Transplanted Mesenchymal Stem Cells Accelerate Glomerular Healing in Experimental Glomerulonephritis

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Bone marrow–derived cells contribute to glomerular cell turnover and repair, but the cell types involved are unknown. Whether rat mesenchymal stem cells (MSC) can accelerate recovery from damage in rat mesangioproliferative anti-Thy1.1 glomerulonephritis was studied. After injection into the left renal artery on day 2 after disease induction, fluorescently labeled MSC were detected in 20 to 50% of glomeruli and rare intrarenal vessels but not in the tubulointerstitium, in contralateral kidneys, or in medium controls. In control experiments, injected mesangial cells were detected less frequently in glomeruli in comparison with injected MSC. In nephritic outbred Wistar rats, MSC injection led to an approximately 50% reduction of mesangiolysis on days 4 and 6 after disease induction, accompanied by three- to four-fold higher intraglomerular cell proliferation on day 4 and more rapid mesangial reconstitution as detected by α-smooth muscle actin expression. Injection of MSC into tail veins or intra-arterial injection of mesangial cells instead of MSC failed to reproduce any of these findings. In inbred Lewis rats, anti-Thy1.1 nephritis followed an aggravated course with transient acute renal failure. Acute renal failure was ameliorated by MSC injection into the left renal artery on day 2 after disease induction. Again, MSC led to more rapid recovery from mesangiolysis, increased glomerular cell proliferation, and reduction of proteinuria by 28%. Double immunostaining of 5-bromo-2′-deoxyuridine–labeled MSC for endothelial, mesangial, or monocyte/macrophage antigens showed that 85 to 95% of MSC that localized in glomeruli on day 6 failed to express these markers. *In vitro*, MSC secreted high amounts of vascular endothelial growth factor and TGF-β1 but not PDGF-BB. In conclusion, even low numbers of MSC can markedly accelerate glomerular recovery from mesangiolytic damage possibly related to paracrine growth factor release and not to differentiation into resident glomerular cell types or monocytes/macrophages.

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Bone marrow–derived stem cells contribute to cell turnover and repair in various tissue types, including the kidneys (1,2). Using transgene enhanced green fluorescence protein (EGFP) mice as bone marrow donors, differentiation of mouse bone marrow cells into glomerular cells that expressed desmin was reported (3). In rats with anti-Thy1.1 nephritis, differentiation of invading bone marrow cells into endothelial or mesangial cells (4,5) was shown. These data were interpreted to reflect a regular contribution of bone marrow to glomerular cell turnover that is modified during disease. The ability of bone marrow cells to convert into mesangial-like cells also has been demonstrated in cell culture, including administration of PDGF-BB in the presence of type IV collagen (6). Furthermore, rat bone marrow provides the kidney with resident, pluripotent Hoechst^{low}/side population cells (7).

Despite the above data, it currently is unknown, which subtype(s) of the heterogeneous bone marrow population, including mesenchymal and hematopoietic stem cells, carries the largest potential for renal repair. A recent study even suggested a new stem cell type without apparent bone marrow origin, the "slow-cycling cell of the inner renal medulla," to promote renal repair in ischemia (8).

Mesenchymal stem cells (MSC) are attractive candidates for renal repair, because nephrons are of mesenchymal origin and because stromal cells are of crucial importance for signaling, leading to differentiation of both nephrons and collecting ducts (9). MSC commonly are defined as bone marrow–derived fibroblast-like cells, which despite the lack of specific surface markers can be selected by their adherence characteristics *in vitro* and which have the ability to differentiate along the three principal mesenchymal lineages: Osteoblastic, adipocytic, and chondrocytic (10,11). The potential of MSC for renal repair was reported recently by Herrera *et al.* (12), who ameliorated glycerol-induced acute renal failure (ARF) by injecting EGFP-labeled MSC intravenously into mice; Lange *et al.* (13) improved recovery from renal arterial clamping–induced ARF by injection into the carotid artery of rat MSC.

In this study, we sought to determine whether intrarenal

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injection of MSC can alter the course of anti-Thy1.1 nephritis and by which mechanisms this might occur. Anti-Thy1.1 nephritis, a model of mesangioproliferative glomerulonephritis, is characterized by initial mesangiolysis followed by repair *via* endothelial and mesangial cell proliferation and accumulation of mesangial matrix. As others have shown little engraftment of mouse MSC after injection in an allogeneic as opposed to a syngeneic setting (14), we investigated the effects of MSC on the nephritis in both outbred Wistar and inbred Lewis rats.

Materials and Methods

Animals were housed under standard conditions in a light-, temperature-, and humidity-controlled environment with free access to tap water and standard rat diet. All animal protocols were approved by the local government authorities.

Harvest and Culture of MSC

Male Wistar rats that weighed 170 to 200 g were obtained from Charles River (Borken, Germany), and male Lewis rats that weighed 170 to 200 g were obtained from Harlan (Horst, The Netherlands). Rats were killed, and femurs and tibias were removed aseptically. First, bones were washed in 70% ethanol, then at 4°C in sterile PBS and complete DMEM-LG medium (Cambrex, BioWhittaker Europe, Verviers, Belgium), supplemented with L-glutamine, 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (all supplements obtained from Life Technologies Invitrogen, Paisley, UK). Metaphyses of bones were cut off, and the diaphyses were flushed with complete medium. Cells then were passed through a 21-G needle several times, spun down at 1200 rpm at 4°C for 5 min, followed by a washing step in PBS and resuspended in complete medium. Cells were seeded onto six-well plates (nine wells per donor) and cultured at 37°C in a humidified atmosphere that contained 5% CO_2. Medium was changed after 2 d and every 3 d thereafter. Nonadherent hematopoietic cells were removed when medium was changed. After a mean of 6 d, cells reached subconfluence and were detached with trypsin/EDTA, reseeded at 4×10^3 cells/cm^2, and used for experiments after the third passage.

In a control experiment, primary Wistar rat mesangial cells (passage 3) were injected instead of MSC. Mesangial cells were obtained and cultured as described (15).

Osteogenic and Adipogenic Differentiation and Immunocytochemical Characterization of MSC

Osteogenic differentiation of MSC from Wistar and Lewis rats was tested following the protocol of Bruder *et al.* (US patent 5,736,396). In brief, MSC were expanded in complete medium containing 10% FCS until subconfluence after the second passage. Complete medium then was replaced by medium that was composed of DMEM-LG, 10% FCS, 100 nM dexamethasone, 50 μM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate (all from Sigma-Aldrich, Taufkirchen, Germany). After 10 d, cells were fixed in 10% buffered formalin for 1 h followed by von Kossa staining.

Adipogenic differentiation of MSC from Wistar and Lewis rats was tested following the protocol of M. Pittenger (US patent 5,827,740). Subconfluent MSC after the second passage were incubated in adipogenic differentiation medium (Cambrex). After 15 d, Oil Red O staining was performed.

For analysis of our MSC *in vitro* for presence of the Thy1.1 antigen, cells were grown on coverslips until confluent. After fixation in Methacarn solution for 20 min, cells were incubated with mouse anti-Thy1.1 mAb (clone OX-7), followed by a biotinylated anti-mouse antibody and an immunoperoxidase reaction. Mesangial cells were used as positive controls.

Fluorescence Labeling for In Vivo *Tracking of Cells*

Cells were labeled using the PKH26 red fluorescence cell linker kit (Sigma-Aldrich, Saint Louis, MO) according to the manufacturer's protocol. In brief, subconfluent MSC or mesangial cells after the third passage were detached and washed three times with serum-free medium. Cells were resuspended at 1×10^7 cells in 1 ml of diluent C plus dye, followed by incubation at 25°C for 5 min. To stop the reaction, 2 ml of FCS was added. Cells were supplemented with 4 ml of complete medium and washed three times. Cells were resuspended at 1×10^6 cells/250 μl complete medium and used within 30 min.

For assessment of vitality and preservation of differentiation capacity of PKH26-labeled MSC, they were reseeded onto plates and then underwent differentiation testing upon reaching confluence. For testing viability of PKH26-labeled primary mesangial cells, they were recultured.

Experimental Model and Experimental Design

Anti-Thy1.1 mesangioproliferative glomerulonephritis was induced in outbred Wistar or inbred Lewis rats as described (16). On day 2 after disease induction, rats were anesthetized with ketamine/xylazine and the abdominal aorta was prepared. After the aorta was clamped above and below the left renal artery, a 27-G needle was inserted into the distal aorta, and 2×10^6 MSC, 2×10^6 mesangial cells, or an equal volume of control medium was injected into the left kidney. After this, the aortic puncture site was closed with fibrin glue and blood flow was restored, total renal ischemia being <1 min. In additional experiments, in both Wistar and Lewis rats, intra-arterial was replaced by intravenous injection of 2×10^6 PKH26-labeled MSC ($n = 5$) or medium ($n = 5$) into tail veins. Animals were killed at day 4 (both strains), day 6 (both strains), or day 10 (Lewis) after induction of anti-Thy 1.1 nephritis.

For studying the intrarenal localization of MSC, fluorescence labeling was replaced by marking of the cells by incorporation of the thymidine analogue 5-bromo-2′deoxyuridine (BrdU; Sigma-Aldrich, Taufkirchen, Germany). For this, 30 μg/ml BrdU was added daily to the cell medium of third passage MSC starting 7 d before harvest and injection. Rats that received BrdU-labeled MSC ($n = 5$) on day 2 after induction of anti-Thy1.1 nephritis and the corresponding medium control rats ($n = 5$) were killed at day 6.

Before the rats were killed, they were placed in metabolic cages for 24-h urine collections. BrdU (100 mg/kg body wt) was injected intraperitoneally at 4 h before the rats were killed. Rats that had received BrdU-labeled stem cells did not receive the BrdU injection.

Miscellaneous Measurements

BP was measured by tail-cuff plethysmography on day 5 in conscious rats using a programmed sphygmomanometer (BP-981; Softron, Tokyo, Japan). Creatinine was measured by autoanalyzer and urinary protein excretion using the VITROS UPRO method (Ortho-Clinical Diagnostics, Rochester, NY).

Renal Morphology

Tissue for light microscopy was fixed in methyl Carnoy's solution and embedded in paraffin. Four-micron sections were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. In PAS-stained sections, the number of total or endothelial mitotic figures within 100 to 150 glomerular cross-sections was determined as described (16). Mesangiolysis was graded on a semiquantitative scale (0,

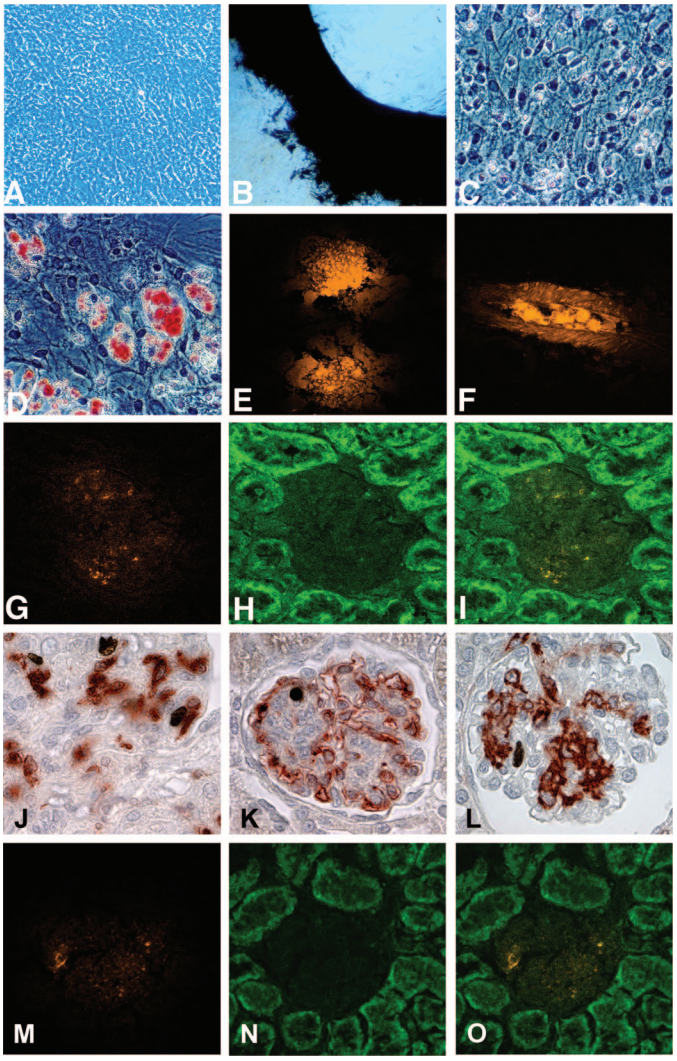

Figure 1. (A through D) *In vitro* differentiation of mesenchymal stem cells (MSC) along adipogenic and osteogenic lineages. (A) Wistar MSC cultured for 10 d with complete medium growing in spindle-shaped morphology. Negative Von Kossa staining for calcium-hydroxyapatite. (B) Wistar MSC cultured for 10 d with complete medium that contained osteogenic supplements. Osteogenic differentiation is shown by formation of calcium-hydroxyapatite–positive areas (Von Kossa staining). (C) Lewis MSC cultured for 15 d with complete medium. Negative Oil Red O staining for lipids. (D) Lewis MSC cultured for 15 d with complete medium after induction with adipogenic supplements. Adipogenic differentiation is visualized by Oil Red O staining for intracellular lipid vacuoles. Differentiation experiments were performed three times with identical findings in both Wistar- and Lewis-derived cell lines. (E through I) Detection of fluorescence specific for PKH26-labeled MSC. (E) Frozen section of a Wistar rat kidney immediately after intra-arterial injection of 2×10^6 PKH26-labeled MSC. Specific fluorescence is detected throughout all glomeruli and in some intrarenal arteries (F). (G) Wistar rat glomerulus 4 d after intra-arterial injection of 2×10^6 PKH26-labeled MSC with bright fluorescence within mesangial fields. (H) Autofluorescence detection. Settings show typical bright green in tubular areas, whereas the glomer-ulus does not show significant autofluorescence. (I) Overlay of both channels demonstrating PKH26 fluorescence inside the MSC-treated glomerulus. (J through L) Double immunostaining for MSC that were labeled *ex vivo* by 5-bromo-2′deoxyuridine (BrdU) and other cell markers on day 6 after induction of anti-Thy1.1 nephritis and day 4 after their injection into a renal artery. (J) Double labeling for BrdU (black) and a monocyte/macrophage marker (ED-1; red). (K) Double labeling for BrdU (black) and an endothelial marker (JG12; red). (L) Double labeling for BrdU (black) and a mesangial cell marker (α-smooth muscle actin [α-SMA]; red). No double staining is present in any of the three images. (M through O) Detection of rare fluorescence specific for PKH26-labeled mesangial cells. (M) Frozen section of a Wistar rat kidney 4 d after intra-arterial injection of 2×10^6 PKH26-labeled mesangial cells. Fluorescence is observed in some mesangial fields. (N) Autofluorescence detection. Settings show typical green in tubular areas, whereas the glomerulus does not show significant autofluorescence. (O) Overlay of both channels demonstrating PKH26 fluorescence inside the mesangial cell–treated glomerulus. Magnifications: ×100 in A and B; ×400 in C, D, F through I, M through O; ×200 in E; ×600 in J through L.

no mesangiolysis; 1, segmental mesangiolysis; 2, global mesangiolysis; 3, microaneurysm) as described previously (16).

Immunoperoxidase Stainings

Four-micrometer sections of methyl Carnoy's–fixed biopsy tissue were processed by an indirect immunoperoxidase technique as described previously (16). Primary antibodies were identical to those described previously (17,18) and included a murine mAb (dilution 1:50; clone BU-1; Amersham, Buckinghamshire, UK) against BrdU-containing nuclease in Tris-buffered saline; a murine mAb antibody to rat endothelial cells (JG12, dilution 1:200 plus tyramide signal amplification [TSA] enhancement; Bender MedSystems, Vienna, Austria) (19); a murine mAb (dilution 1:500; clone 1A4, DAKO Corp., Capinteria, CA) to α-smooth muscle actin (α-SMA); a murine monoclonal IgG antibody (dilution 1:500; clone ED-1; Serotec, Oxford, UK) to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells; and a goat polyclonal antibody (dilution 1:100; Southern Biotech, Birmingham, AL) to human type I collagen cross-reacting with rat type I collagen plus appropriate negative controls using irrelevant antibodies.

In sections stained for the ED-1 antigen or BrdU, total numbers of positive cells per 100 glomeruli were counted. To determine the glomerular areas that were occupied by endothelial or mesangial cells, the immunostains for JG12 and α-SMA were evaluated using a point-counting method. For this, a grid composed of 100 dots was superimposed on an average of 25 consecutive glomeruli (magnification 600-fold), and the percentage of dots overlying stained areas were counted. The same method was applied to analyze glomerular type I collagen expression.

For *in vivo* tracking of BrdU-marked MSC and evaluation of potential differentiation of these cells into endothelial cells (JG12), monocytes/macrophages (ED-1), and/or mesangial cells (α-SMA), double immunostaining for the respective antigen plus BrdU was performed using TSA (NEN Life Science Products, Boston, MA) enhancement. Briefly, sections first were stained with the murine antibody against BrdU followed by a biotinylated secondary antibody and an immunoperoxidase procedure that generated a brown color product. Next, sections were incubated with the murine monoclonal anti-endothelial cell antibody (JG12), the murine monoclonal anti–α-SMA antibody, or the murine mAb to ED-1 followed by a biotinylated secondary antibody

Table 1. Mesangial cell control group[a]

Day 6 after Induction of Anti-Thy1.1 Nephritis	Mesangial Cell Injection ($n = 6$)	Medium Injection ($n = 6$)	*P*
Serum creatinine (μmol/L)	41 ± 8	38 ± 10	NS
Proteinuria (mg/d)	61 ± 22	64 ± 40	NS
Systolic BP (mmHg)	112 ± 13	119 ± 18	NS

[a]Rats received either intra-arterial injection of mesangial cells or control medium into the left kidney on day 2 and were examined on day 6 after induction of anti-Thy1.1 nephritis.

and an immunoperoxidase procedure that generated a red color product. TSA was used for enhancement. Total numbers of double-positive cells per glomerular cross-section were counted. All slides were evaluated by an observer who was unaware of the origin of the slides.

Detection of PKH26-Specific Fluorescence in Glomeruli

Frozen kidney samples were cut into 5-μm sections and air dried, followed by fixation in 2% paraformaldehyde for 20 min. After three PBS washes, sections were covered using immumount (ThermoShandon, Pittsburgh, PA) and stored at 4°C until analysis. Specific fluorescence *versus* tubular background autofluorescence was analyzed using laser scanning microscopy (LSM 510 Meta confocal scanning laser microscope; Carl Zeiss, Jena, Germany). The 543-nm line of the Helium-Neon Laser was used for PKH excitation. PKH fluorescence was detected using the Meta-detector as a 552- to 595-nm bandpass filter. Renal tissue was visualized by its autofluorescence by excitation with the 488-nm line of the Argon laser and a bandpass filter of 505 to 530 nm. There was no cross-bleeding between the two channels. All slides were evaluated by an observer who was unaware of their origin.

ELISA Analyses of Growth Factors in Cell Culture Supernatant

For the analysis of growth factor production in MSC cultures, supernatant was collected upon confluence in the third passage, *i.e.*, before use of the cells for intra-arterial injection. Samples were frozen at −80°C until analysis. Amounts of vascular endothelial growth factor (VEGF), PDGF-BB, and TGF-β1 in cell culture supernatants were quantified by ELISA (Quantikine ELISA for rat VEGF, human PDGF-BB, rat TGF-β1; R&D Systems, Minneapolis, MN). Fresh DMEM with all additives including FCS was used as a control. All experiments were performed in triplicate.

Flow Cytometric Analysis of Mesangial and MSC Cell Size

Flow cytometry was performed using FACSCalibur and CellQuest-Pro software (Becton Dickinson, Franklin Lakes, NJ). Primary rat mesangial cells and MSC were harvested simultaneously and uniformly according to standard procedures and analyzed immediately. The mean of forward scatter values of the cell-population gate was used to compare the size of the cells.

Statistical Analyses

All values are presented as mean ± SD. Statistical significance was evaluated using one-way ANOVA with modified *t* test performed with the Bonferroni correction. Paired *t* test was used to compare directly the left and the right kidney of an animal.

Results

Characterization of Rat MSC

MSC that were derived from Wistar and Lewis rats exhibited typical spindle-shaped morphology. MSC identity was proved by differentiation into osteogenic (Figure 1, A and B) or adipogenic cells (Figure 1, C and D). The number of differentiated cells that were obtained at the end of the experiments was similar to those described by others (US patent 5,827,740). PKH26 labeling did not affect MSC or mesangial cells, because reseeding of such cells yielded >95% viable cells, which in the case of MSC could be induced to differentiate like the unlabeled cells.

Some hematopoietic cell types, such as rat thymocytes, express the Thy 1.1 antigen on their cell surface (20), and variable Thy1.1 expression can be detected on bone marrow cells under culture conditions that promote conversion into mesangial cells (6). Therefore, residual circulating anti-Thy1.1 antibody might bind to injected MSC. However, immediately before injection, immunohistochemical analysis of our MSC failed to detect the Thy1.1 antigen on >90% of the cells, whereas >98% of cultured rat mesangial cells stained positively (data not shown).

Flow Cytometric Analysis of Mesangial Cell and MSC Size

Mean cell volume of primary Wistar mesangial cells was determined as a mean forward scatter of 398. The same value was calculated for MSC, thus indicating that rat mesangial cells and MSC have a comparable mean cell volume.

MSC Localize to Ipsilateral Glomeruli up to 10 D after Injection into a Renal Artery

More than 98% of PKH26-labeled MSC exhibited fluorescence. When one Wistar rat was killed immediately after the injection of 2×10^6 PKH26-labeled MSC, the localization of multiple fluorescence cells was confirmed in all glomeruli and some intrarenal arteries but not in tubulointerstitial areas (Figure 1, E and F). No clinical or histologic signs of embolism were noted.

Next, we assessed the intrarenal residence time of labeled MSC. In MSC-injected left kidneys, between 20 and 50% of glomeruli showed focal PKH26-specific fluorescence between days 4 and 10. Most positive glomeruli contained one to two positive areas (Figure 1, G through I). The frequency and the pattern of glomerular fluorescence areas did not differ between days 4, 6, or 10, but fluorescence intensity decreased slightly over time (data not shown). No PKH26-specific fluorescence

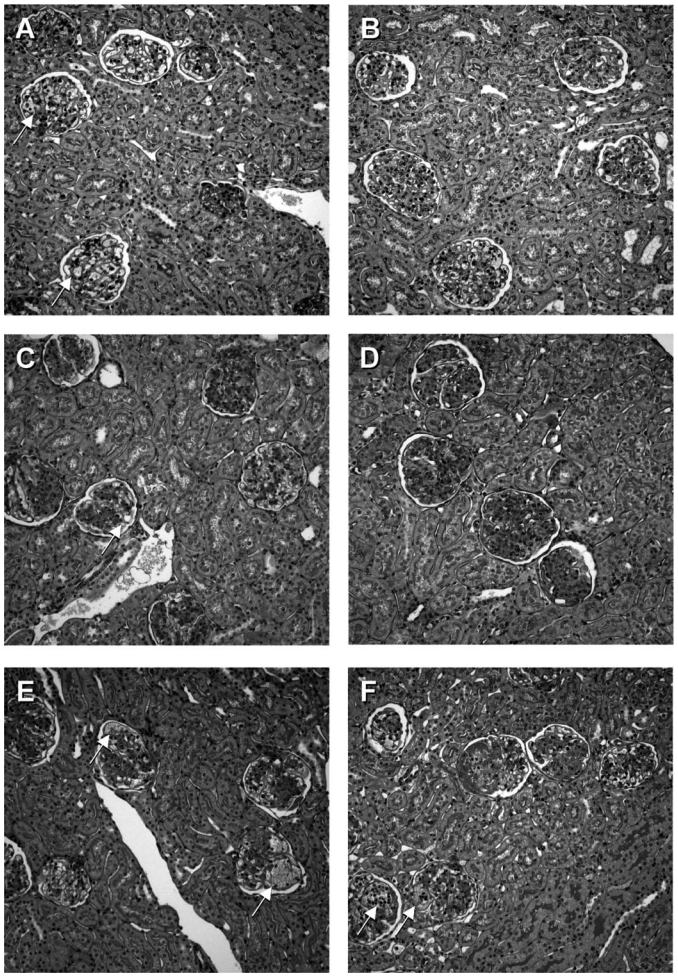

Figure 2. Renal histology in Wistar rats that had anti-Thy1.1 nephritis and received MSC, mesangial cells, or medium into the left renal artery on day 2 after disease induction. (A) Medium-injected kidney, day 4, showing widespread mesangiolysis (arrows). (B) In the same experiment, mesangiolysis is reduced in a MSC-injected kidney on day 4. (C) Apart from mild persistent mesangiolysis (arrow), a medium-injected kidney on day 6 does not exhibit markedly different histology as compared with an MSC-injected kidney from the same experiment (D) on day 6. (E) Control group experiment: Medium-injected kidney on day 6 with persistent mesangiolysis (arrow), comparable to a mesangial cell–injected kidney (F) from the same experiment on day 6. Magnification, ×100 (periodic acid-Schiff [PAS] staining).

was found in medium- or mesangial cell–injected control kidneys at any time point. Contralateral right kidneys of rats that received an injection of PKH26-labeled MSC into the left renal artery failed to exhibit positive fluorescence.

In a control experiment, Wistar rats received 2×10^6 PKH26-labeled mesangial cells instead of MSC into the renal artery. Six days later, we failed to detect significant specific fluorescence in such kidneys by laser scanning microscopy. A very rare positive area is shown in Figure 1, M through O.

When nephritic rats received MSC *via* a tail vein instead of the renal artery, no specific intrarenal PKH26 fluorescence was present 6 d later. Labeled MSC also could not be detected in livers or spleens but occasionally were detected in lung samples.

Anti-Thy1.1 Nephritis in Outbred Wistar Rats

Intrarenal Injection of Primary Wistar Mesangial Cells Does Not Yield Significantly Different Results from Medium Injection. In Wistar rats that received 2×10^6 PKH26-labeled mesangial cells ($n = 6$) or medium injection ($n = 6$), serum creatinines and BP were normal on day 6, and proteinuria in both groups increased similarly (Table 1). Intrarenal injection of primary mesangial cells did not lead to a significant reduction or increase of mesangiolysis scores in comparison with the right, noninjected kidney as well as with rats that received medium (Figures 2, E and F, and 3). The only significant difference between medium-treated kidneys and mesangial cell–treated kidneys was a higher number of mitotic figures on day 6 in medium-treated kidneys (Figure 3). Given that BrdU incorporation, monocyte/macrophage counts, and α-SMA–positive glomerular area all were very similar between the groups (Figure 3), the former result likely represents a sampling error.

Intrarenal Injection of MSC Accelerates Resolution of Mesangiolysis. After the induction of nephritis in Wistar rats, serum creatinine increased mildly on day 4 after disease induction and returned to normal on day 6 (Table 2). A transient increase of proteinuria occurred on day 4 (Table 2). BP remained normal on both days. None of these three parameters was affected significantly by injection of MSC into the left renal artery on day 2 after disease induction (Table 2).

Mesangiolysis in medium control Wistar rats was prominent on day 4 (Figures 2 and 4) and decreased on day 6 (Figures 2 and 4). Intrarenal injection of MSC led to an approximately 50% reduction of mesangiolysis scores on both time points in comparison with the right, noninjected kidney as well as with rats that received medium only into a renal artery (Figures 2 and 4). Reduced mesangiolysis in MSC-treated kidneys (*i.e.*, accelerated glomerular reconstitution) was accompanied by a transient, 3.8-fold higher glomerular cell proliferation, as reflected by total mitotic figures, on day 4 (Figure 4). This difference did not persist on day 6 (Figure 4). Similar data were obtained when we counted BrdU-labeled cells (Figure 4). Injection of mesangial cells instead of MSC failed to reproduce these results (Figures 2 and 3).

Accelerated glomerular healing in MSC-treated rats was associated with more rapid repopulation of the glomerulus by activated mesangial cells. Therefore, the glomerular area that was positive for α-SMA was significantly higher in the MSC-treated left *versus* untreated right kidney on day 4 (Figure 4) and in the MSC-treated *versus* medium-treated kidney on day 6 (Figure 4). Glomerular monocyte/macrophage influx was affected by MSC treatment only on day 4, when cell counts were significantly lower in the left, MSC-treated kidney as compared with the right control kidney (Figure 4). None of these MSC-related effects was reproduced when MSC were injected into a tail vein instead of the left renal artery.

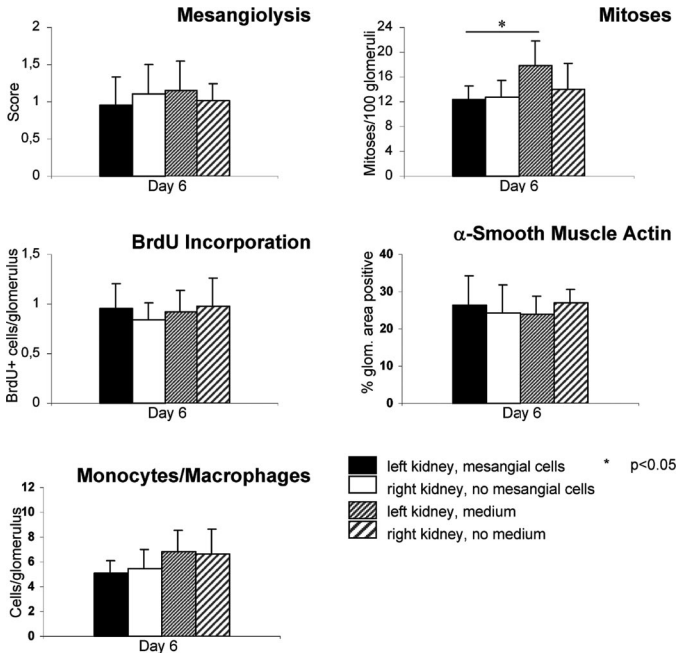

Figure 3. Renal histology and quantitative comparison of histologic changes in control Wistar rats that had anti-Thy1.1 nephritis and received mesangial cells or medium into the left artery on day 2 after disease induction.

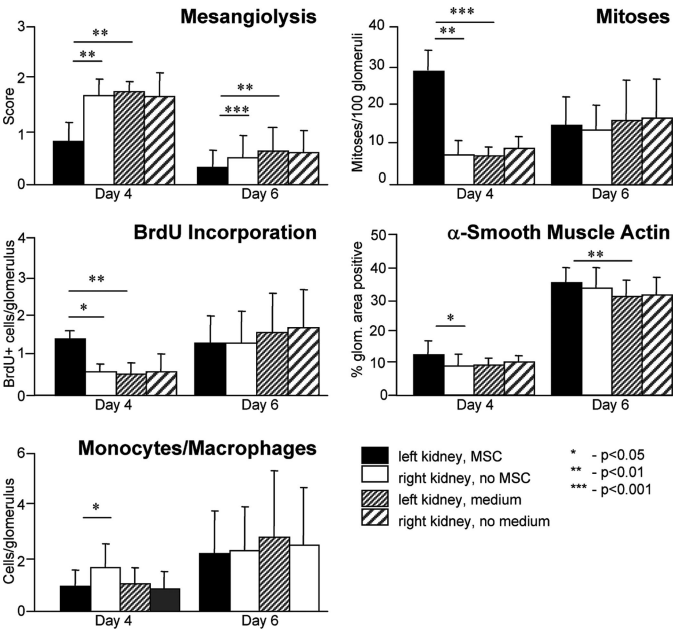

Figure 4. Comparison of Wistar rats that had anti-Thy1.1 nephritis and received MSC or medium into the left renal artery on day 2 after disease induction. Quantitative analysis of renal histologic changes.

Anti-Thy1.1 Nephritis in Inbred Lewis Rats: Intrarenal Injection of MSC Ameliorates ARF and Accelerates Resolution of Mesangiolysis

Compared with Wistar rats, the course of anti-Thy1.1 nephritis in Lewis rats was aggravated with prolonged ARF that persisted until day 10 (Table 3). MSC injection into the left renal artery significantly reduced the peak serum creatinine on day 6 after disease induction (Table 3). In parallel, the peak of proteinuria was reduced by 28% in rats that received MSC into one kidney only (Table 3). BP remained normal in all groups at all time points (Table 3).

In PAS-stained renal sections on day 6, ARF was evidenced by widened and flattened tubular cells and intratubular cast formation (Figure 5C). In MSC-treated kidneys, these changes were markedly reduced on day 6 after disease induction (Figure 5D). Fluorescently or BrdU-labeled MSC were not detected in tubular locations (data not shown), suggesting that the effect of MSC in nephritic Lewis rats with ARF was not due to replacement of damaged tubular cells by MSC.

Extensive mesangiolysis on days 4 and 6 after disease induction was significantly reduced by MSC treatment as compared with medium-treated rats and untreated right control kidneys (Figures 5 and 6). On day 10, both the left and the right kidneys of rats that received MSC into the left renal artery only were significantly less mesangiolytic than kidneys of rats that received medium only (Figure 6), suggesting that on day 10, the MSC-mediated repair processes are no longer strictly limited to the site of injection. Reduced mesangiolysis in MSC-treated kidneys was associated with a significant, transient 2.2-fold increase of glomerular mitotic figures on day 4 (Figure 6), mildly increased numbers of BrdU-positive cells (Figure 6), and increased α-SMA expression, the last of which, however, failed to reach statistical significance in most comparisons (Figure 6).

On day 6, neither absolute numbers of endothelial cell mitoses per 100 glomeruli nor their relative contribution to total mitoses exhibited significant differences among the groups (data not shown). In contrast, endothelial (*i.e.*, JG12-positive) area in MSC-treated left kidneys was significantly higher than in medium-treated left controls (Figure 6).

Glomerular monocyte/macrophage influx was not affected by MSC on days 4 and 6 after disease induction (Figure 6). On day 10, MSC-treated left kidneys showed a significant increase in ED-1–positive cells when compared with left medium control kidneys or right internal control kidneys (Figure 6).

De novo glomerular expression of type I collagen was significantly increased on day 6 in MSC-treated left kidneys as compared with right internal control kidneys ($P < 0.05$) but not in comparison with medium-treated left kidneys ($P = 0.06$; Figure 6). As in Wistar rats, none of these MSC-related effects was reproduced when MSC were injected into a tail vein of Lewis rats instead of the left renal artery (data not shown).

More Than 85% of Glomerular BrdU-Labeled MSC Fail to Exhibit Endothelial, Mesangial, or Monocyte/Macrophage Markers on Day 6 after Disease Induction

By daily administration of BrdU to cultured Lewis MSC for 7 d, >90% of the cells became labeled. Four days after BrdU-labeled MSC were injected into left kidneys of five nephritic Lewis rats, these cells were detected in an average of 17% of glomeruli of the treated (left) kidneys. The staining pattern was exclusively nucleic, thus excluding endocytosed material. Right

Table 2. Wistar rats, days 4 and 6 after induction of anti-Thy1.1 nephritis[a]

	MSC Injection	Medium Injection	*P*
Day 4 after induction of anti-Thy1.1 nephritis	($n = 5$)	($n = 4$)	
serum creatinine (μmol/L)	57 ± 8	54 ± 5	NS
proteinuria (mg/d)	82 ± 88	88 ± 65	NS
systolic BP (mmHg)	116 ± 7	110 ± 5	NS
Day 6 after induction of anti-Thy1.1 nephritis	($n = 22$)	($n = 23$)	
serum creatinine (μmol/L)	43 ± 7	39 ± 9	NS
proteinuria (mg/d)	20 ± 24	20 ± 31	NS
systolic BP (mmHg)	106 ± 13 ($n = 9$)	112 ± 16 ($n = 7$)	NS

[a]Functional measurements data in rats that received either intra-arterial injection of mesenchymal stem cells (MSC) or control medium into the left kidney on day 2.

Table 3. Lewis rats, days 4, 6, and 10 after induction of anti-Thy1.1 nephritis[a]

	MSC Injection	Medium Injection	*P*
Day 4 after induction of anti-Thy1.1 nephritis	($n = 5$)	($n = 5$)	
serum creatinine (μmol/L)	65 ± 6	59 ± 6	NS
proteinuria (mg/d)	92 ± 55	100 ± 47	NS
systolic BP (mmHg)	117 ± 7	120 ± 7	NS
Day 6 after induction of anti-Thy1.1 nephritis	($n = 9$)	($n = 6$)	
serum creatinine (μmol/L)	57 ± 19	76 ± 9	<0.05
proteinuria (mg/d)	81 ± 27	112 ± 27	<0.05
systolic BP (mmHg)	116 ± 10	110 ± 9	NS
Day 10 after induction of anti-Thy1.1 nephritis	($n = 9$)	($n = 8$)	
serum creatinine (μmol/L)	36 ± 9	40 ± 6	NS
proteinuria (mg/d)	9 ± 3	8 ± 3	NS
systolic BP (mmHg)	113 ± 8	119 ± 12	NS

[a]Functional measurements data in rats that received either intra-arterial injection of MSC or control medium into the left kidney on day 2.

kidneys consistently were BrdU negative. BrdU-positive cells outside of glomeruli were an extremely rare event in all five MSC-treated kidneys.

Double staining of the five MSC-treated kidneys for BrdU and the monocyte/macrophage marker ED-1 revealed a total of five double-labeled cells (6% of all BrdU-positive cells) with ED-1 positivity along 20 to 50% of the cell circumference, and one BrdU-positive cell nucleus was completely surrounded by ED-1–positive cytoplasm (Figure 1J, Table 4). BrdU and the endothelial cell marker JG12 were coexpressed in only three cells with JG12 positivity along 20 to 50% of cell circumference (Figure 1K, Table 4). In the case of BrdU and the mesangial cell marker α-SMA, seven (9%) cells showed α-SMA positivity along 20 to 50% of cell circumference, and three BrdU-positive cell nuclei were completely surrounded by α-SMA–positive cytoplasm (Figure 1L, Table 4).

MSC in Culture Produce VEGF and TGF-β1 but No Detectable PDGF-BB

Finally, we assessed cell culture supernatants of MSC after 72 h of culture during the late third passage (*i.e.*, before injection) for the presence of growth factor secretion. In the case of TGF-β1, mean concentrations were 1929 ± 522 pg/ml in the supernatant as opposed to 1040 ± 65 pg/ml in fresh medium that contained heat-inactivated FCS. For VEGF, mean supernatant concentrations were 1152 ± 387 pg/ml as opposed to <3 pg/ml in fresh medium. PDGF-BB concentrations in cell culture supernatants were lower than in fresh medium (3.6 ± 2.2 *versus* 18.6 ± 5.9 pg/ml), indicating consumption and/or degradation.

Discussion

In this study, we first established that adherent, bone marrow–derived, spindle-shaped cells exhibit two key features of MSC, namely adipogenesis and osteoblastogenesis. We also ascertained that they are Thy1.1 negative, which is important in this model, in which some Thy1.1 antibody might have persisted in the circulation. Third, we demonstrated that fluorescence labeling did not affect MSC features.

Our first major finding was an almost exclusive distribution of MSC in glomeruli of the ipsilateral kidney for up to 10 d after

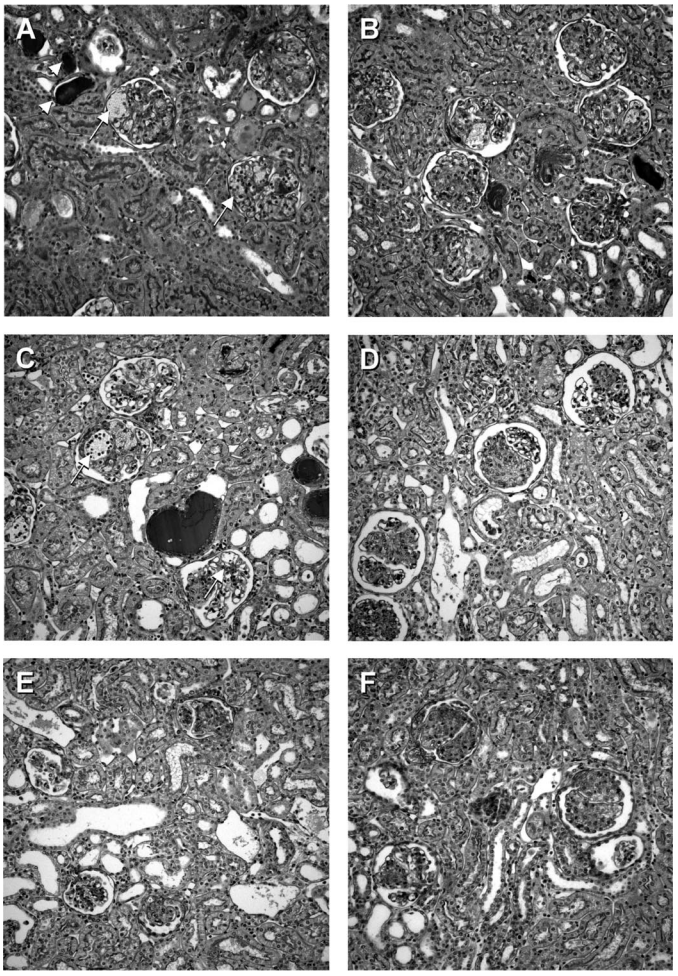

Figure 5. Renal histology in Lewis rats that had anti-Thy1.1 nephritis and received MSC or medium into the left renal artery on day 2 after disease induction. (A) Medium-injected kidney, day 4, showing prominent mesangiolysis (arrows) and tubular casts (arrowheads). (B) Mesangiolysis and tubular cast formation is reduced in a MSC-injected kidney on day 4. (C) Marked, persistent mesangiolysis (arrow) and tubular cast formation are noted in a medium-injected kidney on day 6. (D) An MSC-injected kidney on day 6 exhibits mostly mesangioproliferative changes and little persistent mesangiolysis. (E) Apart from mild persistent mesangiolysis, a medium-injected kidney on day 10 does not exhibit markedly different histology as compared with an MSC-injected kidney (F) on day 10. Magnification, ×100 (PAS staining).

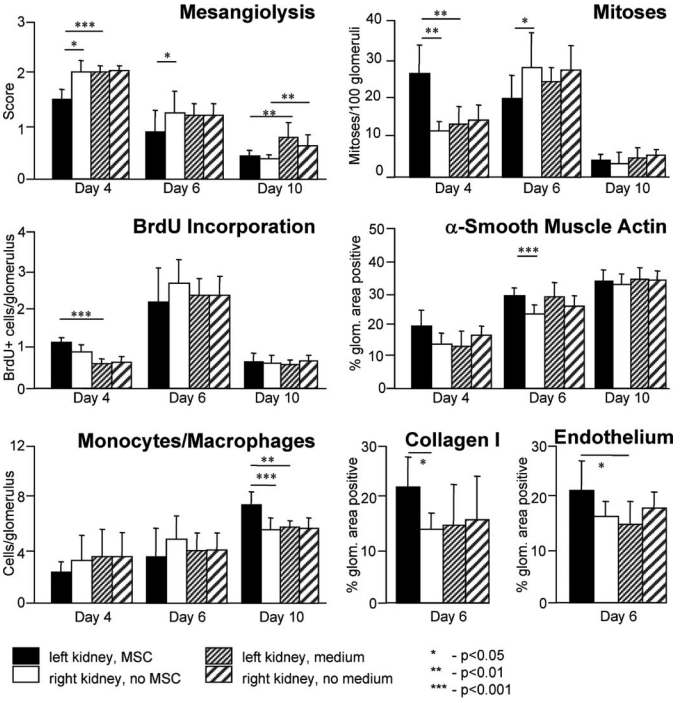

Figure 6. Comparison of Lewis rats that had anti-Thy1.1 nephritis and received MSC or medium into the left renal artery on day 2 after disease induction. Quantitative analysis of renal histologic changes.

injection into a renal artery. This was a specific feature of MSC, as mesangial cells, despite their similar size, in general failed to reproduce these findings. More important, after injection into the renal artery of nephritic Lewis rats with acute tubular damage, our MSC did not populate the damaged tubulointerstitium as well. This observation contrasts with a number of studies (21–23) that showed homing of intravenously injected rat MSC to injured areas in several disease models in rodents. A potential explanation for these seemingly contradictory findings is provided by the study of Rombouts *et al.* (14), which describes loss of homing capacity of mouse MSC after *ex vivo* culture. In addition, we cannot exclude that MSC injection at another time point after induction of anti-Thy1.1 nephritis induction might have resulted in different homing characteristics.

Our second major finding was that MSC ameliorated mesangiolytic damage in two different rat strains after induction of anti-Thy1.1 nephritis. In particular in Lewis rats, that exhibit an aggravated course of the disease, functional improvement of the kidney also was notable (*i.e.*, proteinuria and serum creatinine were lowered). Our data thereby extend previous findings in cisplatin- or glycerol-induced ARF in mice, in which MSC promoted recovery as well (12,24). In this context, it is important to stress that our effects were observed despite that only the left kidney received MSC, whereas the course of the nephritis hardly was affected in the contralateral, non–MSC-injected kidney. Accelerated healing of mesangiolytic lesions by MSC involved both faster capillary restoration, as reflected by higher glomerular areas covered by endothelium, and more rapid repopulation of the mesangium. The latter was evidenced by upregulated expression of glomerular α-SMA and type I collagen, both of which can serve as markers of activated mesangial cells (25). This effect could not be reproduced by injection of an equal number of Wistar-derived primary mesangial cells, although rat mesangial cells are known to secrete PDGF-B. The long-term consequences of the more rapid mesangial repopulation and matrix accumulation as seen with MSC treatment are presently unknown and are the subject of ongoing studies. However, others have shown that intravenous administration of MSC immediately after bleomycin-induced

Table 4. Double immunostaining of BrdU-labeled MSC for markers of monocytes/macrophages (ED-1), endothelial cells (JG12), and mesangial cells (α-SMA)[a]

	ED-1−	ED-1+	JG12−	JG12+	α-SMA−	α-SMA+
BrdU + nuclei (relative frequency)	75 (93%)	6 (7%)	93 (97%)	3 (3%)	71 (88%)	10 (12%)

[a]Numbers of 5-bromo-2′deoxyuridine (BrdU)-positive nuclei are total counts in >100 glomeruli each of five rats. α-SMA, α-smooth muscle actin.

lung injury resulted in significantly reduced inflammation, collagen deposition, and matrix metalloproteinase activation (21).

Our observations were consistent in an inbred, syngeneic (*i.e.*, Lewis rats) as well as an outbred, allogeneic (*i.e.*, Wistar rats) setting. Apart from its obvious implications for human therapy, these data shed further light on the stem cell capacity to induce immunologic tolerance. For example, in a xenogeneic model, Allers *et al.* (26) reported survival and engraftment into bone marrow, spleen, and mesenchymal tissues of human MSC after intravenous injection into unconditioned mice. A number of other studies also confirmed persistence of allogeneic MSC *in vivo*, which is mediated in part by suppressing T cell activation (27,28). If our MSC similarly inhibit activation of T cells, then this also could explain some of the effects on the course of anti-Thy1.1 nephritis. However, before day 2 (the time point of MSC injection in our experiments), neutrophils and macrophages mediate renal damage, whereas T cell activation has not been noted until day 5 after disease induction (29).

What are the mechanisms underlying the benefit of MSC in our model? First, injected MSC might have differentiated into resident glomerular cells. We therefore labeled our MSC by letting them incorporate BrdU *in vitro*. Oliver *et al.* (8) described slow-cycling putative stem cells in the renal papilla that were labeled by BrdU pulses in rat pups. These cells were characterized as stem cells, and they showed proliferation and disappeared from the papilla upon transient ischemia, thereby indicating that incorporation of BrdU did not inhibit their "stemness" and responsiveness to injury. Another reason to use BrdU is that others observed fluorescence leakage from damaged EGFP-positive cells that underwent glomerular filtration and subsequently labeled tubular epithelial cells in a granular pattern (7). In this study, by double immunostaining of BrdU-labeled MSC for endothelial, mesangial, and monocyte/macrophage markers, we were able to show that the vast majority of MSC failed to differentiate into either cell type. Therefore, we can largely exclude glomerulus-specific differentiation of our MSC or even fusion of our donor bone marrow cells with nonhematopoietic cells of the recipient (30). In this respect, our data differ from findings showing that Lewis rat MSC differentiated into vascular/endothelial and myocardial cells after intravenous injection in a rat myocardial infarction model (23). They also differ from a report that bone marrow can give rise to new mesangial cells in the setting of experimental glomerulonephritis when transplanted without any previous culture (4).

A second possibility to explain how MSC ameliorated mesangiolytic damage is intraglomerular proliferation of these cells to fill more rapidly the cellular gaps. Again, this seems unlikely given our data with *ex vivo* BrdU-labeled MSC: Four days after injection, immunostaining of renal sections for BrdU revealed either brightly positive cells or no staining at all. This indicates that the majority of our MSC did not proliferate in the glomeruli and therefore not dilute the BrdU content of their DNA during nuclear division. In addition, intrarenal injection of cultured mesangial cells did not result in similar effects as injected MSC, indicating that efforts to replace mesangial cells directly in this disease model cannot improve function and histology.

In view of the above, possibility 3—paracrine effects of the MSC in the glomeruli—seems most likely to underlie their action. This was already indicated by the fact that engraftment of fluorescence MSC was a comparatively rare event in glomeruli, visible in only 17 to 50% of glomeruli per section. Given a section thickness of 4 μm, this would translate into no more than a total of one to six MSC per glomerulus. Such low or even lower cell numbers also were noted previously: Iwatani *et al.* (7) transplanted Hoechst low/side population cells that were derived from kidneys of EGFP-positive rats into kidneys of wild-type rats after induction of Thy1.1 nephritis and at follow-up rarely observed these cells in diseased glomeruli. However, such low cell numbers might create an altered microenvironment at sites of engraftment. In this respect, it is noteworthy that our MSC released high amounts of proangiogenic VEGF and profibrotic TGF-β1 *in vitro*. Our findings are in line with data of others showing that MSC release VEGF and basic fibroblast growth factor and thereby exert beneficial effects in experimental ischemia/reperfusion-induced ARF (31,32). Furthermore, bone marrow mononuclear cells, preconditioned toward endothelial progenitor cells *in vitro*, were injected into nephritic kidneys and likely mediated their therapeutic effect by secreting VEGF (33). Apart from paracrine effects of MSC on neighboring glomerular cells, it also is possible that intrarenally injected MSC served as chemoattractors and "feeders" to circulating hematopoietic stem cells and thereby further promoted resolution of injury.

It is interesting that in our studies on paracrine actions of MSC, we failed to detect PDGF-BB, a growth factor that is essential for mesangial cell development. This may explain in part our observation that MSC seemed not to differentiate into mesangial cells, as Suzuki *et al.* (6) showed that PDGF-BB is an important factor for the conversion of bone marrow–derived stem cells into mesangial cells.

For future studies, a recent report by Burns *et al.* (34) added a cautious note to results that were obtained by thymidine analogue labeling (*i.e.*, BrdU) of transplanted stem cells. They

observed incorporation of graft-derived thymidine analogues into dividing host neural precursors and glia, suggesting differentiation of grafted cells. However, because this could be reproduced by injecting fibroblasts or even dead cells, they concluded that thymidine analogues can be "passed on" to resident cells. In our case, the strong positivity for BrdU, limited to single intraglomerular cells without bordering positive cells, argues against reuptake of BrdU by resident cells. Otherwise, we would expect clusters of double-stained cells with a diminished positivity for BrdU. Furthermore, the observation that most of our BrdU-positive cells failed to exhibit differentiation markers of endogenous glomerular cells also argues against rapid cell death of injected, BrdU-labeled MSC with passing on of BrdU to glomerular cells.

Conclusion

We demonstrate that intra-arterial as opposed to the commonly used intravenous injection of adult MSC provides a tool to accelerate glomerular healing in a rat model of mesangioproliferative glomerulonephritis. Our approach worked in both allogeneic and syngeneic settings and is technically reproducible in human medicine *via* renal artery catheterization. The benefits that are exerted by our MSC include lowering of proteinuria, prevention of ARF, and improved resolution of mesangiolytic damage in MSC-treated kidneys.

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