Bioluminescence imaging to monitor the in vivo distribution of administered mesenchymal stem cells in acute kidney injury

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Regenerative medicine is a rapidly developing field in which bone marrow derived cells are administered for the treatment of various disorders (41). Animal studies have provided insight into the fate of administered cells, but such data largely end-point studies, thereby only providing little dynamic information regarding cell distribution and long-term engraftment (9, 21). BLI offers thus a unique possibility to repeatedly track and quantify administered cells in vivo.

Acute kidney injury (AKI) is a serious and common clinical problem with high morbidity and mortality (18). Cell therapy is a promising new experimental treatment for renal diseases, and bone marrow-derived stem cells have been shown to be promising cell therapeutic vehicles (22, 28, 35). Although there is controversy about hematopoietic stem cells (11, 12, 23, 29), multipotent mesenchymal stromal cells or MSCs have been shown to be effective in preventing injury and enhance regeneration of kidneys after AKI (2, 17, 26, 33, 39, 40). Although administered MSCs have been shown to differentiate to variable extent and late after injury into renal cells in AKI (33), their early renoprotective activity is primarily mediated by paracrine mechanisms such as immunomodulation and growth factor secretion (2, 19, 20, 33, 39, 40). MSCs have also been shown to be effective in the treatment of a rat model of glomerulonephritis and in a model of Alport’s syndrome (14, 24, 25).

The current study was designed to test the utility of BLI to track MSC after intravenous or intra-arterial injection in mice with AKI as well as in normal animals, and to investigate their distribution and survival kinetics over time. Our data show that BLI is a sensitive and specific tool to track cells in vivo, demonstrating that administered MSC distribute in distinctive patterns when infused either intra-arterially or intravenously. We did not detect long-term engraftment of a significant number of cells. In conclusion, BLI is a sensitive tool that allows the in vivo definition of cellular distribution and survival data, thereby substantially aiding in the preclinical testing and optimization of various cell-based treatment protocols.

METHODS

Animals and Cells

Animal experiments were performed following approval from the University of Utah Institutional Animal Care and Use Committee. Adult C57Bl6 mice of either sex, weighing 20–25 g, were purchased from Charles River laboratories (Wilmington, MA). Animals were housed in a temperature-controlled environment with a 12:12-h day-night cycle. All mice ate regular chow and had free access to water.

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AKI in mice was induced by clamping both renal pedicles for 30 min. In brief, mice were anesthetized with Avertin, and kidneys were exposed after abdominal midline incision. Microvessel clamps were applied to both renal pedicles, and cessation of blood flow was confirmed by visual inspection (color change). Clamps were released after 30 min, and reflow was visually confirmed before closure of the abdominal incision.

Multipotent MSCs were kindly provided by Dr. Claudia Lange (University of Hamburg). Bone marrow from anesthetized C57/B16 mice was obtained by flushing of femurs with saline. Obtained cell suspensions were washed and plated in T25 flasks (Corning) containing α-minimal essential medium and preselected 10% FBS (GIBCO). MSCs were infected with pMMP-LucNeo and selected with G418. Supernatant was kindly provided by Dr. Lessnick, Huntsman Cancer Institute, University of Utah (3). Wild-type and luciferase-neomycin (luc-neo) MSCs were both subjected to differentiation protocols, showing identical differentiation patterns in adipocytes, chondrocytes, and osteocytes (data not shown). This indicates that the transfection of MSCs does not interfere with in vitro differentiation. Following selection with G418, $1 \times 10^5$ MSCs were injected in C57/B16 mice. Intrajugular injection was carried out after dissecting the neck with a midline incision, preparation of the internal jugular vein, and injecting 100 μl of saline containing MSCs slowly directly in the jugular vein. Carotid injection was carried out after dissection and distal ligation of the left carotid artery and introduction of a PE-10 tube in the proximal carotid artery. Thereby, MSCs were delivered directly in the descending aorta.

Mice were imaged using a Xenogen IVIS 100 imaging system, per the manufacturer’s directions. For in vivo imaging of cells, mice were anesthetized with Avertin and injected intraperitoneally with 50 mg/kg d-luciferin (Caliper, Hopkinton, MA) at 5–10 min before imaging. Regions of Interest (ROIs) were drawn using Living Image 2.5 software (Caliper). In vivo biodistribution of luciferin after intraperitoneal injection is fast, with high blood levels after 5 min and high uptake in liver and kidneys according to one publication (27). We tested imaging 5, 10, and 15 min after intraperitoneal luciferin injection in preliminary experiments and found no difference in signal intensity (data not shown) at these time points; therefore, an imaging time point between 5 and 10 min after injection of luciferin was chosen. To control for background photon emission, obtained data were subjected to average background subtraction, using data from control animals that were only injected with identical doses of luciferin.

Binning increases the pixel size on the CCD, which delivers higher sensitivity at the expense of spatial resolution. Binning a luminесent image results in a significant improvement in the signal-to-noise ratio. Although spatial resolution is degraded at high binning, this is often acceptable for in vivo images where light emission is rather diffuse: binning 1 = 16 CCD pixels/field; binning 2 = 4 CCD pixels/field (4 pixel summed together); binning 4 = 1 pixel/CCD field (16 pixels together).

**Real-Time RT-PCR**

Total RNA for real-time PCR was extracted with an RNeasy kit (Qiagen, Valencia, CA), including a DNase digestion step to exclude contaminating DNA. Reverse transcription was performed using Moloney murine leukemia virus RT (Invitrogen, Carlsbad, CA) for 60 min at 42°C. Real-time PCR with relative quantification of target gene copy numbers in relation to β-actin transcripts was carried out using the following primers: luciferase forward: 5’-gagagatcttcctggtt-3’; reverse: 5’-ctactgtaggtgcaagatg-3’; β-actin forward: 5’-catctgttggtggcatagaggtc-3’; reverse: 5’-agagggaaatgctgccgtgaca-3’.

**In Vitro Determination of Bioluminescence Sensitivity**

Imaging of dilution series containing 10,000–100 luc/neu MSCs in vitro showed an excellent linear correlation between photon emission intensity and cell numbers ($P = 0.0016$, Fig. 1). As few as 100 cells were easily detected by this in vitro system. Photon emission was neither detected in transfected cells that were not treated with luciferin substrate nor in untransfected cells (data not shown), demonstrating the sensitivity and specificity of BLI. In vivo sensitivity was tested in a model system whereby 1,200 and 12,000 cells in an Eppendorf tube were placed in the retroperitoneal space of a dead mouse. Twelve hundred cells were readily detected (Fig. 1C). There was some scattering of the signal, but the location corresponded well to the placement location, and 1–5% of the in vitro signal intensity for both cell numbers was detected after retroperitoneal placement (Fig. 1C).

**In Vivo Studies**

Intra-arterial injection. To determine the distribution of luc/neu MSCs infused in the aorta via the left carotid artery, in normal and AKI animals, BLI measurements were always performed after prior intraperitoneal administration of luciferin. Measurements were carried out immediately after the single administration of luc/neu MSC and repeated at 24 and 72 h and 7 days. At 10–15 min after cell infusion, total body luminescence was 681,400 in AKI ($n = 5$, SD 415,000) and 622,500 photons·s$^{-1}$·cm$^{-2}$·sr$^{-1}$ in normal animals ($n = 4$, SD 388,000) ($P = 0.83$). AKI animals showed distinct accumulation of infused cells in the areas corresponding to the location of the kidneys (Fig. 2A), whereas normal animals showed a diffuse, whole body distribution with greater accumulation in the lungs in some animals (Fig. 2B). Renal location of injected cells in AKI animals was further confirmed by lateral imaging.
as well as by imaging excised organs directly at 24 h after injection (Fig. 2C). After cell infusion (24 h), reassessment of total animal luminescence showed a significant decline to 203,000 (SD 72,500) in AKI animals and to 156,000 (SD 41,000) photons s⁻¹ cm⁻² sr⁻¹ in normal animals. Total animal bioluminescence decreased further at 72 h to 64,300 (SD 2,500) in AKI and 44,000 (SD 35,000) photons s⁻¹ cm⁻² sr⁻¹ in normal animals. There was no statistically significant difference between the 24- and 72-h time points as determined by ANOVA. At 7 days after injection, total photon emission fell to 25,000 (SD 2,200) and 26,000 (SD 28,500) photons s⁻¹ cm⁻² sr⁻¹ in AKI and normal mice, indicating that most of the cells had vanished. RT-PCR for luciferase expression was used to detect cells in RNA extracts from kidneys 7 days after injection. This was done to determine whether there were cells remaining that were below the detection limit of the BLI method. After luc-neo MSC administration (7 days), mRNA transcript numbers of luciferase, determined by quantitative RT-PCR, in lungs, kidneys, liver, and spleen were at least nine log fold lower than in 10,000 luc/neomycin MSC cells, indicating the virtual absence of residual cell numbers in the examined organs.

**Intravenous injection.** Administration of luc/neomycin MSCs in the jugular vein revealed almost exclusive accumulation of cells in the lungs of normal animals (Fig. 3A), whereas only one AKI animal showed renal signal immediately after injection (Fig. 3B). Positive signals from administered cells gradually decreased over the next 3 days, and 7 days after injection no positive signal was detectable above background level in normal animals. In one animal in the AKI group, a midabdominal signal of 1,287,000 photons s⁻¹ cm⁻² sr⁻¹ was seen, corresponding to a poorly healing abdominal wound (data not shown).

**Intrarenal apoptosis of MSC.** To assess whether administered CFDA-labeled luc/neomycin MSC undergo intrarenal apoptosis, kidney

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**Innovative Methodology**

**BOLUMINESCENCE CELL TRACKING IN AKI**

Fig. 1. A: in vitro determination of sensitivity and specificity of bioluminescence imaging (BLI) measurements of luciferase/neomycin (luc/neom)-transfected mesenchymal stem cells (MSCs). Transfected cells were incubated with luciferin, and photon emission was measured with the Xenogen IVIS system. Tubes containing 100, 1,000, and 10,000 cells were suspended in culture medium containing luciferin. The region of interest (ROI) indicates photon emission measurement of the red-encircled area (units: photons s⁻¹ cm⁻² sr⁻¹); 100 cells were readily detected in vitro, emitting a signal of 961,000 photons s⁻¹ cm⁻² sr⁻¹. Note: binning is set at 1, giving a higher spatial resolution. Red, highest photon density; blue, lowest photon density. B: regression analysis revealed significant linear increase of luminescence with cell numbers. C: in vitro placement of tubes containing 1,200 and 12,000 cells in the retroperitoneal space of a dead animal. Tubes, containing 1,200 and 12,000 cells, as shown on right, emitted 442,390 and 1,282,600 photons s⁻¹ cm⁻² sr⁻¹, respectively, as indicated by the corresponding ROI area measurements. After placement of the tubes in the retroperitoneal space of a dead animal (areas indicated by circles) 5,378 and 64,309 photons s⁻¹ cm⁻² sr⁻¹ were detected, respectively. A signal intensity corresponding to 1 and 5% of the original in vitro signal that was significantly above background levels outside the encircled areas allowed the detection of 1,200 and 12,000 cells. There is some light scattering beyond the area of tube placement. Note: binning is set at 4, thereby increasing sensitivity and the signal-to-noise ratio at the expense of spatial resolution, which results in increased pixilation of the signal.
sections from mice killed at 2 and 24 h after injection were examined by TUNEL assay. MSCs were easily detectable by their bright green fluorescence and were localized mainly in glomerular and less commonly in peritubular capillaries. Consistently, ~1% of identified MSCs were TUNEL positive, indicating low-level apoptosis of intrarenal MSCs (Fig. 4). This is in contrast to an apoptosis rate of ~10% of tubular cells (counting total and apoptotic cells in 10 high-power fields/cortical section; n = 3) at each time point (2 and 24 h), a response that is expected in an ischemic model of AKI.

DISCUSSION

The aim of the current study was twofold: first to determine if BLI is a sufficiently sensitive and specific method for in vivo cell tracking of administered single cell suspensions and second, to determine the distribution kinetics over time of cells infused by different routes (ia or iv) to normal and AKI animals. We demonstrate here that BLI is a suitable tool for sensitive and specific in vivo cell tracking. Despite expected scattering and signal attenuation through overlaying tissues,
cells over time, information that is critical in the design of cell therapies.

The method, site, and route of cell administration are important determinants in the development of effective, disease- or organ-specific cell therapies. This is illustrated by MSCs that were administered in the present study. This cell type requires attachment to extracellular matrix for survival and growth and is thus not naturally found in the circulation. Accordingly, when such cells are no longer attached to an extracellular matrix or vascular endothelial cells, they may undergo anoikis, a form of apoptosis. We hypothesize that anoikis is important in this context because MSCs are by their nature not circulating cells and require extracellular matrix-derived signals for survival, and deprivation of these signals in the vasculature might induce apoptosis/anoikis of MSCs. The extent to which this phenomenon applies to the here-demonstrated disappearance of administered MSC suspensions was elucidated. Because MSCs gradually disappear from injured kidneys, we assessed whether they undergo apoptosis (13, 32). We found that ~1% of MSCs within glomerular and peritubular capillaries were TUNEL positive, indicating ongoing apoptosis and suggesting that this process may contribute to the declining number of intrarenal MSCs after administration. Although this number might seem low at first, continuing attrition of small numbers of cells eventually leads to the disappearance of all cells if there is no substrate supporting permanent attachment. Accordingly, strategies that reduce apoptosis might improve effectiveness of cell therapy as has been shown by Mangi et al. (31). Strategies to genetically engineer MSCs to withstand apoptosis, such as transduction with protein kinase B or incubation with lysophosphatidic acid, might prove valuable in the future to enhance effectiveness of MSC therapy (6, 34).

Furthermore, there is concern of causing capillary clogging when larger cell types such as MSCs are infused, a complication that could result in hemodynamic compromise, interference with pulmonary gas exchange, and respiratory distress (15). Schreper et al. (37) have shown that intravenously injected MSCs localize mainly to the pulmonary capillary bed, which can, however, be prevented by the administration of vasodilators. Our data confirm this observation, showing pulmonary localization of intravenously administered MSC, a pattern not seen with intra-aortic administration. Specifically, our data demonstrate prompt homing of MSCs to the injured kidney after intra-arterial administration. However, the robust renal signal started to decline already within 24 h of administration, indicating either redistribution and dilution or death of administered cells. Using highly sensitive PCR analysis, we demonstrated that this decline in renal signal intensity was the result of cell loss from the kidneys and, at least in part, due to apoptotic cell loss. Although we and others showed that the therapeutic effectiveness of MSCs in AKI is primarily mediated by paracrine mechanisms, prolonged presence of MSCs at sites of injury might improve their effectiveness (2, 20, 40).

Strategies, therefore, that enhance attraction, engraftment, and that improve MSC recruitment to specific organs or compartments are being developed and include genetic engineering of surface properties (36).

In summary, our data demonstrate that BLI is a sensitive and specific in vivo method to monitor the distribution and fate of administered cells over time. We conclude that its application in preclinical studies provides a new tool for the development

Fig. 4. TdT-dUTP nick end-labeling (TUNEL) staining (red fluorescence) of a representative kidney section containing CFDA-stained MSCs (green) in a glomerulus (encircled). Nuclei were counterstained with Hoechst 33342 dye (blue). MSCs are readily identified by bright green staining (arrow). An apoptotic MSC (arrowhead) 24 h after injection is depicted in red.
and testing of optimal organ- and disease-specific treatment protocols.

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