Early-Outgrowth Bone Marrow Cells Attenuate Renal Injury and Dysfunction via an Antioxidant Effect in a Mouse Model of Type 2 Diabetes

Yanling Zhang, Darren A. Yuen, Andrew Advani, Kerri Thai, Suzanne L. Advani, David Kepecs, M. Golam Kabir, Kim A. Connelly, and Richard E. Gilbert

Cell therapy has been extensively investigated in heart disease but less so in the kidney. We considered whether cell therapy might also be useful in diabetic kidney disease. Cognizant of the likely need for autologous cell therapy in humans, we sought to assess the efficacy of donor cells derived from both healthy and diabetic animals. Eight-week-old db/db mice were randomized to receive a single intravenous injection of PBS or 0.5 × 10^6 early-outgrowth cells (EOCs) from db/db or db/db mice. Effects were assessed 4 weeks after cell infusion. Untreated db/db mice developed mesangial matrix expansion and tubular epithelial cell apoptosis in association with increased reactive oxygen species (ROS) and overexpression of thioredoxin interacting protein (TxnIP). Without affecting blood glucose or blood pressure, EOCs not only attenuated mesangial and peritubular matrix expansion, as well as tubular apoptosis, but also diminished ROS and TxnIP overexpression in the kidney of db/db mice. EOCs derived from both diabetic db/db and nondiabetic db/m mice were equally effective in ameliorating kidney injury and oxidative stress. The similarly beneficial effects of cells from healthy and diabetic donors highlight the potential of autologous cell therapy in the related clinical setting. *Diabetes* 61:2114–2125, 2012
Bone marrow harvesting and cell culture. EOCs were cultured as previously described (8). In brief, bone marrow cells were collected from the femur and tibiae of 3- to 4-week-old male db/m or db/db mice and cultured in endothelial growth medium-2 (EGM-2; Lonza, Walkersville, MD) at 37°C with 5% CO₂ for 7-10 days to produce EOCs.

Cell infusion. EOCs were washed with DPBS to remove all medium components. Viable cells were analyzed by trypan blue exclusion and counted by a hemocytometer. Cells were resuspended in DPBS at a final concentration of 2 x 10⁶ EOCs/mL. Eight-week-old-db/m mice received an infusion of 5 x 10⁶ db/m EOCs, 5 x 10⁶ db/db EOCs, or DPBS by tail vein injection.

Renal and metabolic parameters. Body weight and glucose levels (Accu-Check Advantage; Roche, Mannheim, Germany) were determined biweekly. Urinary albumin excretion was determined after 24 h of metabolic caging at baseline and study end with an AssayMax mouse albumin ELISA (Assaypro, St. Charles, MO). At the end of the study, invasive blood pressure was measured in anesthetized mice (2% isoflurane). Renal histology. Four-micrometer sections were used for periodic acid–Schiff staining or immunostaining. Frozen tissues embedded in cryostat matrix were cut into 30-μm sections prior to staining and confocal microscopic analysis. Mesangial expansion was assessed as previously described (19). Immunohistochemistry was performed as previously described (19). Stained sections were imaged with the Aperio Ultra-High-Resolution Digital Scanning System (Aperio Technologies, Vista, CA), with the acquired images analyzed using Aperio ImageScope software. Type IV collagen degradation was quantified in 30 randomly selected glomeruli and 15 random, nonoverlapping 20× fields for each animal in a masked fashion.

Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling. Renal cell apoptosis was assessed using terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL). TUNEL-positive cells were counted in 50 randomly selected glomeruli and in 15 random, nonoverlapping 20× fields in the cortical tubulointerstitium for each kidney section.

Renal oxidative stress. Superoxide ion generation was assessed by quantification of the conversion of dihydroethidium to ethidium, as previously described (20). Images were obtained with a Leica TCS SL confocal microscope. Fluorescence was detected with a 568-nm long-pass filter. Laser settings were identical for acquisition of images from all specimens. Kidney sections were placed in 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2-DCFDA; Invitrogen), as previously described (18). The intracellular ROS was measured by measuring 10,000 events per sample following excitation with a 488-nm wavelength laser and reading through a 525/50 filter using a flow cytometer (FACSCalibur). Flow cytometry data were analyzed using WinMDI 2.9 (Scripps Institute, La Jolla, CA).

Real-time quantitative RT-PCR. Real-time quantitative PCR was used to determine the relative expression levels of Trx and TrxIP transcripts as described previously (16). Primers were obtained from Sigma-Aldrich. Primer sequences were as follows: mRPL13a forward GCT CAC AAG GGT CTG CTG G and reverse AGA TCT TCT TCT TCC GAT A; mouse TrxIP forward TCA AGG GCC CCT GGC AAG ATC C and reverse GAC ACT GGT GCC ATT AAG TCA G; and mouse Trx forward CAA ATA ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT
or db/db donor mice showed no significant progression (157 ± 5 vs. 104 ± 5 mg/day for db/db EOC recipients, 8 vs. 12 weeks, P = NS; 140 ± 6 vs. 185 ± 9 mg/day for db/m EOC recipients, P = NS).

**Cell surface markers.** To further determine the attributes of the EOCs, we examined their cell surface markers by fluorescence-activated cell sorter analysis. Among freshly isolated nucleated bone marrow cells, 65% were Ly6C⁺, 20% CD11b⁺, 6% CD34⁺, 7% CD133⁺, and 3% VEGFR²⁺ (Fig. 1A–H). In contrast, virtually all EOCs were CD34⁺ and VEGFR²⁺, albeit weakly so. No cell surface expression of either CD133 or the monocyte marker CD11b was noted among EOCs, and although the vast majority of cells were Ly6C⁻, a small proportion (0.42%) was strongly positive for this cell surface marker (Fig. 1I–P).

**Histopathology.** When compared with their nondiabetic, db/m counterparts, PAS-stained kidneys of db/db mice displayed prominent mesangial expansion and glomerular hypertrophy, which was ameliorated although not entirely normalized by EOC administration (Fig. 2A–F). Collagen type IV immunolabeling was localized to the glomerular mesangium. When compared with nondiabetic db/m mice, the kidneys of diabetic db/db animals demonstrated substantially increased abundance of immunostainable collagen IV in mesangial areas. Treatment with EOCs from either db/m or db/db donor mice attenuated these changes (Fig. 2G–K).

![Graphs](image-url)

**FIG. 1.** Cell surface markers. Cell surface markers were examined by flow cytometry in freshly isolated nucleated bone marrow cells (A–H) and cultured EOCs (I–P). Freshly isolated nucleated bone marrow cells were mostly Ly6C⁻ (65%) with other cell surface markers present on a smaller proportion of cells (20% CD11b⁺, 6% CD34⁺, 7% CD133⁺, and 3% VEGFR²⁺). Almost all EOCs were weakly CD34⁺ and VEGFR²⁺. A small proportion of EOCs (0.42%) was strongly positive for Ly6C. Negative control for Ly6C (A and I); Ly6C (B and J); negative control for CD11b (C and K); CD11b (D and L); negative control for CD34, CD133, and VEGFR (E and M); CD34 (F and N); CD133 (G and O); and VEGFR (H and P). Cells incubated with conjugated irrelevant IgGs were used as negative controls.
FIG. 2. Glomerular pathology is ameliorated by EOC administration. Kidney sections were stained with PAS (A–D) or immunolabeled with anticollagen type IV antibody (G–J). Representative photomicrographs from db/m mice (A and G), PBS-treated db/db mice (B and H), db/m EOC-treated db/db mice (C and I), and db/db EOC-treated db/db mice (D and J) are shown along with graphs displaying the magnitude of glomerular volume (E), mesangial expansion (F), and glomerular collagen IV immunostaining (K). Original magnification: ×400. *P < 0.05 vs. db/m animals; †P < 0.05 vs. PBS-treated db/db animals. (A high-quality digital representation of this figure is available in the online issue.)
Peritubular matrix expansion and tubular epithelial cell apoptosis are attenuated by EOC administration. At 12 weeks of age, when compared with db/m mice, the kidneys of db/db mice demonstrated significantly increased collagen IV accumulation in peritubular regions, which was reduced in the kidneys of both db/m EOC- and db/db EOC-treated animals (Fig. 3A–D and I). TUNEL-positive apoptotic cells were only rarely seen in db/m mouse kidneys (Fig. 3E). In contrast, numerous TUNEL-positive cells were readily apparent within the tubules of db/db mice (Fig. 3F). Diabetic db/db mice that had received EOCs from either db/m or db/db donor mice, however, showed a marked reduction in the extent of TUNEL labeling in their tubules (Fig. 3G and H). Quantification of apoptotic cells in tubules is shown in Fig. 3J. Apoptotic cells were not present in glomeruli of either db/db or db/m mice.

EOCs reduce superoxide generation in the diabetic kidney. Kidney sections, labeled with dihydroethidium, which produces a red fluorescence when oxidized to ethidium in the presence of O$_2^-$, demonstrated an approximately threefold increase in fluorescence within the cortical tubules and glomeruli of db/db mice when compared with nondiabetic db/m animals ($P < 0.05$). These diabetes-related changes were substantially diminished in animals that had received EOCs from either db/m or db/db donor mice (Fig. 4A–J).

Renal expression of Trx and TxnIP. To investigate the mechanisms underlying the increased oxidative stress observed in the kidney of db/db mice, we examined the expression of Trx and TxnIP along with other enzymes that modulate ROS. TxnIP mRNA was 6.5-fold higher in untreated diabetic db/db mice than in db/m controls ($P < 0.05$). EOC infusion from either db/m or db/db donor mice
FIG. 4. EOC treatment reduces oxidative stress and \textit{TxnIP} overexpression in the kidney of db/db mice. Representative photomicrographs showing ethidium fluorescence (red) in glomeruli and tubulointerstitium of db/m mice (\(A\) and \(E\)), PBS-treated db/db mice (\(B\) and \(F\)), db/m EOC-treated db/db mice (\(C\) and \(G\)), and db/db EOC-treated db/db mice (\(D\) and \(H\)). \(I\) and \(J\): Quantification of ethidium fluorescence in glomeruli and tubulointerstitial area also are shown. EOC-induced reduction in ethidium fluorescence was accompanied by attenuation in the overexpression of \textit{TxnIP} in the kidneys of EOC-treated db/db mice (\(K\)), as assessed by quantitative RT-PCR. \(L\): Renal \textit{Trx} mRNA was similar in all four groups. \(M\): Nox-4 mRNA was elevated in the kidneys of db/db mice and was unaffected by EOC treatment. Ethidium fluorescence and mRNA levels of \textit{TxnIP}, \textit{Trx}, and Nox-4 were all expressed relative to db/m mice levels (that were arbitrarily assigned a value of 1). DHE, dihydroethidium; original magnification in glomeruli: \(\times 400\) (\(A-D\)); in tubulointerstitial areas \(\times 40\) (\(E-H\)). *\(P < 0.05\) vs. db/m animals; †\(P < 0.05\) vs. PBS-treated db/db animals. (A high-quality digital representation of this figure is available in the online issue.)
reduced the magnitude of TcxnIP mRNA overexpression to a similar extent (Fig. 4K). Renal Trx expression, on the other hand, was similar in all four groups, and although Nox-4 expression was increased twofold in db/db mice kidneys, it was unaffected by EOC treatment (Fig. 4L and M). No difference in catalase, Cu/Zn SOD, or MnSOD expression in the kidneys between the groups was noted (data not shown).

**Administered EOCs are not retained in the kidney.**
EOCs that had been labeled with the fluorescent marker, CMTMR, were notably absent from the kidneys of db/db mice at both early and later time points after EOC infusion. In contrast, abundant labeled cells were present in the liver, spleen, bone marrow, and lung, persisting in all but the latter during the 4 weeks of study (Fig. 5). However, no CMTMR-labeled fibroblasts were detected in any of these organs 4 weeks following their infusion.

**EOC-conditioned medium attenuates oxidative stress in cultured proximal tubular epithelial cells.** Given the lack of EOC retention within the kidney, we considered whether these cells may be secreting factors that diminish oxidative stress. The accumulation of ROS within NRK
cells, as demonstrated by increased CFDA fluorescence (Fig. 6A and B), increased ninefold when cells were cultured in 25 mmol/L glucose compared with those in 5.6 mmol/L glucose \((P < 0.001)\). Coincubation of NRK cells with EOC-conditioned medium from both db/m mice and db/db mice attenuated this high glucose–induced effect \((P < 0.001)\).

**EOC-conditioned medium inhibits high glucose–induced upregulation of TxnIP in cultured proximal tubular epithelial cells.** Given the alterations in renal TxnIP expression with EOC infusion, we next examined whether EOC-derived factors might regulate TxnIP expression in NRK tubular cells that had been cultured in high glucose–containing medium. TxnIP mRNA level was fivefold higher in cells cultured in 25 mmol/L glucose-containing medium when compared with cells cultured in 5.6 mmol/L glucose. This robust high glucose–associated increase in TxnIP mRNA level was nearly normalized by coincubation with EOC-conditioned medium \((P < 0.05)\) (Fig. 6C).

**Secreted factors.** Given the dearth of labeled EOCs in the kidney, we assessed their secretory activity using a cytokine antibody array (Fig. 7). Among the 40 different cytokines assayed, 11 were detected, several of which were in particularly high abundance, such as interferon γ–induced protein 10 (CXCL10/CRG-2), a chemokine for mesenchymal stem cells (21); interleukin (IL)-1ra (IL-1F3), the endogenous antagonist of the proinflammatory IL-1

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**FIG. 6.** EOC-conditioned medium reduces glucose-induced ROS and TxnIP overexpression in cultured proximal tubular epithelial cells. A: The intracellular ROS production was measured by flow cytometry after staining cells with CM-H2-DCFDA. NRK tubular cells were cultured in normal glucose (NG; 5.6 mmol/L) or high glucose (HG; 25 mmol/L) in the presence of endothelial cell basal medium-2 (EBM-2) or EOC-conditioned medium. A total of 25 mmol/L mannitol served as osmotic control. Both db/m EOC CM and db/db EOC CM attenuated high glucose–induced ROS \((B)\) and TxnIP mRNA overexpression \((C)\). TxnIP mRNA was expressed relative to the normal glucose group that was arbitrarily assigned a value of 1. Samples were run in triplicate. *\(P < 0.001\) vs. normal glucose; †\(P < 0.001\) vs. high glucose.
FIG. 7. EOC-secreted factors. EOC-secreted factors were assessed using a cytokine antibody array. Ten cytokines were detected among the 40 different analytes (A) and the magnitude of expression quantified using image analysis (ImageJ; National Institutes of Health) (B). IL-1ra, IL receptor antagonist; IP-10, interferon-γ–induced protein 10; JE, also known as MCAF, TDCF, and SMC-CF; KC, keratinocyte-derived chemokine; M-CSF, macrophage colony stimulating factor; MCP-1, monocyte-specific cytokine; MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1β; MIP-2, macrophage inflammatory protein-2; SDF-1, stromal cell–derived factor-1; sICAM-1, soluble intercellular adhesion molecule-1; TIMP-1, tissue inhibitor of metalloproteinase-1.

|       | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|-------|----|----|----|----|----|----|----|----|----|----|----|----|
| A     | PC | blank | blank | blank | blank | blank | blank | blank | blank | blank | blank | PC |
| B     | BLC | C5a | G-CSF | GM-CSF | I-309 | Eotaxin | sICAM-1 | IFN-γ | IL-1α | IL-1β | IL-1ra | IL-2 |
| C     | IL-3 | IL-4 | IL-5 | IL-6 | IL-7 | IL-10 | IL-12 | IL-16 | IL-17 | IL-23 | IL-27 |
| D     | IP-10 | I-TAC | KC | M-CSF | JE | MCP-5 | Mig | MIP-1α | MIP-1β | MIP-2 | RANTES | SDF-1 |
| E     | TARC | TIMP-1 | TNF-α | TREM-1 |       |       |       |       |       |       |       | NC |
receptor (22); and tissue inhibitor of metalloproteinase-1, an inhibitor of matrix metalloproteases with antiapoptotic action (23). Together these factors, among others, indicate the potential for the secretory output of EOCs to mobilize other reparative cell types, dampen inflammation, and protect cells from injury and death.

**DISCUSSION**

Using a well-established rodent model of diabetic nephropathy, a single infusion of EOCs not only ameliorated oxidative stress but also reduced extracellular matrix deposition and tubular epithelial cell apoptosis. In addition to these structural effects, the progressive rise in albuminuria, characteristic of the db/db mouse model of experimental nephropathy, also was attenuated. Despite these benefits, however, only minimal retention of administered cells was noted in the kidneys of diabetic animals, contrasting with their relative abundance in the liver, spleen, and bone marrow. These findings, in conjunction with the ability of EOC-conditioned medium to abrogate high glucose–induced oxidative stress in vitro, suggest that this form of cell therapy may mediate its effects by mechanisms that include, at least in part, the elaboration of soluble factors with antioxidant activity that act at distant sites.

The initial description of circulating bone marrow–derived EOCs by Asahara and colleagues (24,25) more than a decade ago has been followed by a deluge of studies, many using a range of different cell types although still referring to them all as endothelial progenitor cells. To date, however, no unique cell surface marker(s) have been shown to specifically identify the cell types responsible for the reparative activity. Instead, many investigators have used a combination of cell surface markers or alternatively, as in the current study, described them primarily according to the culture techniques used to produce them. With the latter system of classification, adherent bone marrow cells maintained in endothelial media are referred to as either EOCs or late-outgrowth cells, depending on their length of culture, differing with regard to their lineage, phenotypic markers, and function (7,26). Although late-outgrowth cells more closely resemble mature endothelial cells, EOCs are secretory, tend not to integrate into pre-existing structures, and exhibit a phenotype that seems most closely related to immature monocytes (7,27). In accordance with this nomenclature, the cells used in the current study, having been cultured for 7–10 days, would be classified as EOCs. Their cell surface markers, although substantially different from freshly isolated bone marrow cells, expressed markers such as CD34 and VEGFR-2 that have been typically used to define so-called endothelial progenitor cells (28).

The mechanisms by which progenitor cells mediate organ repair and regeneration remain speculative. Although their differentiation into mature parenchymal cells or their fusion with such cells was initially believed to account for their beneficial effects, later studies have emphasized the paracrine actions of bone marrow–derived cells. However, with regard to their effects in the kidney, still other mechanisms may be operational. In particular, several very recent studies have reported the near-complete absence of the administered cells in the kidney, despite notable improvements in organ function and structure. For instance, in ischemic and cisplatin-induced acute kidney injury, three different groups have reported the dearth or absence of infused marrow stromal cells in the kidney, although renal repair was enhanced in each (3,29,30). Likewise, Yuen et al. (8) recently reported the absence of infused EOCs in the remnant kidney model of chronic renal disease, despite a reduction in proteinuria and improvement in glomerular filtration rate. Although the findings in some of these studies had been interpreted as being the consequence of a paracrine effect that might occur during the cell’s brief sojourn through the kidney, other interpretations also are plausible. In particular, using different cell types and in both acute and chronic kidney injury, both Bi et al. (3) and Yuen et al. (8) have provided evidence of an endocrine mechanism to explain the beneficial effects of cell therapy. Consistent with these studies, we also noted the near-total absence of labeled EOCs in the kidneys of diabetic mice, contrasting with their abundance in the liver, lung, spleen, and bone marrow, suggesting that these cells may be exerting their effects by the distant secretion of renoprotective factors.

Hyperglycemia-induced overproduction of superoxide by the mitochondrial electron transport chain provides a unifying and coherent theme for understanding the pathogenesis of diabetes complications (11,31). Molecular species vary in their propensity to oxidative damage. For instance, the sulfur-containing amino acid residues cysteine and methionine are exquisitely sensitive to oxidative stress (32), although they also can be readily reduced by the enzyme, Trx (33). This highly and ubiquitously expressed di-thiol protein efficiently reduces oxidized sulfhydryl groups. Its activity, however, is tightly regulated by an endogenous inhibitor, TxnIP, a small 38–amino acid protein (34) that not only impedes the reductive capacity of Trx but also promotes apoptosis (35). Among the factors known to regulate TxnIP expression, the induction of its transcription in response to high glucose, reflecting the carbohydrate response element in its promoter (36), is particularly dramatic and widespread (12–17). As previously reported (16), we also noted increased TxnIP mRNA in the kidneys of diabetic animals. This overexpression was, however, substantially ameliorated in diabetic mice that had received EOCs. Likewise, incubating kidney cells in high glucose resulted in increased TxnIP. However, although still in 25 mmol/L glucose, incubating these same cells in the presence of culture medium that had been used to grow EOCs resulted in normalization of TxnIP mRNA. These findings suggest that the secretory product(s) of EOCs may dampen oxidative stress in the diabetic kidney by abrogating the high glucose–induced upregulation of TxnIP overexpression. Although reduced, the increased ROS in animals that received EOCs was not normalized, possibly reflecting the absence of any demonstrable effect of EOC treatment on Nox-4 expression.

Diabetic nephropathy is characterized histopathologically by glomerulosclerosis, interstitial fibrosis, and tubular atrophy (37,38). Unfortunately, like all rodent models of diabetes complications, the db/db mouse is imperfect. It does, however, display a range of structural and functional features akin to human disease. For instance, mesangial expansion, a close correlate of kidney dysfunction in patients with diabetic nephropathy (39), is a prominent histopathological feature of db/db mice. In the current study, we found that EOC administration diminished mesangial expansion in diabetic mice, and although the peritubular collagen deposition seen in the db/db mouse is relatively modest, this too was reduced by EOC treatment.
In addition to fibrosis, tubular atrophy also is a feature of disease progression in diabetic nephropathy (37,40). Although the mechanisms responsible for the atrophy are incompletely understood, apoptosis is likely to contribute (37). Like humans (37,41), tubular apoptosis is a feature of diabetic kidney disease in rats and mice (20,42,43), where ROS has been shown to play a key role in its development (44,45). In the current study, tubular epithelial cell apoptosis, recognized by the presence of DNA fragmentation, was increased in the kidneys of db/db mice. EOC administration reduced the extent of TUNEL labeling to that seen in nondiabetic db/m mice, in association with reduction in ROS.

The equally beneficial effects of EOCs derived from diabetic as well as nondiabetic mice, demonstrated in the current study, highlights the potential for autologous therapy in humans. Of note, the cells were administered intravenously and not directly into the kidney parenchyma. Indeed, the beneficial effects were apparent in the absence of their retention within the kidney, a finding that is consistent with the secretory activity of these cells that included the ability to diminish glucose-induced stimulation of TrxIP expression. Whether similar mechanisms apply to other cell types, to other models of diabetic nephropathy, or ultimately to humans, however, remains speculative.

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