Role for Granulocyte Colony Stimulating Factor in Angiotensin II–Induced Neutrophil Recruitment and Cardiac Fibrosis in Mice

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BACKGROUND
Granulocyte colony stimulating factor (G-CSF) is a key mediator of neutrophil infiltration and is profibrotic in the liver, lung, and infarcted heart, but its roles in angiotensin II (Ang II)–induced hypertension and cardiac remodeling have not been fully determined. Thus, we sought to investigate the causal relation of G-CSF to neutrophil recruitment and cardiac fibrosis in C57BL/6J mice.

METHODS
Hypertension and cardiac fibrosis were induced in wild-type (WT) mice receiving continuous infusion of Ang II (1,500 ng/kg/min). After 7 days, heart sections were stained with hematoxylin and eosin, Masson’s trichrome, and immunohistochemistry. The mRNA expression of cytokines was detected by real-time polymerase chain reaction analysis. The protein levels were measured by Western blot analysis.

RESULTS
After Ang II infusion, myocardial G-CSF expression was significantly elevated in the hearts. Moreover, WT mice exhibited increased blood pressure, marked neutrophil accumulation, proinflammatory cytokine expression, reactive oxygen species production, and cardiac fibrosis after 7 days of Ang II infusion. However, administration of anti-G-CSF neutralizing antibody, but not with control immunoglobulin G, significantly attenuated these effects. In addition, neutralizing G-CSF antibody reversed Ang II-induced activation of ERK1/2, STAT3, and AKT signaling pathways in the hearts.

CONCLUSIONS
This study demonstrates that G-CSF plays a critical role in hypertension and cardiac fibrosis and targeting this cytokine may be a novel therapeutic strategy to ameliorate hypertensive heart disease.

Keywords: angiotensin II; blood pressure; cardiac fibrosis; granulocyte colony stimulating factor; hypertension; neutrophils.

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and bone marrow transplantation.\textsuperscript{10,11} Importantly, G-CSF markedly promotes the proliferation of developing cardiomyocytes\textsuperscript{12} and improves cardiac function and reduces mortality after myocardial infarction in mice.\textsuperscript{13–15} Some clinical trials have supported the idea that G-CSF could be effective in patients with acute myocardial infarction with late repertusion.\textsuperscript{13} In contrast, some studies have indicated that G-CSF may have adverse effects; for example, G-CSF treatment exacerbates cardiac fibrosis after myocardial infarction or the lung toxicity caused by pneumotoxic agents.\textsuperscript{16,17} However, little is known about the therapeutic potential of targeting G-CSF in Ang II–induced hypertension and cardiac fibrosis.

In this study, we hypothesized that blockage of G-CSF would have therapeutic benefits against Ang II–induced cardiac inflammation and fibrosis. Surprisingly, our results demonstrated that targeting G-CSF with neutralizing antibodies markedly attenuated Ang II–induced hypertension, inflammation, and cardiac fibrosis. The beneficial effects resulted from the inhibition of accumulation and activation of neutrophils and fibroblasts through inactivation of ERK1/2, STAT3, and AKT signaling pathways. These results confirm that inhibition of G-CSF signaling is of great potential to be applied in the prevention and treatment of hypertensive heart disease.

**METHODS**

**Antibodies and reagents**

The antibodies against transforming growth factor beta (TGF-β), alpha-smooth muscle actin (α-SMA), Mac-2, CD3, and horseradish peroxidase–linked antimonu or antirabbit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibody against myeloperoxidase (MPO) was from Abcam (Cambridge, MA); antibody against Ly6G/Gr-1 was from Biolegend (San Diego, CA); antibodies against G-CSF and immunoglobulin G (IgG) control were from R&D Systems (Minneapolis, MN); antibodies against phospho-ERK1/2, ERK1/2, phospho-STAT3, STAT3, phospho-AKT, AKT, and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Ang II was from Sigma (Sigma-Aldrich, Louis, MO). Penicillin, streptomycin, and fetal bovine serum were obtained from Invitrogen Life Technologies (Carlsbad, CA).

**Animals and treatment**

Wild-type (WT) mice (C57BL/6J; aged 10–12 weeks; n = 6 per group) were infused for 3 or 7 days with vehicle (saline) or a dose of Ang II (1,500 ng/kg/min) with an osmotic minipump (Alzet, Cupertino, CA) as previously described.\textsuperscript{18,19} Anti-G-CSF neutralizing antibody (anti-G-CSF) or control IgG (30 μg/mouse/day, intraperitoneally) was administered every day from 1 day before the operation to block G-CSF function based on our preliminary results and previous publications.\textsuperscript{20,21} The systolic blood pressure was measured in conscious mice by tail-cuff method (Softron BP-98A, Tokyo, Japan). The mice were handled gently and not forced to enter the restrainer, and the ambient temperature was maintained at warm room temperature (25–30 °C). All mice were maintained in specific pathogen-free conditions and given free access to a normal diet (0.3% sodium chloride). After the study period, mice were anesthetized, and the hearts were removed and prepared for further histological and molecular analysis. All procedures were approved by the Institutional Animal Care and Use Committee of Capital Medical University. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

**Histology and immunohistochemistry**

Heart tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 5 μm. Hematoxylin and eosin (H&E) and Masson’s trichrome staining were performed using standard procedures as previously described.\textsuperscript{18,19} Immunohistochemistry was performed with primary antibodies, including TGF-β (1:200), α-SMA (1:200), Gr-1 (1:200), MPO (1:200), Mac-2 (1:200), and CD3 (1:200). Digital photographs were taken at X200 magnification of over 20 random fields from each heart, and the positive areas were analyzed by Image Pro Plus 3.0 (ECLIPSE 80i/90i; Nikon, Tokyo, Japan).

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted by using TRIZOL (Invitrogen) according to the manufacturer’s instructions, and first-strand cdNA was synthesized with Superscript II (Invitrogen). The transcript levels of G-CSF, collagen I, collagen III, TGF-β, α-SMA, IL-1β, TNF-α, IL-6, and CXCL1 were detected by quantitative real-time polymerase chain reaction analysis.\textsuperscript{19} The primer sequences are described in the Supplementary Methods.

**Western blot analysis**

Protein samples were prepared from heart tissues with lysis buffer as described.\textsuperscript{19} Western blot analysis was performed with indicated primary antibodies, including phospho-ERK1/2, ERK1/2, phospho-STAT3, STAT3, phospho-AKT, AKT, and β-actin as previously described.\textsuperscript{19,22} The blots were developed by use of a chemiluminescent system, and densitometry analysis involved a Gel-pro 4.5 Analyzer (Media Cybernetics, Rockville, MD).\textsuperscript{19}

**Detection of malondialdehyde, glutathione peroxidase, and hydroxyproline in the heart tissue**

The malondialdehyde (MDA) level as an indicator of lipid peroxidation was measured by colorimetric assay kit according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Glutathione (GPx) was measured using an assay from Calbiochem (San Diego, CA).\textsuperscript{23} The levels of hydroxyproline were examined by chemical colorimetry according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute).
Enzyme-linked immunosorbent assay

The levels of monocyte chemoattractant protein 1 (MCP-1) and G-CSF in plasma and supernatants of hearts were analyzed with enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses involved use of 1-way analysis of variance followed by t test for multiple comparisons within treatment groups with use of SPSS version 13.0 (SPSS, Chicago, IL). P < 0.05 was considered statistically significant.

RESULTS

G-CSF expression was elevated in the heart and serum of mice by Ang II infusion, and anti-G-CSF efficiently blocked the activity

To explore the role of G-CSF in Ang II–induced hypertensive hearts, we first detected the expression of G-CSF in WT mice by quantitative real-time polymerase chain reaction analysis. Ang II infusion significantly increased G-CSF mRNA expression on days 3 and 7 (Figure 1a). ELISA assay showed that serum G-CSF levels increased markedly at day 7 after Ang II infusion (Figure 1b). These results suggest that Ang II induces myocardial G-CSF expression that may have a critical role in cardiac remodeling.

To detect the efficiency of anti-G-CSF neutralizing antibody, we first performed experiments of dose–response relationship: 3 different doses at 10, 30, and 50 μg/mouse/day of anti-G-CSF and control IgG were administered every day in mice (n = 3 per group) for 7 days. The levels of serum G-CSF were detected by ELISA. We found that the dose of anti-G-CSF at 30 μg/mouse/day significantly decreased the level of serum G-CSF compared with the IgG group (Figure 1c). Moreover, Western blot analysis showed that the levels of G-CSF protein in the heart were markedly lower in the anti-G-CSF–treated mice than in IgG-treated mice after Ang II infusion (Figure 1d). In contrast, anti-G-CSF did not affect G-CSF mRNA expression in the heart (Figure 1e). To further confirm the effect of anti-G-CSF on G-CSF bioactivity, circulating neutrophils (Gr-1+) were measured by flow cytometry. Consistent with previous findings,26,21,25,35 anti-G-CSF antibody markedly decreased the number of circulating neutrophils compared with the IgG group after Ang II infusion (42% ± 5% vs. 68% ± 8%; P < 0.01). These results indicate that anti-G-CSF at 30 μg/mouse/day can effectively inhibit G-CSF function in vivo.

G-CSF neutralization attenuates Ang II–induced high blood pressure and myocardial fibrosis

To determine the effect of G-CSF inhibition on Ang II–induced hypertension, WT mice were infused with Ang II and then injected with anti-G-CSF neutralizing antibody or IgG control. Seven days after Ang II infusion, no adverse effects were observed in mice treated with anti-G-CSF or IgG. Moreover, the elevation of systolic blood pressure was significantly lower in anti-G-CSF–treated mice than in IgG-treated mice (Figure 2a). There was no difference in the blood pressure between the 2 groups after saline infusion (Figure 2a).

To further determine the effect of G-CSF inhibition on Ang II infusion–induced cardiac fibrosis, we examined cardiac fibrotic areas in the hearts by Masson’s trichrome staining. Ang II infusion markedly increased fibrotic areas in cardiac tissues of IgG-treated mice, whereas administration of G-CSF antibody markedly attenuated this effect (Figure 2b). Moreover, the levels of fibrotic markers, including collagen I and III mRNA expression, and hydroxyproline content were lower in anti-G-CSF–treated mice than in IgG-treated mice after Ang II infusion (Figure 2c, d). Finally, cardiac expression of α-SMA (a marker for myofibroblasts) and TGF-β was significantly decreased in anti-G-CSF–treated mice compared with IgG-treated mice (Figure 2e, f). These results demonstrate a critical role for G-CSF in cardiac fibrosis induced by Ang II infusion.

To further assess the effect of anti-G-CSF antibody on cardiac function and hypertrophy, echocardiography was performed. Ang II infusion significantly enhanced cardiac function (left ventricular fractional shortening (%LVFS) and left ventricular ejection fraction (%LVEF)) compared with saline-treated groups (Supplementary Figure S1a, b, c). Moreover, Ang II infusion markedly induced cardiac hypertrophy reflected by heart weight to body weight ratio and the myocyte size compared with saline-treated control mice after IgG treatment (Supplementary Figure S1d, e). Although there was a trend toward decrease in heart weight to body weight ratio and the myocyte size, there was no statistically significant difference between anti-G-CSF–treated and IgG–treated mice after Ang II infusion (Supplementary Figure S1d, e). Thus, inhibition of G-CSF does not affect Ang II–induced cardiac function and hypertrophy at 7 days after Ang II infusion.

Depletion of G-CSF inhibits neutrophil recruitment, proinflammatory cytokine expression, and oxidative stress in the heart

G-CSF has been shown to promote recruitment of neutrophils during the inflammatory response, and neutrophils display more G-CSF receptors than immature cells.24 To examine whether G-CSF blockade decreases neutrophil accumulation in the heart, H&E staining and immunohistochemistry were performed. Seven days after Ang II infusion, the numbers of Gr-1+ (a marker for granulocytes), MPO+ (a marker for neutrophil activation), CD3+ (a marker for T cells), and Mac-2+ (a marker for macrophages) cells in the hearts were significantly increased in saline-treated mice. In contrast, these alterations were markedly reduced in anti-G-CSF–treated mice (Figure 3a, b; Supplementary Figure S2). There was no significant difference in the number of these cells between the 2 groups after saline infusion (Figure 3a, b; Supplementary Figure S2). Moreover, as expected, neutralization of G-CSF significantly reduced the expression of TNF-α, IL-1β, IL-6, MCP-1, and CXCL1 in the heart.
G-CSF contributes cardiac fibrosis compared with the IgG-treated group at 7 days after Ang II infusion (Figure 3c, d; Supplementary Figure S3). G-CSF is known to stimulate reactive oxygen species (ROS) production in neutrophils and other cell types. We then compared the levels of 2 oxidative stress markers, MDA and GPx, in heart tissues. Ang II infusion significantly increased MDA level and decreased GPx activity in the hearts of mice treated with IgG as compared with saline-treated mice. In contrast, these changes in MDA level and GPx activity were attenuated in anti-G-CSF–treated mice on day 7 (Figure 3e). Thus, G-CSF is essential for Ang II–induced neutrophil infiltration, inflammation, and oxidative stress in the heart.
Effect of G-CSF neutralization on activation of ERK1/2, STAT3, and AKT signaling pathways

Several classes of signaling pathways, including ERK1/2, STAT3, and AKT, have a central role in modulating neutrophil accumulation, activation, and chemotaxis. We thus examined the activation of ERK1/2, STAT3, and AKT in the hearts by Western blot analysis. The relative levels of phosphorylated ERK1/2, STAT3, and AKT were significantly increased in Ang II–treated mice compared with saline-treated mice, whereas these effects were markedly attenuated in anti-G-CSF–treated mice (Figure 4a–c). The relative levels of phosphorylated ERK1/2, STAT3, and AKT were similar among groups with saline treatment (Figure 4a–c).

Figure 2. Neutralization of granulocyte colony stimulating factor (G-CSF) inhibits hypertension and myocardial fibrosis. Wild-type (WT) mice were injected with anti-G-CSF neutralizing antibody or control immunoglobulin G (IgG) (30 μg/mouse/day, intraperitoneally) every day from 1 day before angiotensin II (Ang II) infusion (1,500 ng/min/kg) for 7 days. (a) Noninvasive blood pressure was measured (n = 6 per group). (b) Representative Masson trichrome staining (top panels) and quantification of heart sections (bottom panels). (c) Quantitative real-time polymerase chain reaction (qPCR) analysis of collagen I and III mRNA expression. (d) The levels of hydroxyproline in the hearts. (e) Immunohistochemistry (left panels) and quantification (right panels) of heart sections for transforming growth factor beta (TGF-β) and alpha-smooth muscle actin (α-SMA). qPCR analysis of TGF-β and α-SMA mRNA expression (right). Bar: 50 μm. Data are mean ± SEM (n = 6 per group). *P < 0.05 vs. saline-treated mice; **P < 0.05 vs. IgG + Ang II–treated mice. Abbreviations: C, control; T, treatment.
Thus, G-CSF may be involved in Ang II–induced neutrophil recruitment and activation by the ERK1/2, STAT3, and AKT signaling pathways.

**DISCUSSION**

G-CSF is a hematopoietic cytokine, and its role in hematopoiesis, stem cell mobilization, heart development, and cardiac repair has been investigated in detail.\textsuperscript{11,12,14} However, there is still no evidence that inhibition of G-CSF has therapeutic benefits against hypertension and cardiac fibrosis. In this study, we found that Ang II infusion induced hypertension and increased cardiac neutrophil recruitment, proinflammatory cytokine expression, ROS production, and cardiac fibrosis. In contrast, blockade of G-CSF by neutralizing antibody markedly attenuates Ang II–induced effects. These changes were associated with inactivation of ERK1/2, STAT3, and AKT signaling pathways.

Accumulating evidence suggests that G-CSF is produced by various cell types, including bone marrow stromal cells, fibroblasts, endothelial cells, macrophages, and others.\textsuperscript{10,11} G-CSF has been reported to repair cardiac tissues and improve left ventricular remodeling and function after acute myocardial infarction.\textsuperscript{13–15} Recently, a study reported that G-CSF administration improves cardiac hypertrophy and fibrosis after 4 weeks of Ang II infusion through inhibition of osteopontin expression and p70S6 activation.\textsuperscript{28} However, other contradictory studies have indicated that G-CSF treatment can exacerbate rat cardiac fibrosis after myocardial infarction and the lung toxicity caused by pneumotoxic agents.\textsuperscript{16,29} Thus, we would expect that anti-G-CSF antibody would reduce cardiac fibrosis. Indeed, neutralization of G-CSF remarkably attenuated Ang II–induced expression of TGF-β and the accumulation of α-SMA+ myofibroblasts leading to decreased cardiac fibrosis at 7 days (Figure 2) but did not affect cardiac function and hypertrophy (Supplementary Figure S1). Collectively, these results indicate that G-CSF is essential for Ang II–induced cardiac fibrosis.

Infiltration of inflammatory cells (including neutrophils, monocytes/macrophages, and T cells) is the early inflammatory event and plays a key role in triggering the fibroinflammatory process in hypertensive cardiac remodeling, infarction, and other inflammatory diseases.\textsuperscript{30} This process is thought to be mediated by chemotactic factors that are released by the damaged myocardium, including G-CSF, MCP-1, CXCL1, and CXCL2.\textsuperscript{10,11,30} It is reported that G-CSF receptor is highly expressive on neutrophils, monocytes/macrophages, and T cells,\textsuperscript{10,11,31,32} indicating that G-CSF is
Figure 3. Neutralization of granulocyte colony stimulating factor (G-CSF) attenuates neutrophil infiltration, proinflammatory cytokine expression, and oxidative stress. (a) Hematoxylin and eosin (H&E) staining of heart tissues in immunoglobulin G (IgG)–treated and anti-G-CSF–treated mice at 7 days after saline or angiotensin II (Ang II) infusion. (b) Immunohistochemistry of heart sections for Gr-1, myeloperoxidase (MPO) (left). Quantification of Gr-1, MPO-positive cells (right) (n = 6 per group). Bar = 50 μm. (c) Quantitative real-time polymerase chain reaction analysis of the mRNA expressions of tumor necrosis factor alpha (TNF-α), interleukin (IL)–1β, and IL-6 in heart tissues from IgG-treated and anti-G-CSF–treated mice after saline or Ang II infusion. (d) Monocyte chemoattractant protein 1 (MCP-1) concentration in heart tissues was measured by enzyme-linked immunosorbent assay using Quantikine Mouse MCP-1 immunoassay kit. (e) Malondialdehyde (MDA) level and glutathione (GPx) activity were measured in heart homogenates from IgG-treated and anti-G-CSF–treated mice after saline or Ang II infusion. Data are mean ± SEM (n = 6 per group). *P < 0.05 vs. saline-treated mice; **P < 0.05 vs. IgG + Ang II–treated mice.
G-CSF Contributes Cardiac Fibrosis

Figure 4. Neutralization of granulocyte colony stimulating factor (G-CSF) reverses activation of ERK1/2, STAT3, and AKT induced by angiotensin II (Ang II). Western blot analysis of protein levels of total and phosphorylated ERK1/2 (a), STAT3 (b), AKT (c) and β-actin from heart tissues (left) at 7 days after Ang II infusion and quantification relative to β-actin level (right). Data are mean ± SEM (n = 3 per group). *P < 0.05 vs. saline-treated mice; **P < 0.05 vs. IgG + Ang II–treated mice.

crucial for recruitment of these inflammatory cells. Indeed, G-CSF treatment results in a considerable infiltration of neutrophils and release of IL-1β in the heart after transverse aortic constriction. Moreover, neutrophils can express inflammatory cytokines, including IL-1α, IL-1β, IL-8/MIP-2, and TNF-α, after blood loss or endotoxin. Here our results demonstrated that neutralization of G-CSF by anti-G-CSF antibody significantly decreased the number of myocardial neutrophils, macrophages, and T cells and expression of inflammatory cytokines (such as TNF-α, IL-1β, IL-6, and...
CXCL1) compared with IgG-treated WT hearts after Ang II infusion (Figure 3; Supplementary Figure S2 and S3). Thus, these important findings suggest that G-CSF plays a critical role in mediating the inflammatory process and is a promising therapeutic target for preventing cardiac fibrosis.

Several proinflammatory mediators (such as IL-1β, IL-6, and TNF-α) stimulate various pathways that induce neutrophil NADPH oxidase activation to produce ROS.36,37 Besides destroying microbes, ROS can induce cell apoptosis, growth, transformation, and inflammatory response.44 It has been reported that G-CSF induces activation of NADPH oxidase leading to ROS production in neutrophils.30 In this study, our results showed that neutralization of G-CSF markedly reversed Ang II–induced MDA production and decrease of GPx activity (Figure 3), indicating that G-CSF is a stimulator for the generation of oxidative stress in the hearts.

Emerging evidence demonstrates that binding of G-CSF to the G-CSF receptor is an important mechanism for survival, proliferation, differentiation, and function of neutrophil precursors, mature neutrophils, cardiomyocytes, and other cell types.10,11 G-CSF regulates them using multiple signaling pathways, including AKT, MAPKs, and STAT3.35 Recently, G-CSF was found to play an important role in innate immunity. Our results suggest that with Ang II infusion, ERK1/2, STAT3, and AKT signaling pathways were activated in IgG-treated WT mouse hearts, as demonstrated by the increased levels of phosphorylated AKT, ERK1/2, and STAT3, but were markedly attenuated in anti-G-CSF–treated mice (Figure 4). These data indicate that inhibition of G-CSF effectively inhibited Ang II–induced inflammation and cardiac fibrosis at least in part through the inhibition of ERK1/2, STAT3, and AKT signaling pathways.

Notably, we observed administration of anti-G-CSF antibody lowered Ang II–induced blood pressure in mice; however, a contradictory study showed G-CSF treatment had no effect on blood pressure.28 Recently, several studies have demonstrated that proinflammatory cells, including T cells and monocytes/macrophages, but not B cells, in mouse vascular contribute to Ang II–induced hypertension.30,37 Our results indicate that anti-G-CSF treatment markedly decreased infiltration of neutrophils, macrophages, and T cells into the hearts (Figure 3; Supplementary Figure 2); however, it is unclear whether anti-G-CSF has the similar effect in vascular tissue. Thus, we will be working on how G-CSF regulates the vascular infiltration and activation of inflammatory cells, such as T cells or monocytes/macrophages, leading to vascular or renal dysfunction and high blood pressure in future studies.

Potential limitations of our investigation include the following. First, the blood pressure of mice was measured using the tail-cuff method, but it is difficult to detect small differences in blood pressure. It is better to use indwelling catheters or telemetry to strengthen our results in future experiments. Second, this study tested therapeutic effect of anti-G-CSF neutralizing antibody on the development of fibrosis. There are several advantages of the antibody-based treatment, including versatility, low toxicity, target specificity, and favorable pharmacokinetics compared with that in knockout approach, but it will be important to confirm the effects of G-CSF in knockout mice.

In conclusion, our results provide evidence for the critical role of G-CSF in Ang II–induced hypertension, cardiac inflammation, and fibrosis. Blocking of G-CSF function not only attenuated inflammation and ROS production but also reduced collagen deposition and the subsequent cardiac fibrosis in Ang II–induced hypertensive hearts. The alterations were associated with inhibition of ERK1/2, STAT3, and AKT signaling pathways. These results suggest that G-CSF may be a new potential target for preventing hypertensive heart disease.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at American Journal of Hypertension (http://ajh.oxfordjournals.org).

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DISCLOSURE

The authors declared no conflict of interest.

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Overexpression of Nrdp1 in the heart exacerbates doxorubicin-induced ischemia and reperfusion-induced cardiac injury.


