Allogenic stem cell therapy improves right ventricular function by improving lung pathology in rats with pulmonary hypertension

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Am J Physiol Heart Circ Physiol 297: H1606–H1616, 2009. First published September 25, 2009; doi:10.1152/ajpheart.00590.2009.—Pulmonary arterial hypertension (PAH) is a chronic lung disease characterized by increased pulmonary artery pressure, pulmonary vascular damage, and medial hypertrophy of pulmonary arterioles, leading to right ventricular (RV) hypertrophy (RVH), RV failure, and, eventually, death (8). In the present study, we used a well-established model of experimental PAH in rats induced by a single injection of monocrotaline (MCT) (25, 33). MCT, a pyrrolizidine alkaloid derived from Crotalaria spectabilis, causes a pulmonary vascular syndrome in rats characterized by proliferative pulmonary vasculitis, PAH, and cor pulmonale. The consequences of two doses of MCT (30 and 80 mg/kg) on RV function at 28 days after MCT administration were dose-dependent 1) RVH, 2) RV dilatation, and 3) impairment of RV systolic function, without marked effects on RV diastolic function (15).

In rats with MCT-induced PAH, the elevation of pulmonary arterial pressure is correlated with thickening of the medial wall of small pulmonary arteries and arterioles due to the proliferation of vascular smooth muscle cells (12, 50).

PAH has been shown to be refractory to most of the conventional pharmacological therapies. Stem cell therapy may constitute a new treatment modality for patients with PAH. Particularly, mesenchymal stem cells (MSCs) are unique in possessing "prosurvival factors," such as growth factors or cytokines, hence improving the tissue’s condition by paracrine mechanisms (24).

Different modes of administration of stem cells, including intravenous (21), intratracheal (2), and direct implantation of cells into the lungs (48), have been investigated. We used the intravenous route of administration as it is a safe and attractive method of cell injection. We hypothesized that, when administered intravenously, MSCs filter through the lungs and may engraft at the sites of lung parenchymal or vascular damage. Once these cells reside in damaged tissue, they start secreting “prosurvival factors,” such as growth factors or cytokines, hence improving the tissue’s condition by paracrine mechanisms (24).

Our purpose was to treat recipient rats with MCT-induced PAH with bone marrow-derived MSCs obtained from donor rats with MCT-induced PAH to mimic autologous stem cell transplantation. To analyze the effects of MCT-induced PAH and cell therapy in this model, we studied the 1) thickness of pulmonary arteriolar walls, 2) thickness of alveolar septa, 3) pulmonary expression of genes associated with inflammation and angiogenesis, 4) RV pressures, 5) RV volumes and RV ejection fraction (RVEF), 6) RVH, 7) RV myocardial expression of genes associated with myocardial hypertrophy, and 8) RV myocardial expression of extracellular matrix (ECM) proteins. All measurements were obtained in normal rat hearts (control), hearts of rats with MCT-induced PAH without cell therapy (MCT60), and hearts of rats with MCT-induced PAH with cell therapy using MSCs (MCT + MSC) from donor rats with PAH.
MATERIALS AND METHODS

Animals

All animals were treated in accordance with national guidelines and with permission from the Animal Experiments Committee of Leiden University Medical Center. The investigation conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

Study Design

We used 8-wk-old female Wistar rats weighing 200–250 g (Harlan, Zeist, The Netherlands). On day 1, animals received a single subcutaneous injection of MCT (60 mg/kg body wt) diluted in PBS to induce PAH (n = 20) or PBS alone to serve as a control (n = 10). On day 14, MCT-treated rats received an intravenous injection into the jugular vein of 10⁶ Dil ( Molecular Probes, Invitrogen, Breda, The Netherlands)-labeled MSCs suspended in 1 ml PBS (MCT + MSC, n = 10) or 1 ml PBS alone (MCT60; n = 10). Control rats also received an intravenous injection of PBS. Animals were housed at 2 animals/cage with a 12:12-h light-dark cycle and an unrestricted food and water supply. Rats were weighed 3 times/wk. After 4 wk, RV function was analyzed, and the rats were killed.

MSC Isolation and Culture

To obtain MSCs, female donor rats were injected subcutaneously with MCT (60 mg/kg, n = 5). After 28 days, rats were anesthetized with isoflurane and killed by an intravenous injection of KCl (100 mmol/l). Their femurs and tibiae were removed and cleaned. The proximal ends were clipped, and the bones placed in microfuge tubes consisting of a regular culture medium (DMEM supplemented with 10 mmol/l glutamine, 50 μg/ml ascorbic acid, and 10 nmol/l dexamethasone) supplemented with 50 μl/ml streptomycin, 1 μmol/l dexamethasone, 50 μmol/l indomethacin, and 0.5 μmol/l amphotericin B solution) supplemented with 5 mg/ml DNase-I (Sigma-Aldrich). Cells were plated in a 25-cm² culture flask (Becton Dickinson, Franklin Lakes, NJ) and cultured in a humidified hypoxic incubator (5% CO2-5% O2) at 37°C. Nonadherent cells were replated after 6 h. Two days later, nonadherent cells were removed, and cells were frozen to confluence.

In Vitro Assays of MSCs

Previously it has been reported that MSCs cultured in 2–5% O2 have an increased proliferation rate and upregulated VEGF expression compared with MSCs cultured in ambient air, while maintaining their multilineage potential (13, 14, 17, 27, 41, 43).

As far as the differentiation capacity of MSCs is concerned, hypoxia has been shown to have positive effects (14) as well as negative effects on osteogenic differentiation (41). MSCs from MCT-treated rats and healthy rats (passages 3 and 4) grown under hypoxic or normoxic conditions were tested for 1) proliferation rate, 2) membrane protein repertoire by FACS, 3) production and secretion of VEGF, and 4) capacity of adipogenic and osteogenic differentiation. In addition, the effects of the major metabolite of MCT (dehydro-MCT) on VEGF secretion by MSCs incubating MSCs with 50 μmol/l dehydro-MCT in vitro.

Proliferation rate of MSCs. MSCs isolated from the bone marrow of healthy rats and the bone marrow of rats with MCT-induced PAH were grown in 96-well plates at 5% and 20% O2. At two moments in time, spaced 4 days, media were poured off, and cells were analyzed with a cell proliferation assay (XTT, Roche, Almere, The Netherlands). The assay is based on the cleavage of the tetrazolium salt XTT into a soluble formazan salt. This conversion only occurs in viable cells. The measured absorbance directly correlates to cell number.

Flow cytometry of MSCs. MSCs isolated from the bone marrow of healthy rats and the bone marrow of rats with MCT-induced PAH were grown at 5% and 20% O2 and analyzed for surface marker expression by flow cytometry. MSCs were detached using trypsin-EDTA (Bio-Whittaker Europe, Verviers, Belgium), resuspended in PBS containing 0.5% BSA (Sigma-Aldrich), and divided in aliquots of 5 × 10⁶ cells. Cells were then incubated for 30 min at 4°C with FITC- or phycoerythrin-conjugated antibodies against rat CD34, CD29, CD44, CD45, CD106 (all from Becton Dickinson), and CD90 (Serotec, Oxford, UK). Labeled cells were washed and analyzed using a FACSSort flow cytometer (BD Pharmingen, San Jose, CA) equipped with a 488-nm argon ion laser and a 635-nm red diode laser. Isotype-matched control antibodies (BD Pharmingen) were used to determine the background fluorescence. At least 5,000 cells/sample were analyzed, and data were processed using CellQuest software (BD Pharmingen). Generally, MSCs have been found to be positive for CD29, CD44, CD45, CD106, and CD105 and negative for CD34 and CD45 (22).

Production and secretion of VEGF by MSCs. MSCs isolated from the bone marrow of healthy rats and the bone marrow of rats with MCT-induced PAH were grown at 5% O2 in 96-well plates with and without dehydro-MCT (50 μmol/l). At two moments in time, spaced 4 days, media were poured off, and cells were analyzed for protein content by the BCA assay (Pierce, Perbio Science, Etten-Leur, The Netherlands). Media were assayed for VEGF using a rat VEGF assay kit (kit-based kit supplied with rat VEGF microparticle concentrate, R&D Systems Europe, Oxford, UK). VEGF concentrations in the media are expressed as nanograms per milligram of cellular protein.

Adipogenic and osteogenic differentiation of MSCs. MSCs isolated from the bone marrow of healthy rats and the bone marrow of rats with MCT-induced PAH grown at 5% O2 were characterized using established differentiation assays (40). Briefly, 5,000 MSCs/well were plated in a 12-well culture plate and exposed to adipogenic or osteogenic induction medium. Adipogenic differentiation medium consisted of a regular culture medium (DMEM supplemented with 15% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B solution) supplemented with 5 μg/ml insulin, 1 μmol/l dexamethasone, 50 μmol/l indomethacin, and 0.5 μmol/l IBMX (all from Sigma-Aldrich) and was refreshed every 3–4 days for a period of 3 wk. Lipid accumulation was assessed by oil red O staining of the cultures (15 mg oil red O/ml of 60% isopropanol) and light microscopy. Osteogenic differentiation medium consisted of culture medium supplemented with 10 mmol/l β-glycerophosphate, 50 μg/ml ascorbic acid, and 10 mmol/l dexamethasone (all from Sigma-Aldrich) and was refreshed every 3–4 days for a period of 2 wk. Afterward, calcium deposits were visualized by staining with 2% alizarin red S in 0.5% NH4OH (pH 5.5).

Effects of Dehydro-MCT on MSCs In Vitro

Dehydro-MCT was prepared from MCT by chemical oxidation using o-chloranil according to Mattocks et al. (31). Mass spectrometric analyses have shown that 25–30% of the input MCT is converted to dehydro-MCT by this method (46). Dehydro-MCT is the major metabolite of MCT and is formed after the conversion of MCT by cytochrome P-450 monoxygenases in the liver. Therefore, it is more relevant to study in vitro experiments with MSCs the effects of dehydro-MCT than the effects of MCT.

Cell Injection

Before injection into recipient rats, MSCs from donor rats with MCT-induced PAH (passages 3 and 4) were trypsinized and labeled with the viable fluorescent dye CM-DiI according to the manufacturer’s recommendations (CellTracker, Molecular Probes, Invitrogen). On day 14, each recipient rat was anesthetized with isoflurane and
placed in a supine position. The jugular vein was surgically exposed, and a 20-gauge cannula (Biovalve, Vygon Nederland, Valkenswaard, The Netherlands) was introduced. Subsequently, 1 ml of cell suspension (10⁶ MSC/ml) or PBS alone was injected slowly. After removal of the cannula, the vein was compressed for 5 min to allow for closure of the puncture, and the skin was closed.

**Hemodynamic Measurements**

On day 28 after MCT or PBS administration, RV pressure was measured as previously described (15). Briefly, rats were sedated by the inhalation of a mixture of isoflurane (4%) and O₂. Subsequently, general anesthesia was induced by an intraperitoneal injection of a fentanyl-fluanison-midazolam mixture at a dose of 0.25 ml/100 g body wt. The mixture consisted of two parts Hypnorm (0.315 mg/ml fentanyl + 10 mg/ml fluanison, Vital-Pharma, Maarheeze, The Netherlands), one part Dormicum (5 mg/ml midazolam, Roche, Mijdrecht, The Netherlands), and one part saline. Animals were placed on a controlled warming pad, intubated, mechanically ventilated with a mixture of air and oxygen, and then placed under a stereomicroscope (Zeiss, Hamburg, Germany). After a midsternal thoracotomy was performed, a combined pressure-conductance catheter (model FT212, SciSense, London, ON, Canada) was introduced via the apex into the RV and positioned toward the pulmonary valve. The catheter was connected to a signal processor (FV898 Control Box, SciSense), and RV pressures and volumes were recorded digitally. Parallel conductance was assessed by the hypertonic saline method as previously described (15). All data were acquired at a sample rate of 2,000 Hz and analyzed offline by custom-made software. To quantify hemodynamic conditions, we determined heart rate, stroke volume, cardiac output, RV end-diastolic volume, RV end-systolic pressure, RV peak systolic pressure (RVPSP), and RV end-systolic pressure-volume relations and preload recruitable stroke work (relation between stroke work and end-diastolic volume) were obtained as the area of the pressure-volume loop, and the maximal rates of RV pressure increase and fall (dP/dt max). RV end-systolic pressure from steady-state pressure-volume loops. In dynamic conditions, we determined heart rate, stroke volume, cardiac output, RV end-diastolic pressure, RV end-systolic pressure, and dP/dt max, respectively) were calculated. The relaxation time constant was assessed as the time constant of the monoexponential decay of RV pressure during isovolumic relaxation. To quantify RV afterload, effective pulmonary arterial elastance was calculated as end-systolic pressure divided by stroke volume. RV end-systolic and end-diastolic pressure-volume relations and preload recruitable stroke work (relation between stroke work and end-diastolic volume) were determined from pressure-volume loops recorded during transient occlusion of the inferior vena cava by external compression of the vessel. The slopes of these relations (end-systolic elastance and end-diastolic elastance) are load-independent parameters of intrinsic myocardial function (3).

**Tissue Preparation**

After hemodynamic measurements, rats underwent either rapid excision of the lungs and heart or lungs were fixed in situ via the trachea cannula with buffered formaldehyde (4% paraformaldehyde in PBS, pH 7.4) at a pressure of 25 cmH₂O for 5 min. The freshly excised lungs and heart were snap frozen in liquid nitrogen and stored at −80°C. The acutely fixed lungs were additionally fixed in paraformaldehyde for 24 h and embedded in paraffin after dehydration in a graded alcohol series and xylene.

**Real-Time RT-PCR**

Total RNA was isolated from lung tissue homogenates and from homogenates of the RV myocardium (RNA-Bee, Tel-Test, BioConnect, Huispen, The Netherlands). The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically. First-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, The Netherlands). For real-time quantitative PCR, SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) was used. Primers designed with the Primer Express software package (Applied Biosystems) for the expression experiments in lung homogenates and for the expression experiments in myocardial homogenates are shown in Table 1.

**Table 1. Primers used for expression experiments in lung homogenates and for expression experiments in myocardial homogenates**

<table>
<thead>
<tr>
<th>Primers for lung homogenates</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>5′-ATATGTTCATCAAGGAGATCTTTGGAA-3′</td>
<td>5′-TGAGTGATGCTGTTGATGAA-3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5′-CAAGGGTCGCCGCGCTGAT-3′</td>
<td>5′-CTCCTGATGTTGAAGTGGCATT-3′</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>5′-TCTGCTCTACAAAAAAGACAAAGA-3′</td>
<td>5′-GTTGATGGTGCCGCTCAC-3′</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>5′-GCCCTGAAACCCCTCAGCACA-3′</td>
<td>5′-GCCCTAGCGGAATGTTCC-3′</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>5′-TGACAGCTCATCTGATGTGC-3′</td>
<td>5′-GCCGGACAGCATGACCA-3′</td>
</tr>
<tr>
<td>Matrix metalloproteinase-2</td>
<td>5′-GCCAAAGACGCTTCTCTCC-3′</td>
<td>5′-GAAAGCAAACGCGCTAC-3′</td>
</tr>
<tr>
<td>β-Act</td>
<td>5′-GGCTCTAAGCAGCACCAGTGAAGATC-3′</td>
<td>5′-GGACCAAAATCCTCAACAGA-3′</td>
</tr>
</tbody>
</table>

**Primers for myocardial homogenates**

<table>
<thead>
<tr>
<th>Primers for myocardial homogenates</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-atrial natriuretic peptide</td>
<td>5′-CCAGGCAATTTGGAGGAA-3′</td>
<td>5′-AGGTCTTGGAAATCTCAGCATGTC-3′</td>
</tr>
<tr>
<td>Pro-B-type natriuretic peptide</td>
<td>5′-GAAGCCTGCTGGAGTAAAG-3′</td>
<td>5′-TGGAGCTCCTGGGCTTCTTTG-3′</td>
</tr>
<tr>
<td>Collagen type 1</td>
<td>5′-CCAGGCTGATCCTTGGAG-3′</td>
<td>5′-AAAGCTGCTGATCGAGACACAT-3′</td>
</tr>
<tr>
<td>Collagen type 2</td>
<td>5′-ATTCTGCTATACTTGGAA-3′</td>
<td>5′-ATTCCATGCTGACGTGTG-3′</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>5′-TCAAGCTCTAGTGGCATC-3′</td>
<td>5′-TGGCAGGACGCGCTGCAG-3′</td>
</tr>
<tr>
<td>β-Act</td>
<td>5′-GGCTCTAAGCAGCACCAGTGAAGATC-3′</td>
<td>5′-GGACCAAAATCCTCAACAGA-3′</td>
</tr>
</tbody>
</table>
Sections were counterstained briefly with hematoxylin and mounted with DPX (BDH, Brunschwig Chemie, Amsterdam, The Netherlands). Dilabeled MSCs in lung tissue sections were studied after being stained with 10 μg/ml Hoechst-33342 solution (Molecular Probes, Invitrogen) and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Sections were examined under a microscope (Nikon Eclipse, Nikon Europe, Badhoevedorp, The Netherlands) equipped with ×10, ×20, ×40, and ×100 objectives and a digital camera (model DXM1800, Nikon). This microscope performed both light microscopy and fluorescence microscopy.

Quantitative Image Analysis

For the determination of the alveolar septum thickness, hematoxylin-phloxin-saffron-stained lung tissue was photographed at the highest magnification. At locations distant from branch points and arterioles, the alveolar septum thickness was measured with calipers in Image-Pro Plus (Media Cybernetics, Silver Spring, MD). For the determination of the precapillary arteriolar wall thickness, α-smooth muscle actin-stained lung tissue was photographed at the highest magnification. The wall thickness of arterioles localized at branch points of the alveolar septa was measured with calipers in Image-Pro Plus.

Cardiomyocyte Isolation Procedures

A separate series of 15 rats was given the same experimental treatments (control, MCT60, and MCT + MSC; n = 5 rats/group) as described above. On day 28, rats were anesthetized, and the thorax was opened. The heart was taken out quickly, immediately put into ice-cold oxygenated Tyrode solution, and perfused in a Langendorff setup (AD Instruments, Spechbach, Germany) with oxygenated Tyrode solution at constant pressure (70 mmHg) at 37°C. After perfusion setup (AD Instruments, Spechbach, Germany) with oxygenated low-Ca²⁺ Tyrode solution and mounted in Vectashield (Vector Laboratories, Burlingame, CA). After that time, collagenase (0.06%, Worthington, Lakewood, NJ) was added. Thirty min later, the heart was removed, and the RV was separated from the left ventricle (LV) including the interventricular septum (IVS). Next, the RV and LV were cut in small pieces, incubated in fresh collagenase (0.06% solution, and dissociated in a water bath shader at 37°C. Thereafter, sedimented cardiomyocytes were resuspended and stored at 37°C in fresh HEPES-buffered salt solution (in mmol/l) 125 NaCl, 5 KCl, 1 MgSO₄, 1 K₂HPO₄, 1.8 CaCl₂, 10 NaHCO₃, 20 HEPES, and 5.5 glucose (pH 7.4). The average fraction of rod-shaped cardiomyocytes was 80%. Per heart, roughly 50 intact, noncontracting, rod-shaped cardiomyocytes from the RV as well as the LV were photographed, followed by measurements of length and width with a calibrated ruler. The cardiomyocyte area was calculated by multiplying the length and width, and the cylindrical cell volume was calculated as follows: cylindrical cell volume = (π/4) × (width)² × length.

Assessment of RVH

To quantify the degree of RVH, hearts were harvested followed by the removal of the left and right atrium. The RV free wall was dissected and weighed separately from the LV including the IVS. The parts were frozen in liquid nitrogen, stored at −80°C, and fixed in buffered paraformaldehyde. The RV mass, weight ratio of the RV/ (LV + IVS), and dimensions of isolated RV cardiomyocytes were used as indicators of RVH.

Statistical Analysis

Group differences were evaluated by one-way ANOVA followed by Bonferroni’s post hoc test. As to the dimensions of isolated cardiomyocytes, we analyzed the data on a per animal basis, with n = 5 animals/group for mean width, length, area, and volume of cardiomyocytes from the RV and LV. SPSS 16 for Windows (SPSS, Chicago, IL) was used for the statistical analysis. Differences were considered significant at P ≤ 0.05. Values are means ± SD unless otherwise stated.

RESULTS

Proliferation Rate of MSCs

If grown at 21% O₂, MSCs from healthy rats proliferated at roughly the same rate as MSCs from MCT-treated rats (P = 0.576). If grown at 5% O₂, MSCs from both groups showed higher proliferation rates, being approximately threefold higher (P < 0.01) for healthy rats and twofold higher (P = 0.061) for MCT-treated rats (Fig. 1A). If grown at 5% O₂, MSCs from healthy rats proliferated roughly twice as rapid as MSCs from MCT-treated rats (P < 0.03).

FACS Analysis of MSCs

A comparison of FACS data obtained from MSCs isolated from rats with MCT-induced PAH with those obtained from MSCs from healthy rats revealed minor differences in the repertoire of surface markers (Fig. 1B). Both types of MSCs were cultured in a 5% O₂-5% CO₂ incubator.

VEGF Production and Secretion From MSCs

MSCs from healthy rats and MSCs from rats with MCT-induced PAH were grown in 5% O₂ in 96-well plates for 4 days. MSCs from rats with MCT-induced PAH had approximately twofold higher VEGF secretion than MSCs from healthy rats (P < 0.02; Fig. 1C). The addition of 50 μmol/l dehydro-MCT, the toxic metabolite of MCT, showed only a tendency toward depressed VEGF secretion from both types of MSCs.

Differentiation Potential of MSCs

MSCs from healthy rats and from rats with MCT-induced PAH, both grown in 5% O₂, were investigated for adipogenic and osteogenic differentiation capacities. Adipogenic and osteogenic differentiation capacities present in MSCs obtained from healthy rats were well preserved in MSCs obtained from MCT-treated rats (Fig. 1D).

Body Weights

Body weight gain on day 28 was 28.1 ± 8.2 g in control rats. PAH animals showed a significantly reduced weight gain (18.1 ± 6.9 g, P < 0.05 vs. control). The weight gain in MSC-treated (PAH-treated) animals was intermediate (20.6 ± 8.2 g) and not significantly different from control rats and MCT60 rats (Fig. 2A).

Lung Weights

Lung weight was 0.96 ± 0.15 g in control animals. In the MCT60 group, lung weight was significantly increased to 1.66 ± 0.32 g (P < 0.01 vs. control). The increase in lung weight observed in the MCT60 group was symptomatic for an increased remodeling of lung tissue in this group rather than to pulmonary edema (15). With stem cell therapy, lung weight had decreased to 1.16 ± 0.24 g (P < 0.05 vs. the MCT60 group). Lung weights normalized for body weight were 3.85 ± 0.68 mg/g in the control group, 6.40 ± 1.40 mg/g in the
Fig. 1. A: proliferation of mesenchymal stem cells (MSCs) at 4-day intervals. MSCs from healthy rats and MSCs from rats with monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) were grown in an incubator with 5% O₂-5% CO₂-90% N₂ (5% O₂) or in an incubator with 5% CO₂ and air (21% O₂). Values are means ± SD. B: surface marker profile of MSCs from the bone marrow of healthy rats and from the bone marrow of rats with MCT-induced PAH. Both types of MSCs were cultured in a 5% O₂-5% CO₂ incubator. C: VEGF concentration in the culture medium of MSCs isolated from healthy rats (healthy MSCs) and isolated from rats with MCT-induced PAH (MCT MSCs). MSCs were grown for 4 days in culture medium in the absence (PBS) and presence of 50 µmol/l dehydro-MCT (dhMCT). After that time, the medium and cells were split, the medium was analyzed for the VEGF concentration, and cells were analyzed for protein content. The medium VEGF concentration is expressed as picograms of VEGF in the medium per milligram of cellular protein. Values are means ± SD. D: differentiation into the adipogenic phenotype (top) and the osteogenic phenotype (bottom) of MSCs isolated from healthy rats (left) and from rats with MCT-induced PAH (right).

MCT60 group (P < 0.01 vs. control), and 4.48 ± 0.95 mg/g in the MCT + MSC group (P < 0.05 vs. MCT60; Fig. 2B).

RVH

In the MCT60 group, RV mass was significantly higher than in control rats (233 ± 53 vs. 143 ± 26 mg, P < 0.001). In the MCT + MSC group, RV mass was 162 ± 25 mg (P < 0.001 vs. the MCT60 group), which was not different from the control group. RV weights normalized for body weight were 0.55 ± 0.11 mg/g in the control group, 0.93 ± 0.21 mg/g in the MCT60 group (P < 0.001 vs. control), and 0.64 ± 0.10 mg/g in the MCT + MSC group (P < 0.001 vs. the MCT60 group; Fig. 2C).

RVH, quantified by the weight ratio of RV/(LV + IVS), showed the same pattern, changing from 0.25 ± 0.04 in the
Table 2. Dimensions of cardiomyocytes isolated from the hearts of rats from the control, MCT60, and MCT + MSC groups

<table>
<thead>
<tr>
<th></th>
<th>RV Cardiomyocytes</th>
<th>LV Cardiomyocytes</th>
<th>P Value, RV vs. LV</th>
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<tbody>
<tr>
<td>Cardiomyocyte length, μm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>98.6 ± 4.1</td>
<td>102.1 ± 4.1</td>
<td>0.318</td>
</tr>
<tr>
<td>MCT60 group</td>
<td>108.5 ± 11.7</td>
<td>99.1 ± 4.8</td>
<td>0.062</td>
</tr>
<tr>
<td>MCT + MSC group</td>
<td>102.4 ± 7.9</td>
<td>102.2 ± 5.2</td>
<td>0.851</td>
</tr>
<tr>
<td>Cardiomyocyte width, μm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>16.9 ± 0.3</td>
<td>17.7 ± 0.2</td>
<td>0.011</td>
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<tr>
<td>MCT60 group</td>
<td>18.2 ± 0.6*</td>
<td>17.6 ± 0.6</td>
<td>0.110</td>
</tr>
<tr>
<td>MCT + MSC group</td>
<td>17.3 ± 0.3*</td>
<td>17.8 ± 0.3</td>
<td>0.003</td>
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<tr>
<td>Cardiomyocyte area, μm²</td>
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<tr>
<td>Control group</td>
<td>1.660 ± 0.40</td>
<td>1.797 ± 0.52</td>
<td>0.021</td>
</tr>
<tr>
<td>MCT60 group</td>
<td>1.960 ± 0.17*</td>
<td>1.735 ± 0.54</td>
<td>0.019</td>
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<tr>
<td>MCT + MSC group</td>
<td>1.779 ± 0.12</td>
<td>1.810 ± 0.88</td>
<td>0.305</td>
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<tr>
<td>Cylindrical cell volume, μm³</td>
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<tr>
<td>Control group</td>
<td>22.607 ± 414</td>
<td>25.527 ± 262</td>
<td>&lt;0.001</td>
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<tr>
<td>MCT60 group</td>
<td>28.559 ± 2.034*</td>
<td>24.574 ± 880</td>
<td>0.017</td>
</tr>
<tr>
<td>MCT + MSC group</td>
<td>25.116 ± 1.956†</td>
<td>26.050 ± 1.359</td>
<td>0.109</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5 animals/group, with 40 cardiomyocytes per right ventricle (RV) and per left ventricle (LV) analyzed morphologically. MCT60, 60 mg/kg monocrotaline; MSC, mesenchymal stem cells. *P < 0.05 vs. the control group; †P < 0.05 vs. the MCT60 group.

-control group to 0.47 ± 0.12 in the MCT60 group (P < 0.001 vs. the control group) and to 0.32 ± 0.07 in the MCT + MSC group [P < 0.01 vs. the MCT60 group and P = not significant (NS) vs. the control group; Fig. 2D].

Dimensions of RV cardiomyocytes in the control group were smaller than those of LV cardiomyocytes (significant difference for cell width, area, and volume; Table 2). In MCT60 rats, the width, area, and volume of isolated RV cardiomyocytes had increased compared with the corresponding dimensions of RV cardiomyocytes from control rats, and area and volume became even larger than those of LV cardiomyocytes of MCT rats. In the MCT + MSC group, RV cardiomyocyte width and volume were significantly smaller than the corresponding dimensions of RV cardiomyocytes from MCT60 rats (Table 2).

RVPSP

As expected, RVSPs were significantly higher in the MCT60 group than in the control group (42 ± 17 vs. 27 ± 5 mmHg in control rats, P < 0.05). In the MCT + MSC group, mean RVSP showed a tendency toward lower levels (31 ± 4 mmHg) compared with the MCT60 group (Table 3).

mRNA in Pulmonary Tissue

Compared with control rats, the relative concentrations of mRNA encoding IL-6, TNF-α, tenascin-C, and matrix metalloproteinase (MMP2) were increased in the lungs of rats from the MCT60 group, although significant increases were observed only for IL-6, tenascin-C, and MMP2 (Fig. 3). However, in the lungs of rats from the MCT60 group, mRNA encoding endothelin (ET)-1 and VEGF-A had decreased significantly. In the lungs of rats from the MCT + MSC group, the relative concentrations of mRNA did not differ significantly from those in the MCT60 group (Fig. 3).

Table 3. RV hemodynamic data of rats in the control, MCT60, and MCT + MSC groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MCT60</th>
<th>MCT + MSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>330 ± 26</td>
<td>341 ± 36</td>
<td>327 ± 26</td>
</tr>
<tr>
<td>Stroke volume, μl/min</td>
<td>228 ± 50</td>
<td>234 ± 64</td>
<td>287 ± 37</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>74 ± 13</td>
<td>79 ± 20</td>
<td>93 ± 10*</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>56.2 ± 11.2</td>
<td>42.8 ± 6.2†</td>
<td>52.1 ± 5.2</td>
</tr>
<tr>
<td>End-systolic volume, μl</td>
<td>200 ± 103</td>
<td>323 ± 132</td>
<td>270 ± 75</td>
</tr>
<tr>
<td>End-diastolic volume, μl</td>
<td>427 ± 150</td>
<td>556 ± 183</td>
<td>557 ± 99</td>
</tr>
<tr>
<td>End-systolic pressure, mmHg</td>
<td>24 ± 5</td>
<td>38 ± 15*</td>
<td>28.4</td>
</tr>
<tr>
<td>End-diastolic pressure, mmHg</td>
<td>1.3 ± 1.2</td>
<td>3.9 ± 1.8†</td>
<td>1.9 ± 0.9‡</td>
</tr>
<tr>
<td>Peak systolic pressure, mmHg</td>
<td>27.2 ± 4.9</td>
<td>41.5 ± 16.9*²</td>
<td>30.7 ± 4.4</td>
</tr>
<tr>
<td>dP/dtmax, mmHg/s</td>
<td>1.565 ± 383</td>
<td>2.215 ± 1.040</td>
<td>1.832 ± 0.455</td>
</tr>
<tr>
<td>Negative dP/dtmax, mmHg/s</td>
<td>1.334 ± 385</td>
<td>1.912 ± 0.860</td>
<td>1.675 ± 0.493</td>
</tr>
<tr>
<td>Stroke work, mmHg·μl</td>
<td>5.071 ± 1.415</td>
<td>7.044 ± 2.238</td>
<td>6.682 ± 1.117</td>
</tr>
<tr>
<td>Relaxation time constant, ms</td>
<td>13.7 ± 3.8</td>
<td>14.0 ± 2.9</td>
<td>12.9 ± 4.7</td>
</tr>
<tr>
<td>Arterial elastance (afterload), mmHg/μl</td>
<td>0.11 ± 0.04</td>
<td>0.19 ± 0.12</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>End-systolic elastance, mmHg/μl</td>
<td>0.19 ± 0.17</td>
<td>0.17 ± 0.20</td>
<td>0.10 ± 0.07</td>
</tr>
<tr>
<td>End-diastolic elastance, mmHg/μl</td>
<td>0.008 ± 0.005</td>
<td>0.010 ± 0.004</td>
<td>0.007 ± 0.004</td>
</tr>
<tr>
<td>Preload recruitable stroke work, mmHg</td>
<td>21 ± 7</td>
<td>25 ± 15</td>
<td>19 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SD. Data were collected 28 days after MCT (or control) treatment. *P < 0.05 and †P < 0.01 vs. the control group; ‡P < 0.05 vs. the MCT60 group.

Recovery of DiI-Labeled MSCs in the Lung

In each rat that received cell therapy with MSCs (on day 14), the lungs were found to contain DiI-labeled MSCs on day 28. Labeled MSCs were located in or near the pulmonary arterioles.

Pulmonary Arteriolar Wall Thickness

Pulmonary arteriolar wall thickness was significantly increased in the MCT60 group to 343 ± 60% (P < 0.001 vs. the control group). With MSC therapy, the arteriolar wall thickness had decreased to 120 ± 28% (P < 0.001 vs. the MCT60 group and P = NS vs. the control group; Fig. 4, A–D). The increase in pulmonary arteriolar wall thickness observed in the MCT60 group represents arteriolar medial hypertrophy in these rats, which was apparently prevented largely by treatment with MSCs.

Pulmonary Alveolar Septum Thickness

Pulmonary alveolar septum thickness was significantly increased in the MCT60 group to 353 ± 56% (P < 0.001 vs. the control group). With MSC therapy, the alveolar septum thickness was only 109 ± 20% (P < 0.001 vs. the MCT60 group and P = NS vs. the control group; Fig. 4, E–H). The increase in the mean pulmonary alveolar septum thickness observed in the MCT60 group was prevented largely by treatment with MSCs.

RV Function

Hemodynamic findings are shown in Table 3. Consistent with previous studies, MCT induced PAH, as evidenced by the significant increases in RV peak pressure and RV end-systolic pressure compared with the control group. The increased RV afterload was reflected by an increase in arteriolar elastance,
although this index just failed to reach statistical significance. RV volumes tended to increase, particularly end-systolic volume, whereas stroke volume was maintained. Consequently, RVEF decreased significantly. RV end-diastolic pressure increased significantly. MCT did not change end-systolic elastance or end-diastolic elastance in a significant way.

The MCT + MSC group did not show any significant differences from the control group, suggesting that MSC treatment reversed or prevented the effects of MCT. Compared with the MCT60 group, the MCT + MSC group showed higher RVEF and lower RV end-diastolic pressure (Table 3).

Fig. 3. Lung tissue concentrations of mRNA encoding IL-6, VEGF-A, TNF-α, endothelin (ET)-1, tenascin (TN)-C, and matrix metalloproteinase (MMP)2 in the control (Cont), MCT60, and MCT + MSC groups. For each mRNA, the mRNA concentration was normalized for the mRNA concentration found in the lungs of PBS-treated rats (controls). Values are means ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control group.

Fig. 4. Immunoperoxidase images of paraffin sections of lung tissue stained with anti-α-smooth muscle actin antibody. A–C: representative pulmonary arterioles of control (A), MCT60 (B), and MCT + MSC (C) rats. Images were acquired using a ×100 objective. D: pulmonary arteriolar wall thickness (in %) of control, MCT60, and MCT + MSC rats. Values are means ± SD. ***P < 0.001 vs. the control group; ###P < 0.001 vs. the MCT60 group. E–G: immunoperoxidase images of paraffin sections of lung tissue stained with anti-α-smooth muscle actin antibody. The lung histology of rats in the control (E), MCT60 (F), and MCT + MSC (G) is shown. Images were acquired using a ×20 objective. H: pulmonary alveolar septum thickness (in %) of control, MCT60, and MCT + MSC rats. Values are means ± SD. ***P < 0.001 vs. the control group; ###P < 0.001 vs. the MCT60 group.
and RV end-diastolic volume did not reach statistical significance.

**RV ECM Proteins**

In the RV myocardium of MCT60 rats, mRNA of collagen type I, collagen type III, and tenascin-C increased significantly compared with control rats (all \( P < 0.05 \) vs. the control group). Stem cell therapy was associated with almost normal mRNA levels of collagen type I, collagen type III, and tenascin-C (Fig. 5, A–C). RV myocardial concentrations of mRNAs encoding pro-atrial natriuretic peptide and pro-B-type natriuretic peptide in MCT60 rats showed a trend to higher values than observed in control rats (Fig. 5, D and E).

In the normal RV and LV myocardium, tenascin-C concentrations were very low (Fig. 5F). However, in the RV myocardium of MCT60 rats, the tenascin-C concentration was increased fourfold (\( P < 0.005 \) vs. the control group), whereas in the corresponding LV myocardium, tenascin-C concentrations remained low. In the RV myocardium of rats from the MCT + MSC group, the tenascin-C concentration was as low as the tenascin-C concentration in the corresponding LV myocardium (Fig. 5, F–I).

Myocardial collagen I and III concentrations were elevated in the RV myocardium of the MCT60 group compared with the LV myocardium of the same hearts. Upon MSC therapy, RV myocardial concentrations of collagen I remained elevated compared with the LV myocardium of the same hearts, but for collagen type III, RV and LV concentrations were not significantly different (data not shown).

**DISCUSSION**

In the present study, we have shown that the intravenous injection of MSCs obtained from donor rats suffering from PAH into recipient rats with PAH has beneficial effects on the lungs and heart, such as reduced pulmonary arteriolar narrowing, reduced alveolar septum thickening, decreased RVPSP, less RVH, and improved RV function compared with rats with PAH not receiving cell therapy. We used female rats as donors of MSCs and as recipients of MCT and MSCs. The ability of MSCs to protect the ischemic heart after intracoronary administration appeared to be dependent on the gender of the MSC donor, with MSCs from females being more cardioprotective than MSCs from males (5). On the recipient side, the development of pressure overload-induced cardiac hypertrophy is...
also dependent on gender, with the female mouse showing less LV hypertrophy upon aortic constriction than the male mouse (47). Several studies using animal models of heart failure have reported that females have better survival than males (20, 36).

Pulmonary hypertension often occurs as a consequence of an isolated pulmonary arteriolar vasculopathy and is then called PAH (8), which leads to increased pulmonary vascular resistance, RVH, and, ultimately, RV failure. Although the distribution of ventilation/perfusion relationships in PAH patients is close to normal, arterial PO2 is usually low, as is venous O2 saturation (6, 32, 45).

Many treatment options for PAH have been tested so far, but an effective therapy is lacking. We used a well-established model of MCT-induced PAH in rats. MCT causes endothelial cell injury in the lungs, as observed in the reduced expression of VEGF and ET-1, and subsequently an infiltration of mononuclear cells into the perivascular regions of arterioles and muscular arteries. Rats develop severe PAH after exposure to MCT (35). In the present study, the expression of VEGF-A and ET-1 was significantly lower in the lungs of the MCT60 group than in the lungs of the control group, indicating endothelial damage.

Dorfmüller and coworkers (7) reported that inflammatory mechanisms play a significant role in MCT-induced PAH in rats and in PAH of various origins in humans. As for the MCT model in the rat, the primary importance of inflammation is illustrated by successful therapies using an IL-1 receptor antagonist (49) and antibodies to monocyte chemotactic protein-1 (23). In the present study, we found a significantly increased pulmonary expression of IL-6 in rats with MCT-induced PAH, but after MSC therapy, these levels were not significantly different from those in control lungs. MCT-induced endothelial cell dysfunction is associated with the deregulated expression of vasoactive, mitogenic, and proinflammatory mediators that may cause these changes (29, 30).

In our study, we observed remodeling of lung tissue in MCT-induced PAH associated with an increase in lung weights in the MCT60 group, which is indicative for increased ECM deposition in the lung tissue in these groups rather than pulmonary edema (15). In addition, there was an increase in the mean alveolar septum thickness along with a higher incidence of discontinuous alveolar septa leading to larger alveolar spaces. On further histological analysis, the mean pulmonary arteriolar wall thickness was significantly increased in the MCT60 group. In lung tissue, we found that MMP2 and tenascin-C expression were significantly increased in the MCT60 and MCT + MSC groups. Increased expression of tenascin-C in lung tissue has been shown to be associated with the progression of clinical and experimental PAH (18, 19). Furthermore, MMP expression and activity are increased in experimental PAH (10). Cell proliferation, ECM accumulation, and ECM remodeling in the lungs are also prominent features of idiopathic PAH (1).

In the present study, the decreased RVEF in MCT60 rats was associated with ECM remodeling, as evidenced by increased mRNA concentrations in the RV myocardium of collagen type I, collagen type III, and tenasin-C and increased protein levels compared with the control RV myocardium.

Cell therapy constitutes a novel therapeutic option for PAH patients. In experimental models of PAH, several groups have shown that cell therapy using bone marrow-derived endothelial-like progenitor cells (51), unfractionated bone marrow-derived cells (42, 44), bone marrow-derived MSCs (21), and umbilical cord blood mononuclear cells (34) given intravenously to rodents with MCT-induced PAH resulted in lower pulmonary artery pressure, less RVH, and improved survival. MSCs are unique in possessing 1) a potential to differentiate into other cell types and 2) an ability to secrete paracrine factors leading to improvements in tissue injury (37).

To mimic a therapeutic strategy in which a patient with PAH is treated by an intravenous administration of autologous bone marrow-derived MSCs, we used MSCs that were isolated from the bone marrow of donor rats with MCT-induced PAH and injected intravenously to recipient rats that had MCT treatment 14 days earlier. MSCs isolated from the bone marrow of rats with MCT-induced PAH differed from MSCs isolated from the bone marrow of healthy rats in several aspects. In MSCs from rats with MCT-induced PAH, the proliferation rate was lower and VEGF secretion was higher compared with MSCs from healthy rats, but the potential of adipogetic and osteogenic differentiation and the surface marker profile of MSCs did not differ between these two sources. In in vitro experiments with MSCs from healthy rats and from rats with MCT-induced PAH, the presence of dehydro-MCT, the pneumotoxic metabolite of MCT, did not modify the synthesis and secretion of VEGF. However, the differences between properties of MSCs from healthy rats and from rats with MCT-induced PAH with regard to the proliferation rate and VEGF production are considered to be the effect of dehydro-MCT in vivo, which is formed after the conversion of MCT by cytochrome P-450 monooxygenases in the liver. Dehydro-MCT inhibits mitosis by DNA cross-linking (16), leading to a lower in vitro proliferation rate of MSCs from rats with MCT-induced PAH compared with MSCs from healthy rats, particularly during culture at 5% O2.

In the MCT + MSC group, we found antiremodeling effects in the lungs, including a normalization of lung weights, alveolar septal thickness, and arteriolar wall thickness combined with an improvement in pulmonary architecture. The mechanism of action of intravenous cell therapy with MSCs has been recently elucidated. Lee and coworkers (26) reported that after an intravenous injection of 2 × 106 human MSCs into mice, most of the cells cleared from the circulation were trapped in the lung. These cells disappeared from the lung with a half-life of ~24 h.

As to the localization of Dil-labeled MSCs in the lung, 14 days after the injection of MSCs, we noted an association and integration of MSCs with pulmonary arterioles. Lee and coworkers (26) reported that after an intravenous injection of 2 × 106 human MSCs into mice, most of the cells cleared from the circulation were trapped in the lung. These cells disappeared from the lung with a half-life of ~24 h.

The therapeutic actions of MSCs engrafted in injured lungs are considered to be the result of paracrine effects (38). Lee and coworkers (26) showed that when intravenous MSCs are embolized in the lung of mice with myocardial infarction, they enhance myocardial repair without cardiac engraftment. These lung-engrafted MSCs do so because they upregulate the expression, and subsequent secretion, of the anti-inflammatory protein TNF-α-induced protein-6. Thus, whereas our study
describes the beneficial effects of lung-engrafted MSCs after an intravenous administration on structure and/or function of the lung and RV, the study by Lee and coworkers (26) explains (one of) the way(s) by which lung-engrafted MSCs after intravenous administration produce functional improvement of the infarcted heart.

Another important paracrine factor is VEGF, which promotes angiogenesis by inducing the proliferation, differentiation, and chemotaxis of endothelial cells (9, 28). The administration of pulmonary artery-derived smooth muscle cells to rats with MCT-induced PAH had therapeutic effects only if transduced in vitro with the VEGF-A gene (4). Likewise, the intravenous administration of fibroblasts transfected with the VEGF-A gene was effective in reversing established PAH, whereas untransduced fibroblasts were not effective (52). The relationship between pulmonary vascular remodeling and lack of VEGF was demonstrated by decreased VEGF mRNA in the lung tissue of mice with PAH due to TNF-α overexpression (11). Surprisingly, the stem cells used in the present study, MSCs from the bone marrow of rats with MCT-induced PAH, produced even more VEGF in vitro than MSCs from the bone marrow of healthy rats. This finding may explain the potent therapeutic effects of the MSCs obtained from MCT-treated rats.

In conclusion, the intravenous administration of bone marrow-derived MSCs obtained from donor rats suffering from PAH into recipient rats with PAH decreased RVSP, pulmonary arteriolar narrowing, alveolar septum thickening, and RV hypertrophy and improved RV function. Based on these results, the use of autologous bone marrow-derived MSCs to treat PAH in humans is recommended.

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