Am J Physiol Renal Physiol 315: F861–F869, 2018.
First published March 7, 2018; doi:10.1152/ajprenal.00633.2017.

RESEARCH ARTICLE

Endothelial progenitor cells accelerate endothelial regeneration in an in vitro model of Shigatoxin-2a-induced injury via soluble growth factors

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Submitted 20 December 2017; accepted in final form 4 March 2018

Patry C, Betzen C, Fathalizadeh F, Fichtner A, Westhoff JH, Fleming T, Eckstein V, Bruckner T, Bielaszewska M, Karch H, Hoffmann GF, Tönshoff B, Rafat N. Endothelial progenitor cells accelerate endothelial regeneration in an in vitro model of Shigatoxin-2a-induced injury via soluble growth factors. *Am J Physiol Renal Physiol* 315: F861–F869, 2018. First published March 7, 2018; doi:10.1152/ajprenal.00633.2017.—Endothelial injury with consecutive microangiopathy and endothelial dysfunction plays a central role in the pathogenesis of the postenteropathic hemolytic uremic syndrome (D + HUS). To identify new treatment strategies, we examined the regenerative potential of endothelial progenitor cells (EPCs) in an in vitro model of Shiga toxin (Stx) 2a-induced glomerular endothelial injury present in D + HUS and the mechanisms of EPC-triggered endothelial regeneration. We simulated the proinflammatory milieu present in D + HUS by priming human renal glomerular endothelial cells (HRGECs) with tumor necrosis factor-α before stimulation with Stx2a. This measure led to a time- and concentration-dependent decrease of HRGEC viability of human renal glomerular endothelial cells as detected by a colorimetric assay. Coincubation with EPCs (10^4–10^5 cells/ml) under dynamic flow conditions led to a significant improvement of cell viability in comparison to untreated monolayers (0.45 ± 0.06 vs. 0.16 ± 0.04, $P = 0.003$). A comparable regenerative effect of EPCs was observed in a coculture model using cell culture inserts (0.41 ± 0.05 vs. 0.16 ± 0.04, $P = 0.003$) associated with increased concentrations of vascular endothelial growth factor, insulin-like growth factor I, fibroblast growth factor-2, and hepatocyte growth factor in the supernatant. Treatment of Stx2a-injured monolayers with a combination of these growth factors imitated this effect. EPCs did not show distinct sings of migration and angiogenic tube formation in functional assays. These data demonstrate that EPCs significantly improve endothelial viability after Stx2a-induced injury in vitro and that this effect is associated with the release of growth factors by EPCs.

endothelial progenitor cells; hemolytic-uremic syndrome; intercellular signaling peptides and proteins; pediatric nephrology; regeneration

INTRODUCTION

The postenteropathic hemolytic uremic syndrome is the leading cause of acute renal failure in children beyond the neonatal period. Most of the cases are caused by infection with Shiga toxin (Stx)-producing strains of *Escherichia coli*. There is currently no specific treatment available for Stx-mediated hemolytic uremic syndrome (HUS). Despite its sporadic occurrence, recent large-scale outbreaks, most notably in Germany in 2011 (8), underline the urgent medical need for new therapeutic approaches.

Stx-induced endothelial dysfunction, leading to thrombotic microangiopathy, plays a pivotal role in the pathogenesis of HUS (14, 24, 37). Of the two major types of this toxin, Stx1a and Stx2a (32), the latter is most commonly associated with postenteropathic HUS (9, 21). Stx particularly injures the microvascular beds of the gut, kidneys, and brain, causing bloody diarrhea, renal failure, and neurological complications (7, 13, 14). Currently, there is no specific treatment for Stx-induced HUS available, and most often, patients become only symptomatic when Stx has already induced organ damage. This is why we need potential novel treatment strategies to especially target this late time point of the disease. Therefore, enhancement of endothelial regeneration might have a beneficial effect on the course of HUS. In this regard, endothelial progenitor cells (EPCs) may be a potential novel treatment strategy since they have a beneficial effect on the course of other microvascular diseases (35). While EPCs initially were thought to be recruited and incorporated into sites of active neovascularization during, e.g., tissue ischemia, vascular trauma, tumor growth, and inflammation (2), more recent work suggests different populations of EPCs with distinct functions (28). Endothelial colony-forming cells (ECFCs) display the ability to form vessels that become part of the host's systemic circulation (20). In contrast, the population of proangiogenic hematopoietic cells (30) do not directly differentiate into persistent vascular endothelial cells or display de novo in vivo vasculogenic potential but rather have potent paracrine properties regulating new vessel formation via angiogenesis (19).

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We have previously demonstrated that transplantation of bone marrow-derived EPCs improved gas exchange, inhibited the synthesis of proinflammatory cytokines, attenuated of pulmonary inflammation, and reduced mortality in a rat model of acute respiratory distress syndrome (25, 27). There are three possible strategies to enhance endothelial repair on the basis of EPCs or paracrine factors release by EPCs: *1*) exogenous administration of EPC; *2*) stimulation of endogenous EP release by mobilizing factors such as granulocyte macrophage colony-stimulating factor, angiopoietin-2, and vascular endothelial growth factor (VEGF); and *3*) administration of EPC-released paracrine factors with intrinsic proangiogenic potential.

In the present study, we hypothesized that EPC treatment attenuates glomerular cell injury in an in vitro model of D + HUS. Furthermore, we hypothesized that this effect is mediated by paracrine release of EPC-derived growth factors.

MATERIAL AND METHODS

Cell culture under static and dynamic flow conditions. Human renal glomerular endothelial cells (HRGECs) were obtained from ScienCell Research Laboratories (Carlsbad, CA). HRGECs were grown at 37°C in culture well plates for static condition experiments. Shigatoxin 2a (Stx2a) was purified as previously described by Bauwens et al. (5). The purified toxin was free of lipopolysaccharide as determined with the Limulus assay (Bactimm, Nijmegen, The Netherlands; detection limit: 0.006 ng/ml); its protein concentrations were 7.6 mg/ml and its 50% cytotoxic dose (CD_{50}) for Vero cells was 0.072 ng/ml. Tumor necrosis factor-α (TNFα) was purchased from Sigma (Deisenhofen, Germany). For dose-response experiments, HRGECs were sensitized with TNF-α (100 ng/l) for 24 h and then incubated with various concentrations of Stx2a (1 ng/l to 1 μg/l) for 48 h as described by Betzen et al. (6). After identification of the LD_{50}, HRGECs were treated with either *1*) 100 ng/l TNFα for 24 h, *2*) 100 ng/l Stx2a for 48 h, or *3*) sequentially by prestimulation with TNFα for 24 h, followed by a change of medium and incubation with 100 ng/l Stx2a for an additional 48 h, and *4*) unstimulated cells served as controls. For dynamic flow condition experiments, HRGECs were seeded in μ-Slide 0.6 Luer, ibidiTreat, tissue culture-treated, sterile, channel slides (Ibidi, München, Germany). A concentration of 1×10^6 cells/ml was prepared, and 1×10^5 cells/cm^2 in 150 μl medium were filled into the channel of the slide, which then was incubated for 1–2 h at 37°C and 5% CO_2. Then, the channel slides were connected to a perfusion set of an Ibidi pump system that contained 12 ml of endothelial growth basal medium EBM-2 (Lonza, Walkersville, MD). The Ibidi pump system consists of an air pressure pump and a fluidic unit. Cells were treated according to the Ibidi application note. In brief, the fluidic unit with the attached channel slide was connected to the pump and placed in an incubator (37°C, 5% CO_2). A shear stress of 5 dyn/cm^2 was applied.

Viability assay. Endothelial injury was detected by a colorimetric assay {3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromid (MTT assay); Sigma, Schnelldorf, Germany} for cell viability.

After incubation of HRGECs with either medium, TNF-α, Stx2a, or TNF-α + Stx2a, cells were washed with 50 μl MTT solution in 200 μl medium and incubated in the dark for 4 h at 37°C. After incubation, the solution was discarded and cells were resolved in 200 μl DMSO on a plate shaker at room temperature. Viability of cells was determined at 590 nm with a 96-micro plate reader (BMG LABTECH, Ortenberg, Germany).

EPC isolation and cultivation. The isolation and cultivation of EPCs was performed according to the UC-EOC Culture Protocol from Dr. Guido Krenning (Groningen, The Netherlands; May 2009 version): Briefly, peripheral blood mononuclear cells were isolated by Ficoll-density gradient centrifugation from cord blood, provided by our department for stem cell transplantation. Then, 1×10^6/cm^2 umbilical cord peripheral blood mononuclear cells were plated on fibronectin-coated culture wells in sufficient Medium-207, which was replenished every 3 days. The cells were passaged at 80–90% confluence (40–50 000 cells/cm^2).

Endothelial tube formation assay (angiogenesis assay). Angiogenic properties of EPCs were assessed with μ-Slide tube formation assays (Ibidi, München, Germany): the slides were coated with 10 μl of Matrigel (BD Biosciences, Heidelberg, Germany) and incubated at 37°C for 30 min. Then, 50 μl of a freshly isolated EPC suspension (3×10^6 EPC/ml) were placed into the well. Images/time lapse were taken at 10-min intervals over a period of 24 h using the JuLI Live Cell Analyzer (Peqlab Biotechnologie, Erlangen, Germany).

Migration assay (chemotaxis assay). The chemotactic properties of EPCs were assessed with a two-dimensional μ-slide chemotaxis assay chamber (Ibidi): 6 μl of a freshly isolated EPC suspension (3×10^6 EPC/ml) were placed into the chamber. We used either FCS (PAA, Pasching, Austria) or VEGF (PeproTech, Rocky Hill, NJ) as chemoattractant. Images/time lapse were taken at 10-min intervals over a period of 24 h using the JuLI Live Cell Analyzer (Peqlab Biotechnologie, Erlangen, Germany). Chemotaxis plots and migration velocities of each cell were determined with the automated cellular analysis system (MetaVi Laboratories; Ibidi).

Fluorescence-activated cell sorting analysis. The expression of cell-surface antigens was determined by immunofluorescence staining: 100 μl of HRGECs (containing 1×10^6 cells) were incubated with 20 μl of FcR-blocking reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 10 min to inhibit nonspecific bindings. Thereafter, the cells were incubated at 4°C for 30 min with 10 μl of FITC-conjugated anti-human CD77 monoclonal antibodies (BD Biosciences) after titration experiments of the antibody. Isotype-matched immunoglobulin G1 and immunoglobulin G2a antibodies (Dako-Cytomation, Hamburg, Germany) were used for each measurement as negative controls. The cells were washed three times to remove unbound antibodies and finally resuspended in 400 μl of fluorescence-activated cell sorting analysis (FACS) solution (BD Biosciences). FACS analysis was performed on a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using WinMDI 2.8 software (Scripps Research Institute, La Jolla, CA).

Treatment with EPCs. To analyze the effects of EPCs on TNF + Stx2a-induced endothelial injury in HRGECs under dynamic flow and static conditions, 1 ml of a freshly isolated EPC suspension (3×10^6 EPC/ml) was added to HRGECs. Images/time lapse were taken at 1-h intervals over a period of 7 days using the JuLI Live Cell Analyzer (Peqlab Biotechnologie). The monolayers of HRGECs were again assessed for viability (see above).

Coculture experiments. To analyze the paracrine effects of EPCs on injured glomerular endothelium, HRGECs and EPCs were cocultured in 24-well plates using transwell culture inserts (Thincert; Greiner Bio-One, Frickenhausen, Germany) with a permeable membrane (pore size: 0.4 μm). First, variable cell counts of HRGECs (1×10^3 to 1×10^4) were seeded in 800 μl/well EGM-2 medium in 24-well plates and incubated at 37°C for 24 h, followed by prestimulation with TNF-α (100 ng/l) for 24 h and incubation with Stx2a (100 ng/l) for 48 h. The medium was then changed, the Thincert was inserted, and the medium was seeded with a EPC cell suspension of 200 μl (25 000 EPC/ml). The cells were cultured at 37°C and after 72 h, and the supernatant was collected to measure the concentrations of relevant growth factors. The monolayers of HRGECs were assessed for viability (see above).

Enzyme linked immunosorbent assay. The concentrations of VEGF, insulin-like growth factor (IGF)-I, fibroblast growth factor (FGF)-2, and hepatocyte growth factor (HGF) were assessed in triplicate samples in the supernatant of the coculture experiments using enzyme-linked immunosorbent assay kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) following the manufacturer's instructions.

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Growth factor treatment experiments. To analyze the effects of the growth factors, released by EPCs, on TNF + Stx2a-induced endothelial injury in HRGECs in dynamic flow and static conditions, 3 ml of a special combination of growth factors including 10 ng/ml basic-FGF-2), 20 ng/ml HGF, 10 ng/ml IGF-I, and 10 ng/ml VEGF165 (all PeproTech) (adapted from the UC-EOC Culture Protocol from Dr. Guido Krenning, May 2009 version) or each growth factor alone was added to the cells. Images/time lapse were taken at 10-min intervals over a period of 72 h using the JuLI Live Cell Analyzer (Peqlab Biotechnologie). The monolayers of HRGECs were assessed for viability (see above).

Statistical analyses. Comparisons for growth factor measurements were done using one factorial ANOVA for multiple groups (followed by Sidak's multiple comparisons test) and two-sided paired *t*-tests for two groups. Results from dynamic flow experiments with EPCs (3 and 7 days old) have been analyzed using a two factorial ANOVA (followed by Turkey test for multiple comparisons). Statistical analyses have been performed using SAS Version 9.4. Results are given as means ± SD. The significance level for differences was set as $P < 0.05$. All findings of the statistical tests are descriptive due to the nature of the study.

RESULTS

Stx2a-induced endothelial injury in HRGECs. The incubation of HRGECs with Stx2a alone already reduced cell viability, but the sequential incubation with TNF-α followed by Stx2a resulted in a more pronounced reduction of cell viability (Fig. 1). Flow cytometry demonstrated an increased expression of the Stx-receptor Gb3/CD77 (globotriaosylceramide) by TNF-α but not by Stx2a stimulation (data not shown). In addition, incubation with TNF-α followed by Stx2a resulted in an increased expression of the adhesion molecules ICAM-1 and VCAM-1 compared with TNF-α alone (Fig. 2). Incubation with Stx2a alone did not alter adhesion molecule expression (Fig. 2).

Tube forming and migratory activity of early EPCs, HRGECs, and HUVECs. Primary colonies of EPCs started to grow after 5–7 days (early EPCs). Late-outgrowth endothelial cells (OECs) grew after 14–21 days. For our experiments we used early EPCs at *day 7* and late-outgrowth endothelial cells at *day 21*. Early EPCs used in our study and HRGECs showed no distinct formation of angiogenic tubes compared with HUVECs (Fig. 3). Furthermore, early EPCs did not show any distinct differences in migration compared with HRGECs and HUVECs (Fig. 4). Also OECs did not show any tube-forming or migratory activity (data not shown).

EPCs accelerate endothelial regeneration. Monolayers of Stx2a-treated HRGEC, which were incubated with EPC, had a significantly improved viability compared with Stx2a-treated HRGECs alone after *day 3* (0.4 ± 0.06 vs. 0.23 ± 0.09, $P = 0.0004$) and after *day 7* (0.56 ± 0.08 vs. 0.32 ± 0.05, $P < 0.0001$, significant treatment-effect in two factorial ANOVA) (Fig. 5). There was no significant difference in viability of untreated HRGECs between *days 3* and *7* (Fig. 5), but we observed a significant difference in viability of treated HRGECs between *days 3* and *7* (0.4 ± 0.06 vs. 0.56 ± 0.08, $P = 0.0003$, significant time effect in two factorial ANOVA). Two factorial ANOVA showed that there was no interaction between time and treatment. The same experiments were also performed using OECs: here, a trend for accelerated HRGECs regeneration was observed, but this difference was not statistically significant compared with Stx2a-treated HRGECs, which were not incubated with OECs (data not shown).

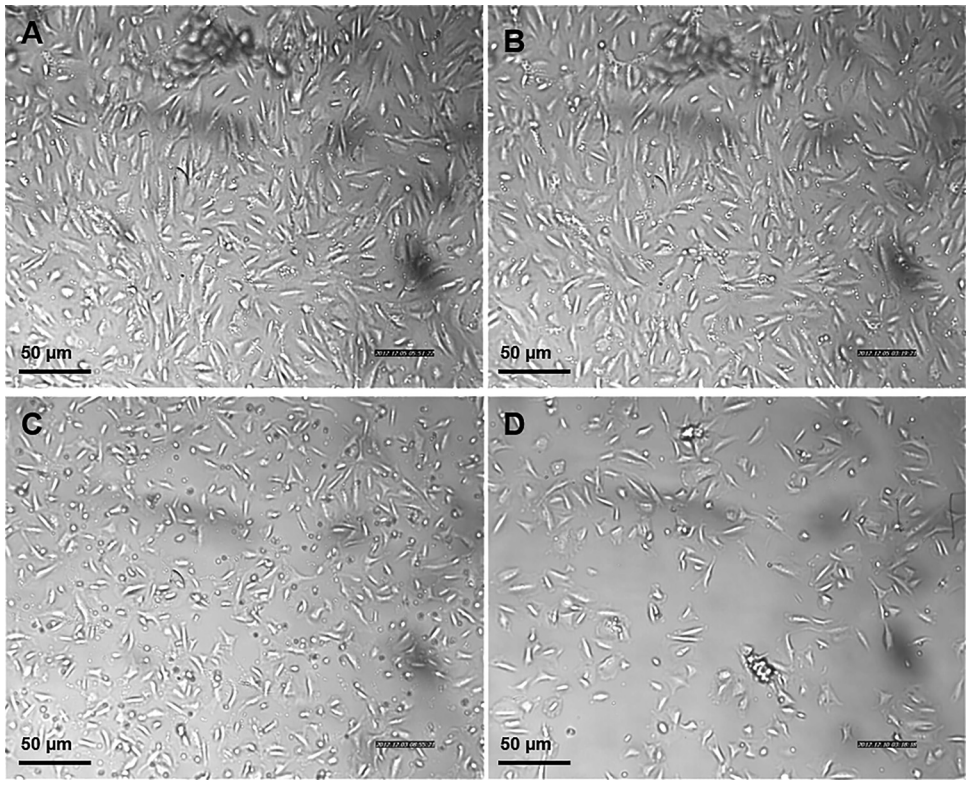

Fig. 1. Microscopic analysis of viability of Shiga toxin 2a (Stx2a)-induced endothelial injury in human renal glomerular endothelial cells (HRGECs) under static conditions. As controls, HRGECs remained untreated (medium) ($0.59 \pm 0.05 \times 10^6$ cells/cm^2, $n = 3$) (*A*). Monolayers of HRGECs were either incubated with tumor necrosis factor-α (TNF-α) (100 ng/l) for 24 h ($0.45 \pm 0.07 \times 10^6$ cells/cm^2 after treatment, $n = 3$) (*B*), Stx2a (100 ng/l) for 48 h ($0.36 \pm 0.03 \times 10^6$ cells/cm^2 after treatment, $n = 3$) (*C*), or both in subsequent order ($0.22 \pm 0.02 \times 10^6$ cells/cm^2 after treatment, $n = 3$) (*D*). While incubation with Stx2a alone already reduced cell viability, the sequential incubation with TNF-α followed by Stx2a resulted in a more pronounced reduction of cell viability. Scale bars are depicted in *A–D*.

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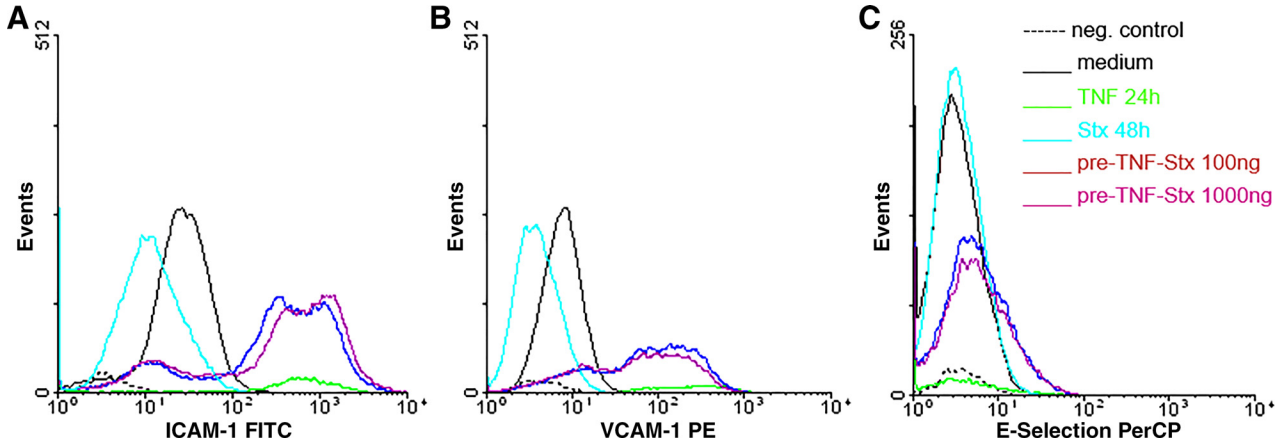

Fig. 2. Upregulation of adhesion molecules in human renal glomerular endothelial cells (HRGECs). Monolayers of HRGECs were either incubated with tumor necrosis factor-α (TNF) (100 ng/l) for 24 h ($n = 3$), Shiga toxin 2a (Stx2a) (100 ng/l) for 48 h ($n = 4$), ot TNF (100 ng/l) for 24 h followed by Stx2a (100 ng/l or 1,000 ng/l) ($n = 3$) or remained untreated ($n = 3$). After the cells were harvested, the expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin was evaluated via flow cytometry. While incubation of HRGECs with Stx2a did not result in an upregulation of the adhesion molecules, TNF and TNF incubation followed by Stx2a led to an upregulation of ICAM-1 (*A*) and VCAM-1 (*B*); E-selectin (*C*) was not affected. The expression of ICAM-1 and VCAM-1 was increasingly expressed in HRGECs incubated with TNF and Stx2a.

Coculture of EPCs leads to endothelial regeneration. Coculture of EPCs and HRGECs resulted in an improved viability of the TNF + Stx2a-treated HRGECs compared with TNF + Stx2a-treated HRGECs alone (0.41 ± 0.05 vs. 0.16 ± 0.04, $P = 0.003$). This was comparable with the effect of direct EPCs treatment (0.45 ± 0.06) (Fig. 6). There was no significant difference between coculture of EPCs and direct treatment (0.41 ± 0.05 vs. 0.45 ± 0.06, $P = 0.36$) (Fig. 6).

Stx2a-triggered release of growth factors by EPCs. We detected a significant increase in levels of VEGF ($P < 0.001$), IGF-I ($P = 0.004$), FGF-2 ($P < 0.001$), and HGF ($P = 0.03$) in the supernatant of HRGECs treated with TNF + Stx2a (Fig. 7). We found similar results for VEGF ($P < 0.001$), IGF-I ($P = 0.01$), FGF-2 ($P = 0.02$), and HGF ($P < 0.001$) in the supernatant of EPCs after treatment with TNF + Stx2a (Fig. 7). Furthermore, Stx2a-treated HRGEC, which were also incubated with early EPC, showed a significant increase of the growth factors VEGF, IGF-I, HGF, and FGF-2 in their supernatant compared with EPCs (VEGF, $P < 0.001$; IGF-1, $P = 0.003$; HGF, $P = 0.006$; FGF-2, $P = 0.01$) and HRGECs (VEGF, $P < 0.001$; IGF-I, $P = 0.002$; HGF, $P = 0.01$; FGF-2, $P = 0.03$) only treated with TNF + Stx2a (Fig. 7).

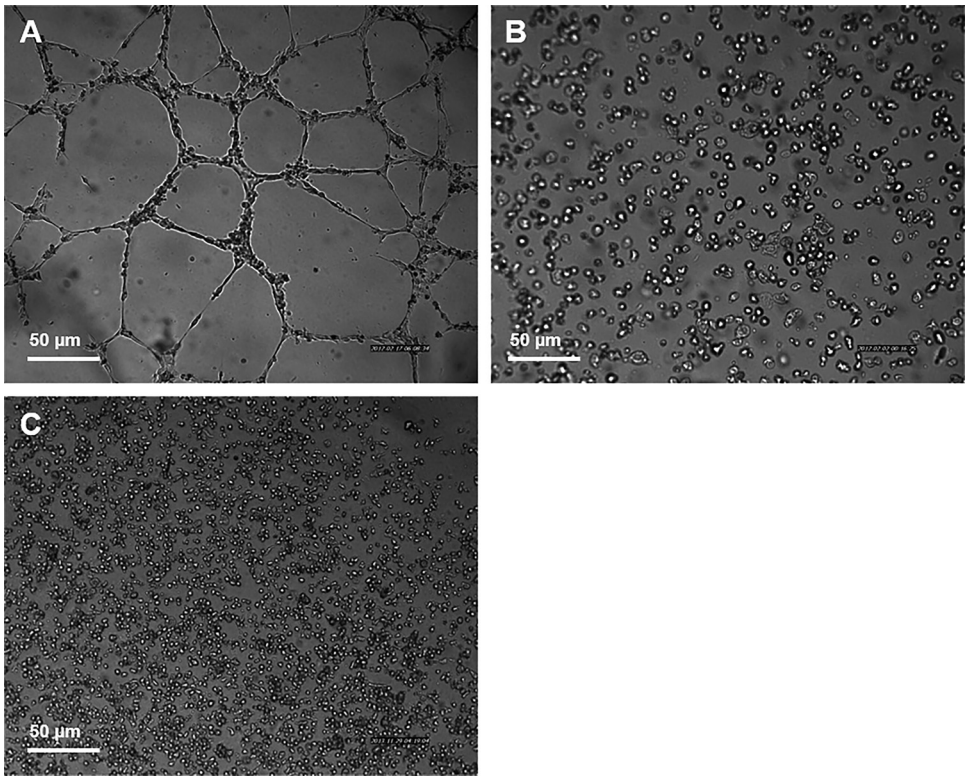

Fig. 3. Microscopic imaging of angiogenesis assays performed on human umbilical vein endothelial cells (HUVECs), human renal glomerular endothelial cells (HRGECs), and early endothelial progenitor cells (EPCs). HUVECs show distinct tube formation on Matrigel ($n = 4$) (*A*), HRGECs ($n = 4$) (*B*), and early EPCs ($n = 4$) (*C*) showed no patterns of angiogenic sprouting on matrigel. Scale bars are depicted in *A*–*C*.

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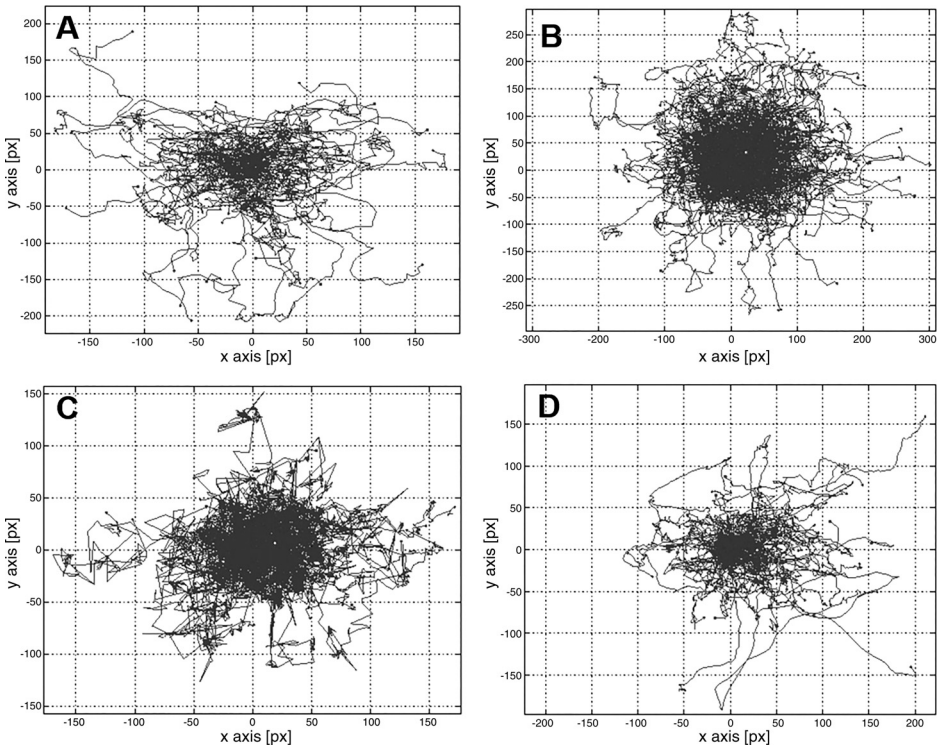

Fig. 4. Trajectory plots of chemotaxis assays performed on human umbilical vein endothelial cells (HUVECs), human renal glomerular endothelial cells (HRGECs), and early endothelial progenitor cells (EPCs). HUVEC + VEGF (*A*), HUVEC + fetal calf serum at 20% (FCS20%) (*B*), EPC + vascular endothelial growth factor (VEGF) (*C*), and HRGEC + FCS20% (*D*) (each $n = 4$) showed no enhanced patterns of chemotaxis. Center of mass is indicated by central dots. *A*: forward migration index *x*: −0.020612; forward migration index *y*: −0.040643; center of mass *x*: −7.2973; and center of mass *y*: −15.3378. *B*: forward migration index *x*: 0.058574; forward migration index *y*: 0.091081; center of mass *x*: 23,1457; and center of mass *y*: 33.4548. *C*: forward migration index *x*: 0.044022; forward migration index *y*: 0.019313; center of mass *x*: 18.906; and center of mass *y*: 6.0336. *D*: Forward migration index *x*: 0.14761; forward migration index *y*: −0.0034027; center of mass *x*: 29.7986 and center of mass *y*: −1.1223.

Growth factor combination mimics the effect of EPCs. The incubation of TNF-Stx2a-treated HRGECs with EPCs as well as with a growth factor combination (GFc), including VEGF, IGF-I, HGF, and FGF-2, resulted in a significantly improved viability (EPCs, $P = 0.002$; GFc, $P < 0.001$) (Fig. 8). There was no significant difference between EPCs and GFc treatment ($P = 0.27$). When each growth factor was added separately to Stx2a-treated HRGEC monolayers, only VEGF showed a trend to improved cell viability, but this was not significant compared with Stx2a-treated HRGEC monolayers without EPCs or GFc (data not shown).

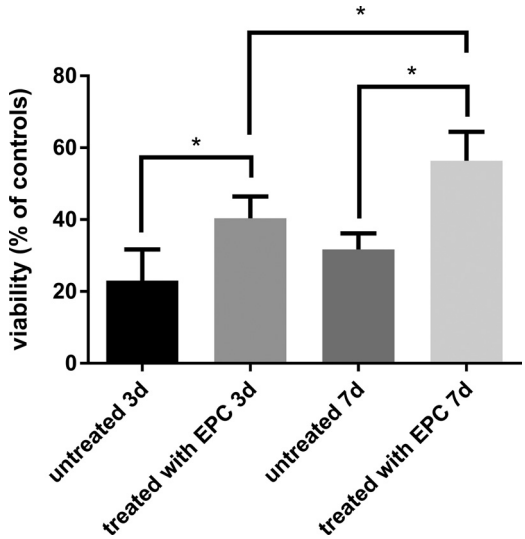

Fig. 5. Endothelial progenitor cells (EPCs) accelerate endothelial regeneration. Monolayers of tumor necrosis factor-α (TNF) + (Stx2a)-treated human renal glomerular endothelial cells (HRGECs) were incubated with early EPCs, assessed for viability on *days 3* and *7* and compared with TNF + Stx2a-treated monolayers of HRGEC, which were not treated with EPC. Monolayers of TNF + Stx2a-treated HRGEC, which were incubated with EPC, had a significantly improved viability compared with untreated monolayers after 3 and after 7 days. In addition, there was a significant difference in viability of EPC-treated HRGECs between *days 3* and *7*. The difference in viability of HRGECs not treated with EPCs between *days 3* and *7* was not significant. The results are expressed as means ± SD. $*P < 0.05$; $n = 5$.

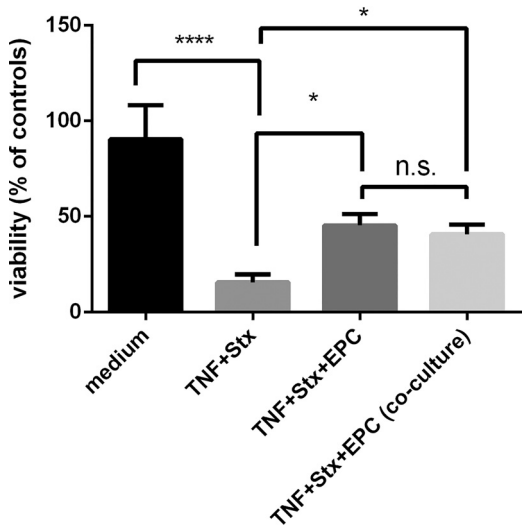

Fig. 6. Coculture of human renal glomerular endothelial cells (HRGECs) with early endothelial progenitor cells (EPCs) enhances regeneration of Shiga toxin 2a (Stx2a)-induced endothelial injury. The paracrine effects of early EPCs on Stx2a-treated HRGECs was assessed in a model of tumor necrosis factor-α (TNF) + Stx2a-induced endothelial injury. Coculture of HRGECs with early EPCs resulted in an improved viability of the HRGEC monolayer, comparable with the effect of direct EPC treatment. The results are expressed as mean simple linear regression ± SE. $*P < 0.05$, $****P < 0.0001$.

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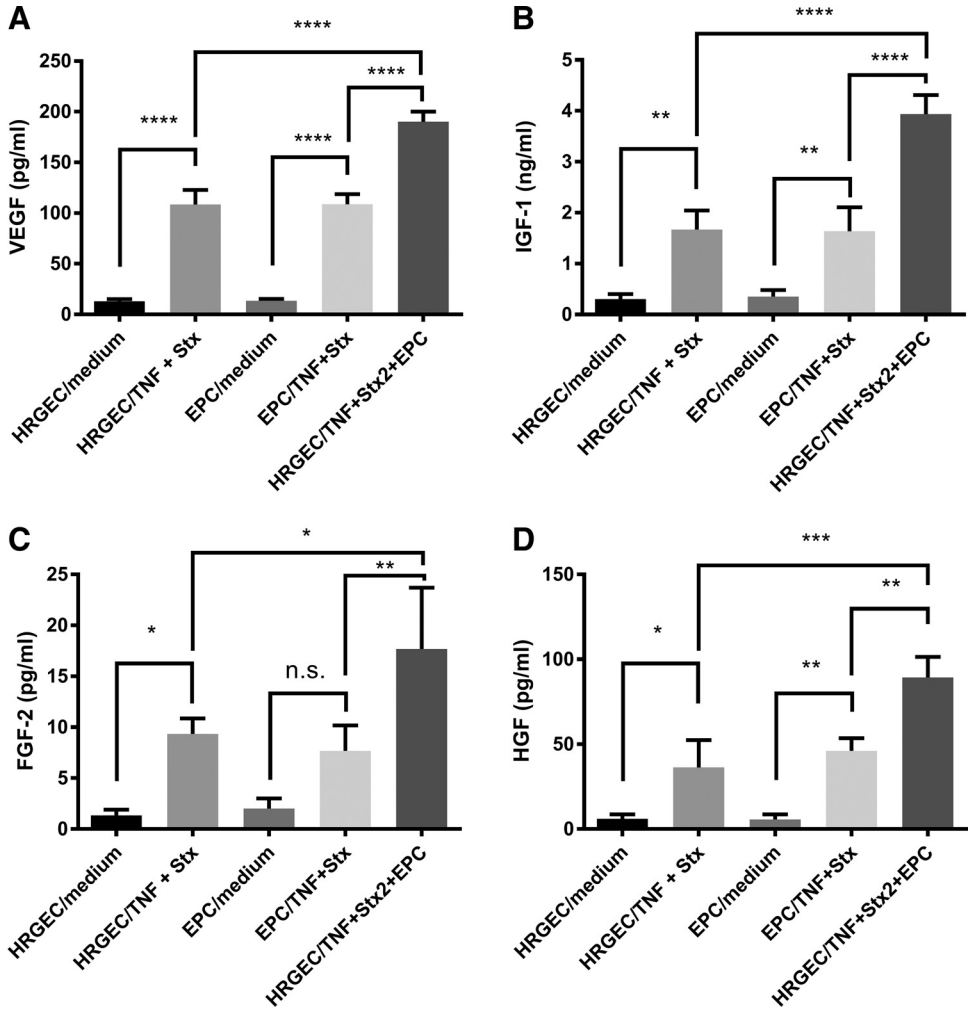

Fig. 7. Stx2a-triggered release of growth factors by EPCs. To assess the release of growth factors by early EPCs in a Stx2a milieu, the concentrations of VEGF (*A*), IGF-I (*B*), FGF-2 (*C*), and HGF (*D*) were measured in the supernatant of TNF + Stx2a-treated monolayers of HRGECs and in the supernatant of early EPCs and compared with monolayers of TNF + Stx2a-treated HRGEC, which were treated with EPC. Treatment with TNF + Stx2a resulted in a significant increase of VEGF, IGF-I, FGF-2, and HGF by HRGECs compared with medium. Similar results were found for VEGF, IGF-I, FGF-2, and HGF when early EPCs were treated with TNF + Stx2a. Incubation of Stx2a-treated HRGECs with EPCs synergistically increased the release of VEGF, IGF-1 and HGF compared with monolayers of EPCs and HRGEC, which were only treated with TNF + Stx2a. In this setting an increased concentration of FGF-2 was found in the supernatant, which, however, was only marginally significant compared with EPCs and HRGEC, which were only treated with TNF+Stx2a. The results are expressed as mean simple linear regression ± SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. FGF-2, fibroblast growth factor; HGF, hepatocyte growth factor; HRGEC, human renal glomerular endothelial cells; IGF-I, insulin-like growth factor I; TNF, tumor necrosis factor-α; Stx2a, Shiga toxin 2a; VEGF, vascular endothelial growth factor.

DISCUSSION

The data derived from our study support our initial hypothesis that EPCs attenuates Shigatoxin-induced glomerular endothelial cell injury in an in vitro model of D + HUS. Furthermore, our results suggest that this regenerative effect might be mediated by paracrine release of EPC-derived growth factors rather than by cell-cell interactions between EPCs and glomerular endothelial cells.

EPCs as therapeutic agents have already been investigated in several settings of vascular disease and endothelial injury (10, 26, 40, 41). The idea of using EPC-based therapies to treat kidney disease was raised first in 2006. Patschan et al. (23) demonstrated nephron-protective effects of transplanted EPCs in a murine model of acute kidney disease. In our study, application of EPCs increased viability after Stx2a-induced glomerular endothelial injury in both flow experiments and coculture experiments. Since there was no physical contact between EPCs and glomerular endothelial cells in the coculture experiments, this indicates that the beneficial effects of EPCs are associated with the release of paracrine effectors. For confirmation, the conditioned medium of EPCs was analyzed after Stx2a-stimulation. Elevated levels of the growth factors VEGF, IGF-I, FGF-2, and HGF were found. The application of a combination of these growth factors also increased glomerular endothelial cell viability in our model of Stx2a-induced endothelial injury. The extent of this growth factor-induced improvement of viability was comparable to the effects of direct EPC application. EPCs used in our project showed no distinct angiogenic sprouting or chemotactic activity. These data are suggestive that EPCs beneficially influence glomerular endothelial cell injury in this in vitro HUS model via the release of the specific growth factors VEGF, IGF-I, FGF-2, and HGF and not by differentiation into mature endothelial cells.

The secretion of paracrine mediators has been proposed by Sieveking et al. (33) as one potential mechanism by which EPCs exert their beneficial and proangiogenic effects on resting endothelial layers. The group of Sieveking et al. (33) differentially analyzed so called "early EPCs" and "late outgrowth EPCs". "Early EPCs" appear in culture during the first week of cultivation; late outgrowth EPCs form after 2 to 3 wk (12). Sieveking et al. (33) demonstrated, that especially early EPCs do not integrate into preexisting vascular structures but rather promote angiogenesis in a paracrine manner, whereas late outgrowth EPCs incorporate into the existing vessel and thereby promote de novo vessel formation. Our results correspond partly to this concept as the early EPCs used in our experiments showed no distinct patterns of angiogenic tube formation or chemotactic activity in vitro. Surprisingly, the

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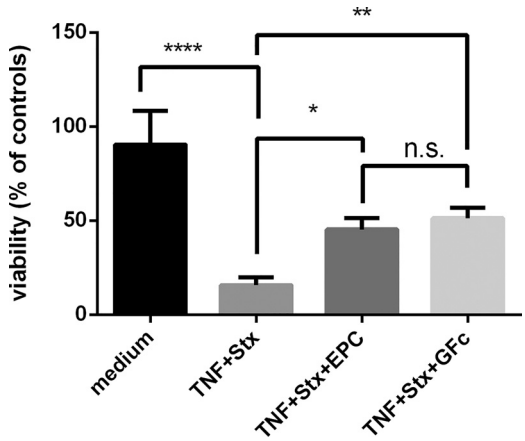

Fig. 8. Combination of growth factors mimics the regenerative effect of EPCs on Stx2a-induced endothelial injury. The effect of GFc including VEGF, IGF-I, HGF, and FGF-2 on TNF + Stx2a-treated HRGECs was compared with the effects of early EPC. Treatment with EPCs as well as GFc resulted in a significantly improved viability compared with TNF+Stx2a. But there was no significant difference between EPCs and GFc treatment. The results are expressed as mean simple linear regression ± SE. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. EPCs, endothelial progenitor cells; GFc, combination of growth factors; FGF-2, fibroblast growth factor; HGF, hepatocyte growth factor; HRGECs, human renal glomerular endothelial cells; IGF-I, insulin-like growth factor I; TNF, tumor necrosis factor-α; Stx2a, Shiga toxin 2a; VEGF, vascular endothelial growth factor.

OECs used in our study also did not show patterns of angiogenic tube formation or chemotactic activity. The beneficial effects of early EPCs on glomerular endothelial cell viability in HUS settings appear to be predominantly associated with EPC-released soluble factors. Currently, the promotion of angiogenesis by such EPC-released soluble growth factors is an emerging concept of translational EPC research (31). Urbich et al. (38) found that the respective abundance of VEGF-A, VEGF-B, stromal cell-derived factor-1, and IGF-I mRNA was distinctly elevated in EPCs and concluded that EPC-based growth-factor secretion may improve vessel formation and cardiac regeneration after cell therapy. Recently, further evidence of paracrine effector mechanisms regarding EPCs has been provided by studies on EPC-derived exosomes (16, 17, 43). In our in vitro model of D + HUS, we identified the growth factors VEGF, IGF-I, FGF-2, and HGF to be released by EPCs and demonstrated that these factors exert beneficial effects on resting glomerular endothelial cells injured by TNF-α and Stx2a.

With respect to a potential clinical application of these results in the future, we analyzed two distinct experimental settings where these growth factors were released by Stx2a-stimulated EPC. First, we observed regeneration of glomerular endothelium in a coculture model without attachment of EPCs to the resting glomerular endothelial monolayer. Hence, cell-cell contact between EPCs and the resting endothelial layer is not a prerequisite for growth factor release. However, it remains to be determined which intracellular signaling mechanisms lead to Stx2a-triggered release of growth factors in circulating EPC. Second, in flow experiments, EPCs very likely come into contact to the resting glomerular endothelial cells. Consistent with the current knowledge about endothelial adhesion molecules in inflammatory settings (18), we demonstrated that TNF-α stimulation results in an upregulation of the adhesion molecules VCAM and ICAM in HRGECs. Both adhesion molecules are likely to be involved in integrin-based EPC homing to the resting glomerular endothelial layer (4, 28). According to our current understanding of EPC biology, homing of endothelial progenitors and of neutrophils follows similar principles (11, 39). After attachment to the activated endothelial layers, EPCs are thought to transmigrate into the surrounding tissue and promote angiogenesis or vasculogenesis (22). Yet, the early EPCs used in our study demonstrated neither distinct migratory nor tube formation capabilities. Hence, it is unlikely that early EPCs exert their beneficial effects on glomerular endothelial cells in HUS settings based on transmigration. Rather, our data suggest a paracrine mode of action by production and subsequent release of the above mentioned growth factors.

Regarding the EPC mediated-growth factor release, we tried to determine if the presence of growth factor-producing EPCs is a prerequisite for improvement of glomerular endothelial cell viability. Our results show that the beneficial effects of EPC treatment on one hand and the effects of the EPC-released growth factors VEGF, IGF-I, FGF-2, and HGF on the other hand were similar. In both settings, we observed a comparable improvement of glomerular endothelial cell viability under HUS conditions, while the application of each growth factor alone did not lead to significant endothelial regeneration. Thus there seems to be an intrinsic protective effect of the growth factor combination on glomerular endothelial cells, which is independent of the presence of the growth factor-providing EPCs. Consistent with this hypothesis, IGF-I, HGF, and VEGF have already been demonstrated to be nephron protective in other experimental settings. IGF-I has been shown to protect tubular epithelial cells in a mouse model of unilateral ureteral obstruction (42) and promotes migration and tube formation of endothelial cells (3). Furthermore, there are indications that HGF also protects against acute proximal tubule injury (36). VEGF-A, expressed by podocytes, appears to protect the nephron filtration apparatus since anti-VEGF-A-mediated tumor therapy can lead to proteinuria and acute renal insufficiency (15). Furthermore, it has been demonstrated by Suga et al. (34) that VEGF can preserve glomerular endothelium in a rat model of renal microangiopathy. Also FGF-2 seems to be related to renal disease mechanisms. Ray et al. (29) showed that serum and urinary concentrations of basic FGF-2 correlate with the severity of postenteropathic (D + HUS) in children. Our results, which demonstrate a beneficial effect of these growth factors on glomerular endothelial cells under HUS conditions, integrate well into these previously published data about the relevance of VEGF, IGF-I, FGF-2, and HGF for kidney regeneration. However, our results suggest that the application of these growth factors in combination might be superior compared with the application of each growth factor alone in treating Stx2a-induced injury of HRGEC.

The limitations of this study are the limited number of experiments and the lack of growth factor receptor blockade studies. The results from this study remain therefore associative and loosely mechanistic. Future experiments will test the hypothesis that combinational growth factor treatment is sufficient to improve cell viability, while single treatment is not, more rigorously via individual receptor antagonism. To interpret the results of our study within a clinical context and with respect to the design of future in vivo studies, we also have to

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discuss the limitations of our experimental set-up. We used an in vitro model of Stx2a-induced HUS; thus the interpretation of our results with respect to clinical implications must be made carefully. Our in vitro HUS model simulated primary glomerular endothelial cell injury and dysfunction after Stx2a-stimulation by measuring cellular viability. Reduced glomerular endothelial cell viability after treatment with Stx2a in vitro is generally accepted to resemble endothelial dysfunction in clinical HUS (1). To further approximate physiological conditions encountered in vivo, we performed our experiments under constant flow conditions. Shiga toxin-induced glomerular endothelial cell injury and dysfunction are believed to be the causative processes and the basis of subsequent and ongoing HUS pathology, leading to severe microangiopathy, thrombocyte consumption, acute kidney failure, and uremia. In this respect, it is important to note that our model specifically focuses on Stx2-induced endothelial injury without directly assessing further microangiopathy-related sequelae, therefore limiting the interpretation of our results with respect to the disease progress as a whole. In conclusion, our results demonstrate that addition of early EPCs after Stx2a-induced injury in vitro significantly improves endothelial viability and thus might accelerate endothelial regeneration and that this effect seems to be associated with paracrine signaling of EPC-released growth factors VEGF, IGF-1, FGF-2, and HGF. The application of these growth factors could potentially play a role in the treatment of HUS. Further studies need to be performed to investigate the potential of treatment strategies applying EPCs or EPC-released growth factors to attenuate Stx2a-induced injury in clinical settings of HUS.

ACKNOWLEDGMENTS

We thank Peter Nawroth for kindly providing the laboratory facilities, Dr. Varun Kumar for valuable input and methodical assistance, and Krista Rafat for editing assistance in the preparation of the manuscript.

GRANTS

This study was supported by a research grant from the Doktor Robert Pfleger-Foundation, the German Kidney Foundation, and the Friedrich Fischer-Estate (mediated by the Medical Faculty of the University of Heidelberg). C. Patry, C. Betzen, and N. Rafat were supported by research scholarships from the Physician Scientist-Program of the Medical Faculty of the University of Heidelberg. T. Fleming was supported by the German Research Foundation (DFG; SFB1118).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.P., C.B., A.F., J.H.W., M.B., H.K., G.F.H., B.T., and N.R. conceived and designed research; C.P., C.B., F.F., T.F., and V.E. performed experiments; C.P., C.B., F.F., A.F., J.H.W., T.F., V.E., T.B., M.B., H.K., G.F.H., B.T., and N.R. analyzed data; C.P., C.B., F.F., A.F., J.H.W., T.F., V.E., T.B., M.B., H.K., G.F.H., B.T., and N.R. interpreted results of experiments; C.P. and C.B. prepared figures; C.P. drafted manuscript; N.R. edited and revised manuscript; N.R. approved final version of manuscript.

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