Injured kidney endothelium is only marginally repopulated by cells of extrarenal origin

Holger Schirutschke,1 Regina Vogelbacher,2 Andrea Stief,3 Simon Parmentier,1 Christoph Daniel,3 and Christian Hugo1

1Technische Universitaet Dresden, Carl Gustav Carus Faculty of Medicine, Department of Internal Medicine III, Division of Nephrology and Hypertension, Dresden, Germany; 2University Erlangen-Nuremberg, Department of Nephrology and Hypertension, Erlangen, Germany; and 3University Erlangen-Nuremberg, Division of Nephropathology, Erlangen, Germany

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Schirutschke H, Vogelbacher R, Stief A, Parmentier S, Daniel C, Hugo C. Injured kidney endothelium is only marginally repopulated by cells of extrarenal origin. Am J Physiol Renal Physiol 305: F1042–F1052, 2013. First published July 24, 2013; doi:10.1152/ajprenal.00653.2012.—The role of bone marrow marrow-derived cells after kidney endothelial injury is controversial. In this study, we investigated if and to what extent extrarenal cells incorporate into kidney endothelium after acute as well as during chronic endothelial injury. Fischer F-344 wt (wild type) rat kidney grafts were transplanted into R26-hPAP (human placental alkaline phosphatase) transgenic Fischer F-344 recipient rats to allow identification of extrarenal cells by specific antibody staining. A severe model of renal thrombotic microangiopathy was induced via graft perfusion with antiglomerular endothelial cell (GEN) antibody and resulted in eradication of 85% of the glomerular and 69% of the peritubular endothelium (GEN group). At week 4 after injury, renal endothelial healing as well as recovery of the kidney function was seen. Endothelial chimerism was evaluated by double staining for hPAP and endothelial markers RECA-1 or JG-12. Just 0.25% of the glomerular and 0.1% of the peritubular endothelium was recipient derived. In a second experiment, chronic endothelial injury was induced by combination of kidney transplantation with 5/6 nephrectomy (5/6 Nx group). After 14 wk, only 0.86% of the peritubular and 0.05% of the glomerular endothelium was of recipient origin. In summary, despite demonstration of extensive damage and loss as well as excellent regeneration, just a minority of extrarenal cells were incorporated into kidney endothelium in rat models of acute and chronic kidney endothelial injury. Our results highlight that kidney endothelial regeneration after specific and severe injury is almost exclusively of renal origin.

5/6 nephrectomy; GEN; hPAP; transplantation

MATERIALS AND METHODS

Experimental design. The animal studies were performed in accordance with the internal animal review board (Regierung von Mittelfranken: 54-2531.31-15/03). Thirty-six Fischer F-344 wt (wild type) rat kidney grafts (hPAP negative) were transplanted into R26-hPAP transgenic Fischer F-344 recipient rats to allow histological tracing of recipient-derived cells in the donor kidneys (Fig. 1). Rat kidney transplantation was performed as described by Vogelbacher et al. (26).

In 14 cases, acute endothelial injury was induced by kidney graft perfusion with 0.2 ml of saline followed by 80 mg/kg body wt of antiglomerular endothelial cell antibody (GEN group; Ref. 16). Kidneys were perfused ex vivo via the renal artery by slow and gentle fluid injection with a 1-ml syringe (BD Micro-Fine, San Diego, CA). Appropriate antibody dilution had been tested in pilot experiments to reach maximal endothelial damage. One recipient kidney was removed and one recipient kidney remained as an assist organ to prevent death from acute kidney injury. In 12 corresponding controls, kidney and chronic kidney diseases could not be drawn (10, 11, 19, 25). To study this issue systematically, animal models of experimental kidney injury were combined mainly with bone marrow transplantation to allow discrimination of extrarenal from intrarenal-derived endothelial cell replacement. A highly variable percentage of endothelial chimerism was documented in kidney injury models. Contradicting results ranged from 30% of extrarenal-derived endothelium to the detection of no endothelial chimerism at all (9, 12, 13, 18, 22, 23, 27). Hence, the proportion of incorporated endothelial cells from exogenous, nonrenal sources in kidney diseases is still controversial. It is unknown if there are quantitative differences that account for progression or healing of kidney diseases. Thus the goal of our study was the quantification of incorporated endothelial cells from extrarenal sources in both an acute and reversible, endothelial cell-specific nephritis model (GEN model) and a complex, chronic progressive model of kidney endothelial injury, the 5/6 nephrectomy model (5/6 Nx model; Refs. 15, 16). Syngeneic rat kidney transplantation, in which Fischer F-344 wt (wild type) rat kidney grafts were transplanted into R26-hPAP (human placental alkaline phosphatase) transgenic Fischer F-344 recipient rats, was combined with either antiglomerular endothelial cell antibody perfusion (GEN group) or two-thirds renal mass reduction (5/6 Nx group) of the kidney graft. Despite very severe injury, the endothelium of GEN rats recovered 4 wk after model induction, whereas the endothelium of 5/6 Nx rats progressively decreased until death at week 14. Analysis of endothelial hPAP-chimerism (4 and 14 wk after injury, respectively) revealed that, independently of disease outcome, extrarenal-derived cells were only marginally incorporated into kidney endothelium in either model.
grafts were perfused just with saline where the volume of saline was adjusted to the total fluid volume that was used for perfusion in the GEN group. Kidney perfusion was performed in the same way as in the GEN group indicating that also the pressure of perfusion was equal in both groups. Seven GEN rats and six controls were killed at day 3 for histological assessment of endothelial damage in the grafts (GEN day 3, Fig. 1A). The remaining seven GEN rats and six controls underwent explantation of the assist kidney after 3 wk to prove sufficient functional recovery of GEN-treated grafts. At week 4 (GEN week 4, Fig. 1B), animals were killed and grafts were removed for histology. Blood samples were collected for analysis of serum creatinine.

In another five cases, kidney transplantation was combined with 5/6 nephrectomy to induce chronic endothelial injury (5/6 Nx group, Fig. 1C). Two of the three branches of the graft artery were ligated immediately before explantation from the donor animal. Both kidneys of the graft recipient were removed. In five control cases, kidney transplantation was performed with sham handling of the graft and explantation of both recipient kidneys. The 5/6 Nx rats and controls were killed after 14 wk (5/6 Nx week 14), and grafts were removed for histology. Blood samples were collected for analysis of serum creatinine.

As a control experiment, we studied endothelial hPAP expression during experimental kidney injury in three GEN-induced R26-hPAP transgenic Fischer F-344 rat donor kidneys that were transplanted into R26-hPAP transgenic Fischer F-344 recipient rats (positive controls). Animals were killed after 4 wk, and endothelial hPAP expression was compared between the GEN-induced kidney grafts and not-induced R26-hPAP transgenic Fischer F-344 rat kidneys by RECA-1 and hPAP double immunostaining.

Since recipient-derived endothelium most likely derives from the bone marrow, the degree of hPAP expression was analyzed in bone marrow smears of two R26-hPAP transgenic Fischer F-344 rats and two Fischer F-344 wt rats. The femoral medulla was collected immediately after death, and bone marrow smears were performed by push smear technique.

**Immunohistochemistry.** Kidney tissue for light microscopy and periodic acid-Schiff (PAS) staining was fixed in Carnoy’s solution and embedded in paraffin. Kidney tissue for light microscopy and indirect immunoperoxidase staining, nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) staining, and also immunofluorescence microscopy was fixed in zinc fixative solution (BD Pharmingen, San Diego, CA) and embedded in paraffin. Three-micrometer paraffin sections were used for staining procedures. Bone
marrow smears were air dried and stained within 1 h. PAS and indirect immunoperoxidase staining were performed as described by Iruela-Arispe et al. (8). Indirect immunoperoxidase staining for rat endothelium was performed with a murine monoclonal IgG1 antibody against RECA-1 (AbD Serotec, Oxford, UK). Changes in endothelium expression patterns were confirmed by indirect immunoperoxidase staining of tissue sections with JG-12 (Bender Medsystems, Vienna, Austria), a murine IgG1 monoclonal antibody specific for rat aminopeptidase P. Negative controls for immunostaining included either deleting the primary antibody or substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody. To identify recipient-derived cells, kidney tissue was either enzymatically reacted for hPAP activity using NBT/BCIP (toluidine salt) or stained with specific anti-hPAP antibody for immunofluorescence analysis. For enzymatic treatment, kidney sections and bone marrow smears from Fischer F-344 wt rats served as negative controls for enzymatic staining.

**Immunofluorescence double staining.** For detection of donor-derived endothelium, indirect double immunostaining for RECA-1 and hPAP expression was performed. The endothelial marker RECA-1 was detected by the murine monoclonal IgG antibody (AbD Serotec, Oxford, UK). For CD45 and hPAP indirect double immunostaining, a purified mouse anti-rat CD45 antibody (clone OX-1; BD Pharmingen) was used. Red fluorochrome-labeled secondary antibody was always a highly cross-adsorbed Alexa Fluor 555 goat anti-mouse monoclonal IgG antibody (Invitrogen, Pailey, UK). Primary antibody for hPAP staining was a polyclonal IgG rabbit antibody (AbD Serotec). Secondary antibody was an affinity-purified biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). After addition of streptavidin-horseradish peroxidase (SA-HP), a Fluorescein Tyramide Signal Amplification System (TSA; PerkinElmer, Waltham, MA) was used for green visualization of HRP activity. In a last step, cellular nuclei were stained blue with 4’,6-diamidino-2-phenylindole (DAPI).

For all antibodies, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit IgG. Untreated R26-hPAP transgenic Fischer F-344 rat kidney sections served as positive controls for double immunofluorescence sample staining of RECA-1 (red color) and hPAP (TSA amplified, green color). Specific staining patterns with yellow overlay for glomerular as well as peritubular capillaries demonstrated a high-sensitive detection system for recipient-derived endothelium (Fig. 2, A and E). Untreated F-344 wt rat kidney sections were used as negative staining controls. Negativity for green hPAP signal ruled out unspecific anti-hPAP antibody binding (Fig. 2, C and G).

**Image acquisition.** The technical setup included a Keyence BZ-9000 microscope for light as well as fluorescence microscopy. Digital image acquisition was performed with the built-in camera of the microscope. Glomerular images were obtained using a ×40 objective, whereas images of peritubular capillaries were obtained with a ×20 objective. Exposure times for blue, green, and red fluorescence channels as well as exposure time for light microscopy were manually set with the help of appropriate staining controls. The digitalized images were stored in uncompressed TIFF format (1,360 × 1,024 pixels, 8 bit per color) for later analysis. For each biopsy, 25 consecutive cross sections of cortical glomeruli and 25 visual fields of peritubular capillary area were digitally photographed and stored. Sections of the glomerular tuft, in which the capillary cross sections had a very low cellularity, were excluded from the image acquisition.

**Image analysis with Adobe Photoshop.** Adobe Photoshop (version 9) allows the sampling of the color of any individual pixel and its setting as a target color. The “Select/Color Range” tool selects all pixels in the image having a color similar to that of the target color, which then can be counted with the “Histogram” tool (14). Target colors of enzymatic hPAP staining (purple), immunohistochemical RECA-1 staining (brown), as well as immunofluorescence double staining for RECA-1 (red) and hPAP (green, yellow overlay) were defined by use of appropriate staining controls (Fig. 2, B and F). Positive control of RECA-1 and hPAP double immunostaining; Fig. 2, D and H; negative control of RECA-1 and hPAP double immunostaining.

For glomerular image analysis (25 images per biopsy, magnification ×40), selection of glomerular cross section area was executed by encircling the Bowman capsule with the “Ellipse Selection” tool. Pixels of the staining-specific glomerular target color were counted as described above and divided by the whole glomerular pixel count. Further multiplication with 100 resulted in the glomerular percentage of the corresponding target molecule expression. Pure tubulointerstitial image area (25 images per biopsy, magnification ×20) was selected with the help of the “Reverse Selection” tool where all glomeruli of the image had been selected with the “Ellipse Selection” tool in a previous step. Similar to the glomerular analysis, pixels of the staining-specific tubulointerstitial target color were counted and divided by the whole tubulointerstitial area pixel count. Further multiplication with 100 resulted in the tubulointerstitial percentage of the corresponding target molecule expression. For calculation of endothelial hPAP-chimerism at GEN week 4 and 5/6 Nx week 14, the mean values of glomerular and tubulointerstitial RECA-1 and hPAP overlap (pixel counts of yellow target color of RECA-1/hPAP double immunostaining) were divided by the respective mean values of glomerular and tubulointerstitial RECA-1 expression (pixel counts of single channel analysis of red target color of RECA-1/hPAP double immunostaining). Further multiplication with 100 resulted in the percentage of extrarenal-derived glomerular and peritubular endothelium.

**Statistical analysis.** All values are expressed as means ± SD. After testing for normality with the Shapirow-Wilk normality test, statistical significance (defined as P < 0.05) was evaluated by means of Student’s t-test. The plots were drawn with the aid of the GraphPad Prism software.

**Miscellaneous measurements.** Serum creatinine was measured using picric acid (Jaffé method; WAK-Chemie, Steinbach, Germany).

**RESULTS**

**Development of kidney function after acute and chronic endothelial injury.** To investigate acute primary endothelial injury, we used the GEN model induced by an endothelial-specific antibody at first. During pilot testing of the GEN group, recipient rats died 3 days after transplantation from acute kidney failure. Therefore, we changed the experimental design so that one recipient kidney remained in place until 1 wk before the end of the final experiment. After explantation of this uninjured kidney at week 3, both GEN rats and controls did well and had normal urinary excretion. Analysis of serum creatinine at week 4 showed only a nonsignificant trend of higher serum creatinine in GEN rats indicating the recovery of the kidney function (0.65 ± 0.32 mg/dl in controls, n = 6, vs. 0.84 ± 0.61 mg/dl in GEN rats, n = 7, NS). In contrast, there was an anticipated elevation of serum creatinine in the 5/6 Nx group at the end of the experiment on the investigation of secondary endothelial injury with chronic disease progression (week 14: 0.80 ± 0.38 mg/dl in controls, n = 5, vs. 2.03 ± 0.75 mg/dl in 5/6 Nx rats, n = 5, P < 0.01).

**Histologic changes after induction of GEN model and 5/6 Nx.** Histology of the kidney was examined by PAS staining (images not shown). At day 3 after GEN model induction, severe kidney injury was documented. Glomerular capillaries...
were distorted and dense PAS-positive material was deposited in the glomerular capillary lumina. Peritubular capillaries could hardly be identified and concomitant tubular damage was prominent. At week 4, PAS staining of GEN-induced rats showed almost a complete recovery of tubular architecture with distinct peritubular capillaries. Glomerular tufts were reconstituted and only sparse residual PAS-positive material was seen.

Parallel to the kidney function, marked glomerular sclerosis and peritubular fibrosis were evident after 14 wk in the 5/6 Nx group. Glomerular tufts were dilated and peritubular capillaries were rarefied.

Endothelial rarefaction after acute primary and chronic secondary injury. At GEN day 3, immunohistochemical staining of RECA-1 was negative in a substantial part of the kidney (Fig. 3A) compared with the controls (Fig. 3B) indicating very severe acute injury to the renal endothelium. Analysis of RECA-1 expression revealed an 85% reduction within glomeruli (Fig. 3G, 6.81 ± 7.17% in GEN rats, n = 7, vs. 41.20 ± 6.40% in controls, n = 6, P < 0.05) and a 69% reduction in the
peritubular capillaries (Fig. 3H, 1.93 ± 0.74% in GEN rats, n = 7, vs. 6.27 ± 0.80% in controls, n = 6, P < 0.05) of GEN treated rats. Nevertheless, immunohistochemical staining for RECA-1 showed an almost complete reconstitution of glomerular as well as peritubular endothelium at GEN model week 4 (Fig. 3C). Compared with the controls (Fig. 3D), analysis of RECA-1 expression showed no differences in both glomeruli (Fig. 3G, 30.23 ± 5.37% in GEN rats, n = 7, vs. 38.22 ± 6.63% in controls, n = 6, NS) and peritubular capillaries (Fig. 3H, 5.02 ± 1.20% in GEN rats, n = 7, vs. 5.75 ± 0.63% in controls, n = 6, NS) of GEN-treated rats at week 4.

At 5/6 Nx week 14, immunohistochemical staining for RECA-1 demonstrated an obvious rarefication of peritubular endothelium (Fig. 3E) compared with the controls (Fig. 3F). For glomerular endothelium, only a trend of reduced RECA-1 expression was seen (Fig. 3G). Analysis of RECA-1 expression showed a 49% reduction in the peritubular capillaries (Fig. 3H, 2.43 ± 1.86% in 5/6 Nx rats, n = 5, vs. 4.79 ± 1.34% in
controls, \( n = 5, P < 0.01 \) and a 23% reduction in the glomeruli (Fig. 3G, 24.90 ± 9.57% in 5/6 Nx rats, \( n = 5 \), vs. 32.29 ± 10.11% in controls, \( n = 5 \), NS) of 5/6 Nx rats.

Results of immunohistochemical staining using the monoclonal antibody JG-12 as an endothelial cell marker highly correlated with immunohistochemical staining for RECA-1 and confirmed substantial loss of kidney endothelium after induction of the GEN as well as the 5/6 Nx injury model (data not shown).

Tracing of extrarenal hPAP\(^+\) cells in GEN and 5/6 Nx model. As expected, NBT/BCIP staining of R26-hPAP transgenic Fischer F-344 rat bone marrow smear preparations showed a uniform hPAP expression on all bone marrow cells, whereas Fischer F-344\(\text{wt}\) rat bone marrow smear preparations were completely hPAP negative (pictures not shown).

At GEN day 3, NBT/BCIP staining of hPAP detected only few hPAP\(^+\) cells in the control group (Fig. 4B) but revealed an extensive hPAP\(^+\) cellular infiltrate in glomeruli and tubulointerstitium of the GEN group (Fig. 4A). Therefore, the analysis of hPAP expression resulted in a >200-fold higher hPAP\(^+\) glomerular infiltrate (Fig. 4G, 23.39 ± 9.29% in GEN rats, \( n = 7 \), vs. 0.11 ± 0.01% in controls, \( n = 6 \), \( P < 0.001 \)) and a
calculation showed only 0.05% of recipient-derived glomerular endothelium at 5/6 Nx week 14 (Fig. 5F) and 0.003% in the associated controls. The glomerular endothelial chimerism of the GEN group was therefore fivefold higher than that of the 5/6 Nx group. Detection of peritubular endothelial cells, originating from extrarenal sources, was also a rare finding (Fig. 6, A–D). It was most often observed at 5/6 Nx week 14 and least often in the controls. Analysis of the 5/6 Nx group at week 14 showed a significant increase in the number of recipient-derived peritubular endothelial cells compared with the controls, in contrast to the nonsignificant changes of glomerular endothelium (Fig. 6E, 0.021 ± 0.024% in 5/6 Nx rats, n = 5, vs. 0.001 ± 0.001% in controls, n = 5, P < 0.05). Chimerism calculation resulted in 0.86% of recipient-derived endothelium in the 5/6 Nx group after 14 wk (Fig. 6F) and 0.01% in the respective control group. Analysis of the GEN group after 4 wk showed an increase of recipient-derived peritubular endothelial cells compared with the controls, which did not reach statistical significance (Fig. 6E, 0.004 ± 0.021% in GEN rats, n = 7, vs. 0.001 ± 0.005% in controls, n = 6, NS). Chimerism calculation resulted in only 0.1% of recipient-derived peritubular endothelium at GEN week 4 (Fig. 6F) and 0.02% in the controls.

**DISCUSSION**

The role and importance of endothelial precursor cells from nonrenal sources are controversial as discussed (3, 4). Therefore, we aimed to quantify the incorporation of endothelial cells from nonrenal vs. renal sources after primary selective ablation of kidney endothelium in the GEN model as well as after 5/6 renal mass reduction in a model of secondary endothelial injury. For that purpose, each model was combined with transplantation of Fischer F-344wt rat kidney grafts into R26-hPAP transgenic Fischer F-344 recipient rats for sensitive and reliable detection of incorporated extrarenal-derived hPAP+ cells. Analysis of R26-hPAP transgenic Fischer F-344 rat bone marrow smears revealed complete hPAP positivity of all bone marrow cells. We therefore assumed that putative bone marrow-derived endothelial cells could also be traced by specific hPAP staining. To maximize the chances of endothelial repair from outside the injured kidney, we purposely enhanced endothelial injury in the GEN model by increasing the antibody dose to the point whereupon on day 3 85% of the glomerular endothelial cells and 69% of the peritubular endothelium had disappeared and transplanted rats would not survive without a remaining intact kidney. GEN injury and repair in this model were therefore maximized but still did not prevent an almost complete regeneration of the kidney endothelium within 4 wk as indicated by renal function and histology. In contrast to the chronic progressive 5/6 Nx model, endothelial rarefaction was marked in peritubular capillaries after 14 wk (~49%) and less severe in glomeruli (~23%). Enzymatic hPAP activity revealed a large hPAP+ cellular infiltrate with a maximum at GEN day 3 (23.4% of glomeruli and 10.6% of tubulointerstitium) and at 5/6 Nx week 14 (6.5% of glomeruli and 23.5% of tubulointerstitium). Detailed characterization of hPAP+ cells was not an aim of our study, but others have shown that inflammatory cell types like ED-1-positive macrophages constitute the main infiltrating cell population in the GEN as well as in the 5/6 Nx model (16, 28). This was also verified by our
own results (data not shown). For detection of extrarenal-derived endothelium, RECA-1 and \textit{hPAP} (TSA amplified) double immunostaining was performed. We are aware that the \textit{hPAP} marker has not been used as commonly as enhanced green fluorescent protein (\textit{eGFP}) or \textit{/H9252}-galactosidase (\textit{/H9252}-gal) in previous studies. Nevertheless, sensitivity of \textit{hPAP} detection should be equivalent to \textit{/H9252}-gal detection in antibody-based immunofluorescence histology. Whereas \textit{eGFP} has the advantage of being directly visible during fluorescence microscopy, the feature of being enzymatically active (like \textit{hPAP} or \textit{/H9252}-gal) during immunohistochemistry is lacking. The comparison of our results to positive control experiments (\textit{GEN}-induced \textit{R26-hPAP}/\textit{H11001} transgenic Fischer F-344 rat donor kidneys were transplanted into \textit{R26-hPAP}/\textit{H11001} transgenic Fischer F-344 recipient rats) and the use of positive (untreated \textit{R26-hPAP} transgenic kidney sections) as well as negative (untreated wild type Fischer F-344 rat kidney sections) staining controls demonstrate that \textit{hPAP} served as a very suitable marker protein for our study. Hereby, we found a \textit{GEN}-induced downregulation of endothelial \textit{hPAP} expression in both glomerular (\textit{30%}) as well as peritubular capillaries (\textit{29%}) after \textit{GEN} week 4 when compared with untreated \textit{R26-hPAP} transgenic Fischer F-344 rat donor kidneys. We therefore determined reliable detection of RECA-1 and \textit{hPAP} co-expression after recovery from endothelial damage on the one hand (50% sensitivity) and background noise-adjusted image exposure times for the exclusion of false positive results on the other hand (100% specificity). All samples were assessed by computerized image analysis whereupon observer-based and subjective errors were excluded.

Despite substantial renal endothelial damage as well as abundant infiltration of cells with extrarenal origin were detected, only a minority of the glomerular (0.25% at \textit{GEN} week 4 and 0.05% at 5/6 \textit{Nx} week 14) and the peritubular endothelium (0.1% at \textit{GEN} week 4 and 0.86% at 5/6 \textit{Nx} week 14) was recipient derived. Even if corrected for the technical sensitivity of our RECA-1/\textit{hPAP} double immunostaining of \textit{50%}, the result of a very minor fraction of extrarenal replacement of the renal endothelium remains. Therefore, independently of disease type (acute or chronic) or disease outcome (healing or progression), incorporation of extrarenal precursor cells into the kidney endothelial cell layer was a rare event in our experiment. Additional double immunostaining for \textit{CD45} and \textit{hPAP} revealed that at \textit{GEN} week 4 as well as at 5/6 \textit{Nx} week 14, almost all glomeruli were \textit{CD45} negative whereas almost all peritubular \textit{CD45} cells were \textit{hPAP} (data not shown). We

\begin{figure}
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\caption{Fig. 5. Results of RECA-1 (red) and \textit{hPAP} (TSA amplified, green) double immunofluorescence staining (yellow overlay): analysis of glomeruli. Images of glomerular RECA-1/\textit{hPAP} double immunostaining are shown (A–D: magnification: ×40). \textit{Insets}: enlarged image details of arrow marked capillary \textit{hPAP}/RECA-1 overlays. A and C: glomerular capillary overlay was infrequently found at \textit{GEN} week 4 (A) and 5/6 \textit{Nx} week 14 (C). B and D: sample scanning for yellow target-color confirms extrarenal origin of stained endothelium at \textit{GEN} week 4 (B) and 5/6 \textit{Nx} week 14 (D). E: y-axis shows analysis of relative glomerular RECA-1/\textit{hPAP} overlay in percent (comparison with controls, ** \textit{p} < 0.01). Significant more recipient-derived endothelium was detected at \textit{GEN} week 4 (\textit{P} < 0.01) and 5/6 \textit{Nx} week 14 (\textit{P} < 0.01). Only minor overlay was found at both control groups. F: results of chimerism calculation. Circle charts visualize the small amount of recipient-derived, glomerular endothelial cells at \textit{GEN} week 4 and 5/6 \textit{Nx} week 14.}
\end{figure}
therefore speculate that incorporated RECA-1 and hPAP double-positive cells might be true endothelial progenitor cells lacking CD45 expression although this explanation is speculative and only suggested by the negative results of our glomerular CD45 and hPAP double immunostaining (17). In a similar experimental setup, Rienstra et al. (22) transplanted dark Agouti rat kidneys into R26-hPAP transgenic Fischer F-344 recipient rats to induce allogeneic chronic transplant vasculopathy. In contrast to our results, 62% of all glomeruli contained recipient-derived endothelial cells 12 wk after transplantation. Recipient endothelial chimerism was furthermore observed at low levels in peritubular capillaries (22). Major methodological differences between the study of Rienstra and our study are unlikely since an analogous immunostaining technique for hPAP was used. Although there is the question why no images of TSA amplified hPAP staining controls were published, the fact that the underlying pathomechanisms of chronic allograft vasculopathy are distinct and hardly comparable with the situation of renal endothelial repair during kidney diseases in a syngeneic situation may be most important. Xu et al. (27) detected even >30% of recipient-derived endothelium on day 20 after transplantation of ischemic male Brown Norway rat kidneys into female Lewis rats. In this study, recipient-derived cells were identified by X-chromosome specific DNA samples but double staining with an endothelial marker was not performed.

Overall, analysis at different time points as well as methodological and sensitivity differences in the detection of endothelial chimerism make a comparison of results in rodents or even humans after kidney transplantation quite difficult. For example, several groups analyzed endothelial chimerism after human sex-mismatched kidney transplantation with in situ hybridization. Results ranged from 17.5% to the detection of no endothelial chimerism at all and therefore may be indicative for sensitivity issues of this method (1, 10, 25). Other groups used experimental transplantation of allogeneic or transgenic bone marrow (e.g., eGFP and β-gal) for the detection of bone marrow-derived renal endothelium after kidney injury. Conflicting results ranged from seven donor-derived endothelial cells per glomerular cross section to the detection of no endothelial chimerism at all (9, 12, 13, 23). We are convinced that experimental kidney transplantation is more suitable for investigation of renal endothelial cell chimerism since whole bone marrow transplantation results in transfer of both putative endothelial progenitor cells and also mature endothelial cells of the bone marrow cavity. In addition, the process of bone marrow
transplantation takes ~6 wk and allows infiltration or incorporation of recipient-derived cells before disease model induction.

Nevertheless, the rare event of incorporation of extrarenal-derived cells into renal endothelium as demonstrated in our experiments using very severe and chronic progressive endothesial injury models does not mean that these cells are not important for renal injury repair. However, this result suggests that extrarenal-derived cell recruitment contributes rather indirectly via paracrine effects to kidney endothelial healing than through plain incorporation into the endothelial layer as supported by studies from other groups. For example, during tumor angiogenesis abundant bone marrow-derived cells are recruited close to endothelial cells but did not form part of the endothelium (20). In addition, it has been shown that recruited bone marrow-derived cells can enhance capillary repair and angiogenesis by in situ proliferation of endothelial cells via secretion of proangiogenic factors (7), by expression of several chemokines at growing sites of collateral arteries (29), or by transfer of membrane-derived mRNA-containing microvesicles (6).

In summary, independently of the type and time course of disease (acute vs. chronic progressive) and despite marked severity of renal endothelial cell injury, endothelial regeneration originated almost exclusively from intrinsic kidney cells. While many extrarenal-recipient-derived cells were transiently recruited to the injured kidney, the incorporation of endothelial precursor cells into the renal endothelial layer was increased but still a rare event with <1% of all endothelial cells. Recently, therapeutic transfer of matured endothelial progenitor cells has been effective in ameliorating pathologic kidney changes owing to experimental 5/6 renal mass reduction of the mice and experimental renal arterial stenosis of the swine (5, 24). This suggests that endothelial precursor cells act mostly by exerting paracrine effects. Based on these promising findings and our own results, future research should focus on the probably paracrine interactions between endothelial progenitor cells and renal endothelium to further unravel the mechanisms that lead to kidney regeneration.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.S., C.D., and C.H. conception and design of experiments; H.S., R.V., A.S., and S.P. performed experiments; H.S. and R.V. analyzed data; H.S. and C.H. interpreted results of experiments; H.S. and C.H. edited and revised manuscript; H.S. and C.H. drafted manuscript; H.S. and C.H. revised manuscript; H.S. and C.H. performed data analysis; H.S. and C.H. prepared figures; H.S. drafted manuscript; H.S. and C.H. edited and revised manuscript; H.S. and C.H. approved final version of manuscript.

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