Mobilized Human Hematopoietic Stem/Progenitor Cells Promote Kidney Repair After Ischemia/Reperfusion Injury

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Background—Understanding the mechanisms of repair and regeneration of the kidney after injury is of great interest because there are currently no therapies that promote repair, and kidneys frequently do not repair adequately. We studied the capacity of human CD34+ hematopoietic stem/progenitor cells (HSPCs) to promote kidney repair and regeneration using an established ischemia/reperfusion injury model in mice, with particular focus on the microvasculature.

Methods and Results—Human HSPCs administered systemically 24 hours after kidney injury were selectively recruited to injured kidneys of immunodeficient mice (Jackson Labs, Bar Harbor, Me) and localized prominently in and around vasculature. This recruitment was associated with enhanced repair of the kidney microvasculature, tubule epithelial cells, enhanced functional recovery, and increased survival. HSPCs recruited to kidney expressed markers consistent with circulating endothelial progenitors and synthesized high levels of proangiogenic cytokines, which promoted proliferation of both endothelial and epithelial cells. Although purified HSPCs acquired endothelial progenitor markers once recruited to the kidney, engraftment of human endothelial cells in the mouse capillary walls was an extremely rare event, indicating that human stem cell mediated renal repair is by paracrine mechanisms rather than replacement of vasculature.

Conclusions—These studies advance human HSPCs as a promising therapeutic strategy for promoting renal repair after injury. (Circulation. 2010;121:2211-2220.)

Key Words: angiogenesis ■ capillaries ■ ischemia ■ kidney ■ stem cells

Although the kidney has tremendous capacity for regeneration, chronic kidney disease and kidney failure after acute kidney injury or after both repetitive and chronic kidney injuries are now leading causes of morbidity and mortality in the world.1–3 Furthermore, chronic kidney disease has been identified as a leading independent risk factor for cardiovascular diseases and cardiovascular mortality.4 Chronic kidney diseases may represent unsuccessful or inadequate renal repair after injury, and currently there are few therapies that promote repair and regeneration of the kidney.5

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There has been much interest in the reparative and angiogenic properties of stem cells from bone marrow,6–8 and several studies in mouse models of kidney disease have shown that mouse mesenchymal stromal cells of bone marrow can prevent or attenuate kidney injury, possibly by paracrine or systemic secretory mechanisms.9–11 However, the possible angiogenic role of hematopoietic stem/progenitor cells (HSPCs) in kidney repair has been little explored, and no studies have ascertained the practicability of harvesting human HSPCs in cell therapy to promote organ repair and regeneration.12

The kidney peritubular microvasculature has received increasing attention recently because this fragile vasculature may not regenerate normally after injury. This may predispose to chronic ischemia of the kidney,13–16 triggering chronic inflammation, tubular atrophy, and interstitial fibrosis, hallmarks of chronic kidney disease.13–15 It has been proposed that successful regeneration of peritubular capillaries after injury is central to progression to chronic kidney diseases.13–15

The fact that human stem cells (HSCs) from bone marrow may have angiogenic properties, have the capacity to differentiate into a primitive cell type, known as circulating endothelial progenitor (CEP), which is recruited to sites of blood vessel injury to help repair damage,17–20 and have been shown to
promote vascular regeneration in other organs21–23 led us to study the role of human HSPCs in kidney repair after injury, with particular attention to the peritubular capillary plexus.

In these studies, we show that mobilized human CD34+ stem/progenitor cells are recruited to the injured kidney and promote survival, vascular regeneration, and functional recovery.

Methods
An expanded version of Methods is available in the online-only Data Supplement and includes detailed methods for the following: Animals; Human Peripheral Blood CD34+ Cell Purification and Tracking; Animal Model; Renal Function; Tissue Preparation, Immunostaining, Imaging, and Quantification of Injury and Repair; Flow Cytometric Analysis and Sorting; and Real-Time Polymerase Chain Reaction.

Statistical Analysis
All values are given as mean±SD. The nonparametric Mann-Whitney U test was used for group comparisons. Analyses were performed with the use of Prism software (GraphPad, La Jolla, Calif.). For antibody cytokine arrays, results were normalized to positive controls, and background signal was removed by subtracting the average of the negative controls plus 2 SDs. P values <0.05 were considered significant in all statistical tests.

Results
Characterization of Isolex-Purified Granulocyte Colony-Stimulating Factor–Mobilized HSPCs
CD34+–enriched leukocytes from HSC-mobilized human donors were analyzed for viability and purity. More than 98% of HSPCs were viable when assessed by exclusion 7-amino-actinomycin D (Figure IA in the online-only Data Supplement).20 The characterization, in addition to HSPCs, is consistent with mobilized human peripheral blood CD34+ cells containing small numbers of CEPs and possibly rare circulating endothelial cells.20,24,25

Human HSPCs Are Recruited to Kidney During Repair After Ischemia/Reperfusion Injury
To study the effect of human HSPCs on kidney repair, we initially determined whether they could be recruited to the injured kidney. In preliminary studies, intravenous infusion of 2.5×106 HSPCs labeled with 5-chloromethylfluorescein diacetate (CMFDA) before injury did not result in significant recruitment 24 hours after injection (not shown). Next we infused CMFDA-labeled HSPCs on days 1 and 2 after kidney ischemia/reperfusion injury and examined the kidney 2.5, 3, 5, and 7 days after injury (Figure 1); many recruited HSPCs could be detected in the kidney parenchyma. Many were localized within peritubular capillaries (PTC) (Figure 1C), but some were detected outside of the confines of the capillaries in a perivascular location (Figure 1E and Figure 1B in the online-only Data Supplement). We also noticed that after unilateral ischemia/reperfusion injury, there was a small but significant recruitment of HSPCs to the uninjured kidney (Figure 1B). However, we could not detect any HSPCs in the heart or gut (not shown), indicating that this was specific recruitment of HSPCs to the uninjured and injured kidney. Because of concern that CMFDA might be diluted and become undetectable with time, we infused unlabeled HSPCs into mice on days 1 and 2 after injury. These unlabeled cells were detected by antibodies against human leukocyte antigen (HLA) class I (Figure 1A, 1D, and 1E). HLA–I–positive cells were readily detected in the kidneys at all time points, but notably there was persistence of HLA-I+ cells in the kidney 14 and 28 days after ischemia/reperfusion injury (Figure 1D). As expected, HSPCs were also identified in spleen and bone marrow (Figure 1F through 1H), and there was persistence of HSPCs in the marrow, with evidence on day 7 after ischemia/reperfusion injury that HSPCs in the bone marrow had induced the myeloid marker CD11b (Figure 1I), suggesting that HSPCs had engrafted the mouse bone marrow and that the mice were now chimeric.

Systemic Human HSPC Therapy Reduces Mortality and Improves Kidney Function After Ischemia/Reperfusion Injury
To determine whether HSC recruitment to the injured kidney had any functional consequence during repair, we subjected mice to bilateral ischemia/reperfusion injury (day 0), followed by intravenous infusion of human HSPCs on days 1 and 2. Plasma creatinine was assessed in sham surgery mice (on day 0, plasma creatinine value was 0.05±0.06) and on days 1, 2, 5, and 7 after ischemia/reperfusion injury. Bilateral kidney ischemia/reperfusion injury resulted in a significant increase in serum creatinine at 24 hours and peaked at 48 hours (Figure 2A). Although plasma creatinine levels at 24 hours (time of first injection) were no different in treatment and vehicle groups, there was a marked and significant decrease in plasma creatinine at 48 hours in mice that had received HSPCs (Figure 2A), whereas the vehicle group of mice had persistently highly elevated plasma creatinine levels at this time. Over the subsequent 5 days of recovery, the proportion of mice that showed recovery of kidney function detected by a plasma creatinine ≤0.4 mg/dL was significantly higher in mice treated with HSPCs (Figure 2B). In the vehicle-treated group, only 50% of mice survived to day 7, whereas 90% of mice that received human HSPCs survived to day 7 (Figure 2C). The surviving numbers in the 2 groups can be seen in Figure 2C. These findings indicate that human HSPCs both promote kidney repair/regeneration and enhance survival.

Human HSPC Therapy Attenuates Kidney PTC Loss, Promotes Tubular Epithelial Regeneration, and Prevents Long-Term Fibrosis After Ischemia/Reperfusion Injury
To study the mechanism by which HSPCs promote kidney repair, we analyzed kidney sections for loss of PTCs and
persistence of tubule injury (Figure 3). Analysis of mCD31-labeled PTCs by morphometry revealed that HSC treatment prevented PTC loss (Figure 3A through 3C) during the repair phase through day 7 after ischemia/reperfusion injury. PTC loss after 14 and 28 days was not different in HSC-treated mice, indicating that endogenous factors promote regeneration of PTCs but that HSC therapy attenuates early loss of vasculature. Similarly, HSC therapy attenuated persistence of tubule injury during the repair phase of this model of ischemia/reperfusion injury (Figure 3D through 3F), suggesting that HSPCs promote tubule regeneration by either direct or indirect mechanisms. We have previously demonstrated that kidney ischemia/reperfusion injury can lead to persistent interstitial fibrosis, which is a harbinger of chronic kidney disease and is strongly associated with progressive long-term loss of kidney function.15,26–28 To test whether systemic infusion of HSPCs during repair of the injured kidney affected long-term consequences of injury, we quantified interstitial fibrosis (Figure 3G through 3I). In vehicle-treated mice, interstitial fibrosis accumulated progressively in the 4 weeks after injury, but in those mice that had received HSPCs, interstitial fibrosis was attenuated by day 28.

**Human HSPCs Acquire Endothelial Progenitor Cell Markers in the Kidney After Ischemia/Reperfusion Injury**

HSPCs are the source of myeloid, erythroid, megakaryocyte, and lymphoid lineage cells. We noted that although many HSPCs were recruited to kidneys on days 2 and 3 after injury, the number of retained cells fell progressively through day 7 but thereafter increased up to day 28 after injury (Figure 1). We labeled kidneys for human lymphoid and myeloid commitment markers (Figure 4). CD3 was expressed in a minority of HSPCs recruited to the kidney (Figure 4B and 4E), and this expression occurred several days after recruitment and was proportionately similar in both uninjured and injured kidney. None of the recruited HSPCs expressed the myeloid differentiation marker CD11b in the first 5 days after injury (Figure 4C and 4F), indicating that the number of CMFDA-positive cells per section in the spleen on days 3, 5, and 7 after ischemia/reperfusion injury. H, Graph indicating proportion of human CD45+ cells in mouse bone marrow after adoptive transfer. I, Representative flow cytometric plot for CD11b (detects mouse and human antigens) and human CD45 of whole bone marrow from day 7 post–ischemia/reperfusion injury mouse that received adoptively transferred human HSPCs 1 day after ischemia/reperfusion injury. Values are mean±SD. Bars=50 μm; n=6 to 10 per group.

**Figure 1.** Human hematopoietic stem cells are recruited to post–ischemia/reperfusion injury (IRI) kidneys, spleen, and bone marrow of Non Obese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice. A, Photomicrograph of NOD/SCID mouse day 2.5 post–ischemia/reperfusion injury kidney that received adoptively transferred human HSPCs on day 1, showing HLA-I–positive cells in the interstitium of the post–ischemia/reperfusion injury kidney (arrowheads) between necrotic tubules. B, Graph indicating the number of human CMFDA+ HSPCs identified in post–ischemia/reperfusion injury and control (CON) kidneys with time after ischemia/reperfusion injury. C, Representative confocal image of day 3 post–ischemia/reperfusion injury kidney after intravenous transfer of human HSPCs on day 1 after ischemia/reperfusion injury showing CMFDA fluorescence in human cells (arrowheads) in peritubular CD31+ capillaries. D, Graph indicating the number of human HLA class I cells in post–ischemia/reperfusion injury and control kidneys on days 7, 14, and 28 after ischemia/reperfusion injury. E, Representative confocal image detecting human HLA class I (green, arrow) of day 7 post–ischemia/reperfusion injury kidney of NOD/SCID mice treated with human HSPCs on day 1 after ischemia/reperfusion injury. F through G, Representative confocal image (G) of CMFDA-labeled human HSPCs in spleen 3 days after ischemia/reperfusion injury, adoptively transferred 1 day after ischemia/reperfusion injury and graph (F) indicating the number of CMFDA-positive cells per section in the spleen on days 3, 5, and 7 after ischemia/reperfusion injury. H, Graph indicating proportion of human CD45+ cells in mouse bone marrow after adoptive transfer. I, Representative flow cytometric plot for CD11b (detects mouse and human antigens) and human CD45 of whole bone marrow from day 7 post–ischemia/reperfusion injury mouse that received adoptively transferred human HSPCs 1 day after ischemia/reperfusion injury. Values are mean±SD. Bars=50 μm; n=6 to 10 per group.
lack CD34 (Figure 4L), and occasional human cells with characteristic multilobar nuclei of neutrophils were identified in kidneys at days 14 and 28 after ischemia/reperfusion injury (not shown). Collectively, these findings indicate that the late increase in human cells in the kidney either reflects bone marrow chimerism and recruitment of mature cells from bone marrow or reflects local differentiation of mature cell types in the kidney.

To investigate further the local differentiation of HSPCs in the kidney during the first 7 days, sections were initially colabeled to detect human CD45 with HLA-I (Figure 4G and 4H). Although day 0 HSPCs uniformly expressed both
markers, a proportion of HSPCs recruited to the kidney did not express CD45 at 7 days after ischemia/reperfusion injury. Kidneys were next labeled to detect human CD34 and CEP markers VEGFR2 (KDR), CD146, and CD133, and this expression was compared with day 0 HSPCs (Figure 4I through 4L). Although few mobilized enriched HSPCs expressed KDR or CD146, the majority expressed CD133 before injection. In the kidney, however, at day 3 after ischemia/reperfusion injury, there was a phenotypic switch because nearly all recruited HSPCs expressed CD146, but none expressed CD133 (Figure 4L). The expression of KDR was similar in mobilized enriched HSPCs compared with those recruited to kidney. Because CD146 expression has been associated with CEP functions and because few HSPCs express the T-cell receptor (Figure 4E), our findings suggest that the kidney promotes HSC differentiation toward CEP-type function. Because a small fraction of the purified HSPCs expressed CD146 before injection into mice, we tested whether the apparent phenotypic switch was due to selective recruitment of CD146⁺, CD34⁺ cells to the kidney after infusion. Mobilized purified HSPCs were separated into CD146⁺ fractions and CD146⁻ fractions. Aliquots of these subpopulations were infused intravenously into mice 24 hours after kidney ischemia/reperfusion injury (Table II in the online-only Data Supplement). There was equivalent recruitment of the CD146⁺ or CD146⁻ cells to the injured kidney.

Figure 4. Differentiation of human HSPCs in kidneys. Representative confocal image (A) and epifluorescence images (B and C) of kidney outer medulla showing expression of human CD45 (A), human CD3 (B), and absence of CD11b (C) in cells (arrowheads) in day 5 post–ischemia/reperfusion injury (IRI) kidneys that received intravenous injection of HSPCs 1 day after injury. Images are colabeled (A and B) to show mCD31 (red) of the mouse vasculature. Graphs show the number of human CD45⁺ (D), human CD3⁺ (E), and CD11b⁺ CMFDA⁺ (F) cells identified in post–ischemia/reperfusion injury kidneys and control kidneys. G and H, Confocal split panel image (G) and graph (H) showing the presence of HLA-1⁺, hCD45⁺ cells (arrowheads) in the interstitium of the day 7 post–ischemia/reperfusion injury kidney. T indicates tubule. Inset shows a HLA-1⁺, hCD45⁺ leukocyte (arrow). I to K, Representative epifluorescence images of the outer medulla of day 3 post–ischemia/reperfusion injury kidney of NOD/SCID mice that received human HSPCs on days 1 and 2 labeled with CMFDA (arrowheads) colabeled with antibodies against human CD133 (I), CD146 (J), and KDR (K). Note that anti-KDR antibodies also detected mouse endothelium (arrows). T indicates tubule, outlined with hatched white line in J. L, Graph showing the expression of stem cell/CEP markers by HSPCs before (day 0) and after recruitment to the post–ischemia/reperfusion injury kidney. Bars—50 μm. Data are mean±SD; n=6 per time point.
extremely rare contribution of purified human CD34

Furthermore, the CD146\textsuperscript{−} cells rapidly acquired expression of CD146, confirming that HSPCs rapidly undergo a phenotypic switch in kidney.

**Human HSPCs Contribute to Vascular Repair by Paracrine Mechanisms**
To dissect the mechanism by which HSPCs support neovascularization, we determined initially whether HSPCs had differentiated into endothelial cells. Using the human-specific antibodies against CD31 and human von Willebrand factor (vWF), 2 markers of endothelial cells, we identified human CD31\textsuperscript{+} cells in injured kidneys at days 7, 14, and 28 but not at earlier time points (Figure 5A to 5C). Therefore, CD31 expression did not coincide with maximal repair. Occasional CD31\textsuperscript{+} HSPCs lacked CD45 expression and were found in the PTC wall with morphology consistent with endothelial cells (Figure 5A). However, the vast majority of CD31\textsuperscript{+} human cells also coexpressed CD45 (Figure 5B) or were located in the interstitium with leukocyte morphology, consistent with CD31 expression by lymphocytes and monocytes, indicating that human CD31 is not a specific marker of endothelium. Parallel studies using antihuman vWF antibodies (that did not cross-react with mouse vWF) also identified rare vWF\textsuperscript{−} human cells that lacked CD45 expression (Figure 5D), adding weight to the observation that occasionally human cells do become functioning endothelial cells. Because these investigations provided evidence for only an extremely rare contribution of purified human CD34\textsuperscript{+} cells to direct endothelial replacement, and yet there was marked expression of the CEP marker CD146 in all HSPCs (Figure 4), we tested whether HSPCs were functioning by paracrine mechanisms. This was particularly tractable given the intravascular and perivascular locale of HSPCs in the kidney after injury. Kidneys labeled for cells in the cell cycle with the use of the pan cell cycle marker Ki67 were revealing: Infusion of HSPCs led to a marked increase in the number of parenchymal cells in the cell cycle after injury (Figure 6A to 6C), and this enhancement of cellular proliferation persisted throughout the repair phase. The enhanced proliferation was in both tubule epithelial cells and interstitial cells, indicating that HSPCs were influencing both compartments (Figure 6C). In addition, in the unilateral model of ischemia/reperfusion injury, where there is compensatory growth in the uninjured kidney, the HSPCs also promoted proliferation of parenchymal cells of this kidney. Although proliferation was enhanced, there was no change in the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)\textsuperscript{−} apoptotic cells seen in the kidneys (Figure 6D).

To explore further potential release of cytokines locally, CMFDA-labeled HSPCs that had been recruited to the kidney on day 4 after ischemia/reperfusion injury were purified from whole kidney with the use of established methods,\textsuperscript{30} and their human specific transcriptional profile was analyzed by reverse transcription polymerase chain reaction, comparing it with the transcriptional profile of homogeneic HSPCs before systemic injection into mice (Figure 6E). Mobilized enriched HSPCs generated high levels of transcripts for proangiogenic cytokines including angiopoietin-1, fibroblast growth factor-2, and vascular endothelial growth factor-A and in addition generated high levels of hepatocyte growth factor, recognized for its role in kidney epithelial regeneration (Figure 7). Strikingly, after injection into mice, those HSPCs that were recruited to the kidney exhibited highly similar transcriptional activity for the proangiogenic cytokines, further supporting a paracrine role in angiogenesis.

Because HSPCs were also recruited to the spleen (Figure 1), we determined whether HSPCs secreted circulating angiogenic cytokines in addition to local production of angiogenic factors directly in the kidney. Protein microarray analysis of plasma detected angiogenic cytokines at enhanced levels in mice that had received HSPCs (Figure IIIA in the online-only Data Supplement), and these were context specific (that is, plasma levels of human angiogenic cytokines were affected by the presence of ischemia/reperfusion injury disease in the kidney). However, the levels were low compared with endogenous production of the same cytokines. In addition, cultured spleen cells did not generate angiogenic cytokines (Figure IIIB in the online-only Data Supplement), making the spleen an unlikely source of angiogenic factors and suggesting that the kidney may be the major source of circulating human angiogenic factors. Because human angiogenic factors were nevertheless detected in the circulation, we infused conditioned media from 2.5×10\textsuperscript{6} cultured HSPCs into mice on day 1 after ischemia/reperfusion injury to determine whether HSPCs function as endocrine cells. Whereas HSC infusion promoted a rapid decline in creatinine level (Figure 2), conditioned media resulted in a nonsignifi-
cant improvement of renal function (Figure IIIC in the online-only Data Supplement), indicating that detection of human angiogenic cytokines in plasma likely reflects the paracrine rather than endocrine action of HSPCs in the kidney.

Discussion

Acute kidney injury in humans continues to confer high mortality and has limited therapeutic options; therefore, identifying potential regenerative approaches as new therapeutic strategies is highly desirable. In addition, emerging evidence indicates that acute kidney injury in humans is a harbinger of chronic kidney disease characterized by inflammation, vasculopathy, epithelial atrophy, fibrosis, and progressive loss of function, leading to organ failure.\(^3\),\(^15\),\(^27\),\(^31\) New strategies that attenuate kidney injury or enhance repair and regeneration will not only have short-term impact but conceivably will alter the long-term course for kidney function. The long-term benefits from such therapies will affect not only kidney disease but also cardiovascular diseases because chronic kidney disease is an independent risk factor for cardiovascular diseases.\(^4\) Recently, adult human peripheral blood CD34\(^+\) cells as well as HSPCs have been reported to promote vasculogenesis and osteogenesis after stroke and bone injury.\(^22\),\(^32\) Furthermore, CD34\(^+\) cells are capable of expansion and mobilization into the peripheral circulation in the presence of exogenously applied granulocyte colony-stimulating factor,\(^33\)–\(^35\) making HSPCs readily available and strengthening the rationale of clinical cellular therapy.

In the present study, we demonstrated that human HSPCs administered systemically 24 hours after kidney injury were selectively recruited to injured kidneys and localized prominently within and around vasculature. This recruitment was associated with enhanced repair of the microvasculature, tubule epithelial cells, enhanced functional recovery, and increased survival of mice. Additionally, long-term fibrosis was prevented.
During the first 7 days of regeneration and recovery after ischemia/reperfusion injury, most HSPCs recruited to the kidney did not acquire markers of myeloid or lymphoid differentiation; rather, they acquired markers consistent with CEPs. Furthermore, HSPCs synthesized high levels of angiogenic transcripts, and this pattern of transcription persisted after recruitment to the kidney, which is another characteristic of CEPs. Although mobilized purified HSPCs contained small numbers of CEPs and rare circulating endothelial cells defined by cell surface markers before recruitment to the kidney, the injured kidney environment triggered HSPCs to lose CD133, CD45 and activate expression of CD146, phenotypic features consistent with CEP differentiation at the site of injury. This injury-triggered differentiation toward CEP cell type is highlighted by the fact that HSPCs recruited to spleen did not generate angiogenic cytokines. Nevertheless, despite local CEP differentiation within the kidney, we identified very few human endothelial cells in the mouse capillary walls. Taken together, these data indicate that HSC-mediated renal repair is by paracrine mechanisms rather than replacement of vasculature (Figure 7). The definition of CEPs or even their existence remains controversial. The data presented here show, at least in kidney injury, that human HSPCs can remain in a peripheral organ as a primitive cell type for several days and promote vascular repair and that this cell type loses CD34 and expresses cell surface markers that others have reported in cells performing vascular reparative functions. Our findings are consistent, therefore, with HSPCs becoming CEPs but not becoming angioblasts and replacing lost endothelial cells directly. Our studies rarely identified cells within the capillary wall with endothelial morphology, expression of CD31, vWF, and absence of CD45, consistent with human endothelial cells. CD31 expression alone is insufficient to designate a cell endothelial because it is also expressed by B cells and monocytes/macrophages. Mobilized purified HSPCs contained a minor population of purified circulating endothelial cells (<0.05%). It is therefore possible that the rare human endothelial cells detected in mouse kidney derived from cell fusion of CEPs with endothelial cells, incorporation of rare circulating endothelial cells, or possibly occasional transdifferentiation of CEPs.

Human HSPCs were selectively recruited into injured kidney, indicating that the kidney releases chemoattractants for HSPCs. Systemic administration of HSPCs at the onset of injury (day 0) led to poor recruitment of HSPCs, but delayed administration of HSPCs until the beginning of the repair phase was highly effective in triggering recruitment. This recruitment pattern is similar to monocyte influx to the kidney, suggesting that in addition to stromal cell–derived factor-1, other monocyte chemoattractants may play a role in HSC recruitment. Our prior studies in mice provided no evidence for endogenous HSC mobilization from the bone marrow or recruitment to the kidney simply in response to ischemia/reperfusion injury, although others have reported that endogenous HSC can be recruited to injured kidney, and the differences in these data may be methodological. Regardless of whether endogenous HSPCs traffic to the kidney after injury, our studies indicate that there is an inadequate endogenous signal for recruitment of HSPCs from their normal niche in the bone marrow. Because injection of HSPCs into the peripheral circulation results in effective recruitment to the kidney, HSC therapy overcomes a normal block in release from the bone marrow niche.

HSPCs were detected in the kidney through days 14 and 28 after ischemia/reperfusion injury with the use of antibodies against HLA class I antigens. There was a bimodal distribution of HSC retention in the kidney with time, with the nadir occurring at about 7 days. We noted that the mice developed bone marrow chimerism and that at days 14 and 28 (but not earlier), all of the human cells in the kidney expressed mature lineage markers, and none of them expressed stem cell markers. It is therefore likely that many of the late human cells in the kidney are recruited from bone marrow rather than deriving from the original recruitment of HSPCs. However, our data strikingly point to HSC infusion on days 1 and 2 of disease resulting in long-term impact on fibrosis. It is unclear from the present studies whether a reduction in long-term fibrosis reflects improved early vascular repair or whether it reflects a persistent population of reparative mature human leukocytes in the kidney at late time points.

Ischemic injury in the kidneys is characterized by epithelial injury. Less well described is the loss of PTC. However, data derived from several severe acute kidney injury models (ischemia, toxin, transient angiotensin II) demonstrate capillary loss that typically precedes the development of prominent fibrosis, and neoangiogenesis may be a central process in preservation of vascular structure and restoration of organ function. We also show in these studies that after ischemia/reperfusion injury there is marked loss of PTCs with only relatively mild renal injury and that although...
there is significant regeneration of these PTCs during repair, there is persistent loss of vasculature 1 month after injury, indicating that the kidney has an inherent defect in revascularization after injury, unlike other organs such as skin. Our studies show unequivocally that HSPCs attenuate that loss of PTCs in the kidney during the repair, and this is associated with both rapid functional recovery of the kidney and enhanced survival of mice. In our unilateral ischemia/reperfusion injury model, HSC-mediated regeneration of PTCs did not attenuate the long-term persistent PTC loss at 28 weeks but nevertheless affected recovery and survival seen in the bilateral ischemia/reperfusion injury model, pointing to early vascular repair as a central process in renal repair. Despite the window of HSC-mediated vascular repair being restricted to early time points after injury, there was nevertheless prevention of fibrosis progression in the kidney at 1 month after injury. Further studies will be required to understand whether this long-term effect of early HSC infusion is through early enhancement of pericyte-endothelial cell interactions, which may be a central interaction in the development of interstitial fibrosis.

In preliminary studies, late administration of HSPCs to mice 14 days after ischemia/reperfusion injury resulted in poor recruitment and little evidence of enhanced vascular repair (not shown), indicating that there is a restricted period after injury during which HSPCs are efficacious.

Although the kidney ischemia/reperfusion injury model is characterized by severe injury and repair of the tubule epithelial cells, particularly the S3 segment of the proximal tubule, it is likely that without PTC regeneration those injured tubules will not regenerate successfully because of persistent ischemia. In addition to PTC regeneration, HSPCs promoted epithelial regeneration, as assessed by tubule injury score and functional recovery. The generation of angiogenic cytokines, combined with the intravascular and perivascular locale of HSPCs, suggests a primary role in kidney microvascular repair, which secondarily promotes epithelial repair. However, because HSPCs generate hepatocyte growth factor, which is known to promote epithelial regeneration directly, HSPCs may have a direct paracrine role on epithelial repair, independently of PTC repair.

In conclusion, we demonstrate here that systematically administered peripheral blood mobilized human HSPCs reduce mortality and promote rapid renal repair and regeneration of the kidney by paracrine mechanisms directed at peritubular capillaries. These findings support human HSPCs as a promising therapeutic strategy for treatment of acute kidney diseases and in the prevention of chronic kidney diseases.

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Disclosures
Baxter has a patent pending for the use of HSCs in repair of the injured kidney. Dr Duffield serves on the Scientific Advisory Board for Promedior Inc. The other authors report no conflicts.

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Acute kidney injury, formerly known as acute tubular necrosis, has reached epidemic proportions. It is a harbinger for chronic kidney disease, end-stage kidney disease, cardiovascular events, and untimely death. Despite this, other than supportive management, there are no active therapeutic strategies that promote repair and regeneration. In this article, Li and colleagues identify a promising new strategy for the treatment of acute kidney injury using cellular therapy. Human stem cells were mobilized from healthy donors and purified from peripheral blood. They were infused into mice with acute kidney injury. The human stem cells trafficked to the kidney, where they orchestrated microvascular repair. The consequence of this microvascular repair was enhanced animal survival and prompt resolution of organ failure. One month after injury, the propensity to develop organ fibrosis, a characteristic of chronic kidney disease, was significantly diminished. The kidney repair prompted by human stem cells manifested as enhanced proliferation in both the microvascular and nephron compartments of the injured organ rather than replacement of kidney cells. These compelling studies in mice highlight the urgent need for new therapies focused on vascular repair/regeneration and raise the possibility that donor human stem cells might be indicated prophyactically in patients at high risk of acute kidney injury.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

**Animals**

Male immune deficient non-obese diabetic (NOD/SCID) mice (NOD.CB17-Prkdc<sup>scid</sup>/J) (Jackson Laboratories, Bar Harbor, ME) were used at the age of 8-10 weeks. Note these mice are not diabetic, but lack functional T and B cells. All mice were maintained in filter top cages and received sterilized food and acidified water. All experimental protocols were approved by the Harvard Center for Animal Research and Comparative Medicine.

**Human Peripheral Blood CD34<sup>+</sup> Cell Purification, Characterization, and Tracking**

Human peripheral blood CD34+ stem/progenitor cells were selected from granulocyte colony stimulating factor (G-CSF) mobilized apheresis products obtained from normal healthy adult donors (catalog# mPB026, AllCells, Berkeley, CA). Briefly, G-CSF (10µg/kg/day) was administered to donors for 5 consecutive days to mobilize CD34+ cells from the bone marrow into peripheral circulation. Donors underwent apheresis on days 5 and 6 to collect mobilized peripheral blood mononuclear cells. CD34+ cells were highly enriched from the apheresis product using ISOLEX 300i Magnetic Cell Positive Selection System (version 2.5, Baxter Healthcare, Deerfield, IL, USA) according to the protocol provided with the instrument’s User Manual. Purified cells were characterized by flow cytometry (see below). Enriched, selected cells were maintained in RPMI (Invitrogen) with 0.5% human serum albumin at 25°C and used within 48h. To test
viability, aliquots of 2 x 10^5 cells were labeled with 7-AAD (20µg/ml, 20min, 4°C in 100µl PBS), washed with FACS buffer (PBS/5%BSA), and then analyzed by flow cytometry. To test for clonogenic capacity, the colony forming unit assay (CFU) was performed using modifications to methods previously described. Purified CD34+ stem/progenitor cells were diluted in Iscove’s MDM (IMDM) with 2% FBS (Stem Cell Technologies, Cat # 07700 Vancouver, Canada) to a concentration of 5x10^3 cells/ml. The primary dilution was further diluted in MethoCult GF+ H4435 Medium (Cat # 04445, StemCell Technologies, Vancouver, Canada) (1:10) to a final cell concentration of 500 cells per 35mm dish and cells were cultured in quadruplicate per sample and reported as an average. Fourteen days later, the CFU were scored using bright field microscopy for the presence of Colony Forming Unit-Granulocyte Macrophage (CFU-GM), Colony Forming Unit Erythroid (CFU-E), Burst Forming Unit-Erythroid (BFU-E) and Colony Forming Units with both GM and Erythroid colonies (CFU-GEMM). CFU-E: Colony-forming unit-erythroid produces a colony containing 1 to 2 clusters with a total of 8-200 erythroblasts. BFU-E: Burst-forming unit-erythroid produces a colony containing > 200 erythroblasts, usually present in > 2 clusters. CFU-GM: Colony-forming unit-granulocyte, macrophage produces a colony containing > 40 granulocyte and macrophage cells. CFU-GEMM: Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte produces a colony containing erythroid cells as well as 20 or more granulocyte, macrophage and megakaryocyte cells.

In some experiments, in order to track HSCs following systemic administration, human CD34+ cells were labeled with the green fluorescent tracer 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen) using 1µg/10^7 cells for 30 minutes at 25°C in 10ml of RPMI. Excess CMFDA was quenched after centrifugation (250xg 5min) by resuspending in 10ml 5%BSA (ultrapure) (Sigma) in RPMI. After further centrifugation cells were resuspended in 1%BSA/RPMI (12.5 x 10^6/ml). CMFDA labeling did not yield any changes in viability detected using 7-AAD (not shown).
**Animal Model**

Ischemia-reperfusion injury of the kidney was modified from methods previously described \(^2\). In brief, on day 0, kidneys of anesthetized male mice (8-10 wks) were exposed through surgical incisions to the flanks, and at core temperature of 36.8-37.3°C a surgical clamp was placed across the renal artery and vein of either one or both kidneys. The kidneys were confirmed to become dusky, then replaced in the retroperitoneum for 27 minutes (unilateral model) or 25 minutes (bilateral model). Clamps were removed and reperfusion to kidneys was confirmed visually, and wounds closed. To test the effect of human HSCs, these mice with unilateral IRI kidney injury were divided into two groups. In the treatment group (n = 6-10/group), on days 1 and 2 after kidney injury, 200 µl of cell suspension containing 2.5 x 10^6 human CD34+ cells labeled with CMFDA was infused intravenously through the tail vein. In the control group, mice were only given vehicle. Mice were sacrificed on days 3, 5, 7, 14 and 28 IRI of the kidney.

**Quantification of Renal Function and Circulating Human Cytokines**

To evaluate renal function, mice with bilateral IRI kidney injury (d0) were randomly divided into two groups. The treatment group (n = 10) received 2.5 x 10^6 human HSCs by intravenously tail vein infusion on days 1 and 2. The control group (n = 16) received vehicle only. Plasma creatinine was analyzed from plasma samples were taken from the tail vein on days 1, 2, 5, 7, 14 and 28 after injury using Methods previously described \(^2\). Plasma samples were also assessed for the presence of angiogenic cytokines by human cytokine array (Ray Biotech, Inc.)
Tissue Preparation, Immunostaining, Imaging and Quantification of Injury and Repair

Mice were perfused with ice cold PBS then organs fixed in PLP solution for 2h followed by 18% sucrose 16h, then preserved in OCT (−80°C) 3, or tissue for light microscopy was fixed in 10% neutral-buffered formalin for 12h, transferred to 70% ethanol, then processed for paraffin sections (3mm) and sections and stained with periodic acid-Schiff (PAS) or picrosirius red stain 3. Immunofluorescence labeling was performed on 5mm cryosections. To detect infused human cells in kidneys, spleen and heart, either antibodies against human leukocyte antigens with no cross-reactivity to mouse antigens were used or fluorescence of CMFDA was used (up to d7). The following antibodies were used employing methods described elsewhere 2, 3: anti-human leukocyte antigen class I (HLA)-ABC (FITC, 1:200, eBioscience), anti-human CD45 (FITC, 1:200, eBioscience), rat-anti-human CD45 (1:200, Abcam), anti-human CD3 (FITC, 1:200, eBioscience), anti-human CD31 (FITC, 1:200, eBioscience), rabbit anti-human vWF (1:200, Abcam), anti-human CD146 (FITC, 1:100, Abcam), biotin-anti-human CD34 (1:100, Abcam), rat-anti-mouse cross react with human CD11b (1:100 eBioscience), biotin-anti-human CD133 (1:100, Miltenyi), rabbit-anti-human-CD133 (1:100, CellSignaling), goat-anti-human KDR (1:100, R&D Systems), and rabbit-anti-human KDR (1:100, NeoMarkers), followed by rabbit-anti FITC (1:200, Invitrogen), anti-rat Cy3 or anti-rabbit Cy3 or anti-goat Cy3 (1:400, Jackson Immunosresearch) or anti-avidin Cy3 (1:3000, Jackson Immunosresearch). To label mouse vasculature using rat-anti-mouse CD31 (1:200, eBioscience), which does not cross-react with human antigen was
applied, followed by anti-rat Cy3 (1:400, Jackson Immunosresearch). Detection of the Ki67 antigen and detection of apoptotic cells by T.U.N.E.L was performed as previously described. Sections were post-fixed with 1% paraformaldehyde (PFA), then mounted in Vectashield with DAPI. Peritubular capillary loss and tubule injury were determined by assessing anti-CD31-Cy3 labeled kidney sections or PAS stained paraffin sections respectively using a blinded scoring method as reported previously. In brief, images were captured by digital imaging (X200) sequentially over the entire sagittal section incorporating cortex and outer medulla (10-20 images). Each image was divided into 252 squares by a grid. To calculate peritubular capillary loss, each square without a peritubular capillary resulted in a positive score; the final score presented as a percentage positive score. To assess the tubular injury, each square the presence of tubule injury (tubule flattening, necrosis, apoptosis or presence of casts) resulted in a positive score. The final score is the percentage of squares with positive score per image, which was averaged for all images from the individual kidney. Epifluorescence images were taken with a Nikon TE2000 microscope, CoolSnap camera (Roper Scientific, Germany) and processed using IP lab software (BD Biosciences, San Jose, CA). Confocal images were generated using a Nikon C1 D-Eclipse confocal microscope. Projection images were generated from 10 Z-stack images that were acquired at 0.1 mm steps. To allow comparison between sections, all confocal settings including were kept constant between sections.

Flow Cytometric Analysis and Cell Sorting

Isolex-enriched CD34 cells were analyzed using the following human antibody

Single cells were prepared from kidney, spleen and bone marrow as previously described. In brief, single cells (1x 10^5) from kidney, spleen and bone marrow were resuspended in FACS buffer and incubated with antibodies against human CD45 (FITC, 1:200, eBioscience) and mouse CD11b (PE, 1:200, eBioscience) for 30 minutes. After washing with FACS wash buffer, and resuspending in 200ul FACS buffer, cells were analyzed using BD FACSCalibur flow cytometer. The human HSCs labeled with CMFDA on day 2 after injection were sorted directly by FACS sorting using FACSaria. Sorted CMFDA+ cells from kidney were immediately lysed and RNA purified using RNA Easy (Qiagen) system, for real time PCR. In some experiments ISOLEX purified HSCs were separated into CD146+ and CD146- subpopulations prior to systemic injection, by magnetic activated cell sorting using MACS cell separation system (Miltenyi Biotech, Auburn, Calif., USA) and methods previously described. Briefly, following, incubation with anti-human CD146-FITC antibodies (1:20 dilution) on ice for 20 min. cells were labeled with anti-FITC IgG MicroBeads (Miltenyi Biotech; 1:5) washed applied to the MS column of MiniMACS magnetic separation kit followed by a second column.
Unlabeled, CD146- cells were collected, and then labeled CD146+ cells were collected separately after the magnetic field was removed. Cells were relabeled with anti-CD146-FITC antibodies and compared with unlabeled cells by flow cytometry.

**Real Time PCR**

Total RNA was generated from tissue and cells using a kit (RNA Easy Qiagen), according to the manufacturer’s instructions. Purity determined by A260 to A280. cDNA was synthesized from 1µg of total RNA using iScript and primers comprising random hexamers and poly dT. Real-time PCR of human and mouse samples was performed using an ABI7900HT sequence detection system (PerkinElmer Life Sciences, Boston, Applied BioSystems, Foster City, CA) in the presence of SYBR-Green (SYBR Green PCR kit; Qiagen) using methods previously described. Primer/probe sets specific for human GAPDH, HPRT1, Angiopoietin 1 (ANGPT1), Fibroblast growth factor 2 (FGF2), Hepatocyte Growth Factor (HGF), Insulin-like growth factor 1 (IGF1), interleukin-8 (IL8), Platelet-derived growth factor B (PDGFB), transforming growth factor β1 (TGFB1), Vascular endothelial growth factor (VEGF), TIE1, were from Sabiosciences. Equal amounts of cDNA were used for RT-PCR reaction and mixed with ready to use reaction mix (Sabiosciences). All of the reactions were performed in triplicate. Optimization of the real-time PCR was performed according to the manufacturer’s instructions. For standard curve determination, we used a pool of all the samples, serially diluted in four log₂ steps and run in parallel to the samples. The total volume of each reaction was 20 µl, containing 300nM forward and 300nM reverse primer and 125ng of cDNA. Appropriate negative controls were run for each reaction.
Cell Culture

Human ISOLEX purified HSCs were incubated (2.5x10^6) in 3cm diameter wells (Falcon) in 2ml of medium (X-vivo 10, Lonza) supplemented with SCF and Flt-3 ligand (20ng/ml each, Peprotech) for 48h. Supernatants were separated from cells by centrifugation and filtration then 1ml was administered to mice 24h post IRI (200µl IV and 800µl IP). In other experiments, spleens was harvested from mice 72h post HSC or vehicle infusion and splenocyte single cell preparation made \(^5\). 1x10^6 cells were incubated in 3cm diameter wells in serum free RPMI for 12h. Supernatants were separated from cells by centrifugation and filtration. From the same mice, plasma was collected. Cytokine content from supernatants and plasma were analyzed by antibody cytokine array (Ray Biotech, Inc.).
Supplemental Tables

Table 1. Human CD34+ enriched mobilized peripheral blood stem cells (n = 6/group).

<table>
<thead>
<tr>
<th>Markers</th>
<th>CD34+</th>
<th>CD34+CD45-</th>
<th>CD34-CD45+</th>
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<tr>
<td>Average</td>
<td>96.44</td>
<td>0.08</td>
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<tr>
<td>SD</td>
<td>0.84</td>
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Table 2. Minor subpopulations in human CD34+ enriched mobilized peripheral blood HSCs by cell surface markers (n = 6/group)

<table>
<thead>
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<th>CEP type markers</th>
<th>CECs</th>
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<tbody>
<tr>
<td>Markers</td>
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</tr>
<tr>
<td>CD14+</td>
<td>0.05</td>
</tr>
<tr>
<td>CD133+</td>
<td>0.04</td>
</tr>
<tr>
<td>Average</td>
<td>0.05</td>
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<tr>
<td>SD</td>
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Table 3. Analysis of recruitment to the kidney and *de novo* expression of hCD146+ in the kidney by hCD146+ and hCD146- subpopulations of ISOLEX purified CD34+ HSCs following IV injection and following recruitment to kidney at day 2 post IRI.

<table>
<thead>
<tr>
<th></th>
<th>hCD146- group</th>
<th>CD146+ group</th>
</tr>
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<tbody>
<tr>
<td>hCD146+ on D0</td>
<td>0.02%</td>
<td>7.34%</td>
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<tr>
<td>hCD146+ on D2 in kidney</td>
<td>98%</td>
<td>100%</td>
</tr>
<tr>
<td>hCD146+ cells/section</td>
<td>155.3±56.4</td>
<td>166.7±40.4</td>
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Figure S1. (A) FACS plot showing CMFDA labeled ISOLEX purified human CD34+ cells and uptake fluorescent viability marker 7-AAD. Note only 1.3% of CD34+ cells lack viability. (B) Fluorescence photomicrographs showing HLA-I human HSCs 12h after recruitment to the post IRI kidney showing expanded morphologies ‘bridging’ gaps in the disrupted capillaries (arrowheads) and recruitment within capillaries and in the interstitium (arrows). Marker = 50µm.
**Figure S2.** Evidence of multi-lineage clonogenic capacity of CD34+ ISOLEX purified cells. (A) Low power images of erythrocytic and granulocyte/monocyte colonies growing from single CD34+ cells. (B) Pie chart showing the proportion of colony types cultured from CD34+ cells and (C) graph showing the number of colonies per 500 HSCs plated.
Figure S3. Angiogenic cytokine production by HSCs is dependent on recruitment of HSCs to the injured kidney. (A-B) Detection of human angiogenic cytokines in plasma of mice (A), or in supernatant following 12hr culture of splenocytes (B), collected from mice d3 after IRI mice or from healthy mice infused with either HSCs (2.5x10^6) or vehicle on d1. Cytokine levels are means with SE and are relative to healthy mice with vehicle injection (n=3 per group). (C) Plasma creatinine levels from d1 and d2 in mice subjected to bilateral IRI on d0 and infused on d1 with 1ml of conditioned medium from cultured HSCs (2.5x10^6) or vehicle. The difference between groups at d2 is not significant (n=7 per group).
Supplemental References


