Adult skeletal muscle stem cells differentiate into endothelial lineage and ameliorate renal dysfunction after acute ischemia


We previously demonstrated that endothelial cells are severely damaged during renal ischemia-reperfusion and that transplantation of adult human endothelial cells into athymic nude rats subjected to renal ischemia resulted in a dramatic protection of the kidney against injury and dysfunction. Morphological studies demonstrated the engraftment of transplanted cells into renal microvasculature. The goal of the present study was to determine the potential efficacy of in vitro expanded skeletal muscle-derived stem cells (MDSC) differentiated along the endothelial lineage in ameliorating acute renal injury. MDSC obtained from the Tie-2-green fluorescent protein (GFP) mice were used as donors of differentiated and nondifferentiated stem cells. FVB mice, used as recipients, were subjected to renal ischemia and transplanted with the above MDSC. The differentiation of MDSC along the endothelial lineage was monitored by the appearance of Tie-2 promoter-driven expression of GFP. These mouse endothelial cell antigen-, endothelial nitric oxide synthase (eNOS)-, Flk-1-, Flt-1-, and CD31-positive cells engrafted into renal microvasculature and significantly protected short-term renal function after ischemia. Transplantation of nondifferentiated MDSC characterized by the expression of Sca-1 (low levels of CD34, Flk-1, and cKit, and negative for GFP, eNOS, and CD31) did not improve short-term renal dysfunction. In conclusion, the data 1) provide a rich source of MDSC, 2) delineate protocols for their in vitro expansion and differentiation along the endothelial lineage, and 3) demonstrate their efficacy in preserving renal function immediately after ischemic insult.

Adult skeletal muscle contains unipotent satellite cells, which fuse and differentiate to a multinucleated myotube (2, 16). In addition, there are multipotent stem cells characterized by a broader phenotypical differentiation spectrum as illustrated by the adult bone marrow stem cells capable of differentiating into ectodermal and mesodermal phenotypes (9, 10, 20). One of us previously developed procedures to isolate the latter stem cells from adult skeletal muscle and demonstrated their potential to give rise to cells of different lineages (30, 31). This finding, together with the growing skepticism as to the extent of in vivo transdifferentiation (1, 18), prompted us to investigate the potential of these stem cells to differentiate ex vivo into endothelial progenitor and/or endothelial cells.

We previously observed that endothelial cells are severely damaged during renal ischemia-reperfusion and that transplantation of adult human endothelial cells into athymic nude rats subjected to renal ischemia resulted in a dramatic protection of the kidney against injury and dysfunction (3). This finding served as a proof-of-principle demonstration of the critical role of endothelial dysfunction in the pathogenesis of acute renal injury. Morphological studies demonstrated the engraftment of transplanted cells into renal microvasculature. The choice of the differentiated cells over stem cells was made based on a prediction that acute injury would be best managed by supplying a terminally differentiated cellular substitute. It has previously been demonstrated that bone marrow cells, engrafted into transplanted kidneys in both mice and humans (7, 23), eventually differentiated into epithelial cells and podocytes. More recently, two studies using adult bone marrow-derived stem cells for therapeutic transplantation in the postischemic period, one using stem cells derived from the marrow stroma and the other using hematopoietic stem cells, have been published (12, 15). Transplantation of the stem cells derived from the stroma resulted, within 24-h postischemia, in the increased number of Lin-negative Sca-1-positive circulating stem cells from 1.4 to 23.8%. These cells, 48-h and 1-wk postischemia, were found to repopulate tubular epithelium in the outer medulla but not in the cortex (12). When hematopoietic stem cells from ROSA26 mice were transplanted in the postischemic period, Lin et al. (15) showed the presence of β-galactosidase-positive cells in the regenerating renal tubules 4 wk after the intervention. Although immediate functional benefits of such a procedure remain obscure, it is reasonable to surmise that this therapy may be beneficial for a long-term recovery of tubular function. In neither study were transplanted cells detected in the renal microvasculature.
In an attempt to resolve the emerging controversy as to the optimal degree of differentiation of transplanted cells and the site of engraftment of these cells, we hypothesized that endothelial progenitor cells should have a much higher affinity for homing within the vasculature than less-differentiated adult stem cells, whether stromal or hematopoietic stem cells. Therefore, we undertook a study of adult stem cells isolated from the skeletal muscle of Tie-2-green fluorescent protein (GFP) mice, in vitro expanded and differentiated into the endothelial progenitors and/or differentiated endothelial cells, and transplanted them in mice subjected to renal ischemia. Transplantation of these cells resulted in the microvascular engraftment and amelioration of renal dysfunction, in contrast to the transplantation of nondifferentiated stem cells.

MATERIALS AND METHODS

Animal model. The animal study protocol was in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services Public Health Services, NIH, NIH Publication No. 86–23, 1985) and approved by the Institutional Animal Care and Use Committee. Tie-2-GFP mice on FVB background were obtained from Jackson Labs (Bar Harbor, ME). These mice express GFP driven by an endothelial-specific and -selective promoter for Tie-2 receptor, resulting in specific fluorescence of endothelial cells as previously described (17). FVB mice were used as recipients of transplanted cells obtained from Tie-2/GFP mice. All animals were separately caged with a 12:12-h light-dark cycle and had free access to water and chow throughout the study.

Surgical procedures. After an overnight fast, FVB animals were anesthetized with a combination of 6.0 mg/100 g ketamine hydrochloride and 1.3 mg/100 g xylazine. The kidneys were exposed through a midline incision, and the left kidney was subjected to 90 min of ischemia. After reperfusion, the animals were sacrificed and the kidneys were harvested for histological analysis.

Fig. 1. Immunocytochemical characterization of adult skeletal muscle-derived stem cells. Freshly isolated adult skeletal muscle-derived stem cells were allowed to adhere and spread on gelatin-coated coverslips, fixed, and stained for the expression of indicated markers, as detailed in MATERIALS AND METHODS. Note that these cells are Sca-1 positive, c-Kit negative, and CD34 positive but are almost completely devoid of endothelial cell markers (endothelial nitric oxide synthase (eNOS), CD31, and Tie-2 promoter-driven expression of green fluorescent protein (GFP)). Neg, negative. Magnification ×600.
and 0.77 mg/100 g xylazine hydrochloride, placed on a heated surgical pad, and rectal temperature was maintained at 37°C. A subcutaneous injection of 250 U/kg heparin was given 15 min before the operation. A 1.5-cm midlateralotomy was performed, the right kidney was exposed, and two 3–0 sutures were passed under the renal pedicle. The left kidney was exposed, and the renal artery was separated from the renal vein and underpassed with a 3–0 suture. Renal ischemia was initiated by clamping the left renal artery with microsutures (Fine Science Tools, Forster City, CA). After 30 min, the left renal artery was released, while the right renal pedicle and the right ureter were ligated and right nephrectomy was performed. After the clamp was released, 2 × 10^5 differentiated endothelial or nondifferentiated stem cells suspended in 0.2 ml of Krebs buffer were injected into the aorta (through a catheter inserted into the left carotid artery). The incision was closed with 3–0 suture and surgical staples. Blood was drawn for determination of the serum creatinine (Cr) concentration, using a Raichem kit (San Diego, CA). Sham-operated mice receiving intra-aortic infusion of the same dose of Krebs buffer or cells served as control.

**Cell culture and expansion/stem cell selection.** Hindlimb muscle was harvested from female Tie-2-GFP mice. Muscle tissue was carefully minced with sterile curved scissors in 1 vol of Eagle’s minimal essential medium (EMEM; Gibco, Grand Island, NY). The tissue was then digested in a solution consisting of 1 vol tissue, 4 vol collagenase/disprase, 2 vol 3% bovine serum albumin, and 13 vol EMEM at 37°C for 1–2 h until the tissue was digested. The collagenase solution consisted of 33.3 U type IV collagenase (Gibco) and 33.3 U/ml dispase (Gibco). The suspension was then transferred to centrifuge tubes and centrifuged at 300 g for 20 min. The supernatant was discarded, and the cells were resuspended in 20 ml of EMEM with 10% preselected horse serum. The cells were filtered through a 20-μm Nitex filter to obtain a single-cell suspension, centrifuged at 150 g for 10 min, the supernatant was discarded, and the pellet was resuspended in 10 ml of EMEM with 10% horse serum. The cells were counted in a hemocytometer and plated at 100,000 cells per 100-mm culture dish precoated with 1% bovine gelatin (Fisher).

Cells were expanded to confluence in EMEM supplemented with 10% horse serum with culture medium being replaced every other day (30, 31), then released with trypsin, filtered through a 20-μm filter, and slowly frozen in aliquots of 1 ml containing 10^6 cells in EMEM plus 10% horse serum and 7.5% DMSO to −80°C. After at least 24 h, aliquots of the frozen cells were thawed and replated on gelatin-coated culture dishes in EMEM with 10% horse serum for expansion and later cell culture under differentiation conditions, as detailed below. The above freezing-thawing cycle has been shown to eliminate more than 98% of fibroblasts, thus resulting in the enrichment of stem cells, which preferentially survive this procedure (29).

**Differentiation conditions.** To induce differentiation of endothelial progenitors, expanded stem cells were replated in fibronectin-coated culture dishes and maintained in endothelial cell basal medium-2 (EBM-2; Clonetics) supplemented with 2% fetal bovine serum and EGM supplement kit (bFGF, hydrocortisone, VEGF, ascorbic acid, hEGF, and heparin) for 4 wk. The medium was changed every other day, and cells were examined under fluorescence microscopy for the expression of GFP.

**Immunocytochemical detection of endothelial cell and stem cell markers.** For immunocytochemical analyses, cells were plated in Lab-Tec II chamber slides (Nalge Nunc International) precoated with gelatin (for nondifferentiated cells) or with fibronectin (for differentiated cells). Upon being spread, cells were fixed with methanol at −20°C for 10 min, air dried at room temperature, and washed in PBS. Nonspecific protein binding was blocked by incubation of cells in 2% BSA in PBS for 1 h. Incubation with primary antibody was performed overnight at −4°C. Cells incubated in nonimmune serum instead of primary antibody were processed simultaneously and used as negative control. The following primary antibodies were employed as endothelial cell markers: goat polyclonal anti-Flt-1 (C-17, 1:50), mouse monoclonal anti-Flk-1 (A-3, 1:50), goat polyclonal anti-CD31 (M-20, 1:100; Santa Cruz Biotechnology), mouse monoclonal anti-endothelial nitric oxide synthase (eNOS; 1:50; BD Biosciences), and rat monoclonal anti-mouse endothelial cell antigen (MECA-32, 1:50; Developmental Studies Hybridoma Bank). Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 594 donkey anti-goat IgG, and Alexa Fluor 568 goat anti-rat IgG (1:500, Molecular Probes) were used to visualize mouse, goat, and rat primary antibodies, respectively. To characterize the population of nondifferentiated stem cells, we used FITC-conjugated rat anti-CD34, FITC-conjugated rat anti-CD117 (c-Kit), and R-phycocerythrin-conjugated rat anti-Ly-6A/E (Sca-1; BD Biosciences) antibodies. To visualize the nuclei, cells were counterstained with DAPI (Molecular Probes). Cells were examined using a Nikon compound fluorescence microscope with the appropriate dichroic mirrors.
Labeling and transplantation. Differentiated or nondifferentiated stem cells (2 \times 10^5 cells/animal) were injected into wild-type mice postoperatively. Injected cells were labeled with a Cell Tracker CM-Dil (Molecular Probes). The cells were resuspended and incubated in 5 \mu g/ml of CM-Dil in PBS for 5 min at 37°C, and then 15 min on ice. Cells were pelleted and washed with PBS three times to remove unincorporated fluorophore.

Twenty-four hours after the surgery, mice were euthanized, and the blood and kidneys were obtained. Frozen 20-\mu m-thick sections were prepared and examined using fluorescence microscopy for GFP and Cell Tracker CM-Dil expression. The Cr concentration was measured using Raichem kit.

Statistical analysis. Data are presented as means ± SE. Differences between various treatments were analyzed by one-way ANOVA. Differences were considered significant at \( P < 0.05 \).

RESULTS

Characterization of skeletal muscle-derived stem cells. The cells isolated from skeletal muscle of the Tie-2-GFP mouse were immunocytochemically examined for the expression of Sca-1, CD34, c-Kit, Flk-1, eNOS, and CD31. Data presented in Fig. 1 depict representative images demonstrating that the muscle-derived stem cells expressed markers of stem cells (Sca-1 positive, c-Kit negative), displayed only a faint or rare expression of hematopoietic stem cell markers (CD34 low), and were almost devoid of endothelial markers (eNOS very rare, Flk-1 very rare, CD31-low, and low-to-negative expression of GFP controlled by the Tie-2 promoter). These findings are in partial agreement with those of Huard and colleagues (14),...
who also isolated stem cells from adult mouse skeletal muscle, but by a very different technique. Those stem cells were characterized by the expression of stem cell markers Sca-1 and Flk-1 but were negative for the hematopoietic cell markers like c-Kit and CD45 (17). These cells have been shown to have a potential to differentiate into hematopoietic lineages (4). Adult stem cells isolated from adult mouse skeletal muscle by yet a third method were Flk-1 negative but CD13 positive (11). The stem cells in this study were passaged up to 12 times during expansion of cell culture, with an average of five cell doublings per passage, for a total of 60 cell doublings. Cell doubling time was between 24 and 36 h. No signs of senescence or increased cell doubling time were noted in the cultures.

In vitro expansion and differentiation. After cell expansion followed by 4-wk differentiation in the defined medium (the acquisition of endothelial phenotype was heralded by the uniform appearance of GFP fluorescence, as detailed in MATERIALS AND METHODS), cells were plated in multiwell chambers and the expression of endothelial cell markers CD31, Flk-1, Tie2, and eNOS was examined along with the expression of GFP protein. Cells cultured in EMEM + 10% horse serum did not express GFP, even after 12 passages (data not shown). However, when the cells were placed in EBM medium, Tie-2 promoter-driven GFP expression was uniformly and consistently detected after 4 wk by fluorescent microscopy (Fig. 2). The detailed immunocytochemical characterization of these cells showed that ~90% of the cells were GFP positive (Fig. 3). GFP-positive cells were also positive for the endothelial markers MECA, eNOS, Flk-1, Flt-1, and CD31 (Fig. 3). These findings confirm that 1) the differentiating medium did differentiate the stem cells to endothelial cells or endothelial progenitors and 2) that cells from Tie-2-GFP mice that express GFP also express the other phenotypic markers for endothelial cells. Thus the expression of GFP by stem cells obtained from Tie-2-GFP mice is a reliable augur of cell differentiation along the endothelial lineage.

Morphological and functional consequences of transplantation of endothelial lineage cells. To confirm that these cells are functionally competent and can reproduce the functions of terminally differentiated endothelial cells, as shown in our previous studies using human umbilical vein endothelial cells for transplantation (3), GFP-expressing cells were injected into wild-type animals subjected to renal ischemia. Transplantation of GFP-expressing cells of endothelial lineage after renal ischemia resulted in a partial protection of renal function, as judged by the blunting of increase in Cr level (Fig. 4). When the undifferentiated stem cells were used instead of GFP-positive cells for transplantation in the posts ischemic period, short-term renal dysfunction did not improve compared with vehicle-injected animals subjected to renal ischemia.

Transplanted GFP-expressing cells were detectable along the renal microvasculature: in glomeruli and peritubular capillaries (Fig. 5). Transplanted mesenchymal stem cells, tagged with Cell Tracker, engrafted in the tubular lining and were found within the tubular lumen but were not detectable in the microvasculature of the ischemic kidney.

**DISCUSSION**

The data presented herein provide evidence that stem cells harvested from a skeletal muscle have a potential to differentiate ex vivo into the endothelial lineage and are endowed with the potential to ameliorate acute ischemic renal dysfunction. Adult stem cells were isolated from the skeletal muscle of Tie-2-GFP mice by the same method that has been used to isolate stem cells from tissues of other adult mammals, including humans (30, 31). These stem cells have been previously demonstrated to be capable of differentiating to endothelial cells when stimulated with the nonspecific inductive agent dexamethasone (30, 31). Here, we show that these cells initially express stem cell markers and, only faintly, hematopoietic stem cell markers, but almost completely lack endothelial cell markers and do not express the Tie-2-driven GFP fluorescence. When these stem cells isolated from Tie-2-GFP mice were treated with EBM media for 4 wk, ~90% of the cells became positive for Tie-2 promoter-driven GFP and these same cells were also expressing several endothelium-specific markers (CD31, Flk-1, MECA, and eNOS positivity) (Figs. 1–3), indicating that the adult stem cells have differentiated into cells of endothelial lineage. That ~90% of the adult stem cells differentiated to endothelial cells suggests that EBM is an efficient inducer of adult stem cells differentiation to endothelial cells in vitro. This may have implications for the tissue engineering of blood vessels in vitro that EBM may be a means to induce adult stem cells growing on matrices ex vivo to form endothelial-coated surfaces of grafts. This in vitro differentiation of muscle-derived stem cells into cells of endothelial lineage is consistent with the previous observations of their multipotency (4, 14). In fact, there is growing evidence that these skeletal muscle stem cells originate from the bone marrow-derived stem cells (reviewed in Ref. 27).

Transplantation of undifferentiated adult stem cells immediately posts ischemia had no effect on renal dysfunction 24 h after injury. This is not surprising in light of the time (4 wk) required in vitro for the differentiation of adult stem cells to endothelial lineage. We infer that the undifferentiated adult
stem cells simply did not have sufficient time to differentiate to endothelial cells in vivo. It is possible that transplantation of hematopoietic stem cells has not led to their endothelial differentiation and homing to the microvasculature of ischemic kidneys for the same reason (12, 15). Alternatively, some essential differentiation cues may be lacking in the microenvironment of the ischemic kidney as opposed to the anti-Thy-1.1 glomerulonephritis model, where bone marrow-derived cells participated in glomerular endothelial repair (25). This is in concert with the recent finding by Togel et al. (26) that hematopoietic stem cell mobilization-associated granulocytosis not only does not improve but also significantly worsens acute renal failure. In contrast to the nondifferentiated muscle-derived stem cells, adult stem cells expanded and differentiated in vitro to cells of endothelial lineage and did protect against postischemic renal dysfunction (Fig. 4). However, tracking of the cells in vivo also suggests that undifferentiated and differentiated cells display differential homing patterns. The undifferentiated adult stem cells were found in the tubular lining and within the tubular lumen but were not detectable in the microvasculature of the ischemic kidney. In contrast, adult muscle-derived stem cells that had been differentiated to endothelial lineage before transplantation were found along the renal microvasculature in glomeruli and peritubular capillaries. As has been the case with the renoprotective effect of human umbilical vein endothelial cells (3), we presume that the observed protection of renal function was due to the preservation of the vascular integrity.

Fig. 5. Immunohistochemical localization of transplanted cells in ischemic kidneys. A-C: hematoxylin-stained cryosections of FVB mouse kidneys in ischemic group (A), ischemia with injection of differentiated cells (B), and ischemia with injection of nondifferentiated cells (C). Original magnification ×100. D: representative images depicting colocalization of GFP-positive and Cell Tracker-positive cells in glomerular and peritubular capillaries (left 2 panels) and the lack of microvascular homing of nondifferentiated stem cells, which do not express GFP (right 2 panels). Arrows indicate Cell Tracker-stained and/or GFP-expressing cells. G, glomerulus; PT, proximal tubules. Magnification ×400.
This distribution in vivo is consistent with earlier reports of the localization of injected undifferentiated stem cells and differentiated endothelial cells in the ischemic kidney. The undifferentiated bone marrow stem cells, whether hematopoietic or stromal, were found as epithelial cells or podocytes (12, 15). In contrast, our transplanted cells differentiated along the endothelial lineage were found in the microvasculature. We clearly appreciate that our observations are limited by the short-term duration of follow-up in ischemic renal failure and lack of information on the long-term sequelae of transplanting either differentiated or nondifferentiated cells. Testing at later time points would be essential for determining the ultimate fate of the undifferentiated stem cells injected into the ischemic kidney.

Finally, these studies indicate an alternative source for large numbers of undifferentiated adult stem cells that can be differentiated to endothelial lineage in vitro for use in immediate treatment of ischemic renal injury, as well as a potentially broader range of conditions accompanied by the injury to the tubule. The data 1) identify a rich source of muscle-derived stem cells, 2) delineate protocols for their in vitro expansion and differentiation along the endothelial lineage, and 3) demonstrate their efficacy in preserving renal function 24 h after ischemic insult.

GRANTS

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REFERENCES
