Granulocyte Colony-Stimulating Factor Prevents Progression of Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats

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Background  Regeneration of the lung microvasculature and replacing pulmonary artery lesions with functional endothelial cells could be a novel and effective therapeutic strategy for treating advanced pulmonary arterial hypertension (PAH). In the present study it was postulated that granulocyte colony-stimulating factor (G-CSF), which induces the proliferation of endothelial cells, would stimulate endothelial regeneration in situ at sites of impaired lung vasculature and prevent the development of PAH.

Methods and Results  Daily administration of G-CSF for 48 days did not affect the hemodynamics of normal Fischer 344 rats. PAH was induced with monocrotaline (60 mg/kg) and G-CSF was administered daily (100 μg/kg per day). Echocardiographic findings and an invasive catheter study indicated a significant decrease in the progression of PAH in rats given G-CSF. Furthermore, G-CSF increased Ki-67 positivity in the pulmonary arteries of PAH rats but did not accelerate c-kit positive cell recruitment into peripheral blood. Daily doses of G-CSF at both 2 and 100 μg/kg improved the survival and body weight gain of PAH rats.

Conclusions  G-CSF improved the progression of PAH in a rat model, possibly by stimulating pulmonary endothelial cells to proliferate at sites of impaired lung vasculature. These findings show that cytokine therapy for PAH is valid based on the concept of vascular regeneration.

Key Words: Cytokines; Endothelial cells; Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is characterized by increased thickening of the walls of the pulmonary arteries, narrowing of the pulmonary artery (PA) lumen, increased pulmonary vascular resistance, and right-sided heart failure! Environmental stress in a genetically predisposed host might trigger endothelial cell apoptosis, which could lead to arteriolar occlusion either directly, perhaps by initiating microvascular degeneration, or indirectly by promoting the emergence of hyperproliferative, apoptosis-resistant vascular cells. Because transplantation with exogenous endothelial progenitor cells (EPCs) rescues the survival of rats with monocrotaline (MCT)-induced PAH, the regeneration of lung vasculature or the replacement of PA lesions by functional endothelial cells might be effective therapeutic strategies to improve pulmonary hemodynamics in patients with advanced PAH. These findings suggest that disordered antiproliferative and vasoregulatory functions of endothelial cells play an important role in PAH progression. However, a clinically useful number of autologous EPCs is difficult to obtain from donors or from patients with progressive PAH. Therefore, we postulated that granulocyte colony-stimulating factor (G-CSF), which induces endothelial cells to migrate and proliferate, could stimulate regeneration of impaired lung microvasculature and improve the survival of PAH rats.

Methods

Animals were purchased from CLEA Japan (Tokyo). The Institutional Animal Care and Use Committee of the University of Tsukuba approved the animal protocols and the experiments proceeded according to institutional guidelines.

To estimate the basal effect of G-CSF in intact rats (without MCT injection), G-CSF (100 μg/kg per day) or saline was subcutaneously injected into 9-week-old male Fischer 344 rats. On day 40 of injection, peripheral blood cells sampled via the tail vein were counted and on day 48 we performed an invasive catheter study. After euthanasia, the right ventricle (RV) was excised and weighed.

One subcutaneous injection of MCT (Sigma, St Louis, MO, USA; 60 mg/kg) was sufficient to induce PAH in the 4- to 6-week-old male Fischer 344 rats and then recombinant human G-CSF (100 or 2 μg/kg per day; Kirin, Tokyo, Japan) or saline was subcutaneously injected from day 14 (for echocardiographic study, catheter study, histopathologic study) or day 21 (for survival analysis) thereafter. Intact adult male Fischer 344 rats served as normal controls.

Echocardiography and the catheter study proceeded after 1 week of G-CSF administration. Echocardiographic studies were performed using a 12 MHz transducer (Sonos4500, Philips). At the aortic valve (AV) level in the short-axis...
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view, the RV outflow tract dimension (RVOTD) and aortic dimension (AoD) were recorded. The eccentricity index was measured at end-diastole from the parasternal short-axis views of the left ventricle at the level of the chordae tendineae. The diastolic eccentricity index was defined as the D2/D1 ratio at end-diastole, where D2 is the minor-axis dimension of the left ventricle parallel to the septum and D1 is the minor-axis diameter perpendicular to and bisecting the septum. Pulsed Doppler PA flow velocities were recorded at the AV level in the short-axis view. Acceleration time (AT: time from onset of pulmonary flow to peak pulmonary outflow velocity) and ejection time (ET: time from onset to completion of systolic pulmonary flow) of PA flow velocity tracings were measured, and the ratio of AT to ET (AT/ET) was calculated.

After anesthesia was induced with pentobarbital IP (50 mg/kg), RV systolic pressure (RVSP) and mean aortic pressure (MAoP) were recorded using a polyethylene catheter inserted into the RV and ascending aorta via the right jugular vein and right carotid artery, respectively. Formalin-fixed rat lungs were paraffin-embedded, sectioned and stained with hematoxylin–eosin, as well as with the elastic van Gieson technique. A blinded observer measured all vessels with perceptible media (>35 muscular arteries/rat), under ×20 magnification, and the average was obtained for vessels of 25–50 and 50–100 μm in external diameter for each rat. The medial wall thickness is expressed as follows: % wall thickness = [(medial thickness × 2)/external diameter] × 100.

For immunohistochemical localization of Ki-67, endogenous peroxidase in lung tissue sections was quenched and the sections were heated in antigen retrieval solution (0.01 mol/L citrate buffer, pH 6.0) for 20 min in a microwave oven. After incubation in a casein block, mouse MAb anti-rat Ki-67 (clone MIB-5; Dako) was added to the sections at a dilution of 1:50 and incubated overnight at 4°C. We used a secondary detection kit (Histofine Simplestain Rat Max-Po Multi; Nichirei, Japan) to visualize antibody binding. Staining was developed using 3-amino-9-ethylcarbazole, and then sections were counterstained with hematoxylin. Ki-67 positivity was expressed as % Ki-67 labeling by calculating the average ratio (%) of Ki-67 positive endothelial cells within 20 pulmonary arteries per rat. Histopathological photomicrographs were captured using Axioplan 2/AxioVision 3.1 (Carl Zeiss).

Three weeks after MCT injection, peripheral blood was sampled via the tail vein. Red blood cells were lysed using buffered ammonium chloride. The samples were incubated with rabbit anti-c-kit antibodies (H-300, Santa Cruz Biotech Santa Cruz, CA, USA) for 30 min at 4°C, washed with phosphate-buffered saline, and incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG goat antibodies as secondary antibodies for 30 min at 4°C. The samples were washed, propidium iodide was added and cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA).

Survival was estimated from the date of MCT injection until death or 6 weeks after MCT injection.

Data are presented as means ± SD. Data were statistically analyzed using Student’s t-test. The significance of differents.
ences in survival data was determined using the Kaplan-Meier analysis. A value of $p<0.05$ was considered statistically significant.

**Results and Discussion**

After 40 daily G-CSF injections, the number of leukocytes (WBC) in the peripheral blood approximately doubled in intact rats (without MCT injection) compared with control rats. However, body weight (BW) and peripheral blood cell count of erythrocytes and platelets did not significantly differ (Fig 1A). On day 48, significant differences between the 2 groups in mean systemic pressure, systolic pulmonary arterial pressure and right ventricular weight adjusted to BW (RVW/BW), which reflects chronic overload of pulmonary arterial pressure, were undetectable (Fig 1B). The alveolar walls in most lung tissue samples contained slightly more mature neutrophils without vascular and peribronchial infiltrates and no emphysema or pleural thickening as described by Chang et al (Fig 1C). These findings indicated that G-CSF administered over the semi-long term does not affect the hemodynamics of normal intact rats.

Echocardiography, invasive catheter and histopathologic studies proceeded 3 weeks after MCT injection, with daily injections of G-CSF (100 μg/kg; MCT + G-CSF group) or saline (MCT + Saline group) during the last week. Fig 2A shows that flow velocity accelerated rapidly to a peak in early systole, followed by rapid deceleration to the midsystolic notch in the MCT + Saline group, but not particularly in the MCT + G-CSF group. The diastolic eccentricity index was defined as the D2/D1 ratio at end-diastole, where D2 is the minor-axis dimension of the left ventricle parallel to the septum and D1 is the minor-axis diameter perpendicular to and bisecting the septum (Fig 2B). Fig 2C shows quantitative echocardiographic evaluations. The AT/ET values significantly decreased in the MCT + Saline group compared with the normal control group (0.11±0.02 vs 0.25±0.05, $p<0.01$). In MCT + G-CSF group, AT/ET returned to the level of normal control group (0.22±0.04 vs 0.25±0.05, NS). The ratio of RVOTD to AoD (RVOTD/AoD) and diastolic eccentricity index were significantly greater in the MCT + Saline group than in the normal control group (1.26±0.14 vs 0.81±0.10, $p<0.01$ and 1.22±0.20 vs 0.98±0.07, $p<0.05$, respectively). These increases were suppressed in the MCT + G-CSF group (0.97±0.06, $p<0.01$ and 1.07±0.08, $p<0.05$ vs MCT + Saline group, respectively). The AT/ET value correlated inversely to mean PA pressure and resistance. The ratio of RVOTD/AoD is thought to be a parameter of RV dilatation and the diastolic eccentricity index reportedly increases in patients with both right ventricular volume and pressure overload. These findings suggest that MCT injection promotes, whereas G-CSF improves pulmonary hypertension under noninvasive conditions.

Three weeks after MCT injection, RVSP was increased from 20.7±6.3 mmHg in the normal control group to 43.6±9.5 mmHg in the MCT + Saline group (<0.01, Fig 3A), which was consistent with the development of PAH. In rats treated with G-CSF, RVSP was significantly reduced to 30.0±5.3 mmHg ($p<0.01$ vs MCT + Saline group). However, this strategy did not completely restore the RVSP to normal values ($p<0.01$ vs normal control group). The MAoP was slightly but significantly decreased from 116.0±15.7 mmHg in the normal control group to 93.8±25.3 mmHg in the
MCT + Saline group (p<0.05, Fig 3B), and G-CSF restored the MAoP to normal values (110.9±22.2 mmHg, NS vs normal control group). During this examination, heart rates did not differ statistically among the groups (Normal control, 462±45 beats/min; MCT + Saline, 457±54 beats/min; MCT + G-CSF, 447±45 beats/min; NS).

Fig 4A shows representative sections of lung tissue from normal controls (Left) and from rats in the MCT + Saline (Center) and MCT + G-CSF (Right) groups. Hypertrophy of the media of peripheral muscular arteries was apparent in the MCT + Saline group. Quantitative analysis also demonstrated a significant increase in % wall thickness after MCT injection, but G-CSF obviously attenuated this change in pulmonary vessels of 50–100 µm but not in those of 25–50 µm in diameter (Figs 4B,C).

We immunohistochemically examined Ki-67 in paraffin-embedded rat lung tissue sections (Fig 5). Nuclear immuno-reactivity, as revealed by Ki-67 staining, usually has a reddish violet granular appearance. Few endothelial cells were positive for Ki-67 in normal control rats, whereas positivity was increased in groups injected with MCT (MCT + Saline; MCT + G-CSF). Fig 5B shows the %Ki-67 labeling within the endothelial cells of pulmonary arteries (PAECs). Injected MCT increased Ki-67 positivity approximately 4-fold compared with normal rats (8.3±1.1 vs 2.2±0.6, p<0.01). Furthermore, G-CSF slightly but significantly enhanced the increase (11.1±1.4, p<0.01 vs MCT + Saline). Ki-67 detection is an established immunohistochemical technique used to assess cell proliferation.8 Our findings suggest that MCT injection induces apoptosis within PAECs,13 which involves PAH progression and consequently induces replenishment, but insufficient proliferation, of PAECs and that G-CSF accelerated proliferation to a physiologically effective level.
Markers for rat EPCs have not been established. We therefore evaluated circulating angiogenic stem/progenitor cells presumably recruited from the bone marrow (BM) using flow cytometry to detect c-kit, which is a marker of hemangioblasts and hematogenic angioblasts (Fig 6). The findings indicate that the numbers of c-kit positive cells in the WBC fraction of peripheral blood did not statistically differ between the MCT + Saline and MCT + G-CSF groups (0.12±0.01% and 0.07±0.04%, respectively).

The gain in body weight becomes impaired as PAH progresses, and surviving PAH rats weighed significantly less than intact rats (Fig 7A). Treatment with G-CSF at 100 μg/kg per day from day 21 significantly inhibited the reduction in body weight gain. Treatment with G-CSF at 2 μg/kg did not significantly affect body weight gain.

**Fig 5.** (A) representative photomicrographs of immunohistochemistry of Ki-67. Nuclear immunoreactivity has a reddish violet granular appearance (arrows). Control rats (Upper), MCT + Saline (Middle), MCT + G-CSF (Lower). Original magnification ×40. (B) %Ki-67 labeling within endothelial cells of pulmonary arteries. *p<0.01. G-CFS, granulocyte colony-stimulating factor; MCT, monocrotaline.

**Fig 6.** Flow cytometric analysis of peripheral blood. Peripheral blood samples after 10 days of daily G-CSF injections to PAH rats were analyzed by expression of c-kit and propidium iodide (PI) incorporation after gating for WBC fraction. Dot-plots show representative data of peripheral blood from MCT + Saline and MCT + G-CSF groups and bone marrow of normal rats (positive control). Viable c-kit positive cells are fractionated in R3 region. G-CFS, granulocyte colony-stimulating factor; MCT, monocrotaline; PAH, pulmonary arterial hypertension; WBC, white blood cells.

**Fig 7.** Hemodynamic assessment, body weight change and survival curve of PAH rats given G-CSF from day 21 of MCT injection. (A) Body weight change of surviving PAH rats. Number of rats per group on day 0 is shown. Error bars indicate SD. *p<0.05 compared with intact rats; #p<0.05 compared with PAH control rat (MCT + Saline). (B) Survival curve of PAH rats. *p<0.05 compared with intact rats; #p<0.05 compared with PAH control rats (MCT + Saline). G-CFS, granulocyte colony-stimulating factor; MCT, monocrotaline; PAH, pulmonary arterial hypertension.
2 μg/kg per day elicited a similar trend, but the difference was not statistically significant. The administration of G-CSF did not affect the body weight gain of intact rats. All intact rats regardless of G-CSF administration survived the observation period. Saline-injected control PAH rats began to die from day 28 and none survived beyond day 42 (Fig 7B). Treatment with both doses of G-CSF from day 21 significantly improved the survival of PAH rats. Interestingly, the survival of rats on G-CSF (100 μg/kg per day) from day 0 of the MCT injection did not improve (unpubl. data), suggesting that G-CSF enhances the acute inflammatory response to develop pulmonary arterial muscularization after MCT-induced injury of the pulmonary vessels. Because 1 subcutaneous injection of MCT could produce alterations in the pulmonary vessels and right ventricular hypertrophy after 3 weeks,16 the present results showed that G-CSF administration starting from day 21 improves the prognosis of progressive PAH but does not inhibit MCT toxicity.

Lee et al recently reported that a low dose of G-CSF (2–20 μg/kg) in rats directly stimulates mature endothelial cells to migrate without mobilizing endothelial stem/progenitor cells.17 The present study found that a low dose of G-CSF (2 μg/kg) improved the survival of PAH rats, and that daily G-CSF administration did not increase circulating c-kit positive stem cells, which comprise hemangioblasts and hematogenic angioblasts. These findings suggest that in our model, G-CSF did not mobilize angiogenic stem/progenitor cells, which would engraft to injured pulmonary arteries from the BM into the circulation, but directly stimulated endothelial cells to proliferate and migrate in PAH lesions in situ.

This is the first report to show that G-CSF improves the progression of MCT-induced PAH, and that direct administration (without gene transfer) of a cytokine to PAH, based on the concept of vascular regeneration, is valid. Between 200 and 300 μg/kg per day of recombinant human G-CSF injected for 5 days into rats induced hematopoietic effects that are equipotent to those observed in humans given 10–20 μg/kg per day for a similar period.17 We consider that 100 μg/kg per day of rhG-CSF corresponded to 5 μg/kg per day in humans, which is used as a clinical dose.18–21 Further investigation of other tissue-protective properties of G-CSF in this model, such as anti-apoptotic effects22 of right ventricular cardiomyocytes that undergo apoptosis because of pressure overload23 and of renal protective effects24 against MCT toxicity should be informative.

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References