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Dynamics of mobilization and homing of endothelial progenitor cells after acute renal ischemia: modulation by ischemic preconditioning

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Patschan, Daniel, Krystina Krupincza, Susann Patschan, Zhongtao Zhang, Carl Hamby, and Michael S. Goligorsky. Dynamics of mobilization and homing of endothelial progenitor cells after acute renal ischemia: modulation by ischemic preconditioning. *Am J Physiol Renal Physiol* 291: F176–F185, 2006; doi:10.1152/ajprenal.00454.2005.—Endothelial progenitor cells (EPCs) have been shown to participate in tissue repair under diverse physiological and pathological conditions. It is unknown whether EPCs are mobilized in response to acute renal injury. The aim of this study was to characterize EPC mobilization and homing in the course of acute renal ischemia. Mice were subjected to unilateral renal artery clamping (UC) for 25 min. At 10 min, 3, 6, 24 h, and 7 days after UC, the pool of circulating and splenic $CD34^+$/$Flk\text{-}1^+$ cells within the monocytic population was detected by flow cytometry. For ischemic preconditioning (IPC), the first UC was performed 7 days before the repeated ischemic episode. For EPC detection in the kidney, cryosections were stained for c-Kit^+/$Tie\text{-}2^+$ cells. The number of circulating EPCs was not significantly affected at any time after UC compared with sham-operated or control mice. IPC did not significantly change the circulating pool of EPCs. Splenectomy performed before UC resulted in a surge of circulating EPCs. Accordingly, splenic EPCs were significantly increased after UC at 3 and 6 h, but not at later times. EPC homing to the spleen was absent in IPC animals. Immunohistochemical analysis of the kidneys showed a sixfold increase in the number of c-Kit^+/$Tie\text{-}2^+$ cells localized in the medullopapillary region in mice by *day 7* after ischemia. Enriched population of c-Kit^+/$Tie\text{-}2^+$ cells from the medullopapillary parenchyma of Tie-2green fluorescent protein chimeric mice subjected to IPC was isolated and transplanted to wild-type mice with acute renal ischemia. This procedure resulted in the improvement of renal function in recipients. In conclusion, *1*) renal ischemia rapidly (within 3–6 h) mobilizes EPCs, which transiently home to the spleen, acting as a temporary reservoir of mobilized EPCs; *2*) the late phase of IPC is associated with the mobilization of the splenic pool and accumulation of EPCs in the renal medullopapillary region; and *3*) transplantation of EPC-enriched cells from the medullopapillary parenchyma afforded partial renoprotection after renal ischemia, suggesting the role of the recruited EPCs in the functional rescue.

acute renal failure; EPCs; ischemia-associated mobilization; renal ischemia protection; renal stem cells

A NUMBER OF STUDIES published over the past few years have documented that endothelial progenitor cells (EPCs), either derived from the bone marrow or from other niches, participate in the repair of damaged tissues and restoration of organ function (3, 4, 30). Evidence is continuously growing that EPCs participate in postnatal angiogenesis under physiological conditions and in tissue repair processes in cardiovascular disorders (1, 19, 27). It is not known, however, whether acute renal injury leads to the mobilization of endogenous EPCs, one of the aims of the present study. This problem is of significant importance, because acute renal failure (ARF) remains a serious complication with a diverse etiology occurring in 5% of hospitalized patients and its mortality rate still ranges between 30 and 90% (16). The most common cause of ARF is prolonged regional or total renal hypoperfusion (acute ischemic renal failure) (8, 26).

It was demonstrated three decades ago (13) that renal endothelial cells in ARF undergo an early swelling compromising the patency of the vascular lumen and impairing the microcirculation (20, 28). These vascular changes, described as "no-reflow" phenomenon, were proposed to be responsible for a delayed functional recovery of the postischemic kidney. In an attempt to mitigate these microvascular changes, Brodsky et al. (9) infused human umbilical vein endothelial cells (HUVECs) into athymic nude rats subjected to renal artery clamping and found improvement of renal microcirculation and mitigated dysfunction. Arriero et al. (2) transplanted skeletal muscle-derived ex vivo expanded stem cells, which were differentiated along the endothelial lineage to express the markers of EPCs, and demonstrated their renal engraftment and partial protection of the ischemic kidney.

Ischemic preconitioning (IPC), most extensively studied in the heart, is a phenomenon of acquired resistance to various stressors after a preceeding nonlethal stress. IPC has been shown to be a biphasic process: an early phase lasts up to 2 h, a late phase becomes apparent after 24 h to several days (10). The early phase of IPC does not depend on protein synthesis but requires activation of adenosine receptors, ATP-sensitive potassium channels, and translocation of 5′-nucleotidase to the cell surface (21). The late phase of IPC requires protein synthesis, is NO and/or heat shock protein dependent, and is modulated by protein kinases and NF-κB (10). Although the precise mechanisms of the early phase of IPC probably vary from organ to organ, the late phase of IPC is considered to be a universal response (6). With respect to the murine kidney, IPC is maximal at 1–2 wk after the initial episode (25). Molecular mechanisms of IPC in the kidney have been linked to protective changes in tubular function and to the properties of tissue-infiltrating leukocytes (7). Recently, EPCs have been shown to home to murine myocardium after short periods of IPC and protect myocardium through "imported" nitric oxide

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synthase activity (17). There is no information on the participation of EPCs in the IPC after renal ischemia.

The aim of the present study was, therefore, to analyze the dynamics of mobilization and homing of murine endogenous EPCs in the course of acute renal ischemia. Special attention was focused on EPC dynamics and homing in IPC. Using a mouse model of renal artery clamping, we demonstrated the complex trafficking of mobilized EPCs between the spleen and the ischemic kidney and elucidated the intricate effect of IPC on homing of mobilized EPCs to the renal medullopapillary parenchyma. EPC-enriched mononuclear cells isolated from the medullopapillary parenchyma of preconditioned animals significantly improved renal function when administered to wild-type animals with acute renal ischemia. We hypothesize therefore that renal parenchymal homing of EPCs may participate in the renoprotection of IPC.

MATERIALS AND METHODS

Animal models. The animal study protocol was in accordance with National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (U.S. Department of Health and Human Services Public Health Services, NIH, NIH Publication No. 86–23, 1985) and approved by the Institutional Animal Care and Use Committee. FVB/NJ and Tie-2-GFP mice were obtained from Jackson Laboratories (Bar Harbor, ME). Tie-2-GFP mice express the green fluorescent protein (GFP) driven by an endothelial-specific and -selective promoter for Tie-2 receptor, resulting in specific fluorescence of endothelial cells and their progenitor cells, as previously described (2). All animals were separately caged with a 12:12-h light-dark cycle and had free access to water and chow throughout the study. A subcutaneous injection of 250 U/kg heparin was given 15 min before the operation. After a 1.5-cm midlaparotomy, the left kidney was exposed and clamping of the renal pedicle was performed with microserrefines (Fine Science Tools, Foster City, CA). After 25 min, the clamp was released. The abdominal incision was closed with a 4–0 suture and surgical staples. At different time points after release of the clamp (10 min, 3, 6, and 24 h, and 7 days), the animals were killed, and blood, kidneys, lungs, and spleen were collected for further analysis. For IPC, animals underwent an initial 25-min period of clamping exactly 7 days before the described procedure. For sham operation, midlaparotomy without vascular clamping was performed. Before nephrectomy (NE) and splenectomy (SPE), fat tissue around the kidney and the spleen was removed, and then the renal and splenic vessels were ligated. Injections of isolated mononuclear cells from the renal medulla of preconditioned Tie-2-GFP mice were performed into the right renal vein just before ligation of the vessel.

Cytokine treatment. As a positive control for mobilization of stem and progenitor cells, mice were injected with a superagonistic stem cell factor (SCF) variant (L70C-synthsized by Dr. Z. Zhang, Department of Biochemistry, New York Medical College, Valhalla, NY) at 200 $\mu g \cdot kg^{-1} \cdot day^{-1}$ and recombinant murine G-CSF (Peprotech, Rocky Hill, NJ) at 50 $\mu g \cdot kg^{-1} \cdot day^{-1}$ once a day for 5 days, respectively (23).

Isolation of EPCs from splenic and renal tissue. For preparing splenic tissue homogenates, the whole organ was placed in 2 ml of RPMI medium 1640 (Invitrogen, Carlsbad, CA) at 4°C. The tissue was minced and immediately homogenized, according to a previously published technique with minor modifications (11). Mononuclear cells were isolated by density gradient centrifugation using Histopaque-1077 solution (Sigma Diagnostics, St. Louis, MO). For isolation of EPC-enriched medullopapillary mononuclear cells from Tie-2GFP mice after IPC, kidneys were harvested 7 days after the ischemic insult. The cortical tissue was removed under a dissecting microscope. Pooled medullopapillary tissue was transferred into 1 ml of RPMI medium 1640 and immediately homogenized at room temperature. Tissue homogenates were digested in collagenase type II (Invitrogen), 1 mg/ml in RPMI medium 1640 for 30 min at 37°C in 5% CO_2, followed by a second homogenization. For cell injections $\approx 10^6$ freshly isolated cells were resuspended in 100 μl of RPMI medium 1640, the entire volume was administered intravenously as described above. Control animals received the same volume of cell-free medium. Serum creatinine concentration was measured using a commercially available kit (Raichem, San Diego, CA) according to the manufacturer's protocol.

Flow cytometric analysis. To quantify peripheral circulating and splenic EPCs by fluorescence-activated cell sorter analysis (FACS), mononuclear cells were isolated by density gradient centrifugation using Histopaque-1077 solution from either 500 μl of peripheral blood or from splenic tissue homogenates. Cells were incubated for 30 min on ice with FITC-conjugated anti-mouse CD34 (RAM34) and PE-conjugated anti-mouse Flk-1 (Avas12α1) (BD Biosciences, Rockville, MD) or with FITC-conjugated anti-mouse CD117 (c-Kit) (BD Biosciences) and anti-human Tie-2 (sc-9026) (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody for Tie-2 staining was Alexa Fluor 594 donkey anti-rabbit IgG (A21207) (Molecular Probes, Eugene, OR). After incubation, cells were washed with PBS and fixed in 4% paraformaldehyde. Data were acquired using a FACScan cytometer equipped with a 488-nm argon laser and a 620-nm red diode laser and analyzed using CellQuest software (Becton Dickinson, San Jose, CA). The setup of FACScan was performed according to the manufacturer's instructions using unstained and single antibody-stained cells. To quantify EPCs, the number of CD34/Flk-1 double-positive cells within the monocytic cell population was counted.

Immunohistochemical staining and analysis. Tissue samples of kidney and lung were fixed in a 4% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA), followed by incubation in 30% sucrose overnight at 4°C. Embedding was performed in an OCT compound (Tissue-Tek, Torrance, CA), and embedded samples were stored at 80°C. Frozen samples were cut into 10-μm-thick sections (Cryomicrotom CM 1850, Leica Microsystems, Bannockburn, IL). Nonspecific protein binding was blocked by 1-h incubation with PBS-BSA (1%). The following primary antibodies were employed: FITC-conjugate anti-mouse CD117 (c-Kit, 1:1,000 in PBS-BSA 1%; BD Biosciences) and anti-human Tie-2 (sc-9026, 1:100 in PBS-BSA 1%; Santa Cruz Biotechnology). Secondary antibody for Tie-2 staining was Alexa Fluor 594 goat anti-rabbit IgG (A11012, 1:500 in PBS-BSA 1%; Molecular Probes). Incubations with primary antibodies were carried out overnight at 4°C and incubations with the secondary antibody were performed for 1 h at room temperature. Control samples were stained with secondary antibodies only. To visualize the nuclei, tissue sections were counterstained with DAPI (Molecular Probes). Sections were examined using a Nikon compound fluorescence microscope with the appropriate dichroic mirrors. To quantify EPCs in the renal cortex, the number of c-Kit/Tie-2 double-positive cells per cumulative 100 glomeruli was evaluated. In the renal medulla and in the lung, the number of double-positive cells per view field (300 × 1,000 μm) was counted.

Statistical analysis. The results were expressed as means ± SE. The means of two populations were compared by Student's *t*-test. For multiple comparisons ANOVA was employed. Differences were considered significant at $P < 0.05$.

RESULTS

Differential effects of renal ischemia on peripheral circulating and splenic EPCs. To track the kinetics of peripheral EPCs in acute renal ischemia, the percentages of $CD34^+/Flk\text{-}1^+$ monocytic cells in the peripheral blood and in the spleen were evaluated over time after 25 min of unilateral renal clamping. The choice of UC over bilateral clamping was dictated by the desire to avoid any uremia-induced dysfunction of stem and

progenitor cells. The number of EPCs in the peripheral circulation showed no significant changes at any time point in ischemic animals compared with sham-operated or to untreated control mice (Figs. 1 and 2). This is in sharp contrast to the sixfold increase in the percentage of circulating EPCs observed in mice treated for 5 days with SCF and granulocyte colony-stimulating factor, G-CSF, and serving as a positive control (treated vs. untreated animals: 5.23 ± 1.6 vs. 0.87 ± 0.34%, $n = 3$ per group, $P < 0.05$). Hence, these data rejected the possibility of technical artifacts related to the detection of a surge in circulating EPC. The number of circulating EPCs in mice subjected to renal ischemia 7 days after the IPC not only was not increased but showed a significant decrease compared with baseline or a level detected at 3 h postischemia. These data show that IPC of selected duration does not significantly increase EPC numbers in the peripheral circulation (Fig. 3).

In contrast to the blood, the splenic EPC population was significantly increased in ischemic compared with sham-operated or intact control animals at 3 h [14.8 ± 3.6 vs. 8.2 ± 2.3% ($P = 0.025$) and 3.9 ± 0.5% ($P = 0.004$), respectively; $n = 3$ per group] and 6 h [10.6 ± 1.6 vs. 6.2 ± 1% ($P = 0.049$) and 3.9 ± 0.5% ($P = 0.002$), respectively;

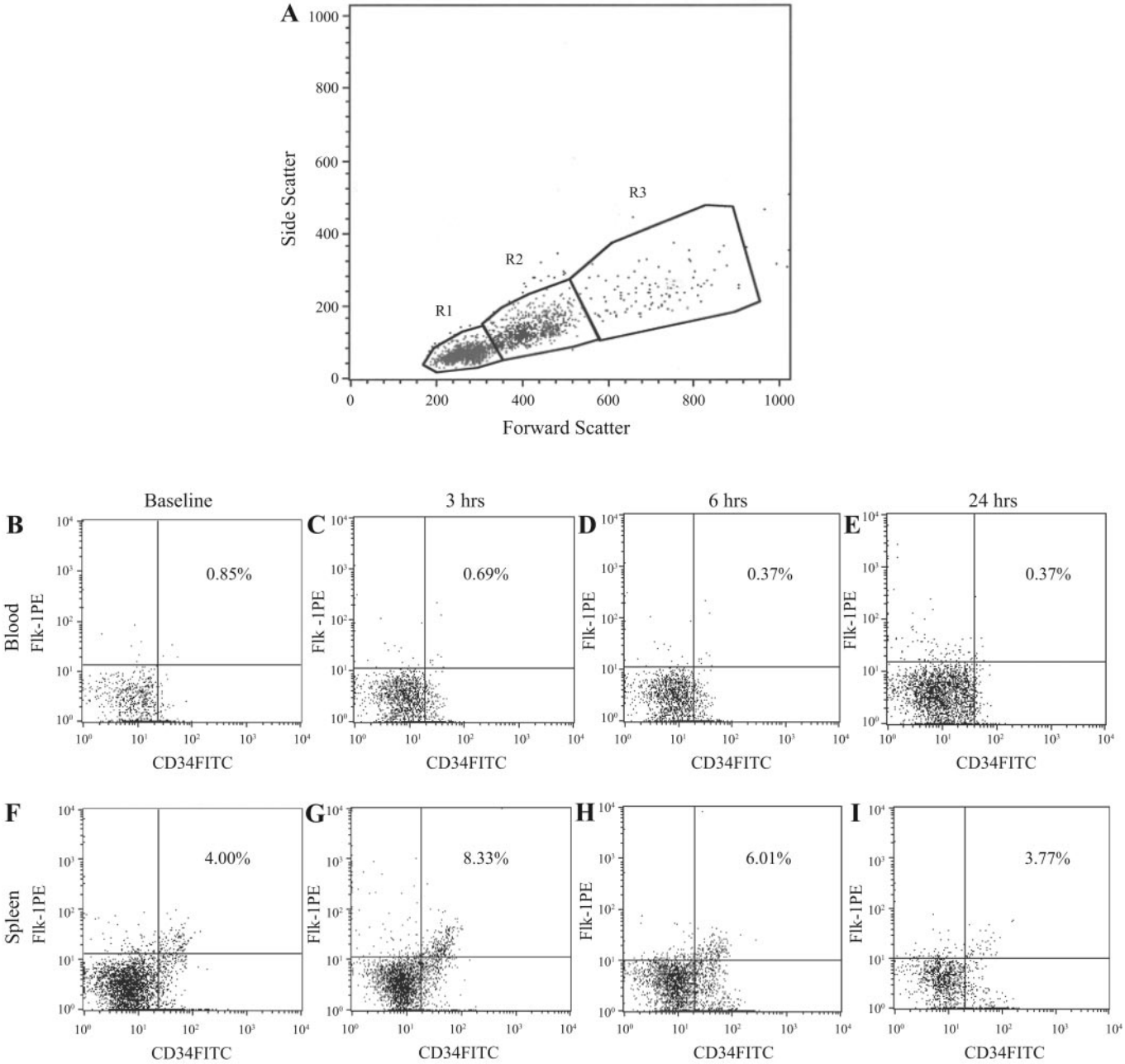


Fig. 1. Quantitative evaluation of circulating endothelial progenitor cells (EPCs) by FACS analysis. Representative FACS data, in which the $CD34^+/Flk\text{-}1^+$ cells within the myeloid mononuclear cell populations (R2 in *A*) of peripheral blood (*B-E*) and spleen (*F-I*) were judged as EPCs, are shown. Analysis of blood and spleen samples was performed at different time points [10 min (not shown), 3 (*C* ± *G*), 6 (*D* ± *H*), 24 (*E* ± *I*) h, and 7 days (not shown) after release of the clamp, *B* ± *F*: baseline values].

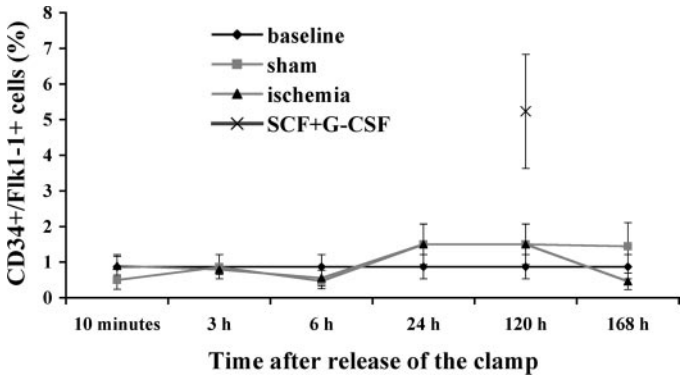


Fig. 2. Percentage of $CD34^+/Flk\text{-}1^+$ cells within the peripheral myeloid mononuclear blood cell population after renal ischemia. After a 25-min period of unilateral renal clamping, animals were killed for measuring circulating EPCs. The percentages of EPCs within the mononuclear blood cell population were not significantly different at 10 min, 3, 6, 24 h, or 7 days after initiation of renal reperfusion. In contrast, 5-day treatment with stem cell factor (SCF) and G-CSF, as detailed in MATERIALS AND METHODS, resulted in a marked increase in the number of circulating EPC. Data are shown as means ± SE.

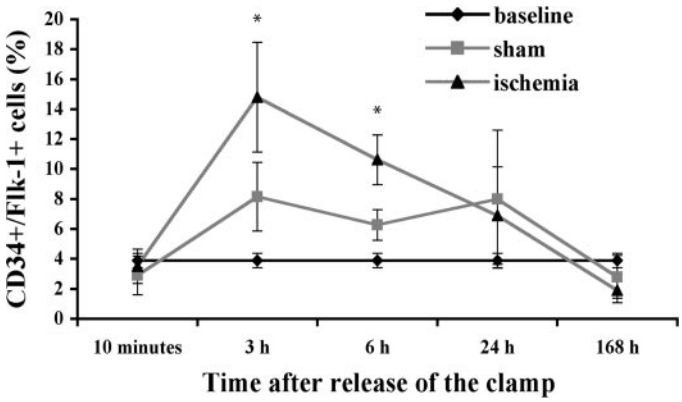


Fig. 4. Increased percentage of $CD34^+/Flk\text{-}1^+$ cells within the splenic myeloid mononuclear cell population after renal ischemia. In contrast to the blood, splenic EPCs surged in animals that were killed 3 and 6 h after reperfusion ($*P < 0.05$). The number of $CD34^+/Flk\text{-}1^+$ cells was higher compared with sham-operated and to nonoperated control mice. Data are shown as means ± SE.

$n = 3$ per group] after release of the clamp. This effect was not only absent but was even reversed in animals that had undergone IPC (0.9 ± 0.2 vs. 14.8 ± 3.6%, $n = 3$ per group, $P = 0.019$; Figs. 4 and 5).

To evaluate whether the spleen serves as a transient niche for the mobilized EPCs, thus explaining its "buffering" effect and the lack of increase in circulating EPCs, SPE was performed simultaneously with UC. At 1 h after the procedure, circulating EPCs were unchanged from the baseline level, but at 3 h after UC and SPE, the number of circulating EPCs was significantly increased, compared with control (2.4 ± 0.4 vs. 0.87 ± 0.34%, $n = 3$ per group, $P = 0.04$; Fig. 6). This latter set of findings establishes the role of spleen as a temporary residence for the mobilized EPC.

Absence of an early surge in circulating EPCs after renal ischemia. Because splenic EPCs were increased within 3 h after renal reperfusion, we examined the possibility of a very early surge in circulating EPCs. At 10 min after reperfusion, EPCs were not significantly increased in blood or in spleen compared with untreated controls (blood: 0.9 ± 0.26 vs. 0.87 ± 0.34%, $n = 3$ per group, $P = 0.89$; spleen: 3.5 ± 1.1 vs. 3.9 ± 0.5%, $n = 3$ per group, $P = 0.76$), thus rejecting the possibility of a very early detectable increase in the circulating pool of EPCs (Fig. 2).

Effects of renal ischemia on EPC accumulation in the kidney. One of the possible sites for entrapment of mobilized EPCs could be represented by the pulmonary microcirculation, as it has been previously shown for the intravenously injected hematopoietic stem cells or HUVECs (7, 22). To test the hypothesis that the blunted increase in splenic EPCs in IPC animals may be a result of pulmonary entrapment of these cells, tissue samples from the lungs were analyzed for the immunohistochemically widely used, convenient markers of EPCs, c-Kit and Tie-2. The number of double-positive cells was not significantly increased compared with sham-operated controls (13.3 ± 0.3 vs. 10 ± 1.7 cells/field, $P = 0.13$). These data rule out the possibility that the depletion in splenic EPCs can be attributed to their trapping in the pulmonary circulation.

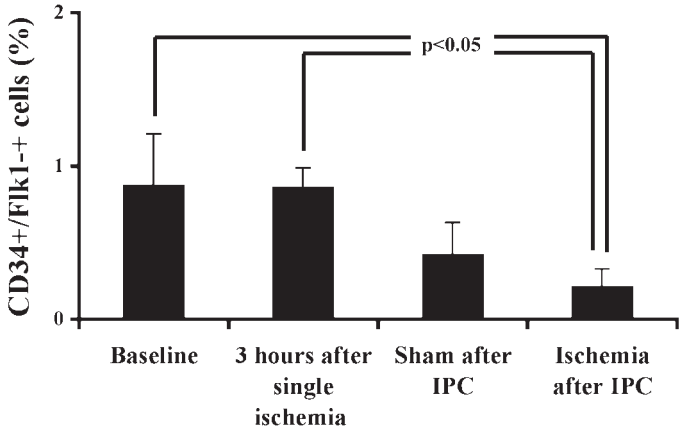


Fig. 3. Percentage of $CD34^+/Flk\text{-}1^+$ cells within the peripheral myeloid mononuclear blood cell population after renal ischemia in preconditioned vs. nonpreconditioned mice. Animals underwent a first 25-min period of unilateral renal artery clamping exactly 7 days before the second ischemic period of the same duration. Three hours later they were killed. The number of circulating EPCs did not significantly increase compared with sham-operated animals but was decreased compared with baseline and to the 3-h time point after a single ischemic episode. Data are shown as means ± SE. IPC, ischemic preconditioning.

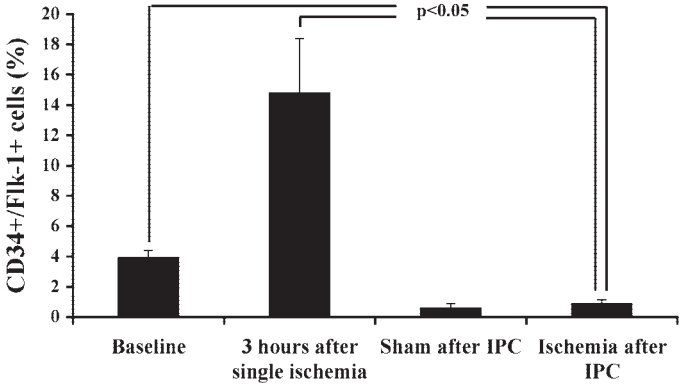


Fig. 5. Decreased percentage of $CD34^+/Flk\text{-}1^+$ cells within the splenic mononuclear cell population after renal ischemia in preconditioned vs. nonpreconditioned mice. In preconditioned animals, the splenic $CD34^+/Flk\text{-}1^+$ cells did not increase but were dramatically reduced compared with baseline animals and to mice that had been killed 3 h after a single ischemic episode. Data are shown as means ± SE.

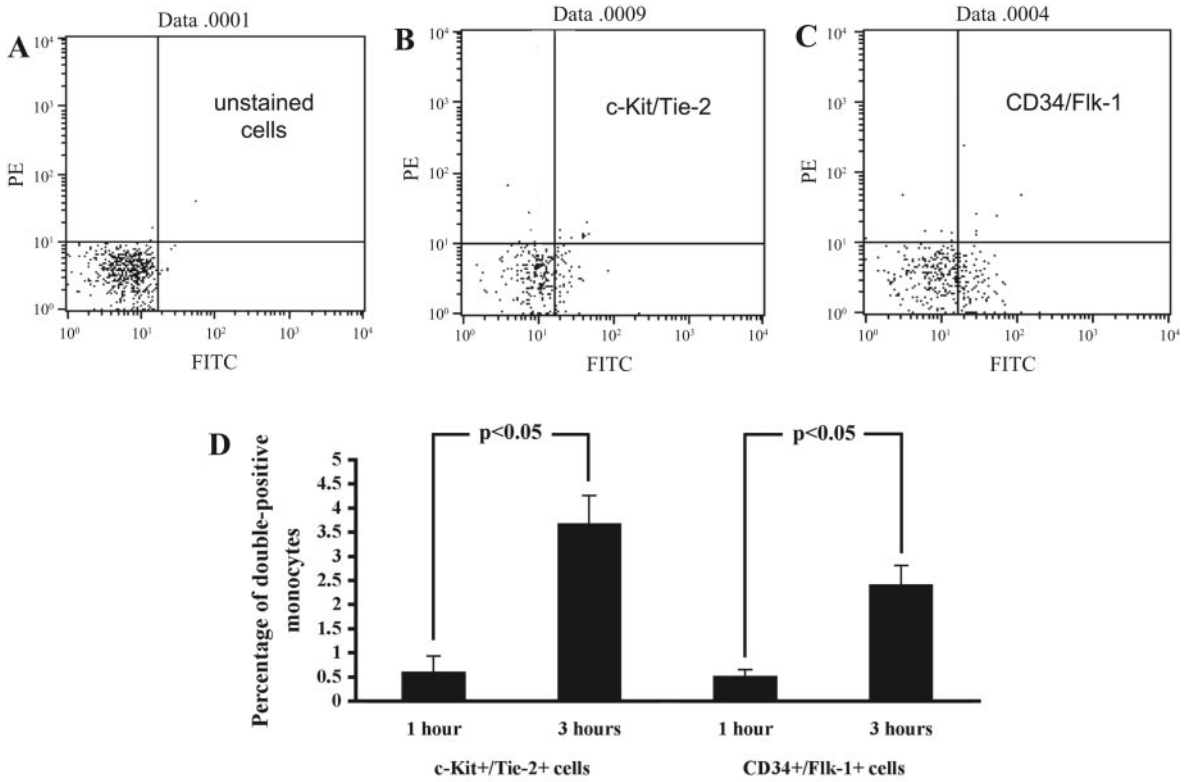


Fig. 6. Quantitative evaluation of circulating cKit$^+$/Tie-2$^+$ and CD34$^+$/Flk-1$^+$ monocytes after UC with simultaneous splenectomy by FACS analysis. Representative FACS data, in which double-positive monocytes were counted, are shown. Analysis of circulating monocytes at 3 h after UC (but not after 1 h) with simultaneous splenectomy showed an increased number of double-positive cells (*D*). *A*: unstained cells. *B*: cells stained with anti-c-Kit-FITC and anti-Tie-2-PE. *C*: cells stained with anti-CD34-FITC and anti-Flk-1-PE.

The same immunohistochemical analysis of kidney tissue sections was performed to elucidate whether changes in the peripheral EPC population after acute renal ischemia are associated with recruitment and homing of the cells to the ischemic organ. EPCs were detected in tissues by staining with antibodies to c-Kit and Tie-2 and by enumerating cells coexpressing these markers. Only a modest representation of c-Kit$^+$/Tie-2$^+$ cells was observed in the kidney cortex of any experimental ischemic group and the differences in the number of double-positive cells between nonpreconditioned and preconditioned animals were not significant. However, in the medullopapillary parenchyma, the number of double-positive cells was significantly higher in preconditioned mice at the late phase compared with animals killed early, at 3 and 6 h after release of the clamp (2.6 $\pm$ 0.3 vs. 0.4 $\pm$ 0.3 cells/view field, $P = 0.001$; Figs. 7 and 8). Preconditioned animals showed similarly increased medullopapillary cell numbers compared with the mice that had been killed 7 days after the first ischemic period, suggesting that the medullopapillary accumulation of EPCs is a gradual process coinciding with the late phase of IPC and that IPC facilitates renal homing of EPCs.

To elucidate the role played by the spleen as a potential transient reservoir of mobilized EPCs, we performed SPE at the time of the maximal accumulation of EPCs in the spleen, at 3 h, and examined the number of EPCs in the kidney. Animals that underwent SPE at the time of maximal transient EPC accumulation in the spleen, 3 h after an ischemic insult of 25 min, did not show significant numbers of c-Kit$^+$/Tie2$^+$ cells within the medullopapillary segment, supporting the idea of the spleen acting as a transient reservoir of mobilized EPCs and suggesting the possibility of splenic-renal EPC traffic during the late phase of IPC. However, animals that were splenectomized at the time of clamping also did not show significant renal cell engraftment, despite the cell numbers were increased in the blood (Figs. 6 and 9).

To obtain an independent set of EPC markers confirming the above findings, we next examined the dynamics of c-Kit and Tie-2, well-established markers of EPCs, after renal ischemia and SPE. This analysis was also dictated by the need to compare the two sets of EPC markers, because we encountered difficulties in staining tissue sections with the available anti-CD34 and anti-Flk-1 antibodies and because immunohistochemical staining of the same tissue sections with anti-c-Kit and anti-Tie-2 antibodies showed lower background and better reproducibility. Hence, we sought to determine whether these two pairs of antibodies detect substantially overlapping cell pools. For this reason, additional FACS analyses of circulating monocytes after incubation with anti-c-Kit and anti-Tie-2 were performed. Three hours after simultaneous unilateral clamping and SPE (but not after 1 h), circulating c-Kit$^+$/Tie-2$^+$ monocytes were significantly increased, in parallel with the similarly increased circulating CD34$^+$/Flk-1$^+$ monocytes (Fig. 6), thus supporting the validity of the performed shift from one set of markers for another.

Transplantation of EPC-enriched medullary mononuclear cells from preconditioned Tie-2GFP mice into wild-type animals with acute ischemic renal failure. To elucidate whether the EPCs accumulated in the renal parenchyma in

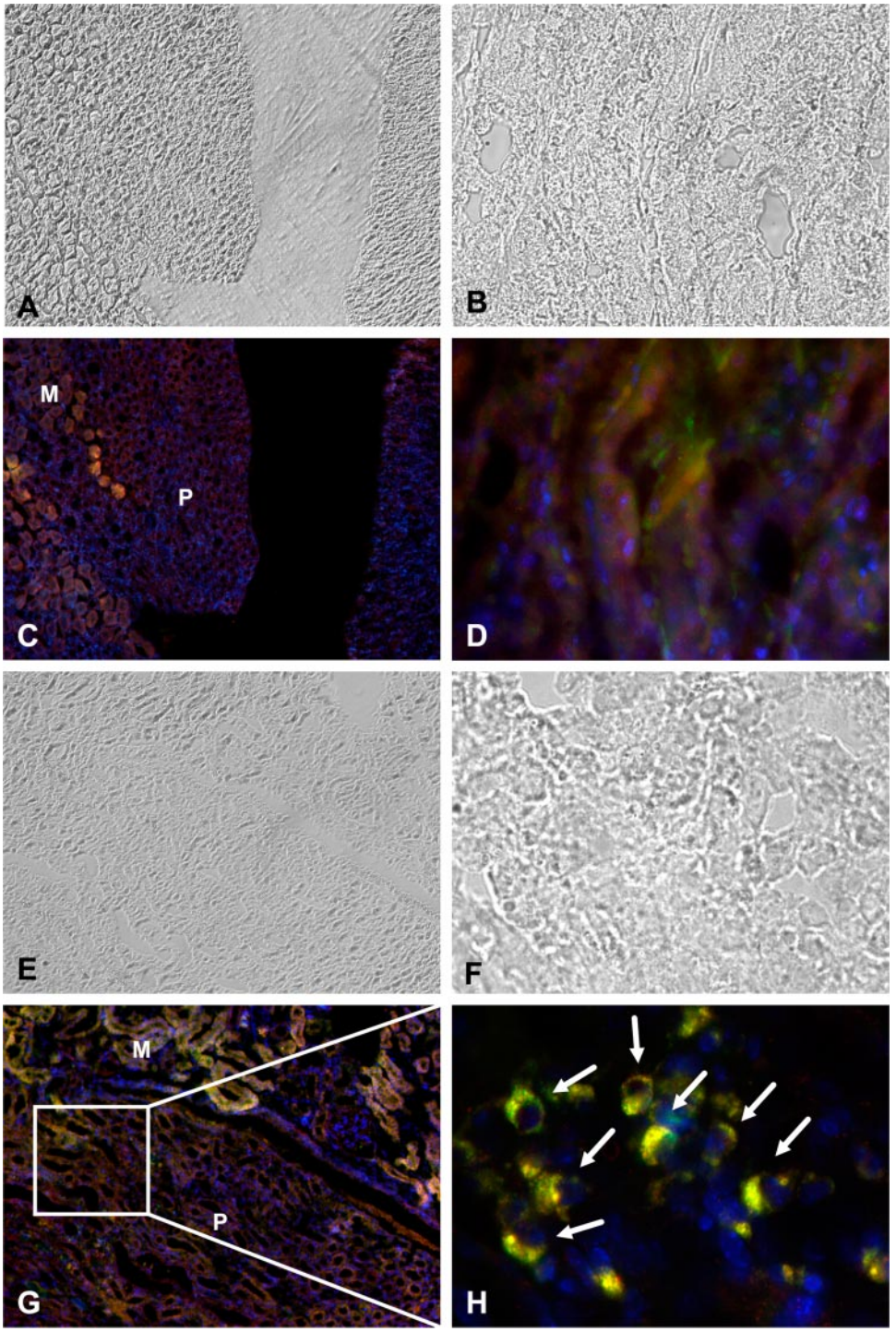


Fig. 7. Accumulation of interstitial c-Kit$^+$/Tie-2$^+$ cells (white arrows) in the medullopapillary region after acute renal ischemia. Brightfield transmitted and fluorescence images of kidneys from untreated mice (*A-D*) and animals killed 1 wk after renal ischemia (*E-H*) are shown. The white square in *G* marks the area with the most intense cell infiltration with c-Kit$^+$/Tie-2$^+$ cells [M, medulla; P, papilla; magnification: ×100 (*A*, *C*, *E*, *G*), ×600 (*B*, *D*, *F*), and ×900 (*H*)]. $P < 0.05$.

the late postischemic period have any potential to rescue the kidney from ischemic injury, we performed the next series of experiments in Tie-2/GFP mice by isolating these cells and transplanting them to wild-type mice subjected to renal ischemia. Because the medullopapillary accumulation of EPCs occurred late after acute renal ischemia, an enriched population of medullopapillary mononuclear cells was isolated from transgenic Tie-2/GFP mice at 7 days postischemia. Freshly isolated cells were injected into wild-type animals, which underwent unilateral renal artery clamping and contralateral nephrectomy. Isolation and injection were performed as detailed in MATERIALS AND METHODS. To evaluate renal function, serum creatinine concentration was measured at 48 h after the surgery. Injection of $\approx 10^6$ mononuclear cells significantly improved renal function in treated animals compared with the controls (0.34 ± 0.08 vs. 0.62 ± 0.09 mg/dl, $P = 0.048$). Histological analysis of kidneys from injected animals showed GFP-expressing cells in the medullary interstitium (Fig. 10).

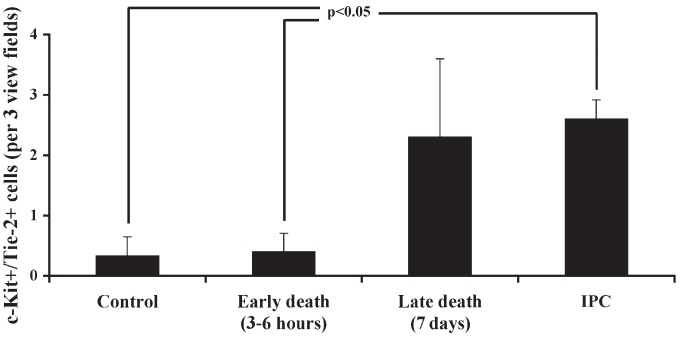


Fig. 8. Increased infiltration of the medullopapillary region by c-Kit$^+$/Tie-2$^+$ cells in preconditioned mice and in ischemic mice killed 7 days postischemia. In preconditioned mice, the number of double-positive cells was significantly higher compared with untreated animals or to animals killed early (3–6 h). There was no significant difference between animals killed late (7 days) and any other group. Data are shown as means ± SE.

DISCUSSION

This study presents for the first time a chronological analysis of the dynamics of EPC mobilization, their homing, and potential biological significance in the course of acute renal ischemia. A 25-min period of unilateral renal ischemia resulted in the accumulation of EPCs in the spleen at 3–6 h but was not accompanied by a significant increase in the number of peripheral circulating EPCs at 10 min, 3, 6, 24 h, or 7 days postischemia, irrespective of whether IPC had been performed 1 wk before it. When SPE was performed simultaneously with the renal clamping, the surge of circulating EPCs was readily detectable, arguing that the mobilized EPCs rapidly home to the spleen. The phenomenon of splenic EPC accumulation was unexpectedly reduced in preconditioned mice subjected to the second ischemic episode. Depletion of splenic EPCs could not be attributed to their trapping in the pulmonary microcirculation, as immunohistochemical analysis revealed no detectable increase of EPCs in the lung tissue. Rather, these preconitioned animals showed an increase in EPCs, as judged by the c-Kit$^+$/Tie2$^+$ marker cells, in the medullopapillary parenchyma of the kidneys, compared with mice that underwent a single ischemic episode and were killed early during the reperfusion period (3 and 6 h after release of the clamp). Of note, animals that had been killed 7 days after a single ischemic episode showed medullopapillary EPC infiltration comparable to that seen in preconditioned mice at the late phase of IPC, suggesting that renal homing of c-Kit$^+$/Tie-2$^+$ cells may be a hallmark of the late phase of preconditioning. Because the data strongly suggested medullopapillary EPC accumulation at the late phase of IPC, EPC-enriched mononuclear cells from preconditioned Tie-2-GFP mice were isolated and injected into FVB wild-type animals with acute renal ischemia. The procedure significantly improved renal function and microscopic analysis revealed GFP-expressing cells in the medullary interstitium of injected animals, consistent with the accumulation of Tie2/GFP-expressing EPCs.

Taken together, the findings suggest that *1*) acute renal ischemia mobilizes EPCs which transiently reside in the splenic niche and later accumulate in the kidney, *2*) IPC facilitates the traffic of EPCs from the splenic niche to the kidney, and *3*) accumulation of EPCs in the late phase is at least partly responsible for beneficial effects of IPC.

In the past few years, a number of studies have addressed the question of the role of circulating EPCs in ischemic diseases and whether these cells are involved in tissue repair after ischemia (31). Their mobilization and participation in microvascular repair have been shown in a number of experimental and clinical studies. Vasa and colleagues (32) reported de-

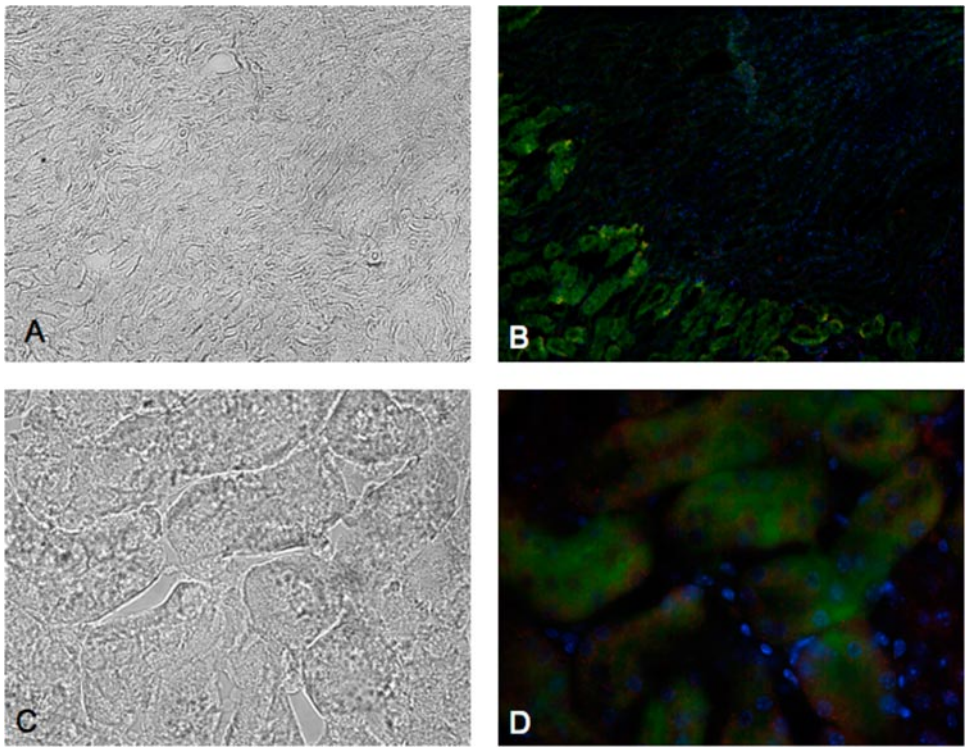
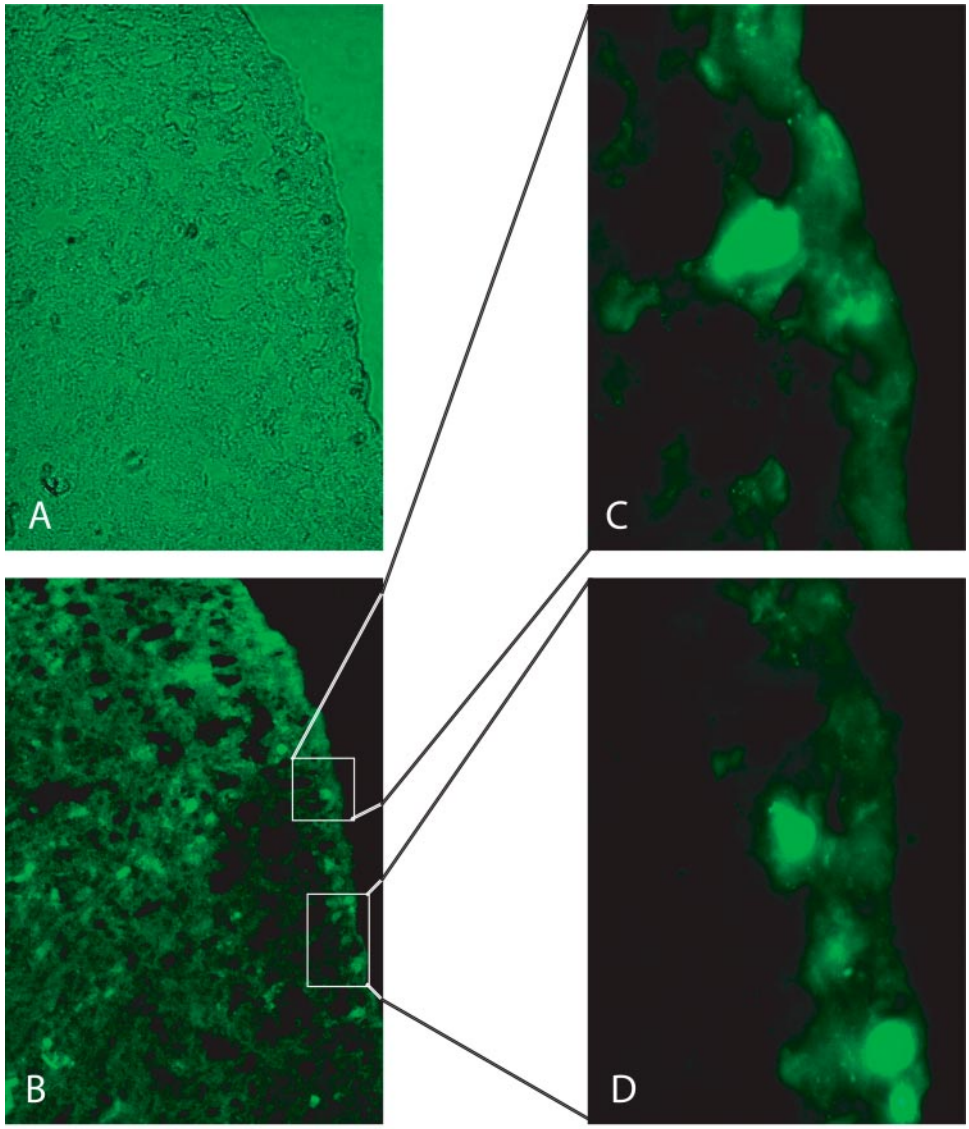


Fig. 9. Renal medullopapillary border zone at 3 h after combined UC and SPE. Despite elevation of circulating EPCs after combined UC and SPE, no cell infiltration was observable in the kidney. Brightfield transmitted (*A* and *C*) and fluorescence (*B* and *D*) images [magnification ×100 (*A* and *B*), ×600 (*C* and *D*)] are shown.



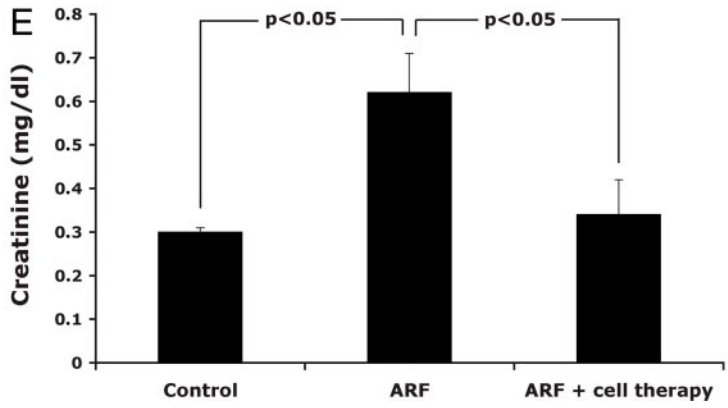


Fig. 10. *A-D*: Tie-2-promotor-driven green fluorescent protein (GFP)-expressing cells in the renal interstitium of FVB mice after transplantation of isolated medullary mononuclear cells from preconditioned Tie-2-GFP mice. *A* and *B*: brightfield transmitted and fluorescence images, respectively, of the medullopapillary region at low magnification (×100). *C* and *D*: fluorescence images of the papilla at high magnification (×900), corresponding to the boxed areas in *B*. *E*: serum creatinine in FVB wild-type mice 2 days after combined UC with NE and injection of medullary EPC-enriched mononuclear cells from preconditioned Tie-2-GFP mice. Renal function was significantly improved in cell injected compared with control animals. Data are shown as means ± SE. ARF, acute renal failure.

creased numbers of circulating EPCs and impaired migratory activity of these cells in patients with coronary artery disease (CAD). Interestingly, the number of cardiovascular risk factors was inversely correlated with the number of circulating EPCs. These oberservations are in accord with results of Lambiase and colleagues (19), who reported that inadequate coronary collateral development in patients with CAD was associated with reduced numbers of circulating EPCs and with impaired chemotactic and proangiogenic activity of these cells. These data suggest that EPCs impart cardioprotection against ischemia. Comparable findings have been presented in relation to the role of EPCs in cerebrovascular disease (14). The proan-

giogenic capacity of EPCs has been documented in the landmark studies by Asahara and colleagues (4), who showed enhanced neovascularization in rabbits with unilateral hindlimb ischemia after the injection of $CD34^+$ cell-derived EPC into the animals' circulation. Since then, further studies have shown comparable results in different animal models as well as in clinical settings (5, 18, 29). A comparably lesser amount of data is available on EPCs in renal diseases, especially in acute ischemic renal failure.

With regard to the studies presented herein, one fundamental question we addressed was whether acute renal ischemia results in the generation of appropriate, albeit yet unknown, signals to mobilize EPCs. Although we did not find changes in the number of circulating EPCs, the increase in EPCs in the splenic niche is equivalent of EPC mobilization (15). Furthermore, the number of circulating EPCs increased when UC was performed together with SPE, thus confirming the fact that UC provides a sufficient signal for EPC mobilization. The lack of a distinct phase of increased circulating EPCs, as has been described in some ischemic cardiovascular and cerebrovascular disease (1, 14, 19, 32), can be attributed to several factors. On the one hand, it is possible that the potency of signaling from ischemic renal tissue to the bone marrow or other niches, resulting in EPC mobilization, is lesser than that occurring in myocardial ischemia. In fact, this is confirmed by the results obtained with the injection of SCF and G-CSF, leading to the surge of circulating EPCs (Fig. 2). On the other hand, it is conceivable that the circulating EPC peak is very transient, thereby "escaping" our analysis. Another possiblity may be the existence of a dynamic equilibrium between circulating and tissue-residing EPCs characterized by a slow and continuous cell migration from the bone marrow and/or other niches via peripheral blood to the spleen and later on to sites of tissue ischemia.

The finding of splenic EPC accumulation early after renal ischemia is also in agreement with the concept of mobilization-associated homing of hematopoietic stem cells (HSC) in the spleen. In studies on stem cell factor and G-CSF-induced HSC mobilization for optimizing myocardial perfusion and/or postinfarct tissue regeneration in mice, a SPE was performed to prevent splenic sequestration of bone marrow cells (12, 23). Because our immunohistochemical analysis of kidneys from early postischemic nonpreconditioned animals showed only mild cortical and medullary infiltration with $c\text{-}Kit^+/Tie\text{-}2^+$ cells, coincident with the significantly increased splenic EPC population, and because the preconditioned mice exhibited increased medullopapillary parenchymal accumulation, coinciding with reduced fraction of splenic EPC and no apparent EPC trapping in the pulmonary circulation, we propose a model in which IPC enables and/or facilitates EPC translocation from the transient splenic niche to the ischemic organ, in this case the kidney (Fig. 11). The finding that the mobilized EPCs in IPC mice show no transient accumulation in the splenic niche and engraft more urgently to the kidney raises the question of "homing memory," which allows for the emergency delivery of these cells to the site of injury and may explain the protective action of IPC. Our observation that SPE performed at the height of EPC accumulation in the spleen, at 3 h postischemia, abolished the accumulation of EPCs in the postischemic kidney by 7 days argues in favor of the fact that the splenic niche represents a transient reservoir and the most abundant source of EPCs destined to later engraft the ischemic kidney.

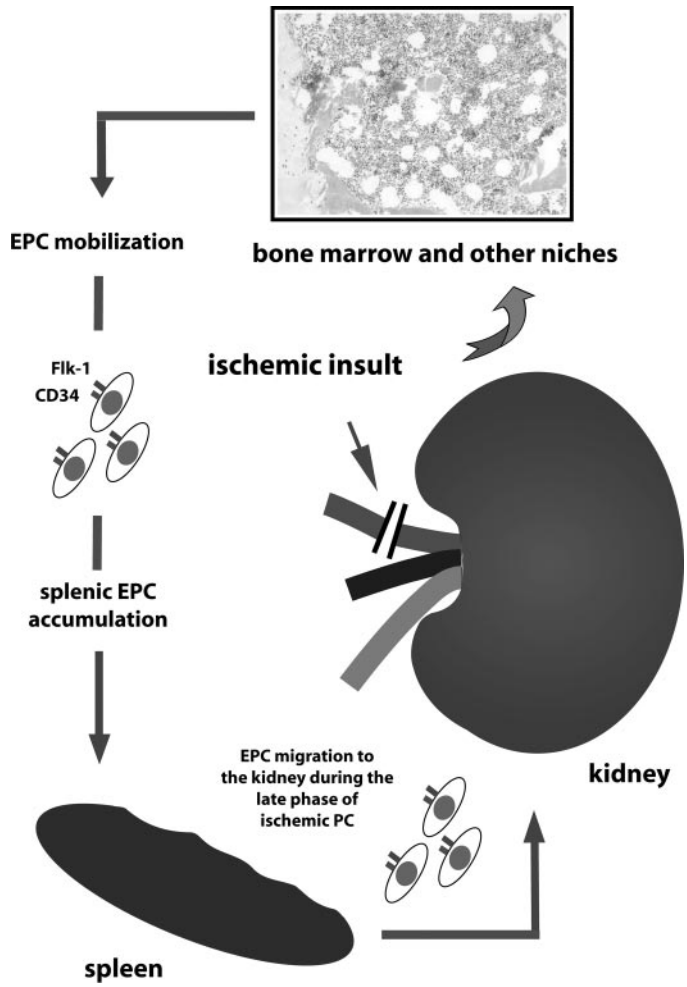


Fig. 11. Hypothetical model of EPC mobilization and migration after acute renal ischemia. Renal ischemic insult rapidly (3–6 h) mobilizes EPC from the bone marrow and/or other niches and establishes a transient splenic niche. During the second late phase of IPC, the cells traffic from this transient niche to the kidney, where they are detectable in the medullopapillary region of the kidney. Ischemic preconditioning appears to facilitate this traffic phase via accelerating a directed trafficking of EPC to the ischemic organ.

The other fundamental question posed by this study was whether ischemia-mobilized EPCs exhibit renoprotective effects in acute renal ischemia. The results of experiments with the transplantation of enriched EPCs to animals with renal ischemia suggest an important biological role for kidney-homed EPCs. The $c\text{-}Kit^+/Tie\text{-}2^+$ or GFP^+ cells were predominatly located in the renal medullopapillary region and were associated with amelioration of renal dysfunction. The mechanism(s) for this effect remain to be elucidated in future studies: is it the result of improved microvascular function or a consequence of paracrine signaling (22).

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