Proliferative potential of human kidney endothelial cells: bone marrow-derived cells may not be required for high proliferation

Kimberly A. Muczynski, Nicolae Leca and Susan K. Anderson

Department of Medicine, Division of Nephrology University of Washington, Seattle, WA 98195, USA

Correspondence and offprint requests to: Kimberly A. Muczynski; E-mail: kzymnski@u.washington.edu

Abstract

Background. Proliferative potential of a single cell, defined as the number of progeny it gives rise to, has been used to define a hierarchy of endothelial progenitor cells in blood. Cells with high proliferative potential are presumed to have greater capacity for endothelium repair. Based on results with commercially available endothelial cells, it has been proposed that a proliferative hierarchy of endothelial cells also exists within blood vessels. It is unknown whether such vessel-derived highly proliferative endothelial cells originate from the bone marrow or whether the supply of precursors is limited to pre-existing cells that reside within vessels.

Methods. In this study, we isolated normal human renal microvascular endothelial cells (RMEC) and larger cortical vessel endothelial cells (EC) by flow cytometry based on differential expression of human leucocyte antigen (HLA)-

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DR, and evaluated the proliferative potential of single cells. To determine if highly proliferative clones might derive from bone marrow recruits, HLA-DR expression on RMEC from transplanted kidneys was evaluated using antibodies that distinguish donor cells from recipient cells.

**Results.** We found the proliferative potential of kidney endothelial cells diverse and variable. Subcloning indicated that proliferative potential was determined by epigenetic events. In transplanted kidneys affected with a variety of different injuries, RMEC were donor derived.

**Conclusions.** We conclude that endothelial cells of high proliferative potential exist within human renal blood vessels, even in individuals into their eighth decade of life, and that highly proliferative endothelial cells are unlikely to be bone marrow derived.

**Keywords:** endothelial cells proliferation; chimerism; flow cytometry; HLA-DR; kidney transplant biopsies

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**Introduction**

Endothelial progenitor cells (EPCs) have become a focus for the treatment of vascular disease in a growing field of angiogenic therapy. Despite the interest in EPCs, a clear phenotypic definition of them is still debated [1,2]. Most of the attention has been on bone marrow-derived cells, defined by expression of CD34, CD133 and the vascular endothelial growth factor receptor-2 (VEGFR-2). Other studies have shown that cells from human cord blood expressing these markers and CD45 fail to form EPCs [3,4]. Further confusing the field is distinguishing circulating bone marrow-derived EPCs that home to sites of new blood vessel formation or repair from endothelial cells shed into the circulation from damaged vasculature.

The lack of a clear definition for EPCs has not impeded the search for these cells under conditions relevant to renal disease. Circulating EPCs have been reported to be deficient in patients with kidney disease [5–7], and their number and function partially restored with dialysis [6,8–10]. ‘Endothelial-specific’ and progenitor-associated genes have been found to be reduced in peripheral blood of children on haemodialysis and normalize after kidney transplantation [11]. Investigations to distinguish circulating detached endothelial cells from bone marrow-derived endothelial progenitor cells have identified an imbalance in the two cell populations in patients with advanced chronic kidney disease compared to healthy individuals [12].

Ingram and Yoder devised an assay to identify endothelial progenitors based on in vitro cloning [13,14]. True EPCs proliferate to form large colonies from a single precursor cell. These investigators found the proliferative potential of blood-derived EPCs to be variable and proposed a hierarchy of EPCs in peripheral blood based on this property. They also found a similar proliferative hierarchy when commercially available human umbilical vein and aortic endothelial cells were cloned, and proposed that EPCs are present within adult human blood vessels in a position where they can be immediately available for repair of local endothelial injury [15,16].

Having endothelial cells with variable potential for proliferation residing in the same vessel is consistent with the literature on endothelial cell heterogeneity [17,18]. Aird proposes that this heterogeneity is required for endothelium health [19]. Resident cells with high proliferative capacity may be useful for maintaining endothelial integrity.

In this study, we examine the hypothesis that endothelial cells with variable proliferative potential exist within human renal blood vessels from normal kidneys. Our focus is not to make a statement about the existence of renal EPCs but to assess the proliferative potential of endothelial cells isolated from capillaries and larger cortical vessels.

We developed procedures for ex vivo characterization and isolation of normal endothelial cells from human kidney cortex using flow cytometry [20]. The microvascular endothelium of the human, but not rodent, kidney normally expresses high levels of major histocompatibility complex (MHC) class II molecules. By sorting for CD45-negative human kidney cells that co-express CD31 and a monomorphic determinant of the MHC class II molecule human leucocyte antigen (HLA)-DR, we isolate renal microvascular endothelial cells (RMEC) from peritubular and glomerular capillaries. The larger vessel endothelial cells (EC) of the renal cortex, both arterioles and venules, do not express HLA-DR and hence can be isolated by sorting for CD45−/CD31+/HLA-DR− cells. Using transplanted kidneys as a model, we also assess whether the origin of RMEC is vessel- or bone marrow-derived using flow cytometry and HLA-DR-specific antibodies that distinguish donor from recipient.

**Materials and methods**

**Human subjects**

The Institutional Review Board of the University of Washington approved all protocols, and informed consent was obtained from donors.

**Immunofluorescence microscopy**

Human kidney sections were fixed and labelled with CD34–FITC (Invitrogen, Carlsbad, CA); L243, an anti-DR which recognizes a monomorphic determinant, conjugated to Alexa 647 (BioLegend, San Diego, CA); rabbit anti-CD31 (Thermo Fisher Scientific) visualized with secondary goat anti-rabbit antibody conjugated to Alexa 568 (Jackson ImmunoResearch, West Grove, PA).

**Endothelial cell isolation**

Endothelial cells were isolated from donors undergoing nephrectomy for renal cell carcinoma. All donors had a normal serum creatinine and urinalyses prior to nephrectomy. Grossly normal cortical tissue from areas away from the renal tumour was obtained within an hour of removal from donors and stored up to 24 h in Hank's balanced salt solution (HBSS), 100 U/ml penicillin and 50 µg/ml streptomycin at 4°C. Tissue was minced with a scalpel and digested for up to 1 h at 37°C in 30 ml of 0.2% collagenase P (Roche Molecular Biochemicals, Indianapolis, IN) in HBSS on an orbital shaker. The digest was centrifuged at low speed (800 g), resuspended in 15 ml phosphate-buffered saline (PBS) containing...
0.05% trypsin and 0.53 mM EDTA, and agitated for 15 min at 37°C. An equal volume of Lonza (Allendale, NJ) EGM-2-MV BulletKit medium was added, and the resulting cell suspension passed through a 20-mesh sieve. Recovered cells were labelled with CD31−PE and L243−FITC and passed through a nylon strainer (40 μm pore size) (Falcon 352340, Fisher, Pittsburgh, PA). Propidium iodide (PI) (5 μg/ml) was added just before sorting on a FACSVantage SE (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon laser. PI-negative cells with the highest level of CD31 and DR expression (RMEC) and single-positive CD31 cells (EC) were collected under sterile conditions in tubes containing EGM-2-MV BulletKit medium, centrifuged and placed in culture with fresh medium. Cells were grown at 37°C in a 5% CO2 incubator.

Cloning and proliferation assay
RMEC and EC were cloned by limiting dilution into 96-well flat-bottom tissue culture plates, at ≤0.5 cells/well. Two to four 96-well plates were prepared for each assay. Fresh media was added every 2 days. Cells were scored 14 days after cloning.

Subcloning was done using cells recovered from a well of the 96-well cloning plate following the protocol described above.

Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) assay
Cells are labelled with 10 μM CFSE (Invitrogen) in PBS for 15 min at 37°C in a 5% CO2 incubator. After labelling, CFSE/PBS is replaced with fresh EGM-2-MV BulletKit medium, and cells are cultured as normal. For cytometry, cells are washed once in PBS, trypsinized, washed and suspended in RPMI.

Flow cytometry of transplant renal biopsies
Portions of 16-gauge core transplant biopsies were obtained from patients needing biopsies for clinical indications. Biopsies were immediately placed in 1.5-ml tubes containing Hank’s balanced salt solution (HBSS), 100 U/ml penicillin and 50 μg/ml streptomycin and stored at 4°C for up to 24 h. The tissue was dissociated into a single-cell suspension using 0.1% collagenase P (Roche Molecular Biochemicals) in HBSS on an orbital shaker with intermittent pipetting for 15 min. This preparation was centrifuged at low speed (600–1000 g), resuspended in 0.5 ml PBS, 0.05% trypsin and 0.53 mM EDTA, agitated for 10 min at 37°C and then neutralized with an equal volume of EGM-2-MV BulletKit medium. For HLA-DR analysis, cell suspensions were labelled for 60 min with primary antibody cocktail containing biotinylated DR-specific antibody (One Lambda, Inc., Canoga Park, CA); CD31−PE, CD34−APC, CD-45−PacificBlue (BioLegend, Inc., San Diego, CA) and L243 conjugated to Alexa 680 per manufacturer’s instructions (Invitrogen). After washing, samples were incubated in streptavidin–FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min. Controls contained no primary biotinylated antibody. RPMI containing DAPI was added prior to flow cytometry on a BD LSRII equipped with a 488-nm argon laser, a 633-nm red diode laser, a 400-nm violet laser and a 355-nm ultraviolet (UV) laser. Files were analysed using FCS Express 3 software (denovosftware.com).

Results

Isolation of human renal cortical endothelial cells
Endothelial cells were isolated from the microvasculature and larger vessels found in human kidney cortex (Figure 1). RMEC, derived from glomerular and peritubular capillaries, express CD34, CD31 and HLA-DR as shown by immunofluorescence microscopy (Figure 1a). Larger vessel endothelial cells from kidney cortex (EC) express CD34 and CD31, but do not express HLA-DR (Figure 1a). RMEC were isolated by flow cytometry as CD31+/HLA-DR+ cells and EC as
CD31+/HLA-DR− cells (Figure 1b). Neither endothelial cell populations expresses CD45 (Figure 1c). Sorted cells were grown in standard tissue culture flasks. Collagen coating of the culture surface was not required for cell growth and had minimal effect on growth rate. Cultured RMEC and EC were morphologically indistinguishable.

Endothelial cell cloning and proliferative potential

Endothelial cells were cloned by limiting dilution (≤0.5 cell/well) into 96-well plates after a confluent T-25 flask was obtained. The number of cells present in each well at the end of 14 days was scored as shown in Figure 2a–d. In donors covering a five-decade age range, the number of endothelial cells growing from a single RMEC or EC precursor cell varied for all donors and in different cloning experiments of an individual donor (Figure 2e). Cloned RMEC and EC which grew to confluency, either loose or tight in colony morphology, represent endothelial cells of high proliferative potential.

Among clones which grew to confluency in the first round of cloning, two phenotypes, cuboidal or elongate, emerged for both RMEC and EC (Figure 3a and b). The proliferative potential of these phenotypically distinct cells was assessed by subcloning cells of each phenotype. For subcloning, cells originating from a single clone were recovered and cloned again by limiting dilution. The proliferative potential of the subcloned cells with cuboidal phenotype was uniform and low, whereas that of the subcloned elongated cells remained diverse (Figure 3c). Some subclones from the elongated cells maintained a high proliferative potential; however, these cells, originating from the elongated clones, developed a cuboidal phenotype. Data were similar for RMEC and EC.

We make two interpretations from these subcloning experiments. First, the cuboidal phenotype denotes cells with less proliferative potential which may be closer to the end of their replicative lifespan. Second, since some cells maintained variable proliferative potential in a second round of cloning, epigenetic events determine proliferative ability. In the subcloning process, each cell originating from a clone should be genetically identical. If genetics were the sole determinant of the replicative lifespan of renal endothelial cells, then cells derived from a single precursor would undergo a similar number of divisions and give rise to equal numbers of progeny. This was not the case for the subclones derived from clones of elongated cells.
Within the RMEC population, glomerular and peritubular capillary endothelial cells can be distinguished on the basis of thrombomodulin expression (CD141). In the normal human kidney, only the latter express thrombomodulin [21], an observation we confirmed by immunofluorescence microscopy. In this study, we used combined glomerular and peritubular capillary RMEC populations. However, when glomerular RMEC (CD141+/CD31+/DR−/CD45−) and interstitial RMEC (CD141+/CD31+/DR+/CD45−) were isolated by flow cytometry and cultured, a mixture of confluent colonies and cells with only a few divisions grew from both cultures, indicating that glomerular and interstitial endothelial cells have similar variability in proliferative potential (data not shown). Therefore, our results of variable RMEC proliferative potential cannot be explained by the RMEC population containing a mixture of glomerular and interstitial capillary endothelial cells.

**Proliferative diversity by CFSE**

The diversity of proliferative potential in RMEC and EC populations was confirmed by CFSE assays. Cells, which had been frozen, were recovered for 2 days in culture, labelled with CFSE and analysed over a period of 4 days. Cells from the same culture were also cloned to determine proliferative potential. Representative examples of CFSE fluorescence and the corresponding cloning data are shown in Figure 4.

One day after labelling, CFSE mean fluorescence was highest for cultures with the fewest confluent clones at the end of the proliferation assay (compare Figure 4a peak fluorescence of 2050 and 2% of clones growing to confluency with Figure 4c peak fluorescence of 508 and 89% of clones growing to confluency). Cytometry analysis 4 days after CFSE labelling identified cells with a broad range of staining for all donors and for both RMEC and EC. Some cultures contained a population of cells which retained relatively high levels of CFSE (Figure 4a), while in others, most of the cells showed reduced CFSE levels (Figure 4c). Still, other cultures had broad and symmetric CFSE staining after 4 days (Figure 4b). In all cases, proliferative potential as reflected in cloning scores paralleled CFSE distribution. RMEC and EC cultures which produced more confluent clones had less intense CFSE staining after 1 day in culture and contained a prominent population of cells with reduced CFSE after 4 days.

**Assessing RMEC chimerism in transplanted kidneys**

We evaluated HLA-DR specificity of RMEC in transplanted kidney biopsies using flow cytometry to determine if the highly proliferative cells within the RMEC population could be bone marrow derived. A protocol was devised for transforming a 16-gauge core biopsy into a suspension of single cells without losing either cells or epitopes of interest. From one-third of a 16-gauge core biopsy, we can identify ~500 RMEC for evaluation. For the final analysis, after staining controls, ~100 RMEC are available for each antibody combination used to distinguish donor from recipient. Specificity of the HLA-DR antibodies was verified using peripheral blood leucocytes and Epstein–Barr virus (EBV)-transformed typing lines of known HLA-DR genotypes. We analysed 12 transplant renal biopsies performed on 11 patients. Three biopsies were from transplants at least 10 years old; two were from 3-year-old transplants; five were from 15- to 17-month-old transplants; one was from a 5.5-month-old transplant; and one was from a 4-week-old transplant.

In Figure 5, representative data from three of the 12 transplant biopsies are shown. The donors covered a spectrum of clinical transplant diagnoses. For each biopsy, we evaluated the HLA-DR specificity of the donor RMEC and the leucocytes found within the transplant after labelling with antibodies to CD31, CD34, CD45, a monomorphic HLA-DR determinant and a specific HLA-DR determinant. Normal transplant kidney biopsies and those with mild acute tubular necrosis (ATN) (Figure 5a) contained only rare donor leucocytes. In older and more compromised transplants (Figure 5b and c), more leucocytes of recipient DR phenotype were present. Recipient peripheral HLA-DR-expressing leucocytes verified specificity of the antibodies. Controls are cells labelled with the same antibodies minus the HLA-DR-specific primary antibody. Lines in the dot plots are drawn to distinguish expression and non-
expression of the HLA-DR-specific epitope based on controls. In all cases, RMEC were predominantly, if not entirely, donor derived, even in cases of injury and prolonged time from transplant.

**Discussion**

Cloning results demonstrate that endothelial cells from normal human kidney blood vessels have variable proliferative potential, and a significant number of cells have high proliferative potential. Within the microvasculature, this is true for both interstitial and glomerular capillary endothelial cells. Furthermore, since the endothelial cells isolated were from donors ranging in age from 34 to 83, highly proliferative endothelial cells continue to be a normal component of renal blood vessels with ageing.

Subcloning endothelial cells gave rise to clones of different proliferative potentials, which we interpret as evidence for epigenetic events determining proliferative potential. An
alternate explanation for the observation relates to the spontaneous cell-to-cell variability within a clonal progenitor population described by Chang and colleagues [22]. Using EML cells, these investigators found phenotypic heterogeneity within the clonal population that was also reflected in the transcriptomes. When phenotypic outlier cells were isolated and continued in culture, phenotype and transcriptomes returned to a parental median state. The authors attribute EML clonal heterogeneity to metastable states of slowly fluctuating transcriptomes within individual cells which may determine reversible stochastic decisions of cell fate. It is possible that the differential proliferation potential of individual RMEC and EC clones relates to cells being in different transcriptome states at the time of cloning. Our system differs from the work of Chang et al. in that endothelial cells are neither immortalized nor pluripotential progenitors. It is unknown whether metastable fluctuating transcriptomes operate in the same manner in differentiated cells with limited replicative lifespans.

We explored the possibility that highly proliferative RMEC might be bone marrow derived using human transplant kidney biopsies as a model. The hypothesis behind the experiments was that highly proliferative endothelial cells might be recent recruits to blood vessels from bone marrow-derived endothelial progenitor cells. This seemed plausible since renal endothelial cell chimerism has been identified in kidney transplants by other investigators using immunohistochemistry [23,24]. The high level of HLA-DR constitutively expressed on RMEC under normal conditions provides a means for distinguishing bone marrow- from blood vessel-derived endothelial cells in a transplanted kidney. Biopsies from transplant recipients where antibodies were available to distinguish donor from recipient RMEC were evaluated using a six-colour flow cytometry analysis. In the data analysis, RMEC, identified on the basis of expression of monomorphic HLA-DR and endothelial, but not leucocyte markers, were assessed for donor or recipient HLA-DR phenotype using HLA-DR-specific antibodies. By this methodology, we found that RMEC were predominantly, if not entirely, donor derived. This leads us to believe that the endothelial cells of the renal microvasculature, including those with high proliferative potential, are unlikely to be bone marrow recruits.

Although we did not find chimerism within RMEC using flow cytometry, it may exist at a frequency too low to be detected with the number of cells available in a biopsy. In our final analyses, ~100 RMEC were available for each of the antibody combinations used to distinguish donor from recipient. This means that, if endothelial chimerism exists within the renal microvasculature of transplanted kidneys, it is present at a frequency of <1%. Our data indicate that 7–45% of cells proliferate to form confluent colonies. Therefore, even if RMEC chimerism occurs in transplanted kidneys as a rare event, the high proliferative potential we observed for some RMEC cannot be accounted for by bone marrow recruits. It is more probable that the renal vasculature contains resident endothelial cells capable of robust proliferation in response to injury.

Our findings predict that endothelium repair in the human kidney primarily utilizes and may be limited by cells pre-existent within the vessel wall. In cases of repeated and/or severe endothelial injury, once the highly proliferative cells within the vessel are used up, without another source of endothelial precursors, the integrity and functionality of the endothelium is lost. Since the donors whose cells were used in the proliferation experiments had no historical or existing renal disease, other than the presence of renal cell carcinomas, the effect of prior renal injury on endothelial cell proliferative capacity could not be assessed.

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Conflict of interest statement. None declared.

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Influence of acidaemia and hypoxaemia on CVVH haemocompatibility in a porcine model

Janis R. Bedarf¹, Martin Russ¹, Sascha Ott¹, Tobias Keckel¹, Michael Kirschfink² and Juliane K. Unger¹

¹Department of Experimental Medicine (FEM), Charité—Universitätsmedizin Berlin, Berlin, Germany and ²Institute for Clinical Immunology, Heidelberg University, Heidelberg, Germany

Correspondence and offprint requests to: Juliane K. Unger; E-mail: juliane.unger@charite.de

Abstract

Background. Reduced haemocompatibility and early filter failure during continuous venovenous haemofiltration (CVVH) can be attributed to various aspects from filter engineering to rheological problems. Still, little is known about the impact of acidaemia and hypoxaemia on the haemocompatibility of a CVVH. In a porcine model, we investigated blood and coagulation parameters, filter performance and blockage of filter capillaries to assess the impact of acidaemia and hypoxaemia on haemocompatibility.

Methods. Pigs were assigned to three groups (n = 6). One group received mixed acidaemia (pH 7.2) by acid infusion (0.2 M of lactic acid and 0.2 M HCl diluted in normal saline) and low tidal volume ventilation (6–8 mL/kg⁻¹) for 3 h based on standardized heparinization. One group underwent an additional hypoxaemia (pH 7.2; PaO₂ <70 mmHg) and another was treated with normal saline and normoventilation (control group; pH 7.4). To accelerate biocompatibility reactions, CVVH was operated with reinfusion of the filtrate to the venous line for 3 h based on standardized heparinization.

Results. Acidaemia led to a contradictory pattern with respect to prothrombin time (prolongation), activated partial thrombin time and activated clotting time (acceleration). In comparison to normal pH homeostasis, acidaemia led to increasing activation markers such as terminal complement complex marker sC5b-9 and thrombin–anti-thrombin complexes (TAT) and D-dimers. Additional hypoxaemia intensified activation with regard to TAT and complement complex marker sC5b-9. Platelet counts suffered from acidaemia and a tendency for higher rates of blocked hollow fibres was found.

Conclusion. Acidaemia led to deteriorated haemocompatibility reactions to a CVVH circuit. The coagulation pattern developed towards complications for the coagulatory state.

Keywords: acidosis; coagulation; continuous venovenous haemofiltration; haemocompatibility; hypoxaemia

Introduction

Early filter failure, low blood flow rates/filter clearance and a deterioration of the coagulation system are common problems during the use of renal replacement therapies (RRT) in the case of acute renal failure (ARF) or multiple organ failure [1–5]. There is one common pathophysiology in most critically ill patients requiring RRT, which is acidosis even when the