycoerythrin (PE)-conjugated-KDR antibody (VEGF Receptor-2; R & D Systems), (4) exclusively received the allophycocyanin (APC)-conjugated-CD45 antibody (BD Biosciences); and (5) labeled with the CD34-FITC, KDR-PE, and CD45-APC antibodies.

The protocol for preparing antibody suspensions consisted of adding each monoclonal antibody to their respective 150 µL blood sample at the manufacturers' recommended concentration and incubating them for 15-20 minutes. Then 1ml of a 1:10 FACS™ Lysis Solution was added and incubation continued for an additional 30-45 minutes, at which time the solutions were washed, centrifuged at 3200 RPMs

for 5 minutes, pelleted, and resuspended in PBS to be put through a cell strainer and eventually a FACSCalibur Flow Cytometer.

FACSComp software and unlabeled, FITC, PE, and APC BD Calibrite™ beads were used to set compensation parameters and distinguish cells from sample debris and/or background signal. CellQuest™ software was used to isolate the lymphocyte population where progenitor cell populations are thought to reside and determine the amount of cells that were CD34+, CD34+/KDR+, CD34+/KDR+/CD45-, CD45+, and CD45+/KDR+ per 500,000 total events (73). Individual antibody control suspensions were initially added to adjust for confounding graphical overlap and potential false-positives of single and multiple labeled cells. In the FSC vs. SSC plot, the forward scatter threshold was set at 200 to exclude debris and non-viable elements (23). Subjects were excluded from study if the percentage of their CD34+ CPCs obtained did not fall within the normally accepted range of 0.01% to 0.1% (63).

PBMNC Isolation and Colony Forming Assay

PBMNC isolation and the colony forming assay were performed as previously described (30). Briefly, 15 ml of each subject's blood obtained intravenously and the equivalent of phosphate buffer saline (PBS) were added to a sterile 50 ml AccuspinTM System-Histopaque® tube (Sigma-Aldrich) containing a porous high density polyethylene barrier and Histopaque® for density-dependent centrifugation. This separated the blood sample into plasma, PBMNC, Histopaque®, and red blood cell layers, so that the PBMNC layer could be removed and washed

with 10ml of PBS. The cell aggregate was then washed again with 5 ml of PBS and resuspended in 1-3ml of growth media containing Medium-199, 20% fetal bovine serum, and 1% streptomycin. Following resuspension, the amount of cells in solution were counted in a 1/20 dilution with Trypan Blue (Sigma-Aldrich) using a hemocytometer, and 5×10^6 cells were added to each well of a 6-well fibronectin-coated plate (Becton Dickson) containing 2 ml of growth media to be incubated at 37°C in a 5% CO_2 environment.

After forty-eight hours in culture, the non-adherent cells were removed, counted in a 1/10 dilution with Trypan Blue and 7-10 million were used for immunophenotyping, while the remainder of cells was added to each well of a 24-well fibronectin-coated plate (Becton Dickson) at a concentration of 1×10^6 cells/well in 1 ml of growth media. Cell culture continued for a period of seven days with the media being changed on days six and nine until fixed and stained on day 10. CFUs, described as multiple, thin, flat cells emanating from a central cluster of cobblestone cells, were counted by two independent investigators in three randomly selected wells (30, 42). Endothelial and myeloid cell phenotype were assessed by incubating CFUs with fluorescent dye-labeled acetylated-LDL (DiL AcLDL; 10ug/mL) at 37°C for 1 hour and fluorscein-isothiocyanate-conjugated Ulex eurpaeus agglutinin (UEA-1; 10ug/mL) for 1 hour.

Non-Adherent Cell Immunophenotyping

On Day 2 of cell culture, 7-10 million non-adherent cells were removed, counted using a hemocytometer, and aliquoted into respective microcentrifuge tubes at

a concentration of 1×10^6 cells/tube to be centrifuged at 3200 RPMs for 5 minutes. The cell aggregates were then washed with PBS and resuspended to be incubated in antibody solutions at the manufacturer's recommended concentrations for 1 hour. Fluorescently-conjugated antibodies of cell surface markers used are associated with cells of hematopoietic, myeloid, and endothelial lineage. These included CD31-FITC (PECAM-1; BD Biosciences), CD34-FITC, CD45-FITC (BD Biosciences), and CD144-FITC (VE-cadherin; AbD Serotec) as well as KDR-PE. CellQuest™ was used to isolate the population of cells representing the majority of cultured cells and count the number and percentage of cells/200,000 gated events that expressed each antibody as compared to unlabeled controls.

Endothelial-Dependent Dilation

An endothelial-dependent FMD fasted measurement using high resolution ultrasound (Titan, Sonosite, Inc.) was performed in accordance with published guidelines (15). All premenopausal women underwent the FMD measurement during the early follicular phase of menstruation.

A pressure cuff was placed just distal to the olecranon process of the non-dominant arm and inflated at 200 mmHg for 5 minutes until released to induce reactive hyperemia. Vasodilatory responses of the brachial artery using a 10 MHz linear phased array ultrasound transducer were recorded at 15 seconds pre- until 2 minutes post cuff release to determine the peak diameter change. Blood velocity, the shear rate stimulus, was also simultaneously assessed with a 4 MHz continuous wave Doppler probe (Multigon 500P) placed just distal to the ultrasound transducer. FMD is

expressed as the percent increase in diameter from baseline (i.e. change in diameter divided by baseline diameter) and the magnitude of the hyperemic stimulus as the area under the curve (AUC) of the shear rate (s-1 Vmean/vessel diameter) profile from cuff deflation to peak diameter. Analysis of brachial artery images and Doppler data collected were recorded and analyzed on custom-made LabVIEW 8.0 software programs.

Statistical Analysis

The statistical design consisted of using unpaired one-tailed t-tests (α = .05) to assess all three hypotheses and determine if the amount of CPCs were reduced and colony forming ability and endothelial-dependent dilation were impaired in the CKD group when compared to the apparently healthy controls. Regression analysis was also conducted to examine the relationships between progenitor cell subpopulations, CFUs, subject characteristics, and endothelial dependent dilation measurements. All statistical tests were carried out in Microsoft Excel and SPSS® statistical software version 17.0. Data are presented as mean values ± SEM.

Chapter 3

RESULTS

Subject Characteristics. A total of 20 subjects were recruited for study including 10 CKD patients in stages 3-5 CKD with a mean eGFR of 34.4 ± 5 and 10 apparently healthy controls (*see Table 1*). All of the CKD patients were on various antihypertensive medications (n = 10), 60% were on statins (n = 6), and 3 were using insulin. Of the apparently healthy controls, two subjects were excluded from the study due to CD34+ CPCs percentages that did not fall within the normally accepted range (>0.3%). *Table 1* depicts the corrected number of subjects in each respective group and compares mean differences in subject demographic, hemodynamic, renal function, and blood measurements.

CKD patients weighed significantly more than healthy controls (71 ± 3.4 vs. 89 ± 5; p = 0.009) and had higher average body-mass indexes (24.7 ± 0.10 vs. 29.6 ± 1.95; p = 0.027). This was also true for hemodynamic measurements of systolic blood pressure (110 ± 3 vs. 140 ± 5; $p < 0.0001$), diastolic blood pressure (65 ± 2 vs. 79 ± 40; $p < 0.004$), and mean arterial pressure (80 ± 2 vs. 99 ± 3; $p < 0.001$), regardless of antihypertensive medication. Renal function was significantly lower in the CKD patients as indicated by blood urea nitrogen (16 ± 1.69 vs. 40 ± 6.8; p = 0.003) and serum creatinine levels ($.888 \pm 0.048$ vs. 2.47 ± 0.391; $p < 0.002$). CKD subjects did not have significantly higher blood cholesterol levels, however blood

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Table 1 *Subject Characteristics*. **Values are means ± SE; n, no of subjects; CKD, chronic kidney disease; BMI, body-mass index; ACE, angiotensin converting enzyme; ANG, angiotensin; *p < 0.05, **p < 0.01, ***p< 0.001, †p < .0001**

	Control	*CKD*
n	8	10
Demographic Information		
Age (yr)	45 ± 5	53 ± 6
Height (cm)	170 ± 2.9	174 ± 3.5
Weight (Kg)	71 ± 3.4	89 ± 5.2**
BMI (Kg/m^2)	24.7 ± 0.10	29.6 ± 1.95*
Hemodynamic Measurements		
Resting HR (BPM)	62 ± 3	65 ± 2
Systolic Blood Pressure (mmHg)	110 ± 3	140 ± 5†
Diastolic Blood Pressure (mmHg)	65 ± 2	79 ± 4**
Mean Arterial Pressure (mmHg)	80 ± 2	99 ± 3†
Renal Function		
Blood Urea Nitrogen (mg/dl)	16 ± 1.69	40 ± 6.8**
Serum Creatinine (mg/dl)	.888 ± 0.048	2.47 ± 0.391**
eGFR (ml/min/1.73m^2)	>60	34.4 ± 5
Blood Lipids, Cells, and Glucose		
Total Cholesterol (mg/dl)	192.5 ± 13.9	208.2 ± 16.7
High-Density Lipoprotein (mg/dl)	52.3 ± 4.05	57.8 ± 8.1
Low-Density Lipoprotein (mg/dl)	122 ± 12.7	122 ± 12.8
Triglycerides (mg/dl)	91.3 ± 11.3	141.9 ± 28.1
Hemoglobin (gm/dl)	13.93 ± 0.25	12.66 ± 0.56*
Hematocrit (%)	40.84 ± 0.706	38.67 ± 1.50
Glucose (mg/dl)	82.5 ± 1.57	102.7 ± 10.3*
Medications (Number of Patients)		
Antihypertensives	0	10
ACE Inhibitors	0	6
ANG II-receptor Antagonists	0	1
Beta-Blockers	0	4
Diuretics	0	5
Calcium Antagonists	0	6
Statins	0	6
Insulin	0	3
Erythropoiesis-Stimulating Agents	0	1
Allopurinol	0	2

glucose levels were higher (82.5 ± 1.57 vs. 102.7 ± 10.3; p = 0.044) and elevated

triglyceride levels approached significance (91.3 ± 11.3 vs. 141.9 ± 28.1; p = 0.06).

Blood hemoglobin was significantly reduced in the CKD patients (13.93 ± 0.249 vs.

$12.66 \pm .560$; p = 0.03).

      EDD, CPC subpopulations, and CFUs. CKD patients had significantly

lower average percent changes in vessel diameter (10.12 ± 1.4 vs. 5.39 ± 1; p = 0.007)

in response to a shear stimulus that was not significantly different between groups

(32687.4 ± 11860.6 vs. 27797.205 ± 9260.5; p = 0.380) (*see figure 1*).

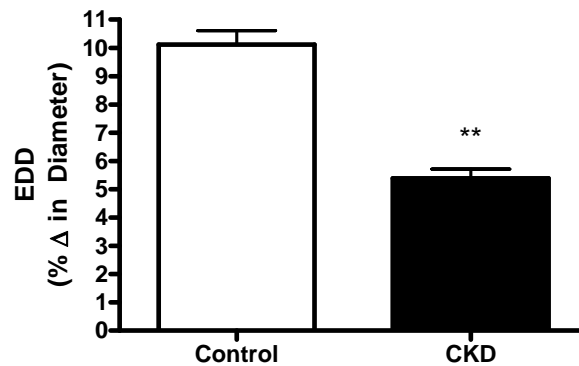*Figure 1*   **Endothelial dependent dilation (EDD) responses in healthy controls (n = 8) and CKD subjects (n = 10). The shear rate stimulus as measured by the area under the curve of the shear rate profile from cuff deflation to peak diameter was not significantly different between groups, whereas % change in vessel diameter was, showing an impaired response in the CKD subjects. **p < 0.01**

The hematopoietic progenitor subpopulations CD34+ (565.8 ± 87.6 vs. 242 ± 40.7; p = 0.004), CD34+/KDR+ (475.2 ± 93.2 vs. 276.9 ± 65.9; p = 0.046), CD34+/CD45- (428.4 ± 100.7 vs. 131.8 ± 30.4; p = 0.004), and CD34+/KDR+/CD45- (298.5 ± 41.2 vs. 107 ± 2.8; p < 0.001) showed a similar trend (*see Figure 2A*), being significantly reduced in the CKD sample, whereas the myeloid precursor populations CD45+ ($1.32 \times 10^5 \pm 1.6 \times 10^4$ vs. $9.9 \times 10^4 \pm 1.3 \times 10^4$; p = 0.07) and CD45+/KDR+ ($4.1 \times 10^4 \pm 1.8 \times 10^4$ vs. $1.1 \times 10^4 \pm 5.9 \times 10^3$; p = 0.07; *see Figure 2 B*), in addition to colony forming units (4.3 ± .953 vs. 2.64± .502, p = 0.065; *see Figure 2C*), were reduced but only approached significance. With *univariate analysis*, flow mediated dilation measurements and hematopoietic progenitor cell subpopulations were significantly associated with a reduction in renal function as measured by increased blood urea nitrogen (FMD: r = -0.627, p = 0.004; CD34+: r = -0.529, p = 0.015; CD34+/CD45-: r = -0.509, p = 0.019; CD34+/KDR+/CD45-: r = -0.588, p = 0.007) and serum creatinine levels (FMD: r = -0.628, p = 0.003; CD34+: r = -0.492, p = 0.019; CD34+/CD45-: r = -0.421, p = 0.041; CD34+/KDR+/CD45-: r = -0.521, p = 0.013; *see Figure 3A-D and Table 2*). FMD was also positively associated with increasing eGFR in CKD patients (r = .647, p = 0.021). In contrast, CPC subpopulations did not significantly correlate with eGFR.

The CD34+/KDR+ subpopulation was negatively associated with increasing serum creatinine (r = -0.313, p = 0.103) and blood urea nitrogen levels (r = -0.359, p = 0.079), but did not reach statistically significant values, and the myeloid precursor subpopulations CD45+ and CD45+/KDR+, and colony forming units, showed no significant relationship with serum creatinine (r = -0.115, -0.265, & -0.281,

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respectively) and/or blood urea nitrogen levels (r = 0.127, -.230, & -.310, respectively).

Other demographic factors that were associated with hematopoietic progenitor subpopulations and flow mediated dilation included body mass index (FMD: r = -0.273, p = 0.173; CD34+: r = -0.41, p = 0.083; CD34+/CD45-: r = -0.421, p = 0.041; & CD34+/KDR+/CD45-: r = -0.499, p = 0.018), systolic blood pressure (FMD: r = -0.393, p = 0.053, CD34+: r = -0.488, p = 0.020; CD34+/CD45-: r = -0.388, p = 0.056, & CD34+/KDR+/CD45-: r = -0.536, p = 0.011) and blood glucose levels (FMD: r = -0.377, p = 0.093; CD34+: r = -0.408, p = 0.052; CD34+/CD45-: r = -0.407, p = 0.053; CD34+/KDR+/CD45-: r = -0.558, p = 0.10) (*see Table 2*) . The CD34+/KDR+ hematopoietic and CD45+ and CD45+/KDR+ subpopulations were not significantly associated with any other demographic criterion (*see Table 2*). Lastly, hematopoietic progenitor cell subpopulations CD34+ and CD34+/KDR+/CD45- were also positively correlated with percent change in diameter as a measured by flow-mediated dilation (r = 0.420, p = 0.041 & r = 0.460, p = 0.047, respectively; *see Table 2 & Figure 4*), and the relationships between CFUs and flow-mediated dilation (r = 0.460, p = 0.057) as well as CFUs and systolic blood pressure (r = -0.420, p = 0.076) approached significance.
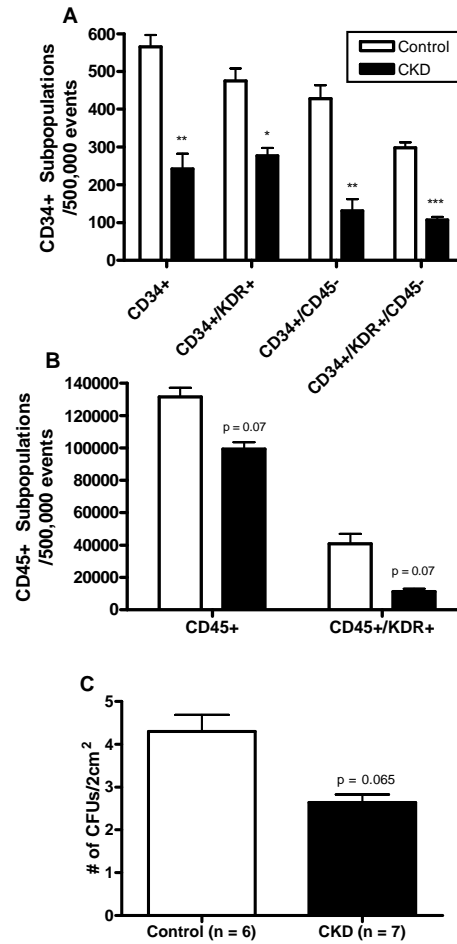
Figure 2 **CPC numbers per 500,000 events in controls (n = 8) and CKD subjects (n = 10). CPCs with hematopoietic and endothelial markers were all significantly lower in the CKD group (A), whereas the myeloid progenitors were lower in the CKD patients but differences only approached significance (B). Reduced CFUs in CKD also approached significance with a p-value of 0.065 (C). *p <0.05, **p <0.01, ***p<.00001**

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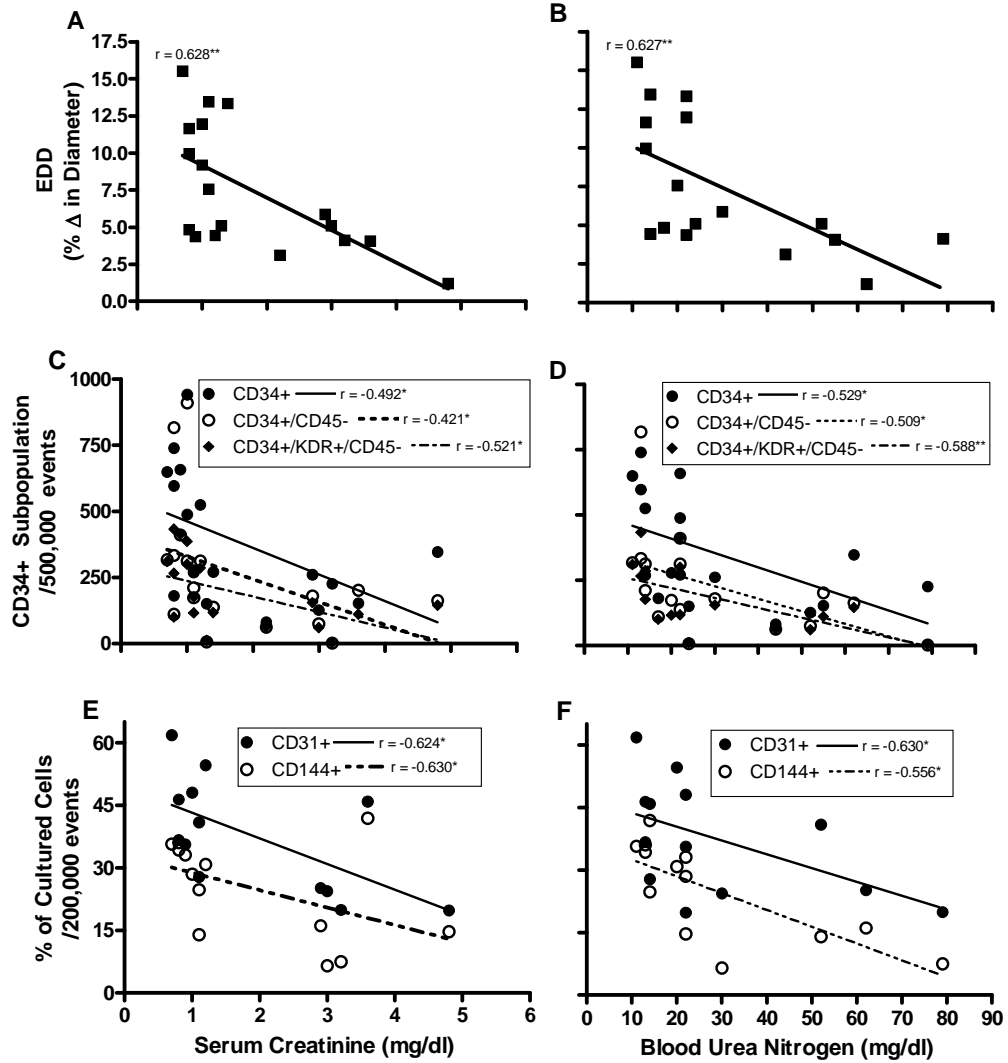
Figure 3   *Serum creatinine and blood urea nitrogen associations* **with EDD (% change in diameter) (A & B; n = 17), hematopoietic CPC subpopulations (C & D; n = 17), and the % of cultured cells expressing endothelial cell surface markers CD31 and CD144 (E & F; n = 13). *p<0.05 and **p<0.01**

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Multivariate analysis was used to assess the relationship between blood urea nitrogen and serum creatinine levels and the hematopoietic CPC subpopulation, CD34+/KDR+/CD45-, in the presence of various other significant influential demographic factors (*see Table 2*). After considering the high collinearity between systolic blood pressure and body mass index (r = 0.564, p = 0.007), blood glucose levels and BUN (r = 0.822, p < .0001), and BUN and serum creatinine (r = 0.844, p < 0.001), body mass index and BUN were chosen to be put into the multiple linear regression equation via forced entry. The adjusted r-values for BMI and BUN are depicted in *Table 2* as -0.478 (p = 0.071) and -0.522 (p = 0.046), demonstrating that BUN was the only factor significantly associated with this CPC subpopulation in the calculated regression model.

Table 2   *Univariate and multivariate analysis* **of demographic factors associated with hematopoietic progenitor cell subpopulations and endothelial-dependent dilation (EDD). *p<0.05, **p<0.01; BUN, blood urea nitrogen.**

					Multivariate Analysis	
		Univariate Analysis			CD34+/KDR+/CD45-	
				CD34+/KDR +/CD45-	Adjusted	
	FMD	CD34+	CD34+/CD45-		r Value	Probability
BMI	−0.273	−0.341	−0.421*	−0.499*	−0.478	0.071
SBP	−0.393	−0.488*	−0.388	−0.536*		
Glucose	−0.337	−0.408	−0.407	−0.558*		
Serum Creatinine	−0.628**	−0.429*	−0.421*	−0.521*		
BUN	−0.627**	−0.529*	−0.509*	−0.588*	−0.522*	0.046*
EDD	——	−0.420*	−0.336	−.406*		

Non-adherent cultured PBMNCs phenotype. The non-adherent PBMNC population obtained from each subject that eventually formed the colony forming cells expressed the endothelial cell surface markers CD31 (37.5% ± 3.5), CD144 (24.9% ± 3.3), and KDR (17.5% ± 3.5); hematopoietic progenitor cell marker CD34 (3.1% ± 0.6), and the myeloid precursor marker CD45 (94.6% ± 0.84). CKD patients demonstrated significantly lower percent CD34+ (4.5 ± 0.978 vs. 1.9 ± 0.536; p = 0.018) and CD144+ cells (32.1±1.8 vs. 18.8 ± 4.9; p = 0.017) than the apparently healthy controls (*see Table 3*). The CD31+ and CD45+ populations were also lower in the CKD group (CD31+: 42.7 ± 4.8 vs. 32.9 ± 5.3, p = 0.104; CD45+: 95.9 ± 1.1 vs. 93.4 ± 1.1, p = 0.076), approaching significance. No significant difference was observed with non-adherent cell KDR expression between groups (19.6±3.3 vs. 15.9±.9; p = 0.323).
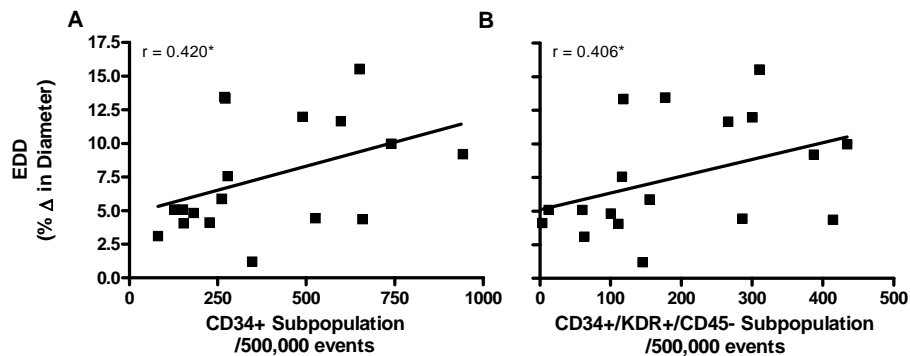

Figure 4   **CD34+ (A) and CD34+/KDR+/CD45- (B) subpopulations as associated with EDD (% change in diameter) (n = 18). *p<0.05**

Pearson correlations revealed that the percentage of cells expressing one of the endothelial, myeloid, and hematopoietic cell surface markers, except KDR, were significantly associated with serum creatinine (CD31+: r = -0.624, p = 0.011; CD34+: r = -0.505, p = 0.039; CD45+: -0.627, p = 0.013; CD144: -0.487, p = 0.046) and blood urea nitrogen levels (CD31+: r = -0.630, p = 0.011; CD34+: r = -0.527, p = 0.032; CD45+: -0.615, p = 0.013; CD144: -0.556, p = 0.024; *see Table 3 and Figure 3*). The percentage of the non-adherent CD31+, CD144+, and CD45+ subpopulations were also significantly associated with blood glucose levels (CD31+: r = -0.519, p = 0.042; CD144+: r = -0.573, p = 0.026; CD45+: r = -0.530, p = 0.038), and the % of CD31+ cells, specifically, was significantly correlated with flow-mediated dilation (r = 0.489, p = 0.045) and estimated glomerular filtration rate (r = 0.791, p = 0.017).

CPCs association with non-adherent cell population. Univariate regressions were performed to establish the relationship between CPC numbers and the expression of the myeloid, endothelial, and hematopoietic cell surface markers in the non-adherent PMNC population. CD34+, CD34+/KDR+, CD34+/CD45-, & CD34+/KDR+/CD45- CPCs were all significant related to cultured cell expression of CD31 (CD34+: r = 0.575, p = 0.20, CD34+/KDR+: r = 0.630, p = 0.010; CD34+/CD45-: r = 0.520, p = 0.034; CD34+/KDR+/CD45-: r = 0.596, p = 0.016), CD34 (CD34+: r = 0.732, p = 0.002; CD34+/KDR+: r = 0.705, p = 0.004; CD34+/CD45-: r = 0.680, p = 0.005; CD34+/KDR+/CD45-: r = 0.696, p = 0.004), and CD144 (CD34+: r = 0.614, p = 0.013; CD34+/KDR+: 0.854, p<.0001; CD34+/CD45-: 0.636, p = 0.10; CD34+/KDR+/CD45-: r = 0.697, p = 0.004). The myeloid precursor population CD45+/KDR+ was significantly correlated with increased CD31 (r =

0.554, p = 0.025) and CD45 (r = 0.525, p = 0.033) expression. The CD34+ and

CD34+/KDR+/CD45- subpopulations were also associated with CD45 expression

(CD34+: r = 0.515, p = 0.036; CD34+/KDR+/CD45-: r = 0.560, p = 0.023), and

CD34+/KDR+ CPCs were significantly related to KDR expression (CD34+/KDR+: r

= 0.849, p<.0001).

Table 3   **Non-adherent PMBNC phenotype as assessed by the hematopoietic, myeloid, and endothelial cell surface markers. Control and CKD groups are expressed as the percentage of cell expressing each marker per 200,000 events. *p<0.05; BUN, blood urea nitrogen**

	Control (n =6)	CKD (n =7)	p-Value	Univariate Analysis Serum Creatinine r Value	BUN r Value
CD31+	42.7±4.8	32.9±5.3	0.104	–.624*	–.630*
CD34+	4.5±.978	1.9±0.536	0.018*	–0.505*	–0.527*
CD45+	95.9±1.1	93.4±1.1	0.076	–0.627*	–0.615*
CD144+	32.1±1.8	18.8±4.9	0.017*	–0.487*	–0.556*
KDR+	19.6±3.3	15.9±0.9	0.323	–0.220	–0.245

Chapter 4

DISCUSSION

The main findings of the present investigation are: (1) EDD is significantly impaired and hematopoietic CPCs CD34+, CD34+/KDR+, CD34+/CD45-, and CD34+/KDR+/CD45- are reduced in the CKD population; (2) myeloid CPCs including CD45+ and CD45+/KDR+ are reduced and colony forming ability of cultured non-adherent PBMNCs is impaired in this diseased population, approaching statistical significance; (3) EDD and decreasing CD34+, CD34+/CD45-, and CD34+/KDR+/CD45- CPCs are associated with a reduction in renal function as assessed by increasing blood urea nitrogen and serum creatinine levels; (4) and the CD34+ and CD34+/KDR+/CD45- CPC subpopulations are positively associated with endothelial-dependent dilation. Taken together, these findings suggest that production, mobilization, and/or differentiation of hematopoietic and myeloid progenitor cell subpopulations are impaired in the CKD population, potentially compromising vascular integrity, with the hematopoietic progenitor cell deficiency being associated with endothelial dysfunction as assessed by endothelial-dependent dilation.

Reduced CPC Subpopulations

Endothelial dysfunction has been reported in different stages of renal insufficiency and associated with not only renal disease progression but also increased

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CVD risk in CKD patients (4, 10, 50, 60, 66, 72). In addition, recent studies have demonstrated deficiencies in progenitor cell number and function in ESRD and with renal disease advancement (13, 36). More specifically, Krenning et al recently showed reductions in the CD34+ hematopoietic progenitor cell population in CKD patients. Our findings are consistent with these results by showing reductions in CD34+/KDR+, CD34+/CD45-, and CD34+/KDR+/CD45- hematopoietic CPCs (36). In contrast, this same group demonstrated that CKD had no affect on myeloid CD14+ cells, whereas we demonstrated reductions in the CD45+ and CD45+/KDR+ subpopulations. Although CD45 is thought to be co-localized with CD14 on myeloid progenitor cells, the replacement of CD14 with this cell surface maker and the addition of the endothelial cell surface marker, KDR, could be responsible for the observed differences (39). Interestingly, CD45+ cells have been shown to be involved in vascular network development during embryogenesis, and bone-marrow derived myeloid cells that enable *in situ* proliferation of endothelial cells via release of proangiogenic factors predominately consist of CD45+ cells (27, 64). Therefore, CD45+ and CD45+/KDR+ cells could be better indicators of vascular myeloid progenitor cell populations in CKD patients. Yet, the CD45+ and CD45+/KDR+ CPCs were not associated with a reduction in renal function or any other measured demographic factors. Hence, further study is needed to determine if CPC subpopulations consisting of CD14+, KDR+, and CD45+ cells, as well as other myeloid markers associated with neovascularization such as CD11b, are impaired in the CKD population and/or associated with renal disease progression (27).

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Mechanisms of CPC Reductions

Reductions in the myeloid and hematopoietic CPC subpopulations of our CKD cohort could be due to both similar and distinctive mechanisms. Stromal-derived factor-1 and its receptor, CXCR4, have been shown to be involved in both myeloid and hematopoietic progenitor cell migration to sites of hypoxia in myocardial infarction and hind limb ischemia (48, 79). In diabetic CKD patients, SDF-1 induced migration of CD34+ cells has been shown to be reduced *in vitro*, a finding that was reversed when cells were treated with diethylenetriaamine NONOate, a nitric oxide (NO) donor (59). These findings suggest that NO induced mobilization of hematopoietic progenitor cells is partially mediated by the SDF-1/CXCR4 axis, which can activate NO through the PI3K/Akt/endothelial nitric oxide synthase (eNOS) pathway (59, 84). Therefore, reductions in hematopoietic progenitor cells in our study could be the result of decreased nitric oxide production and/or bioavailability leading to impaired progenitor cell mobilization. Our findings support this hypothesis by showing impaired endothelial-dependent dilation in CKD patients as compared to controls, a response that is partially mediated by nitric oxide (18). Mechanisms of decreased NO in CKD patients include oxidative stress and increased ADMA levels, which has been shown to be inversely correlated with the number of CD34+ circulating progenitor cells, leading to impaired endothelial dependent dilation and decreased progenitor cell mobilization (40, 74). To our knowledge, nitric oxide regulation of vascular progenitor cell migration via SDF-1/CXCR4 has been limited to CD34+ hematopoietic progenitor cells using *in vitro* studies that do not include myeloid precursors. Thus, further study is needed to examine nitric oxide's role in

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hematopoietic progenitor cell and myeloid precursor mobilization using *in vivo* models of CKD.

Other mechanisms of progenitor deficiencies in the CKD population include decreased erythropoietin (EPO) production and increased blood urea nitrogen levels. Erythropoietin has been shown to mobilize progenitor cells in vitro and in vivo (7, 8). In our investigation, EPO was not directly measured in the blood, but hemoglobin levels were significantly reduced in the CKD patients and hematocrit was decreased, with differences approaching significance. However, a lack of association existed between these two measures and both hematopoietic and myeloid CPC population numbers.

Conversely, hematopoietic CPC subpopulations were negatively associated with blood urea nitrogen levels in this study. Recent investigation has suggested urea to be indicative of uremic-toxin burden in renal disease patients (31). In particular, urea has been shown to induce macrophage proliferation by inhibiting inducible nitric oxide synthase *in vitro* (44). Consequentially, the contribution of myeloid and hematopoietic progenitor cells to re- and neovascularization in CKD patients could be reduced due to transdifferentiation of progenitor cells and differentiation of hematopoietic stem cells into pro-inflammatory monocytes and macrophages instead of vascular progenitors. Supporting this are findings that show little effectiveness of macrophages or dendritic cells in neovascularization *in vivo*, as well as other studies showing increased monocyte-chemoattractant protein (MCP-1) in CKD patients, which is thought to recruit monocytes to atherosclerotic regions in vascular pathology (33, 44, 62, 69). Decreased nitric oxide production and/or

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bioavailability would again be a potential culprit in this process because NO has been shown to inhibit various chemokines responsible for monocyte/macrophage recruitment and proliferation including MCP-1 (17, 52, 82).

Colony Forming Ability and Non-Adherent Cell Immunophenotyping

Consistent with a reduction in CPC subpopulations, colony forming ability was impaired in the CKD population, approaching statistical significance. The colony forming assay that we performed has been proposed to yield a heterogeneous population of cells consisting of monocytes, macrophages, and colonies of CD14+/CD31+/CD45+/CD144+/KDR+ myeloid progenitor cells that are thought to be responsible for tunneling in neovascularization and the release of pro-angiogenic factors facilitating mature endothelial cell migration in angiogenesis and potentially revascularization (24, 37, 39, 78, 80). Although CFUs were not associated with a reduction in renal function or any other subject characteristics in this study, the association between CFUs and flow-mediated dilation in addition to SBP approached significance. Also, expression of CD31, CD34, KDR, CD45, and CD144 in the non-adherent PBMNC population that formed the CFUs was not significant in predicting CFU numbers in similar cell culture conditions between subjects. Therefore, the number of CFUs may be more related to the ability of these myeloid precursors to adhere to fibronectin via $\alpha5\beta1$ and/or $\alpha v\beta3$ integrins, as previously described, and cell-to-cell interactions by means of PE-CAM (CD31) and Ve-Cadherin (CD144) (25, 37). Interestingly, cadherin-mediated adhesion is believed to mediate cell-cell recognition and similar-cell aggregation during development (22). Ve-cadherin,

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particularly, has been shown to be involved in developmental vascularization and suggested to promote CD34+ adhesion to the endothelium to enable transendothelial migration (26, 71). Thus, we hypothesize that the decreased expression of molecules that play key roles in cell-to-extracellular matrix interactions and cell-to-cell signaling may be responsible for impaired CFUs observed in vitro and reflect myeloid CPC impairments in vivo. However, future research is needed to confirm this hypothesis and rule out CD14+ cells and other CPC subpopulations as more significant predictors of CFUs in CKD.

CD31+, CD34+, CD45+, and CD144+ expression in the non-adherent cell population that formed the CFUs was lower in CKD individuals and negatively associated with increasing blood urea nitrogen and serum creatinine levels. Further, the CD34+/KDR+, CD34+/CD45-, and CD34+/KDR+/CD45- CPCs were all significant predictors of both CD31 and CD144 expression in non-adherent PBMNCs, and CD34+/KDR+ also significantly predicted the expression of KDR in these cells. This supports the observed reductions in CPC subpopulations in the CKD population and/or demonstrates the inability of these CPCs to develop a more mature endothelial phenotype *in vitro*.

Limitations and Future Perspectives

Medications were continued during this study including antihypertensive medication and statins, which could have confounding effects on the observed results. Despite the known positive effects of these drugs, EDD was impaired and PC subpopulations were still reduced in CKD patients. The current experimental design

has effectively determined if hematopoietic and myeloid CPC subpopulations are deficient in number in CKD patients. However, the mechanisms responsible still remain unclear and findings are limited to rare event flow cytometric analysis and in vitro cell culture techniques. Although flow cytometry and CFU assays are used consistently in the literature, uncertainty exists about the appropriate identification of progenitor cells and the relevance of these measurements to in vivo physiological functioning. In particular, we did not use CD133, another hematopoietic stem cell marker that is commonly associated with different vascular progenitor cell populations, when determining the amount of progenitor cells in the circulation (21). Lastly, future study is needed to identify and determine the function of progenitor cells using in vivo animal models of CKD in order to elucidate the responsible underlying mechanisms of progenitor cell disparities in this disease population.

Conclusion

      In conclusion, circulating hematopoietic progenitor subpopulations: CD34+, CD34+/KDR+, CD34+/CD45-, CD34+/KDR+/CD45- ; myeloid precursor subpopulations: CD45+ and CD45+/KDR+; and endothelial-dependent dilation are decreased in the CKD population as compared to apparently healthy controls. The CD34+, CD34+/CD45-, and CD34+/KDR+/CD45- CPCs are also negatively correlated with renal functional impairment, and the CD34+ and CD34+/KDR+/CD45- CPCs are positively associated with endothelial-dependent dilation. Reductions in progenitor cells are accompanied by reduced CFU ability and decreased expression of hematopoietic, myeloid, and endothelial cell surface markers

in non-adherent cultured PBMNCs, also negatively related to decreases in renal function. These observations suggest that impaired progenitor cell subpopulations in the CKD population may be related to renal disease progression and CVD risk in the CKD population. Therefore, progenitor-cell based therapies could be important in revitalizing progenitor cell subpopulations in CKD and improving both renal and cardiovascular outcomes.

APPENDIX A

REVIEW OF LITERATURE

According to the National Kidney Foundation, approximately 26 million Americans suffer from chronic kidney disease (CKD) (14). Among them, the risk for cardiovascular disease (CVD) is increased and more are likely to die than reach end stage renal disease (ESRD) (1, 19, 57). Evidence indicates that this cardiovascular mortality may be related to endothelial dysfunction (ED) facilitating not only the progression of renal disease but also increased CVD risk in CKD patients (19, 58).

The endothelial monolayer lining the blood vessels helps maintain vascular homeostasis by regulating vascular tone, vessel wall inflammation, thromboresistance, and cellular adhesion in response to both physical and chemical changes within the vasculature (16, 18). This is accomplished primarily through the production of vasoactive substances such as nitric oxide (NO), endothelial-derived hyperpolarizing factor, prostacyclin, and endothelin-1 (18, 29, 34, 46). Additionally, the endothelium responds to and regulates circulating vasoactive mediators such as thrombin and bradykinin (18). In normal healthy physiological conditions, these vasoactive substances efficiently uphold endothelial integrity and control tissue oxygen perfusion without prolonged activation of host defense responses (18). NO plays a key role in this process by preserving vasodilator tone and preventing extensive periods of inflammation, cellular proliferation, and thrombosis (16, 18, 19).

In contrast, vascular pathology as a consequence of continual and/or repeated exposure to cardiovascular risk factors compromises vascular integrity and results in ED (16, 18, 19, 58).

ED is evident in CKD and characterized by insufficient bioavailability and activity of NO, impaired vasodilation, increased inflammation, and oxidative stress (16, 18, 19, 50, 58). At the kidneys, these pathologic attributes become apparent with the development of a pro-inflammatory phenotype and systemic as well as glomerular hypertension (58). ED may also result in decreased oxygen and nutrient delivery to the tubules promoting renal disease progression, similarly to that seen with atherosclerosis and the advancement of various other cardiovascular diseases (16, 18, 58). Consequentially, ED may facilitate ischemic episodes at not only renal but also cardiovascular tissues leading to end-organ damage and cardiovascular events in CKD patients (9, 18). In this regard, maintenance of endothelial integrity and induction of vascularization by vascular progenitor cell populations become important in limiting endothelial cell apoptosis, ED, and ultimately the effects of hypoxia-induced tissue damage (9, 39).

Re- and neovascularization are no longer thought to exclusively result from migration and proliferation of fully-differentiated resident endothelial cells, but rather different progenitor cell populations are thought to also facilitate these processes (12, 32). Specifically, recent investigation has indicated the presence of two progenitor cell populations of myeloid and hematopoietic lineage potentially involved in new blood vessel growth (39).

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CD14+ myeloid progenitor cells are proposed to migrate to sites of hypoxia, adhere to the endothelium, transcellularly migrate, and at first form scaffolds for angiogenesis (2, 39, 45). After forming these capillary like tunnels, the myeloid progenitors, in conjunction with CD34+hematopoietic progenitor cells, could create a pro-angiogenic environment via the release of various pro-angiogenic factors and enable the migration and proliferation of surrounding mature endothelial cells (39). Another possibility is that the CD14+ cells layering the capillary like tunnels, in combination with CD34+ cells, could differentiate in mature endothelial cells forming new blood vessels (2, 3, 39). These hypotheses are supported by studies showing CD14+ drilling in ischemic myocardium and CD14+/CD34+ induced neovascularization *in vivo* in different disease models (2, 6, 45, 81).

Similarly, CD14+ and CD34+ cells could also contribute to revascularization by incorporating into the endothelium, differentiating into mature endothelial cells, and replacing apoptotic cells and/or enabling resident mature endothelial cells to migrate and proliferate via paracrine signaling (24, 27, 68, 76-78). Both progenitor cell populations secrete the angiogenic factors hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF), and CD14+ cells have been shown to differentiate into mature endothelial cells when cultured with HGF produced by CD34+ cells (6, 38, 43, 81).

CD34+ cells have been shown to be reduced in ESRD and with renal disease progression, whereas CD14+ cells are not significantly different between the different stages of CKD (36, 54). This implies that hematopoietic progenitor cell

production and/or migration from the bone marrow is impaired in CKD potentially contributing to compromised vascular integrity and ED, increased risk of CVD in CKD patients, and renal disease progression. Deficiencies in these CD34+ progenitor cells may be due to impaired migratory ability from the bone marrow as a result of decreased nitric oxide production and/or bioavailability.

Nitric oxide has been shown to be involved in circulating progenitor cell migration via stromal derived factor (SDF-1)/CXCR4 activation of the PI3K/Akt/endothelial nitric oxide (eNOS) pathway (59, 72, 79, 84). This may be accomplished when production of the cytokine SDF-1 is either increased in response to hypoxia, possibly via hypoxia-inducible factor-1 alpha, or as a result of vascular injury (39, 48, 79). Progenitor cells contain the receptor for SDF-1, CXCR4, and when SDF-1 binds to this receptor and activates the PI3K/Akt pathway, eNOS is phosphorylated enabling NO production from the substrate L-arginine. In CKD, increased oxidative stress, hyperuricemia, and uremia could all potentially inhibit this NO production and/or decrease NO bioavailability resulting in impaired progenitor mobilization.

Increased reactive oxygen species, such as NADPH-oxidase, increases superoxide production, which has been shown to oxidize critical cofactors of NO synthesis, impair endothelial nitric oxide synthase, and even reacts with nitric oxide to form peroxynitrite, a powerful oxidant itself (28, 40, 49, 74). Superoxide also increases ADMA expression, which has been shown to inhibit eNOS from using L-arginine as a substrate and is negatively associated with circulating progenitor cell populations (67, 78).

Alternatively, hyperuricemia and uremia inhibit NO by different means. Uric acid has been shown to acutely mobilize progenitor cells in response to ischemia, but with chronic elevation of uric acid in the circulation, as seen in renal disease, uric acid has been associated with decreased progenitor cell mobilizing ability (51). This impaired progenitor cell mobilization could be mediated through uric acid increasing the activity of arginase, the final enzyme in the urea cycle, which competes with NO production for the substrate L-arginine (83). In contrast, uremia mediated decreases in NO production is thought to be related to uremic toxins such as guanidine compounds that inhibit eNOS (13).

Progenitor-cell based therapies that aim to mobilize the hematopoietic progenitor cell population in CKD patients could be implemented to suppress these effects of oxidative stress, hyperuricemia, and uremia on nitric oxide production. In particular, administration of treatments including antioxidants, allopurinol to suppress uric acid levels and others that decrease the effects of uremic toxins may prove to be beneficial. Additionally, statins and erythropoietin that have been shown to increase progenitor cell mobilization could be used (7, 8, 73, 75). Although, future study is needed to further elucidate the potential mechanisms of progenitor cell reductions in CKD patients and determine more effective treatment strategies.

In conclusion, decreased progenitor cell populations in CKD patients could enable the development of endothelial dysfunction resulting in poor renal and cardiovascular outcomes. Recent investigation has shown specifically, CD34+ hematopoietic progenitor cells, to be deficient in ESRD and with renal disease progression, while CD14+ myeloid progenitor cells were not reduced when compared

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between the different stages of CKD (36, 54). However, deficiencies in subpopulations of these different progenitor cells have yet to be determined. Other markers characteristic of vascular progenitor cells such as the immature hematopoietic cell surface marker CD133, endothelial cell surface marker KDR (VEGF-2 Receptor) and CD45, which is co-localized with CD14 on myeloid progenitors, need to be used to identify progenitor cell subpopulation deficiencies. This may be advantageous in progenitor-cell based therapy in order to mobilize and revitalize specific deficient cell populations. Further study is also needed to elucidate the mechanisms of progenitor cell reductions in CKD using in vitro and in vivo methods to enable the development of novel therapeutic strategies and improve poor patient outcomes.

APPENDIX B

INFORMED CONSENT

Research Study: The Effect of Chronic Kidney Disease on Endothelial
Progenitor Cells
Investigators: J. Matthew Kuczmarski, B.S., Dr. David G. Edwards, PhD, Dr.
Carlton Cooper, PhD

Subject Name: _____

1.   PURPOSE / DESCRIPTION OF THE RESEARCH
You are being asked to participate in a research study conducted by the Department of
Health and Exercise Sciences and the Department of Biology at the University of
Delaware. The purpose of this research is to determine the effects of chronic kidney
disease on blood vessel function and a type of cell found in the blood.
You will be one of approximately 40 participants. Two groups will be recruited and
will include:
- 20 chronic kidney disease individuals aged 18-75 years
- 20 apparently healthy individuals aged 18-75 years

Full participation in this study will require 1 visit to the Human Performance Lab, 541
S. College Avenue (the rear section of the Fred Rust Ice Arena) in Newark, DE 19716.
The total time commitment is approximately 1 hour and 30 minutes. Prior to
participation in this study, you will be asked to abstain from food, alcohol, and
caffeine for 12 hours and exercise for 24 hours.

2. WHAT YOU WILL DO
Your visit to the Human Performance Lab will last approximately 1 hour and 30
minutes.
The following will occur:
- You will complete a medical history questionnaire that asks about your current
  and past health.
- Resting heart rate, blood pressure, height, and weight will then be measured.

- 12 electrodes will be placed on your chest to record the electrical activity of your heart.
- A blood sample will then be collected by inserting a needle into an arm vein (approximately 3 tablespoons of blood will be removed). You will also be asked for a urine sample.
- A narrow blood pressure cuff will be placed on your lower arm. Without the cuff inflated ultrasound pictures will be taken of the artery in your upper arm. The cuff will then be inflated for 5 minutes. The ultrasound pictures will be repeated for 2 minutes immediately following deflation of the cuff.
- The urine sample and part of the blood sample will be used to make an assessment of liver and kidney function, a cholesterol profile, red blood cells, and blood sugar level to make sure you are eligible for the study. This will take place at the Christiana Hospital Lab.
- The remainder of the blood sample will be analyzed at the University of Delaware. We will be looking for a specific cell-type that is located in the blood which may be responsible for repairing blood vessels. The blood sample will be used to count these cells and examine their function. Any of the blood sample remaining after these measurements will be disposed of following University of Delaware guidelines for disposal of blood.

3.   CONDITIONS OF SUBJECT PARTICIPATION

Information obtained from this study will be kept strictly confidential. You will not be individually identified, except by a subject number known only to the investigators. All data will be stored in a locked cabinet or password protected computer indefinitely. While the results of this research may be published, neither your name nor your identity will be revealed.

You are free to discontinue participation at any time without penalty.

In the event of injury during these research procedures, you will receive emergency first aid. If you require emergency room or other additional medical treatment, you will be responsible for the cost.

4.   RISKS AND BENEFITS

There are no known risks associated with taking your height, weight, or resting blood pressure. You may have pain and/or bruising at the site where blood is taken, and there is a small risk of infection. Fainting sometimes occurs during or shortly after blood is drawn.

There may be minor discomfort associated with the application and removal of the electrodes. Also, the ultrasound testing requires the inflation of a blood pressure cuff which may result in some temporary mild discomfort similar to that experienced if your arm went numb if you fell asleep on it.

There may be no direct benefit to you for participating in this research study however you will be provided the results of your screening blood work when you complete the study. These results will not be interpreted but are provided for your information. This study may provide new information regarding blood vessel function in chronic kidney disease.

5.   FINANCIAL CONSIDERATIONS
All subjects will receive $30 dollars to offset the cost of transportation and time spent participating.

6.   CONTACTS
Any questions regarding the study can be directed to Dr. David Edwards, Ph.D (302)-831-3363, Assistant Professor, Department of Health, Nutrition, and Exercise Sciences or James M Kuczmarski, B.S. at (302) 831-2911. Questions regarding the rights of individuals who agree to participate in this research may be directed to: Chair, Human Subject Review Board, University of Delaware at (302)-831-2136.

7. SUBJECT ASSURANCES
I have read the above informed consent document. The nature, demands, risks and benefits of the project have been explained to me. I knowingly assume the risks involved, and understand that I may withdraw my consent and stop my participation in this study at any time. By signing this form, I agree to take part in this research study and to allow the use of the described information for the purposes of research until the end of the study.

6.   CONSENT SIGNATURES

_____   Date:_____
Subject's Signature

_____
Subject's Name (Printed)

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