Treatment of pulmonary arterial hypertension with circulating angiogenic cells

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PULMONARY ARTERIAL HYPERTENSION (PAH) is a progressive and life-threatening condition, characterized by increased pulmonary vascular resistance (PVR), ultimately leading to right heart failure and death. Vascular occlusion is caused by smooth muscle and endothelial cell proliferation, obstructive plexiform lesions, thrombosis, and enhanced vasoconstriction (18). Patients with PAH have a poor prognosis and die within 2.8 yr of diagnosis with estimated single-year survival rates of 68% at 1 yr, 48% at 3 yr, and 34% at 5 yr (6).

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) were first described by Asahara et al. (1) as a population of circulating BM-derived circulating cells able to contribute to postnatal neovasculogenesis. These cells contribute to the recovery and maintenance of endothelial function (4, 8, 15), and they can be mobilized from BM in response to tissue ischemia (37) or endothelial injury by the release of chemokines, such as vascular endothelial growth factor (VEGF), stromal-derived factor-1, and tumor necrosis factor-α (10). These cells comprise a heterogeneous group that can be characterized by the expression of surface markers, such as CD34, CD133, and vascular endothelial growth factor receptor 2 (KDR), and by the uptake of acetylated low density lipoprotein (LDL, Dil-Ac-LDL) and by binding lectins (28, 33).

The endothelial damage characteristic of PAH might potentially be further compounded by a decrease in the number and function of the circulating EPCs (9, 21, 27). A 25% decrease in circulating cells has been reported in patients with PAH compared with healthy controls as well as a 50% reduction after ex vivo culture (21). These cells from patients with PAH appear to be significantly impaired in their migratory capacity to VEGF and their ability to adhere to fibronectin (21). In addition, EPCs in a chronic hypoxic-induced PAH model displayed decreased migration to stromal cell-derived factor-1α, adhesion to fibronectin, incorporation into a vascular network, and nitric oxide production (27). Interestingly, an increase in circulating angiogenic cells (CACs) in patients with PAH associated with endothelial damage has also been reported (2), supporting the hypothesis that dysfunction of circulating progenitor cells may be contributing to vascular remodeling (38).

EPCs appear to play an important role in reestablishing endothelial integrity after severe lung injury and may contribute to pulmonary vascular repair (3, 4, 7, 9, 22, 47). Kahler et al. (23) found that peripherally administered BM-derived EPCs homed to sites of injury and appeared to engraf the lung vasculature. Additionally, patients with acute lung injury and higher counts of circulating cells have been shown to have superior survival compared with those with lower counts (4). In clinical studies, autologous administration of adult progenitor cells to patients with idiopathic PAH was associated with a mild improvement in 6-min walk distance and hemodynamics compared with conventional therapy (39).

In animal models of PAH, the benefits of CAC therapy remain unclear. Studies have used cells derived from a variety of sources, including autologous donors (44), BM-derived (35), peripheral blood (30, 36), and umbilical cord (29), and have reported varying success in reducing mortality, and decreasing right ventricular hypertrophy (RVH), right ventricular systolic pressure (RVSP), PVR, pulmonary arterial wall thickness, and other cardiovascular parameters (44, 46).

Our laboratory has expertise in isolation of human peripheral blood CACs and has therefore chosen to work with this cell population (16, 17). Additionally, a variety of therapeutic time points have been examined after monocrotaline (MCT) administration...
tion, from early day 3 therapy (30, 35, 46) to day 28 (44) with variable efficacy of therapy. The importance of timing has been previously shown with BM cell therapy for myocardial infarction (MI) (32).

In this study, we aim to clarify the efficacy of human circulating CAC therapy and the importance of timing in the treatment of PAH by examining two different time points corresponding to different stages of disease severity. In our hands, MCT reliably induces PAH between two and three weeks after administration. We administered CACs before the onset of PAH (on day 14 after MCT, “early” therapy) in one group and after the onset of PAH (on day 21 after MCT, “late” therapy) in another group. Treatment with human CACs either at the early or late time points did not result in increased survival, and therapy did not prevent or reduce the severity of disease compared with control. Histological analysis of RVH and blood vessels showed no benefit with therapy at either time point compared with control.

MATERIALS AND METHODS

Animal model. Six-week-old RNU-1 (Charles River, Wilmington, MA) nude rats were used according to our protocol approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. PAH was induced using the MCT as per previous studies (12, 40). MCT (Sigma-Aldrich, St. Louis, MO) was dissolved in HCl and diluted with distilled water. After adjusting the pH to 7.4, MCT was administered in the intraperitoneal cavity at a dose of 75 mg/kg.

Study protocol. Animals received tail vein injection of 1.5 X 10^6 CACs in 0.5 ml PBS at either day 14 (early therapy, n = 8) or 21 (late therapy, n = 16) or 0.5 ml PBS control solution (n = 31). The number of cells was chosen to obtain maximal effects of transplanted CACs based on dose-response experiments previously described (29). Right heart catheterization was performed at day 0 (“baseline,” immediately before MCT administration), at the time of therapy (day 14 or 21), and on day 35 (Fig. 1). Echocardiography was performed at the times indicated for right heart catheterization and additionally 2 wk after CAC therapy was administered (day 28) in the early treatment group (Fig. 1). Histological evaluation was performed at study termination (day 35). Animals were killed if they showed signs of infection, severe distress, or discomfort not relieved by analgesics as determined by the University of California, San Francisco, Laboratory Animal Resource Center veterinarians.

Cell isolation, culture, and functional assessment. Cell isolation and characterization was performed as previously described by our group (14, 15, 17). Briefly, peripheral blood was drawn from eight young (26.5 ± 4.5 yr) healthy volunteers with no history of hypertension, hypercholesterolemia, diabetes, obesity (body mass index 24 ± 1.5), nor smoking according to our protocol approved by the Committee of Human Research of the University of California, San Francisco. Peripheral blood mononuclear cells were isolated using the Ficoll method (14, 17). The cells were preplated on fibronectin-coated plates for 3 h in EBM-2 MV media (Cambrex, East Rutherford, NJ) with 20% FBS (HyClone Laboratories, Logan, UT). The nonadherent cells were replated at 1 X 10^5 cells/well of a six-well dish for 7 days at 37°C and in 5% CO2 (17, 19).

To confirm the endothelial phenotype of ex vivo differentiated CACs at day 7, fluorescent staining was performed to detect lectin binding and uptake of acetylated LDL. Adherent CACs on fibronectin-coated glass slides were incubated for 1 h with Di-acL DL (Invitrogen, Carlsbad, CA), washed, and fixed in 2% paraformaldehyde (PFA). After blocking with 2% goat serum/PBS, cells were washed and incubated with fluorescein isothiocyanate-conjugated UEA-1 (Sigma-Aldrich). The nuclei were stained with Hoechi 33258 (Invitrogen) and observed using a Nikon E800 fluorescence microscope and Openlab software (PerkinElmer, Waltham, MA). Five fields (×20 magnification) were analyzed for the percentage of double-stained cells (11, 17). After ex vivo expansion, almost all (>95%) adherent cells were positive for UEA-1 and acetylated LDL uptake, confirming the endothelial phenotype of these cells.

CACs were also stained for expression of cell surface markers. At day 7 in culture, media were aspirated, and the dish was washed to remove nonadherent cells. The adherent CACs were removed from the dish with nonenzymatic cell dissociation buffer (Invitrogen) and PBS washes. Staining was performed after Fc-receptor blocking with immunoglobulin G (Zymed, San Francisco, CA). One hundred microliters of 10^5/ml cells in fluorescent-activated cell sorter (FACS) buffer (1% BSA/PBS) were incubated on ice for 30 min with directly conjugated monoclonal antibodies to human CD34, KDR, CD133 (Miltenyi, Bergisch Gladbach, Germany), or isotype controls. After being washed with FACS buffer, cells were fixed with 1% PFA and stored at 4°C until analysis. One hundred thousand mononuclear cells were gated on an LSRRII FACS machine (BD, Franklin Lakes, NJ) and analyzed using Flow Jo software (TreeStar, Ashland, OR) (17, 45).

Before in vivo delivery, CACs were assayed for their functional responses to VEGF. Migration assays were carried out with 2 X 10^5 cells, in triplicate, in Boyden chamber inserts with an 8-µM pore size for 6 h in culture in either minimal media or media supplemented with 100 ng/ml VEGF. Inserts were removed, washed, and fixed in 2% PFA; membranes were cut out and stained with Hoechst 33258; and images were taken on the microscope. Five fields (×10 magnification) were counted for each well (17, 26).

In vivo measurements by right heart catheterization. Under general anesthesia (2% isoflurane/1% oxygen), lidocaine (6 mg/kg) was administered subcutaneously, and the external jugular vein was isolated. A 2-Fr micropip catheter (Millar Instruments, Houston, TX) was guided in the right ventricle (RV) to obtain pressure measurements. RVSP was assessed invasively using a pressure control unit (Millar instruments), a physiological recorder (Powerlab ML786; ADInstruments, Sydney, Australia), and a physiological recorder (Powerlab ML786; ADInstruments, Colorado Springs, CO), and commercial computer software (Chart5; ADInstruments). Pulmonary valve pathology was excluded at baseline using echocardiography; therefore, RVSP was used to evaluate pulmonary arterial pressure (PAP). An average of 15–20 pressure cycles was used to obtain final pressure values. Echocardiography. Under general anesthesia (2% isoflurane/1% oxygen), transthoracic echocardiography was performed by a Sequoia...
C 256 ultrasound device (Acuson, Mountain View, California) with a 15.0-MHz linear transducer. A number of parameters were measured as follows: 1) pulmonary artery acceleration time (PAAT), an indirect measure of PA PAP, was defined as the interval from the onset to the maximal velocity of forward pulmonary artery flow (20); 2) right ventricular thickness was measured at diastole from the parasternal long axis view using M-mode; 3) an apical four-chamber view was employed to measure end-diastolic right ventricular and end-systolic right atrial areas; 4) pulmonary artery flow was measured using pulsed wave Doppler with a sample gate of 1.0 mm; and 5) by combining the pulmonary artery velocity-time integral, pulmonary artery area, and heart rate (HR), echocardiographically derived right ventricular cardiac output was determined as published previously by our group (25, 31).

Histology. Following the final echo and right heart catheterization at day 35, the animals were killed using pentobarbital sodium overdose (>200 mg/kg), and the lung and heart were harvested for histology. The left lung and heart were extracted and fixed in 4% PFA overnight and then processed for paraffin embedding. The right lung was flash-frozen using liquid nitrogen for PCR.

Right ventricular hypertrophy. The RV was removed, and the right ventricular-to-left ventricular plus septal weight ratio (RV/LV + S) was measured as an indicator of the hypertrophic response to longstanding PAH (5).

Arterial muscularization. The left lung was sectioned in the transverse plane (5 μm) at tissue levels separated by 300 μm. Sections were double stained with mouse anti-von Willebrand factor (abD Serotec, Raleigh, NC), an endothelial marker, and rabbit anti-α smooth muscle actin (Abcam, Cambridge, MA), an arterial maker, using horseradish peroxidase- and alkaline phosphatase-conjugated polymers (Biocare Medical, Concord, CA). We assessed vascular remodeling and arteriolar muscularization of small (<100 μm), medium (100-200 μm), and large (>200 μm) blood vessels using the percent medial wall thickness.

Vessels cut tangentially were excluded from analysis. For each artery, the % medial wall thickness was expressed as follows:

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\text{% medial thickness} = \frac{\text{sum of four medial wall thickness measures}}{\text{sum of cross diameters}} \times 100
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Human EPC localization. For analysis of relative human cell concentration within rat tissues, RNA was isolated from total flash-frozen lungs with TRIzol (Invitrogen) using standard methods. To develop a standard expression curve, known numbers of human CACs (2,000–16,000/mg) were added to lung samples before RNA isolation. cDNA was synthesized from isolated RNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) and quantitated using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies). Linear preamplification of the target sequences was accomplished using the Applied Biosystems PreAmp system according to the manufacturer’s instructions. Relative expression was determined using TaqMan Assays (Applied Biosystems) on an ABI 7300 Real-Time PCR system with primers for human nuclear mitotic apparatus protein (Hs00272062_m1) and human CD45 (Hs00174541_m1). Cycle times to detection were normalized against the reference gene, GAPDH (4352339E), and relative changes were calculated using ABI Version 1.4 Sequence Detection Software. Relative expression values were adjusted for tissue mass.

Statistical analysis. Statistical analysis was performed using SigmaStat 3.5 (Systat, Point Richmond, CA). Treatment groups were statistically compared using one-way ANOVA with Dunnett’s post hoc analysis. Kaplan Meier plots were done with Log Rank (Mantel Cox) analysis. The results are expressed as means ± SE. P values <0.05 were considered significant.

RESULTS

CAC characterization. The percentage of circulating CACs determined as CD34/KDR, and CD133/KDR double-positive cells were between the ranges of our previous reports (CD34/KDR⁺: 0.247 ± 0.064%; CD133/KDR⁺: 0.109 ± 0.043%) (14, 15). The migration toward VEGF was 113 ± 37% higher than the control (minimal media), as previously described (14). Before injection, cells were tested for viability. The viability preinjection was found to be 92.3 ± 6%.

Mortality unaffected by CAC therapy. Throughout the experimental protocol, researchers were blinded to treatment groups. Animals were monitored closely for mortality reporting. No mortality was observed before therapy in any group. Mortality was most significant between days 30 and 35 post-MCT but began after day 21 (Fig. 2). Mortality assessment using Kaplan Meier methods showed no difference between animals receiving CAC therapy early or late and the control group (P = 0.31) (Fig. 2).

Animal body weight did not differ significantly between early treatment, late treatment, and control at baseline (Table 1), but we found a trend toward lower body weight in both early and late CAC therapy groups (P = 0.10 and P = 0.17). Additionally, HR was within the same range for treatment groups compared with control at all time points.

MCT induced hemodynamic changes before CAC therapy. At baseline, there were no differences in RVSP, HR, or any echocardiographically derived parameters between the CAC-treated and control groups (Table 1). Invasive RVSP did not differ in either treatment group compared with control just before CAC therapy (early therapy: control 32.4 ± 1.3 mmHg, CAC 30.6 ± 1.3 mmHg P = 0.38; late: control 45.7 ± 4.7 mmHg, CAC 51.0 ± 5.1 mmHg P = 0.39). Additionally, echocardiography showed no significant differences in RV thickness, PAAT, pulmonary artery diameter, or RV area.

At day 14 following MCT administration, right heart catheterization-derived RVSP was not significantly elevated (P =
We investigated the effects of CAC therapy at two time points: early (day 14) and late (day 21) after MCT induction (Table 1). Additionally, arterial muscularization of small, medium, and large vessels remained unchanged in animals receiving early or late therapy compared with those receiving control (small vessels: control 22.4 ± 0.51 mm, early therapy 21.7 ± 0.39 mm, late therapy 20.7 ± 0.33 mm; medium vessels: control 26.5 ± 1.27 mm, early therapy 23.5 ± 0.76 mm, late therapy 27.6 ± 0.53 mm; large vessels: control 27.7 ± 1.36 mm, early therapy 25.6 ± 0.71 mm, late therapy 25.3 ± 0.64 mm) (Fig. 3). Furthermore, PAAT and pulmonary artery deceleration as noninvasive measures of PAP are also supportive of the invasive evaluation in that neither early nor late CAC therapies have a positive effect on PAP at day 35 after MCT induction (Table 1).

Circulating angiogenic cells and pulmonary hypertension. L15

CIRCULATING ANGIOGENIC CELLS AND PULMONARY HYPERTENSION

L15

DISCUSSION

In this study, we investigated the efficacy of early and late human CAC (also termed early EPC) therapy in the MCT rat model of PAH. Our results demonstrate that CAC therapy: 1) does not improve survival; 2) does not significantly impact hemodynamics as assessed by right heart catheterization and echocardiography; 3) fails to prevent or reverse RVH; and 4) has no impact on vascular remodeling.

We investigated the effects of CAC therapy at two time points, 14 and 21 days after MCT administration, reflecting the
times before and after the development of PAH in our model, and found no significant benefit at either time point. Zhao et al. (46) reported prevention of PAH with endothelial-like progenitor cell therapy administered 3 days post-MCT but only slight attenuation with therapy given 21 days post-MCT. Ormiston et al. (30) also reported prevention of PAH at 3 days post-MCT but with a lack of cell persistence within the lungs at 24 h postinjection. Nagaya et al. (29) reported little benefit (14%) of RVSP with EPC therapy alone 7 days post-MCT. It is possible that earlier therapy is critical for the efficacy of cell therapy in PAH. The importance of timing is not new to cell therapy literature. In fact, BM cell-based therapies in the treatment of patients after a MI have shown that timing of therapy is critical (32).

Fig. 3. CAC therapy does not reduce pulmonary hypertension or right ventricular (RV) hypertrophy. Right ventricular systolic pressure (RVSP) significantly increased between days 14, 21, and 35 in animals receiving control therapy. No significant difference was observed between animals receiving CAC therapy on day 14, day 21, or control therapy at either time point (A). RVSP significantly increased post-CAC therapy in animals receiving early CAC therapy but not those receiving late CAC therapy (A). No significant differences were observed in right ventricular hypertrophy (RVH) between groups as assessed by the right ventricular-to-left ventricular plus septal weight ratio RV/LV + S (B). End-diastolic RV area at day 35 indicated more RV remodeling in the early CAC group vs. controls (C), and neither early nor late CAC therapy had an effect on RV ejection fraction (EF) at follow-up (D). NS, not significant.

Fig. 4. Arteriolar muscularization. Paraffin-embedded lung sections from apical, medial, and basal regions of the lung were stained using von Willebrand factor (vWF, brown staining) and smooth muscle actin (SMA, blue staining). Top: control (A), early CAC (B), and late CAC (C). Arteriolar muscularization was assessed using %medial wall thickness of vessels sorted by size. There was no significant difference observed in arteriolar muscularization between the different therapies compared with control.
Fig. 5. Human CACs are not detectable in transplanted lungs. The presence of CACs was assessed by quantitative PCR using primer sets against human nuclear mitotic antigen (NuMA, top) and the CAC-specific marker human CD45 (bottom). As few as 2 CACs/μg lung tissue could be detected in control samples; however, there was no detectable signal for either human transcript in transplanted lungs at 14 or 21 days (d) after cell transplant. Data shown are means ± SE (n = 3).

MI, no clinical benefit is achieved, and, if therapy is delayed until after 5 days post-MI, cell therapy has been shown to have significantly positive benefit on overall cardiac function. In the case of PAH, it is possible that a larger benefit could be achieved if the patients are treated much earlier in the course of their disease, and, once advanced PAH has already developed, cell therapy would be of little or no benefit at all.

In previously published preclinical and clinical studies, endothelial-like progenitor cell therapy has been reported to be beneficial in preventing worsening of PAH. However, the results have been inconsistent depending on the parameter examined and reported. Takahashi et al. (36) showed improvement in mean PAP, cardiac output, PVR, medial thickness of pulmonary arteries, and neovascularization but not RV pressure in MCT-treated dogs with autologous peripheral blood EPC therapy. Nagaya et al. (29), using human umbilical cord-derived EPC treatment for nude rats 7 days post-MCT, showed significantly attenuated PVR, %pulmonary arterial wall thickness, and increased survival but no significant decrease in mean PAP compared with control therapy. Zhao et al. (46) reported significantly reduced RVH and improved pulmonary vasculature but nonsignificant reduction in RVSP and survival with day 21 BM-derived EPCs given to MCT rats (46). This variability is not surprising, considering the different methods involved in inducing PAH, identifying and isolating CACs, and the difficulties involved in measuring hemodynamics invasively in animal models. To overcome some of the potential difficulties with hemodynamic evaluation, our current report used two methods of evaluation, invasive right heart catheterization and noninvasive echocardiography. Our group has expertise in the assessment of PAH parameters using echocardiography (25). With both techniques, we show no benefit of CAC therapy on hemodynamics.

Another significant difference between the studies evaluating the utility of cell therapy in PAH is the cell population used for therapy. Prior studies in rats have used endothelial-like progenitor cells derived either from human umbilical cord (29), derived from rat BM (46), or from human peripheral blood harvested by leukapheresis (30). In contrast, our group utilized CACs derived from human peripheral blood of healthy adult subjects using a validated (14, 16, 17) but different protocol of harvesting and derivation than the previous studies. The discrepancy in findings could relate to the differences in sources, derivation, and methods used to define and isolate CACs, or to species-specific factors that could influence the regenerative capabilities of human cells in an immunodeficient rat model (34). In addition, several reports have suggested that implantation of various cell types may provide functional improvement through paracrine mechanisms leading to release of a wide array of cytokines that exert their effects on surrounding cells (13, 24, 41–43). Therefore, different cell sources may have different profiles and release different factors. This fact could explain our findings of no significant CAC engraftment within pulmonary vasculature, contrary to previous reports using this model. Finally, endothelial-like progenitor cells have shown promise as a gene delivery tool. Several groups have shown benefit with cells modified to overexpress genes of interest (eNOS) (46), adrenomedullin (29), in a rat model of MCT-induced PAH. With the additional data provided by this manuscript, it appears that CACs as a therapy for PAH may have a greater potential as a delivery vehicle and have moderate to no benefit on their own, depending on the timing of cell administration. Additionally, because we could not localize the injected cells in the lung after therapy, there is the possibility of cellular failure postinjection or other unforeseen experimental issues that could have led to the reported lack of therapeutic benefit under the specific experimental conditions.

There are a number of limitations in this study. First, only one dose of CACs was administered given the complexity of the experiments and associated cost of the study. The number of cells was chosen based on dose-response experiments previously described (29). However, the dose-response experiments were conducted using cells derived from human umbilical cord blood mononuclear cells; therefore, a different cell number might have yielded better results. Additionally, we did not compare CACs derived from different origins. Such experiments were out of the scope of this study, but the issue should be addressed in future experiments. Second, only two time points were chosen for cell delivery to keep the study to a manageable size and within budget. Retrospectively, and based on the discussion above reflecting the importance of timing of cell therapy, CAC therapy at even an earlier time point than the one examined would have been a nice additional data point, but this became apparent only after the current study was concluded and the data were analyzed. Future studies should investigate this important point. Third, there are clearly other models of inducing PAH in the rat, and which model best mimics the human condition is arguable (40). We chose the
CIRCULATING ANGIOGENIC CELLS AND PULMONARY HYPERTENSION

MCT model because of our familiarity with the model and in trying to keep the mortality of animals as low as we could in our hands. Other models of inducing in the rodent model can certainly be tested in future studies. Last, we used RVSP as a surrogate to follow the response to therapy rather than direct invasive measurement of pulmonary artery pressures, given the absence of pulmonary valve pathology. This was done to decrease the animal morbidity and mortality associated with this extremely challenging technique of introducing the catheter in the PA position, especially in the frail and sick animals with significant PAH.

In conclusion, treatment with human CACs in MCT-treated nude rats either at the early (14 days) or late (21 days) time points after induction of PAH did not result in increased mortality in nude rats either at the early (14 days) or late (21 days) time with significant PAH.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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