eEOC-mediated modulation of endothelial autophagy, senescence, and EnMT in murine diabetic nephropathy


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Diabetic nephropathy has emerged as the most frequent single cause of end-stage renal disease in our society (21). Microvascular damage is a key event in diabetes-associated organ malfunction. In a complex cascade of molecular events, chronic hyperglycemia induces the glycosylation of vascular proteins in a nonenzymatically manner. The ensuing accumulation of advanced glycation end products causes severe dysfunction of endothelial cells (ECs) in the whole human organ.

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Early endothelial outgrowth cells (eEOCs) act protective in murine acute kidney injury. The aim of the present study was to analyze consequences of eEOC treatment of murine diabetic nephropathy with special attention on endothelial-to-mesenchymal transdifferentiation, autophagy, senescence, and apoptosis. Male C57/B16N mice (8–12 wk old) were treated with streptozotocin for 5 consecutive days. Animals were injected with untreated or bone morphogenetic protein (BMP)-5-pretreated syngeneic murine eEOCs on days 2 and 5 after the last streptozotocin administration. Four, eight, and twelve weeks later, animals were analyzed for renal function, proteinuria, interstitial fibrosis, endothelial-to-mesenchymal transition, endothelial autophagy, and senescence. In addition, cultured mature murine endothelial cells were investigated for autophagy, senescence, and apoptosis in the presence of glycated collagen. Diabetes-associated renal dysfunction (4 and 8 wk) and proteinuria (8 wk) were partly preserved by systemic cell treatment. At 8 wk, antiproteinuric effects were even more pronounced after the injection of BMP-5-pretreated cells. The latter also decreased mesenchymal transdifferentiation of the endothelium. At 8 wk, intrarenal endothelial autophagy (BMP-5-pretreated cells) and senescence (native and BMP-5-treated cells) were reduced. Autophagy and senescence in/cultured mature endothelial cells were dramatically reduced by eEOC supernatant (native and BMP-5). Endothelial apoptosis decreased after incubation with eEOC medium (native and BMP-5). eEOCs act protective in diabetic nephropathy, and such effects are significantly stimulated by BMP-5. The cells modulate endothelial senescence, autophagy, and apoptosis in a protective manner. Thus, the renal endothelium could serve as a therapeutic target in diabetes-associated kidney dysfunction.

Animal models. Animal experiments were performed in accordance with guidelines of the German Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. C57BL/6N mice were bred in the local animal facility of Göttingen University Hospital. For all experiments, male C57BL/6N mice (8–12 wk old) 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.

Streptozotocin treatment. Diabetes mellitus was induced by intraperitoneal injections of streptozotocin (STZ, 50 mg/kg, Sigma, St. Louis, MO) for 5 consecutive days. Successful induction of diabetes was monitored by testing glucose levels in plasma on day 7 and weeks 4, 8, and 12 after the last STZ injection. Injections of 1 × 10⁶ eEOCs in 100 μl endothelial basal medium-2 were performed into the tail vein 2 and 5 days after the last STZ administration.

Culture of mouse-derived eEOCs. The respective procedure has been described in previous studies (10, 11, 15, 17). Briefly, peripheral mononuclear cells were enriched by density gradient centrifugation using Biocoll solution (Biochrom, Berlin, Germany). Cells were mixed, and 4 × 10⁴ cells were plated on 24-well culture dishes coated with collagen I and grown for 7 days.
with human fibronectin (Sigma) and maintained in EC growth medium (EGM-2; Clonetics, Lonza, Walkersville, MD) supplemented with EGM Single-Quots containing 5% FCS. After 4–5 days of culture, eEOCs were identified by the uptake of Dil-labeled acetylated LDL (Invitrogen, Carlsbad, CA) and binding of FITC-labeled Bandeiraea simplicifolia (BS)-1 lectin (Sigma Diagnostics).

In vitro treatment of eEOCs before therapeutic administration. eEOCs used for systemic injections were detached by trypsinization after the first passage. After cells were washed once with PBS, they were resuspended in 100 μl EGM-2 for systemic injection or for further in vitro treatment. For in vitro treatment, eEOCs were incubated with BMP-5 (100 ng/ml in EGM-2, Celprogen Stem Cell Research and Therapeutics, San Pedro, CA) for 60 min at 37°C. After cells were washed once with EGM-2, they were resuspended in 100 μl EGM-2 for systemic injection.

Morphological evaluation of kidneys. For the 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 semiquantitatively assessed by assigning grades of 1 (mild), 2 (moderate), or 3 (severe).

Tissue immunofluorescence microscopy. Formalin-fixed, paraffin-embedded tissue sections were stained with rat anti-mouse CD31 (platelet EC adhesion molecule-1, clone SZ31, Dianova) and rabbit anti-α-smooth muscle actin (α-SMA; EMELCA Bioscience) for primary incubation and Alexa fluor 488 anti-rabbit IgG (Dianova) for secondary incubation, respectively. Primary incubation was performed overnight at 4°C, whereas secondary incubation was performed for 1 h at room temperature. Staining for senescence-associated β-galactosidase (SA-β-Gal) was performed using the SA-β-Gal Staining kit (Cell Signaling Technology) according to the manufacturer’s protocol. Staining of p62 was performed with rabbit anti-p62 (ab91526, Abcam) for primary incubation (4°C overnight) followed by secondary incubation with anti-rabbit 488 (Jackson ImmunoResearch) for 1 h at room temperature. To visualize the nuclei, tissue sections were counterstained with 4,6-diamidino-2-phenylindole. Three view fields per kidney were analyzed for the colocalization of α-SMA and CD31 using ImageJ software.

Endothelial senescence, apoptosis, and autophagy. Cultured mature murine aortic ECs (C57BL/6 mouse aortic EC, Cell Biologics) were used for in vitro experiments. GC and control (NC) treatment were performed as previously described (18). Light chain (LC)3 staining was performed using anti-LC3 (Cell Signaling) for primary incubation (4°C overnight) followed by secondary incubation with NorthernLight 493 anti-rabbit IgG (R&D Systems) for 1 h at room temperature. Perinuclear LC3+ vesicles were enumerated using ImageJ software. For staining of SA-β-Gal, a commercially available kit was used (Cell Signaling Technology) according to the manufacturer’s protocol. Annexin V staining was performed as previously described (16).

Analysis of renal function and proteinuria. The serum creatinine concentration was measured using a commercially available kit (Creatinin PAP, Labor und Technik, Eberhard Lehmann, Berlin, Germany) according to the manufacturer’s protocol. To measure proteinuria, animals were held in metabolic cages for 12 h, and urine was collected over the whole period.

Statistical analysis. Results are expressed as means ± SE. The means of two populations were compared by Student’s t-test. Three or more groups were compared by two-way ANOVA. Differences were considered significant at P < 0.05.
BMP-5-treated eEOCs at 4 and 12 wk. However, at 8 wk, eEOCs inhibit GC-induced senescence and autophagy of cultured mature ECs in an indirect manner. Treatment of mature murine aortic ECs with GC significantly increased the activity of SA-β-Gal over a time course of 96 h (24 h: GC 36 ± 1.7% vs. NC 33 ± 5.8%, P = not significant; 48 h: GC 54 ± 8.9% vs. NC 24 ± 2.7%, P = 0.03; and 96 h: GC 65 ± 3.7% vs. NC 23 ± 4.3%, P = 0.0001; Fig. 3). In contrast, ECs treated with supernatant from native or BMP-5-pretreated cells did not show any SA-β-Gal activity at both 48 and 96 h (Fig. 4). Next, autophagy was evaluated by the quantification of perinuclear LC3+ granules (perinuclear granularity). At 24 h, granularity was most pronounced in GC-treated ECs incubated with medium from untreated eEOCs. The lowest density of perinuclear granules appeared in ECs treated with supernatant from BMP-5-incubated cells (medium: 3.1 ± 0.12 arbitrary units, eEOCs: 0.29 ± 0.11 arbitrary units, and eEOCs + BMP-5: 0.04 ± 0.01 arbitrary units, P = 0.004 for medium vs. eEOCs and P = 0.002 for medium vs. eEOCs + BMP-5; Fig. 5). At 48 and 96 h, perinuclear granularity was generally low with no significant differences between the three groups (48 h: medium 0.14 ± 0.13 arbitrary units, eEOCs 0.23 ± 0.1 arbitrary units, and eEOCs + BMP-5 0.03 ± 0.01 arbitrary units; 96 h: medium 0.5 ± 0.13 arbitrary units, eEOCs 0.13 ± 0.06 arbitrary units, and eEOCs + BMP-5 0.18 ± 0.5 arbitrary units; Fig. 5). Endothelial apoptosis, reflected by positivity for annexin V, increased over time in untreated cells and in ECs incubated with medium from naive eEOCs. It was more pronounced in untreated cells (24 h: untreated NC 22 ± 1%, untreated GC 40 ± 2%, eEOCs + NC 10 ± 1%, and eEOCs + GC 13 ± 1%; 48 h: untreated NC 49 ± 0.8%, untreated GC 49 ± 0.9%, eEOCs + NC 23 ± 1.8%, and eEOCs + GC 21 ± 2.7%; and 96 h: untreated NC 67 ± 0.6%, untreated GC 66 ± 3%, eEOCs + NC 32 ± 0.8%, and eEOCs + GC 27 ± 3.3%). It gradually decreased with the administration of supernatant from BMP-5-pretreated cells (24 h: eEOCs + BMP-5 + NC 19 ± 1.8% and eEOCs + BMP-5 + GC 21 ± 1.3%; 48 h: eEOCs + BMP-5 + NC 15 ± 0.8% and eEOCs + BMP-5 + GC 16 ± 0.8%; and 96 h: eEOCs + BMP-5 + NC 8 ± 0.3% and eEOCs + BMP-5 + GC 6 ± 0.3%). At 24 h, apoptosis was most pronounced in untreated cells incubated with GC (Fig. 5).

**DISCUSSION**

During the last 7 yr, eEOCs, representing one major subpopulation of EPCs, have well been established as a therapeutic measure in murine AKI (10–13, 15, 17). The present study proves that eEOCs can substantially act protective in murine diabetic nephropathy as well. Regarding the cells’ behavior within the perivascular microenvironment, some more recent...
studies have pointed toward new mechanisms involved in eEOC-EC cross-talk. Among those are direct cell-cell communication via so-called nanotubes (23) on the one hand and secretion of microparticles containing certain microRNA molecules (1, 2) on the other hand. It remains to be elucidated to which extent these processes account for EC repair under both physiological and pathological conditions. Nevertheless, there is no doubt that eEOCs (EPCs) predominantly modulate biological properties of endothelial and not of tubular or of certain (nonendothelial) glomerular cells. One essential endothelium-related problem in hyperglycemia, resulting from partial or complete lack of insulin, is aggravated aging or senescence [SIPS (3)] of the cells. This has elegantly been highlighted by Goligorsky and colleagues (19). GC treatment of human umbilical vein ECs induced SIPS after 3 days. While autophagy was significantly stimulated in the early period after GC incubation (1–2 days), it markedly declined later when premature senescence occurred. Autophagy, commonly regarded as endogenous cellular defense mechanism (5, 7), became suppressed by the metabolic abnormalities associated

Fig. 2. Interstitial fibrosis and endothelial-to-mesenchymal transition. Significant fibrosis only occurred at 12 wk but not at 4 or 8 wk (see text). A.1: in this group, administration of BMP-5-treated eEOCs significantly reduced collagen deposition compared with diabetic mice and mice that received native eEOC. A.2-A.4: images depicting interstitial collagen deposition at 12 wk (A.2: DNP, A.3: eEOCs, and A.4: eEOCs + BMP-5). B: increased α-smooth muscle actin (α-SMA) expression in endothelial cells was only detectable at 8 wk. It was also reduced by injection of BMP-5-treated eEOCs. Magnification in A.2–A.4: ×40. Data are given as the relative aberrance from control values in A and as means ± SE in B. *P < 0.05.
with hyperglycemia. Our data confirmed the stimulation of senescence by GC, as indicated by higher SA-β-Gal expression in GC-treated mature murine ECs. Incubation of aortic ECs with supernatant from untreated or BMP-5-pretreated cells completely abrogated increases of SA-β-Gal activity at 96 h. At 8 wk, intrarenal endothelial senescence was dramatically reduced as well. The correct methodologies for analyzing autophagy in cultured cells or in tissues/organs have been recently reviewed in detail (7). We evaluated the dynamics of perinuclear accumulation of LC3+ granules in mature ECs (aortic ECs). While knowing the possible limitations of such approach, we found very low granular densities in aortic ECs treated with supernatant from either untreated or BMP-5-pretreated eEOCs. Granularity was most pronounced in GC-treated aortic ECs incubated with medium alone (at 24 h). Later time points were characterized by very low numbers of perinuclear granules in each group, indicating suppressed autophagocytic activity. It has to be noted that autophagy decreased a little bit earlier than has been reported in a previous study (19), most likely attributable to the other cell type used.

Fig. 3. Intrarenal endothelial autophagy and senescence in the respective groups. A.1–A.3: images depicting the expression of p62 in CD31+ cells. A.4: expression patterns in the different groups. The induction of diabetes increased endothelial p62 levels at 4, 8, and 12 wk. Administration of BMP-5-treated eEOCs reduces endothelial p62 on week 8. B.1–B.3: endothelial senescence-associated β-galactosidase (SA-β-Gal) staining on week 8. B.4: administration of untreated and BMP-5-pretreated cells resulted in lower SA-β-Gal levels in CD31+ cells. NUC, nuclei. Magnification: ×150. Data are given as means ± SE. *P < 0.05.
Nevertheless, if autophagy is understood as self-defensive process, suppressed autophagic activity in cells treated with eEOC supernatant possibly reflects a process of metabolic/functional stabilization of the endothelium. Besides modulating endothelial autophagy and (concomitantly) endothelial senescence in a cell-protective manner, eEOCs also suppressed promesenchymal effects of the diabetic milieu. In recent years, it has come attention that EnMT contributes to tissue fibrosis in the heart and kidney (9, 24, 25). Although the relative amount of EnMT-induced fibrosis may vary, depending on the etiology

Fig. 4. Senescence of cultured mature murine endothelial cells with versus without glycated collagen (GC) treatment. GC induced robust increases of SA-β-Gal over 96 h compared with control treatment (NC). These effects were completely abrogated by incubation of the cells with supernatant from untreated and pretreated eEOCs. Data are given as means ± SE. *P < 0.05.

Fig. 5. Analysis of autophagy and apoptosis in mature GC-treated endothelial cells. A: the perinuclear presence of light chain (LC)3 vesicles was used to evaluate endothelial autophagic activity. B: GC induced autophagy at 6 and 24 h (C) with a subsequent decrease until 96 h (blue line). Cell incubation with supernatant from either untreated or BMP-5-pretreated eEOCs reduced perinuclear granularity at 24 h (green and red lines). C: apoptosis was significantly higher in the presence of medium alone but decreased after cell treatment with supernatant from native or BMP-5-incubated eEOCs. Data are given as means ± SE. *P < 0.05; **P < 0.05 between eEOCs and eEOCs + BMP-5.
of a certain disease and the respective type of tissue involved, chronic nitric oxide inhibitor with N-nitro-l-arginine methyl ester resulted in a significant renal accumulation of collagen type XIII in mice (9). In addition, our more recent study (17) has shown substantial EnMT in 5/6-nephrectomized mice. Several morphological abnormalities that occur in the kidney of patients with chronic kidney disease can also be seen in organs from aging humans, although to a lesser extent (8). One might argue that fibrosis and particularly fibrosis due to EnMT are hallmarks of increased (regular of stress induced) senescence. It can nevertheless not definitely been concluded that EnMT and SIPS are cellular events mechanistically linked to each other in a dynamic or more static manner. However, a new study published by Vasko et al. (22) has shown increased collagen deposition in sirtuin 1 (SIRT1)endo/endo−/− mice restored by the induction of matrix metalloproteinase-4 using Concanavalin A. Since SIRT1 is essential for preventing ECs from SIPS, it has to be realized that senescence and mesenchymal transdifferentiation of the endothelium are at least associated events. A more general conclusion that must be drawn from this study is the apparently essential role of ECs in halting the progression of diabetic nephropathy. By stabilizing the cells’ functional integrity, using eEOCs (or possibly other cell types, such as late endothelial outgrowth cells or mesenchymal stem cells), renal dysfunction and proteinuria were mitigated and mesenchymal transformation of the endothelium was reduced. Therefore, future diabetes-related research should focus even more on the endothelial lineage, possibly allowing to establish therapeutic interventions targeting the inner layer of peritubular and glomerular blood vessels. In this context, it has to be mentioned that therapeutic effects of eEOCs were not detectable over the whole period of postdiabetes induction. For instance, renal function and proteinuria were stabilized/di−/− mished only in week 8 after the last STZ administration. Such effects were not seen in week 12, most likely due to a loss of therapeutic efficiency. Diabetic nephropathy is a long-lasting pathological process that may not be halted by only two individual injections of untreated or BMP-5-preconditioned eEOCs. Chronic diseases require chronic or at least repeatedly performed therapeutic interventions. Therefore, future investigations will also have to focus on the exact schedule of cell administration necessary for optimized disease control.

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