Bone morphogenetic protein-5 and early endothelial outgrowth cells (eEOCs) in acute ischemic kidney injury (AKI) and 5/6-chronic kidney disease

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Bone morphogenetic protein-5 (BMP-5) and early endothelial outgrowth cells (eEOCs) reproducibly have been shown to act protectively in acute ischemic kidney injury (AKI) and chronic kidney injury. Bone morphogenetic protein-5 (BMP-5) acted antifibrotically in human hypertensive nephropathy. The aim of the current study was to analyze effects of BMP-5 treatment in an eEOC-based therapy of murine AKI and 5/6-nephrectomy. Male C57/Bi6N mice were either subjected to unilateral renal artery clamping postuninephrectomy or to 5/6-nephrectomy. Untreated or BMP-5-pretreated murine eEOCs were injected into recipient animals at the time of reperfusion (AKI) or at 2 and 5 days after 5/6-nephrectomy. Analysis of renal function and morphology was performed at 48 h and at 6 wk (AKI) or at wk (5/6 model). Cellular consequences of eEOC treatment were evaluated using different in vitro assays. AKI was mitigated significantly by injecting BMP-5-pretreated eEOCs. Renal function was improved at 48 h and at 6 wk after cell therapy. In 5/6-nephrectomy, the cells failed to protect renal function, but proteinuria was reduced after administering untreated eEOCs. BMP-5 pretreatment resulted in aggravated proteinuria and renal fibrosis. In 5/6-nephrectomized animals, percentages of anti-smooth muscle actin+ /CD31+ cells increased, indicating endothelial-mesenchymal transition (EnMT). In vitro analysis revealed increased cell migration and reduced cell apoptosis/necrosis. Paracrine activity remained unaffected. BMP-5 acts as a potent eEOC agonist in murine AKI in the short and mid to long term. Cell effects in 5/6-nephrectomy are heterogenous, but untreated cells act antiproteinurically and antifibrotically without any impact on EnMT.

Acute renal failure; BMP-5; EPC

MATERIALS AND METHODS

Animal Models

The animal study protocol was in accordance with the guidelines of the German Institute of Health’s Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. C57/Bi6N mice were obtained originally from The Jackson Laboratory (Bar Harbor, ME) and bred in the local animal facility of the Göttingen University Hospital. For all experiments, male, 8- to 12-wk-old C57/Bi6N mice were used. All animals were caged separately with a 12:12-h light-dark cycle and had free access to water and chow throughout the study.

Surgical Procedures

AKI model. Mice were anesthetized (300 µl 6 mg/100 g ketamine hydrochloride plus 0.77 mg/100 g xylazine hydrochloride) and placed on a heated surgical pad. Rectal temperature was maintained at 37°C. After a 1.5-cm midlateralotomy, the right kidney was removed. The left
kidney was exposed, and clamping of the renal pedicle was performed with microserrrefines (Fine Science Tools, Foster City, CA). For induction of acute renal failure, contralateral nephrectomy was performed. First, a suture was placed around the renal artery, vein, and ureter, respectively. The suture was held open until cell injection. A certain volume of eEOC-containing endothelial basal medium (EBM)-2 (0.5 × 10^6 eEOCs in 50 µl) was injected into the right renal vein (systemic circulation). Very shortly after cell injection, the suture was closed to prevent bleeding. The kidney was removed afterward. Cell injections were performed after 40 min of clamping the left renal pedicle. The vascular clamp was removed ~1 min before injecting eEOCs into the right renal vein. The abdominal incision was closed with a 4-0 suture and surgical staples. Two days (48 h) after this procedure, animals were killed, and blood and kidney samples were collected for further analysis. In each experimental group, eight to 10 animals were analyzed. Our previous studies showed that creatinine levels peak at 48 h after unilateral ischemia-reperfusion injury postuninephrectomy (20). In another series of experiments, animals were killed at 6 wk after surgery.

5/6-Nephrectomy. Mice were anesthetised using ketamine hydrochloride plus xylazine hydrochloride as described. Animals were placed on a heated surgical pad, and rectal temperature was maintained at 37°C. After bilateral dorsal, longitudinal incisions, 5/6-nephrectomy was performed by the ablation of two-thirds of the left kidney and removal of the right kidney with preservation of the adrenal glands (38). Injections of 1 × 10^6 eEOCs in 100 µl EBM-2 were performed into the tail vein at 2 and 5 days after surgery.

Culture of Mouse-Derived eEOCs

To perform cell-injection experiments, eEOCs were isolated from C57/B16N mice. Therefore, mouse mononuclear cells (MNCs) were enriched by density gradient centrifugation using Biocoll solution (Biochrom, Berlin, Germany) from peripheral blood and spleen cell extracts. The reason for pooling MNCs was to maximize the total number of cells available for injection. Immediately following isolation, MNCs were mixed, and 4 × 10^5 cells were plated on 24-well culture dishes coated with human fibronectin (Sigma, St. Louis, MO) and maintained in endothelial cell growth medium-2 (EGM-2; Clonetics; Lonza, Walkersville, MD), supplemented with EGM SingleQuots containing 5% fetal calf serum. After 4–5 days of culture, eEOCs were identified by the uptake of 3,3′-diodocetyldeicyldiacarbocyanine (Dil)-labeled acetylated LDL (acLDL; Invitrogen, Carlsbad, CA) and binding of FITC-labeled BS-1 lectin (Sigma Diagnostics, St. Louis, MO). For this purpose, cells were first incubated with 10 µg/ml Dil-acLDL at 37°C for 1 h and later fixed with 2% formaldehyde for 10 min, followed by incubation with BS-1 lectin at 37°C for 1 h. Cells that demonstrated double-positive immunofluorescence in laser-scanning microscopy were defined as eEOCs. Laser-scanning microscopy was performed using an inverted fluorescence microscope IX71 (Olympus Deutschland GmbH, Hamburg, Germany), equipped with the appropriate excitation and emission filters (AHF Analysentechnik,Tuebingen, Germany). Images of respective fluorescence channels were recorded as single, high-resolution, 16-bit, black/white images using a fxview II extensible camera (Olympus Deutschland GmbH). The images from every fluorescence channel were then merged automatically using the multiple fluorescence information-processing module of Cell-F software. Cells that were used for injection experiments were not labeled with acLDL and BS-1 lectin but were incubated with CellTracker (Molecular Probes, Eugene, OR), according to the manufacturer's protocol. For in vitro cell experiments, a murine EPC line was purchased (66110-37; Celprogen; Stem Cells Research & Therapeutics, San Pedro, CA) and cultured, according to the manufacturer's protocol.

**In vitro Treatment of eEOCs before Therapeutic Administration**

eEOCs used for systemic injections were detached by trypsinization after the first passage and after neutralization of trypsin, incubated with CellTracker. After washing the cells once with PBS, they were resuspended in 50 µl EGM-2 for systemic injection or for further in vitro treatment, in which eEOCs were incubated with BMP-5 (100 ng/ml in EGM-2; Celprogen; Stem Cells Research & Therapeutics) for 60 min at 37°C. After washing the cells once with EGM-2, they were resuspended in 50 or 100 µl EGM-2 for systemic injection.

**Morphologic Evaluation of Kidneys**

For quantification of kidney fibrosis, formalin-fixed, paraffin-embedded tissue sections were stained with Masson’s trichrome. The amount of collagen deposition (blue area) was then assessed semi-quantitatively by assigning grades 1 (mild), 2 (moderate), or 3 (severe).

**Immunofluorescence Microscopy**

**Detection of eEOCs.** Tissue samples were fixed in a 4% formaldehyde solution for 1 h, followed by incubation in 30% sucrose overnight at 4°C. Embedding was performed in an optimal cutting temperature compound (Tissue-Tek, Torrance, CA), and embedded samples were stored at −20°C. Frozen samples were cut into 10 µm-thick sections. Nonspecific protein binding was blocked by 1 h incubation with PBS-BSA (1%). Sections were incubated with FITC-conjugated anti-mouse CD117 (c-Kit, 1:1,000 in PBS-BSA 1%; BD Biosciences,Rockville, MD) or with the respective isotype control for 12 h at 4°C. To visualize the nuclei, tissue sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; 1:200 in PBS; Molecular Probes). Sections were examined, as described previously.

**Vascular staining.** Formalin-fixed, paraffin-embedded tissue sections were stained with rat anti-mouse CD31 (platelet endothelial cell adhesion molecule-1, clone SZ31; Dianova GmbH, Hamburg, Germany) and rabbit anti-smooth muscle actin (αSMA; Emelca Bioscience, Breda, The Netherlands) for primary incubation and with Alexa Fluor 488 goat anti-rabbit IgG (Dianova GmbH) and Alexa Fluor 594 goat anti-mouse CD117 (c-Kit, 1:1,000 in PBS-BSA 1%; BD Biosciences, Rockville, MD) or with the respective isotype control for 12 h at 4°C. To visualize the nuclei, tissue sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; 1:200 in PBS; Molecular Probes). Sections were examined, as described previously.

**Analysis of Renal Function and Proteinuria**

Serum creatinine concentration was measured using a commercially available kit (Creatinine PAP; Labor + Technik Eberhard Lehmann GmbH, Berlin, Germany), according to the manufacturer’s protocol.

**Cell Migration Assays**

The eEOC cell migration assay was performed, as published by Shi et al. (28). Briefly, cells were grown on fibronectin-coated six-well plates. As soon as the well area was covered completely by cells (after approximately 5–6 days), an artificial wound was created, using the tip of a syringe. Cells remained in BMP-5-free EBM-2 or in BMP-5 containing medium (100 ng/ml). Incubation time was 1 h in every series of experiments. Each series was performed at least three times. After cell washing with BMP-5-free EBM-2, images of the respective wound areas were taken at 0 and 17 h.

**ELISA Studies**

For all in vitro cell experiments, a commercially available murine “early outgrowth” EPC line was purchased (66110-37; Celprogen;
For analyzing apoptosis, the percentage of Annexin V
were washed, and 24 h later, apoptosis and necrosis were evaluated.
(100 ng/ml). The concentration of TGF-
/ H9252
considered significant at
t populations were compared by Student’s
test. Differences were

Necrosis was measured by cytometric analysis. Briefly, cells with
quantified using a commercially available kit (Becton Dickinson,
/ H11006
0.24
serum creatinine levels of treated mice (1.09
P 0.02). Systemic injection of 0.5 ×
taining medium alone or with TGF-
/H9252
performed as described above. For measuring levels of VEGF, IL-6,
treatment, commercially available ELISA tests were performed, accord-
 to the manufacturer’s protocol. Cell treatment with BMP-5 was
improved acute renal failure [ischemia-reperfusion injury (IRI)]; renal function did not

topkymnephrectomized animals, no cells at all were detectable at 8 wk
after surgery (data not shown).

BMP-5 Does Not Stimulate Homing of Injected eEOCs in AKI or in 5/6-Nephrectomized Animals

There were no differences in intrarenal numbers of injected
CellTracker+/c-Kit+ cells (eEOCs) among the three AKI
groups. As in previous studies (19, 20), cell numbers were
generally low (data not shown). Therefore, BMP-5 did not
stimulate homing of eEOCs in AKI. In kidneys from 5/6-
nephrectomized animals, no cells at all were detectable at 8 wk
after surgery (data not shown).

BMP-5 Does Not Increase Renoprotective Effects of eEOCs in 5/6-Nephrectomized Mice

C57/Bl6N mice, which underwent 5/6-nephrectomy, displayed
significantly impaired renal function at 8 wk after surgery (0.24 ±
0.16 ± 0.008 mg/dl, P < 0.0001; Fig. 2). Renal
function did not improve after administration of untreated or
BMP-5-pretreated syngeneic murine eEOCs (Fig. 2). In contrast
to renal function, significant proteinuria was not detected at 8 wk
after surgery (data not shown).

eEOCs Attenuate Interstitial Fibrosis in 5/6-Nephrectomized Animals

Besides analysis of renal function and proteinuria, kidneys of 5/6-nephrectomized animals were also investigated morpho-
logically. Renal interstitial fibrosis was assessed semiquantita-

**RESULTS**

**BMP-5 Increases Renoprotective Effects of eEOCs in AKI in the Short Term**

Forty minutes of unilateral renal artery clamping (left kidney)
postuninephrectomy (right kidney) significantly increased serum
creatinine levels of treated mice (1.09 ± 0.23 mg/dl vs.
0.24 ± 0.1 mg/dl, P = 0.02). Systemic injection of 0.5 ×
taining medium alone or with TGF-
/H9252
pretreated (hyp. Nephr.) eEOCs neither improved nor
worsened renal function. Administration of BMP-5-treated eEOCs to non-
nephrectomized animals did not worsen renal function (no Nephr. + eEOCs +
BMP-5; data as mean ± SE, *P < 0.05).
tively. 5/6-Nephrectomy induced significant collagen deposition at 8 wk after surgery. Animals receiving untreated eEOCs showed less connective tissue in the interstitial space, and mice injected with BMP-5-pretreated cells displayed a more severe fibrosis than mice receiving untreated eEOCs (Fig. 3). Lately, the process of endothelial-mesenchymal transition (EnMT) has been reported to contribute to chronic renal fibrosis in different murine models of CKD (34). We therefore aimed to investigate EnMT in 5/6-nephrectomized mice undergoing different cell-therapy strategies. EnMT was assessed by quantifying expression of αSMA in CD31+ cells. Expression of αSMA in CD31+ cells was generally low. However, αSMA-expressing endothelial cells were detectable in all animals undergoing 5/6-nephrectomy (Fig. 4). There were nevertheless no differences between mice with no cell treatment and those with injection of untreated or pretreated eEOCs (Fig. 4).

**BMP-5 Stimulates eEOC Migration**

Incubation of cultured murine eEOCs with BMP-5 for 1 h significantly accelerated cell migration, as indicated by smaller wound areas at 17 h after treatment (33.2 ± 0.95% wound-area reduction vs. 8.2 ± 6.12% wound-area reduction, P = 0.01; Fig. 5).

**BMP-5 Promotes eEOC Survival**

Besides stimulating eEOC migration, BMP-5 significantly promoted survival of the cells. This was indicated by lower percentages of Annexin V+/PI+ cells after 1 h of incubation with BMP-5 (25 ± 2.1% vs. 33 ± 1.2%, P = 0.01; Fig. 6).

**BMP-5 Does Not Modulate eEOC Secretion of Proangiogenic/Proinflammatory Mediators**

As eEOCs have been shown to act preferentially by indirect mechanisms (22, 24), cellular production/release of proangio-

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**Fig. 3.** Interstitial fibrosis in kidneys from 5/6-nephrectomized mice at 8 wk after surgery. Collagen deposition was increased significantly after nephrectomy without cell therapy (Nephr.; C). Injection of untreated eEOCs diminished interstitial fibrosis significantly (Nephr. + eEOCs; D). Cell pretreatment with BMP-5 resulted in aggravated fibrosis (Nephr. + eEOCs + BMP-5; E), and collagen deposition was comparable with animals undergoing nephrectomy without cell administration. B: untreated controls are displayed. Control animals injected with BMP-5-pretreated eEOCs did not display aggravated fibrosis (Controls + eEOCs + BMP-5; data as mean ± SE, *P < 0.05).
genic/proinflammatory mediators was evaluated. There were no differences in VEGF, IL-6, and TGF-β between untreated and BMP-5-pretreated eEOCs. Analyses were performed at 1, 3, 6, and 24 h of BMP-5 incubation (Fig. 7).

DISCUSSION

eEOCs have been shown to protect mice reliably from AKI and from chronic hypertensive and ischemic nephropathy (7, 19-21, 23, 26). In addition, different strategies have been established in the past to increase renoprotective cell competence in AKI (19, 23). With this study, BMP-5 has been identified as a novel and very potent eOC agonist in AKI. In 5/6-nephrectomized mice, the protein partly failed to stimulate renoprotective effects of the cells. Increased cell activity in AKI was accompanied by increased cell migration and survival in vitro; cellular production/release of proangiogenic/proinflammatory mediators was not modulated by BMP-5.

The BMP family is represented by at least 15 members (13) that belong to the TGF-β superfamily. They have been shown to play essential roles in diverse physiological and pathological processes, including angiogenesis (13), bone-fracture healing (15), regulation of pulmonary vascular resistance (18), vascular...
calcification (25), and adipogenesis (27), respectively. Meanwhile, BMP-2 and -7 are clinically used in patients with bone fractures (2, 11, 29). In addition, BMP-7 has been shown to directly prevent the kidney from acute and chronic damage (30, 31, 35, 37). Intravenous administration of BMP-7 to rats after bilateral renal ischemia protected the animals from renal failure (31), and interstitial renal fibrosis in diabetic mice was inhibited significantly by the protein (30). More recent data indicated that BMP-5 is another molecule involved in regulating the de novo synthesis of matrix proteins in chronic (hypertensive) nephropathy (4). Such a proregenerative role is most likely not limited to the kidney, since BMP-5 expression has been shown to be increased in synovial tissue of patients with osteoarthritis and rheumatoid arthritis (3). We therefore analyzed in vivo and in vitro consequences of eEOC treatment with BMP-5. The protein significantly promoted migration and survival of treated cells. In the past, comparable effects were induced in eEOCs and in mesenchymal stem cells (MSCs) by using the hormone melatonin (17, 19). While stimulating cell-migratory activity and eEOC survival in vitro, BMP-5 did not increase eEOC homing in AKI. In chronic nephropathy, as a result of 5/6-nephrectomy, eEOCs were not detectable at all at 8 wk after surgery.

Nevertheless, in AKI, BMP-5-treated eEOCs protected renal function significantly, in fact, indicating a proregenerative role for BMP-5. The lack of protective eEOC effects in 5/6-nephrectomized animals in terms of renal function conflicts with data from other investigators. In a study performed by Sangidorj and colleagues (26), EPCs mediated significant reno-protection in 5/6-nephrectomized mice, as indicated by lower blood pressure, lower serum creatinine levels, and less proteinuria, respectively. The cell numbers that were used in this study were equal to cell numbers in our investigation, and cells were administered at days 1 and 7 after surgery. However, animals were analyzed at 12 wk and not at 8 wk after 5/6-nephrectomy. Our study did not show improved renal function after administration of untreated or pretreated eEOCs at 8 wk after surgery, but proteinuria was reduced significantly if untreated
cells had been injected. In this group, interstitial fibrosis was reduced as well. This clearly indicated renoprotective actions of the cells in 5/6-nephrectomy, and one could assume that improvement of renal function may be detectable later after surgery. A critical and yet inexplicable aspect is related to proteinuria and fibrosis in animals injected with BMP-5-treated eEOCs. In AKI, BMP-5 promoted renoprotection of eEOCs in the short term. In 5/6-nephrectomy, renal function remained unaffected, but interstitial fibrosis was increased. At this point, our knowledge of EPC biology in the context of BMP actions is still less than limited. BMP-5 significantly activates eEOCs in vitro. The cells’ migratory activity and survival were improved by the protein, which indicates increased cell competence. At some point, proangiogenic competence must be transduced to endothelial or tubular epithelial or other cells within the kidney. Thus there are, most likely, yet-unknown paracrine mediators produced by the cells that mediate either protective or deleterious actions, depending on the postischemic “paracrine microenvironment.” This is also suggested by a more recent study by Burger and colleagues (5). In an attempt to prevent mice from AKI, CD133+ human cord blood cells were injected after renal ischemia. Such a measure surprisingly resulted in aggravation of renal failure that was attributed to yet-unknown soluble factors.

Hence, BMP-5 could also activate the cells by antagonizing cellular production/release of reactive oxygen species (ROS). In a study by Goligorsky’s group (9), the glutathione peroxidase mimetic ebselen has been documented to restore stem cell competence in obesity-induced diabetes. BMP-7, for instance, has been shown to reduce oxidative stress in rat diabetic nephropathy (32). BMP-2, on the other hand, increased oxidative stress and promoted human coronary artery smooth muscle cell calcification (14). Nevertheless, oxidative stress is being discussed as an essential perpetuating factor in diabetic nephropathy (12), and MSCs have been shown to act renoprotectively in streptozotocin (STZ)-induced diabetes by reducing production of ROS in rats (10).

With regard to interstitial fibrosis in 5/6-nephrectomy, we finally analyzed the process of EnMT. This mechanism has been proposed to contribute to renal fibrosis in experimental Alport syndrome, STZ-induced murine diabetes, and unilateral ureteral obstruction, respectively (34). Percentages of αSMA expression in CD31+ (endothelial) cells were comparable with those in previous reports (34). Cell therapy did, however, not modify percentages of double-positive cells in 5/6-nephrectomy, which indicates that antifibrotic actions of untreated eEOCs are mediated by other mechanisms [e.g., inhibition of epithelial-mesenchymal transition, inhibition of matrix synthesis by local fibroblasts (36)]. Another interesting and yet unexplainable observation was related to the types of vessels that displayed de novo expression of αSMA in endothelial cells. Zeisberg and colleagues (34) detected αSMA in endothelial cells within very small peritubular vessels, such as small arterioles and capillaries. We detected αSMA only in smaller arteries but not within the peritubular microvasculature. These conflicting results may be explained partly by methodological differences, since in the Zeisberg study (34), diabetic mice and animals with Alport renal disease were evaluated at signifi-
cently later time points than in our study. The time-related dynamics of EnMT still need to be determined.

The complex biology of EPCs, which, in part, are represented by eEOCs, is still far from being understood. Nevertheless, this study clearly shows BMP-5 as a potent agonist of eEOCs in AKI and (partly) in 5/6-nephrectomy. Although BMP-5 promotes migration and survival of eEOCs, it still remains to be analyzed by which mechanisms—potentially besides paracrinic actions—the cells communicate with host cells in the kidney.

AUTHOR CONTRIBUTIONS

Author contributions: D.P. conception and design of research; K.S., A.L., N.M., E.H., J.U.B., S.P., and G.A.M. data; D.P. prepared figures; D.P. drafted manuscript; G.A.M. edited and approved final version of manuscript.

DISCLOSURES

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

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