Granulocyte colony-stimulating factor protects cardiac mitochondria in the early phase of cardiac injury

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Am J Physiol Heart Circ Physiol 296: H823–H832, 2009. First published January 9, 2009; doi:10.1152/ajpheart.00774.2008.—Although granulocyte colony-stimulating factor (G-CSF) reportedly plays a cardioprotective role in several models of cardiac injury, clinical use of this drug in cardiac patients has been controversial. Here, we tested, in vivo and in vitro, the effect of G-CSF on cardiac mitochondria, which play a key role in determining cardiac cellular fate and function. Mild stimulation of C57/BL6 mice with doxorubicin (Dox) did not induce cardiac apoptosis or fibrosis but did induce damage to mitochondrial organization of the myocardium as observed through an electron microscope. Cardiac catheterization and echocardiography revealed that Dox did not alter cardiac systolic function or left ventricular size but did induce diastolic dysfunction, an early sign of cardiac damage. Treatment with G-CSF attenuated significantly the damage to mitochondrial organization and rescued diastolic function. In an in vitro model for rat neonatal cardiomyocytes, a subapoptotic dose of Dox induced severe mitochondrial damage, including marked swelling of the cardiac mitochondria and/or decreased mitochondrial membrane potential. These mitochondrial changes were completely blocked by pretreatment with G-CSF. In addition, G-CSF dramatically improved ATP generation, which rescued Dox-impaired mitochondrial electron transport and oxygen consumption mainly through complex IV. These findings clearly indicate that G-CSF protects cardiac mitochondria, which are key organelles in the determination of cardiac cellular fate, in the early phase of cardiac injury.

doxorubicin; mitochondria; cardiac diastolic function; oxygen consumption

HEART FAILURE, the terminal state of various cardiac diseases, remains the leading cause of mortality for both men and women in developed countries (3, 24). In the early phase of hemodynamic overload, such as hypertension or post-myocardial infarction (MI), the heart maintains its systolic ability through adaptive mechanisms that increase the end-diastolic pressure and/or enlarge myocardial cells (51, 54). These early stages of overload are sometimes only clinically evident after the onset of cardiac hypertrophy or diastolic dysfunction. When overloading persists, these adaptive responses fail, resulting in decompensation and subsequent heart failure with impaired cardiac systolic function (8). Despite recent medical progress, current therapies for heart failure are still limited and the prognosis for heart failure is disappointing (33, 34).

Several animal studies have shown that granulocyte colony-stimulating factor (G-CSF) acts as cardioprotection; for instance, it markedly improves cardiac remodeling and function after myocardial infarction or cardiomyopathy in mice (14, 39, 40). G-CSF is a hematopoietic cytokine that has been used for more than 20 years in patients receiving chemotherapy for primarily hematological malignancies, where it mobilizes bone marrow stem cells into the peripheral blood flow (38). One putative mechanism of cardiac repair by G-CSF is the mobilization and homing of bone marrow-derived stem cells to the damaged myocardiun, where they induce angiogenesis (25, 40) or possible myocardial regeneration (25, 35, 39, 49). G-CSF also reportedly enhances cardiomyocyte survival directly by activating specific G-CSF receptors within the heart (13, 16, 31). Administration of G-CSF significantly alleviates adriamycin-induced cardiomyocyte apoptosis as well as high Fas expression in rats (16). Despite this evidence for the cardioprotective role of G-CSF, clinical trials of G-CSF in cardiac patients have not been conclusive. G-CSF treatment of cardiac patients is still controversial (9, 18, 42, 48, 55), suggesting that more studies on the effects of G-CSF on cardiac cellular fate are necessary.

Mitochondria play a central role both physiologically and pathophysiologically in the regulation of cellular death or survival. Because the heart requires a constant high energy supply, cardiac pump function quickly fails when ATP is not efficiently synthesized by oxidative phosphorylation in cardiac mitochondrial electron transport (12, 29). Thus the mitochondrion is a key organelle for cardiac function and cellular fate. However, investigations in vivo of the role played by mitochondrial dysfunction in cardiac pathology have been limited. Cardiac stress as defined in classical experimental models includes myocardial infarction, ischemia-reperfusion, or aorta banding. These stresses induce not only changes in cardiac mitochondria but also lethal damage, such as cardiac dilatation, systolic pump failure, and cardiac cell death (17, 36), which make it difficult to interpret the effects of these stresses on mitochondria. So far, in other cell types, including neurons and glial cells, G-CSF stabilizes and protects mitochondria (5, 46).

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However, the effect of G-CSF on cardiac mitochondria is still not well known.

Here, we induced a cardiac mitochondrial injury model by mild administration of doxorubicin (Dox). Dox, a popular anticancer drug, has been well used in animal heart failure models. Cumulative doses of Dox induce irreversible dilated cardiomyopathy with cardiac pump failure, as well as cardiac cell death, including apoptosis (30, 47, 53). Redox activation of Dox results in the formation of reactive oxygen species that induce cardiac apoptosis via the inactivation of the mitochondrial electron transport chain (27). By mild administration of this drug, which induced changes in cardiac mitochondria without either cardiac systolic failure or apoptosis, we developed a model for the early phase of cardiac injury in vivo and in vitro. We provide here the first evidence that G-CSF directly protects cardiac mitochondria in the early phase of cardiac injury.

MATERIALS AND METHODS

Animals. The study was approved by the Animal Research Committee of the Graduate School of Medicine, Kyoto University, and animals were treated in accordance with the guidelines of the Animal Research Committee of the Graduate School of Medicine, Kyoto University, which conforms to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Male 8-wk-old C57/BL6J mice were randomly divided into four groups: control (n = 11), G-CSF (n = 7), Dox (n = 8), and Dox + G-CSF group (n = 14). Mice were injected intraperitoneally with 5 mg·kg\(^{-1}\)·day\(^{-1}\) of Dox (Kyowa Hakko) in the Dox and G-CSF groups or with saline in the control and G-CSF group. This was performed six times over 2 wk as a first treatment. Then G-CSF (Kirin Pharma) was injected subcutaneously at a dose of 100 µg·kg\(^{-1}\)·day\(^{-1}\) for five consecutive days in the G-CSF and Dox + G-CSF groups. Saline was injected subcutaneously in the control and Dox groups. The mice were killed after hemodynamic measurements, and cardiac tissue was harvested immediately for histological examination (Fig. 1A).

Blood cell count. Peripheral red blood cells, white blood cells, and platelets were counted at days 0 and 49 with an automated hemocytometer (Celltac, Nihon-Kohden).

Hemodynamic measurements in mice. Hemodynamics were measured indirectly via echocardiography with a 15- to 16-MHz phased-array transducer (model 21390A, Philips) and via cardiac catheterization with a 1.4-Fr micromanometer-tipped catheter (Millar Instruments), Nihon-Kohden).

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Molecular Probes) at 37°C for 5 min (22). Fluorescence was captured and analyzed by the JC-1 red/green ratio. The mitochondrial membrane potential was evaluated by the JC-1 red/green ratio.

Assessment for cardiac fibrosis. Cardiac tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with Masson trichrome as previously described (23). Quantification of the fibrotic area was analyzed using computer-assisted planimetry with NIH Image J 1.38 software.

Immunohistochemistry. Using the avidin-biotin complex method with alkaline phosphatase, immunohistochemistry for 8-hydroxy-2′-deoxyguanosine (8-OHdG) was performed as previously described (21). 8-OHdG immunohistochemistry specimens were subjected to densitometric analysis. Quantification of immunological data expressed as the 8-OHdG index was performed. The following equation was used for the quantification of the immunological data: 8-OHdG index = \( \frac{\sum (X - \text{threshold}) \times \text{area (\(\mu m^2\)})}{\text{total cell number, } X > \text{threshold, where } X \text{ is the staining density indicated by a number between 0 and 150 on the gray scale.}} \)

Western blotting. Proteins were extracted from freshly frozen left ventricular myocardium using the Tissue Protein Extraction Regent (Pierce Technology) supplemented with Protease Inhibitor Cocktail for Use with Mammalian Cell and Tissue Extracts (Nacalai Tesque). Western blotting was performed as previously described (21). We probed the membranes with mouse monoclonal anti-4-hydroxy-2′-nonenal (HNE)-modified protein antibody (50) or an anti-actin antibody (Sigma-Aldrich). The blots were visualized by chemiluminescence (ECL, Amersham), and the signals were analyzed using computer-assisted planimetry with NIH Image J 1.38.

Cell culture. Cardiomyocytes were prepared by high-density plating (1,000 cells/min\(^2\)) of cardiomyocytes from the ventricles of 1- to 3-day-old Sprague-Dawley rats as previously described (20, 21, 52). The number of viable cells was determined by trypan blue staining.

Determination of the mitochondrial membrane potential. Cardiac myocytes were incubated in PBS containing 10⁻⁶ M 5,5′,6,6′-tetrachloro-1,1′,3′,3′'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes) at 37°C for 5 min (22). Fluorescence was captured at 529 nm (green) and 590 nm (red) with excitation at 480 nm on a Becton-Dickinson FACScalibur cytofluorimeter. The mitochondrial membrane potential was evaluated by the JC-1 red/green ratio.

- **Control** (n = 11)
- **G-CSF** (n = 7)
- **Dox** (n = 8)
- **Dox + G-CSF** (n = 14)

**Week**

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<td>blood cell count</td>
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**Fig. 1.** Experimental protocol in vivo. C57/BL6J mice were divided into 4 groups, control (n = 11), granulocyte colony-stimulating factor (G-CSF; n = 7), doxorubicin (Dox; n = 8), and Dox + G-CSF (n = 14) group, as indicated. Arrows indicate the time points of treatment or hemodynamic assessment of the mouse heart.
Oxygen electrode measurements. The activities of the mitochondrial electron transport complexes I–IV were measured using a Clarke oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) with a thermostatted chamber, as previously described (1, 22, 28). The cells prepared with air-saturated respiration buffer were permeabilized with digitonin (0.0005%), and substrates and inhibitors were added in the following order and concentrations: 5 mM malate; 5 mM pyruvate; 100 nM rotenone; 5 mM succinate; 50 nM antimycin A; 1 mM ascorbate; 0.4 mM TMPD; and 5 mM KCN. The activity of complex IV was measured after the addition of TMPD. The oxygen concentration was calibrated with air-saturated buffer, assuming 390 ng-atoms of oxygen/ml of buffer. The rates of cyanide-sensitive oxygen consumption are expressed as ng-atoms oxygen per minute per 10^6 cells.

Fig. 2. Histological assessment of the mouse heart. In the mouse heart, low doses of Dox did not induce apoptosis or fibrosis but did induce electron microscopic (EM) changes in mitochondria that were inhibited by G-CSF. A, top: representative photographs of Masson trichrome staining under each indicated condition. The blue-stained area indicates fibrosis. Scale bar, 1 mm. A, bottom: blue-stained area (%) in the myocardium after the indicated treatment. B: terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL)-positive cardiomyocytes in the myocardium (%area) after the indicated treatment. C: representative EM photographs of the treated murine myocardium. Arrows point to examples of impaired alignment of cardiac mitochondria. Scale bar for all photographs in C is 1 μm. ns, not significant.
Statistical analysis. Values shown are means ± SE. The significance of differences between groups was evaluated by ANOVA. Values of \( P < 0.05 \) were considered significant.

RESULTS

Mild administration of Dox induced EM changes in cardiac mitochondria without inducing the typical features of Dox-induced cardiomyopathy. We administered 5.0 mg·kg\(^{-1}\)·day\(^{-1}\) of Dox, a cardiotoxic antitumor drug, into C57/BL6 mice six times over 2 wk (Fig. 1). All of the mice were still alive after 5 wk of Dox treatment. Neither Masson trichrome nor TUNEL staining indicated any histological changes (Fig. 2, A and B). However, EM changes were observed, as evidenced by mitochondrial clustering instead of the regular alignment pattern between myofibril structures (Fig. 2C). Echocardiogram and cardiac catheterization revealed that the typical form of Dox-induced cardiomyopathy with systolic dysfunction and left ventricular dilatation had not occurred (Fig. 3, A and B). However, both LVEDP and \(-\frac{dP}{dt}\) were elevated, indicating diastolic dysfunction, an early sign of heart failure (Fig. 3B) (3). Oxidative stress, which reportedly contributes to the development of Dox-induced cardiac injury (15, 27), did not appear to be involved in these cardiac changes in Dox-treated mice, as evidenced both by Western blotting with an anti-HNEJ antibody and immunocytochemical staining for 8-OHdG (Fig. 4).

G-CSF rescued Dox-induced mitochondrial disorganization as well as diastolic dysfunction in the mouse heart. To test the effect of G-CSF on Dox-induced cardiac damage, 100 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) of G-CSF was administered to C57/BL6 mice for 2 wk after a series of Dox treatments (Fig. 1). No significant differences were observed in the blood cell count or myocardial oxidative stress level, which are reportedly elevated by the inflammatory action of G-CSF (Table 1 and Fig. 4). However, the impaired organization of cardiac mitochondria in Dox-treated mice was dramatically improved by treatment with G-CSF (Fig. 2C). At the same time, both impaired LVEDP and \(-\frac{dP}{dt}\) returned to their control levels, indicating the rescuing of cardiac diastolic function (Fig. 3B).

G-CSF directly protected cardiomyocytes from Dox-induced mitochondrial morphological damage. To investigate whether G-CSF cardioprotection was mediated by the direct action of G-CSF on cardiomyocytes rather than through bone marrow stem cell mobilization, we performed an in vitro assay using cultured rat neonatal cardiomyocytes.

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Fig. 3. Hemodynamic assessment of the mouse heart. In the mouse heart, low doses of Dox did not cause typical Dox-induced cardiomyopathy but did cause diastolic dysfunction, which was rescued by G-CSF. A: echocardiographic parameters. LVDd, left ventricular diastolic diameter; LVDs, left ventricular systolic diameter; %FS, percent fractional shortening. B: hemodynamic results of cardiac catheterization. HR, heart rate; \(+\frac{dP}{dt}\), left ventricular maximum systolic velocity; \(-\frac{dP}{dt}\), left ventricular minimum diastolic velocity; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure. Results are means ± SE of 5 independent experiments.
Treatment with doses of Dox below 0.3 μM did not lead to an increase in TUNEL-positive apoptotic cardiomyocytes (Fig. 5A). However, EM revealed that a Dox concentration even as low as 0.3 μM could induce marked swelling of mitochondria in the myocardium (Fig. 5B). These severe morphological damages in cardiac mitochondria were dramatically inhibited by pretreatment with G-CSF (Fig. 5B). Quantitative analysis of the percentage of swollen mitochondria also indicated that G-CSF rescued mitochondrial morphology after Dox-induced damage.
Fig. 5. Histological assessment of neonatal rat cardiomyocytes in vitro. Subapoptotic doses of Dox induced EM mitochondrial swelling in cardiac myocytes, which was rescued by G-CSF. Neonatal rat cardiomyocytes were incubated under the indicated conditions for 24 h.

A: dose response of Dox on TUNEL-positive cells (%).

B: cardiomyocytes were treated with saline as a control, G-CSF (100 ng/ml), and/or Dox (0.3 µM). Representative EM photographs. Arrows point to examples of normal mitochondria, and arrowheads point to mitochondria that displayed marked swelling. Scale bar for all photographs in B is 1 µM.

C: quantitative analysis of the swollen mitochondria (%). Results are means ± SE of 3 or more independent experiments.

Table 1. Body weight and blood cells counts at baseline

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<td>7 Wk</td>
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<td>Control</td>
<td>23.8±0.9</td>
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<td>G-CSF</td>
<td>24.0±0.3</td>
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<td>Dox</td>
<td>23.6±0.5</td>
<td>26.2±0.4*†</td>
<td>6.0±3.1</td>
<td>4.5±2.5*</td>
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<td>Dox + G-CSF</td>
<td>23.6±0.6</td>
<td>26.1±0.7*†</td>
<td>5.4±2.5</td>
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Values are means ± SE. BW, body weight; 0 Wk and 7 wk indicate 0 and 7 wk from the beginning of the experiment, respectively; G-CSF, granulocyte colony-stimulating factor; Dox, doxorubicin; WBC, RBC, and Plat indicate white blood cell counts, red blood cell counts, and platelet counts, respectively. *P < 0.01 vs. control, †P < 0.01 vs. G-CSF.
G-CSF prevented the Dox-induced drop in mitochondrial membrane potential. We and others (6, 22) have demonstrated that the collapse of the mitochondrial transmembrane potential is one of the earliest features of cardiac cellular injury, as well as a no-return point during the apoptotic process. Therefore, to examine the effects of G-CSF on early damage to myocardial cells, we measured the mitochondrial membrane potential in Dox-treated cardiomyocytes with and without G-CSF by cytofluorimetric analysis. The number of cells with a low JC-1 red/green ratio was higher in Dox-treated cells than in control cells, indicating that Dox reduced the mitochondrial membrane potential (Fig. 6). G-CSF administration completely shifted this ratio back to the control level. These findings indicate that G-CSF protected cardiomyocytes from mitochondrial functional damage in the early phase of Dox-induced cardiac injury.

G-CSF reversed the Dox-induced decrease in mitochondrial electron transport. Finally, we investigated the effect of G-CSF on the mitochondrial respiratory chain, which is critical in controlling cardiac cell fate and function. We measured mitochondrial electron transport as performed by each of the multisubunits belonging to complexes I-IV in mitochondria. Specifically, we used an oxygen electrode to monitor the oxygen consumption produced by a substrate specific to each complex. The total oxygen consumption was lower in Dox-stimulated cardiac myocytes than in control cells, indicating that Dox impaired mitochondrial electron transport. Pretreatment with G-CSF significantly changed oxygen consumption back to the control level (Fig. 7). Notably, throughout the experiment, the most significant changes in oxygen consumption were observed in complex IV. These findings clearly indicate that G-CSF rescued cardiac mitochondrial respiration, a critical process for determining cardiac cell fate.

DISCUSSION

As the heart requires a constant high energy supply, which is provided by the mitochondrial respiratory chain (12, 29), cardiac mitochondria play a central and critical role in cardiac function. The present findings demonstrate direct protective effects of G-CSF both in vivo and in vitro on cardiac mitochondrial function as well as on mitochondrial morphology in the early phase of cardiac injury.

We applied a mild administration of Dox to murine hearts in vivo and a subapoptotic dose to cultured cardiomyocytes in vitro. Therapeutic dose of Dox cause irreversible dilated cardiomyopathy with severe cardiac pump failure and myocardial cell death (30, 47, 53). In the present study, mild administration of Dox in vivo led to myocardial mitochondrial disorganization and cardiac diastolic dysfunction, but to none of the typical Dox-induced cardiomyopathy features. For our in vitro model of cardiac mitochondrial injury, we considered evidence published by us (22) and others (6) demonstrating that mitochondrial damage precedes apparent apoptotic changes. By using such mitochondrial injury models that do not elicit the typical features of cardiomyopathy, we were able to specifically focus on the role played by cardiac mitochondria.

Although a number of experimental animal studies have demonstrated cardioprotection by G-CSF (25, 38–40), the efficacy of this drug in patients with cardiac disease remains controversial (9, 18, 42, 48, 55). It has been suggested that the time point of G-CSF initiation is responsible for these differing outcomes. One study showed that G-CSF treatment of MI patients immediately after percutaneous coronary intervention results in a significant (8%) improvement of ejection fraction (EF) by 12 mo (18). Another study demonstrated improved left ventricular end-diastolic volume and EF in cardiac patients.
treated with G-CSF (48). Conversely, very late treatment with G-CSF after percutaneous coronary intervention did not increase EF (9, 42, 55). These results have raised the hypothesis that the late administration of G-CSF may diminish its potential benefits on the heart (9, 48). The present study demonstrates that G-CSF improves hemodynamic cardiac function as well as cellular mitochondrial function in the early phase of cardiac injury. Although our model differs from the ischemia-reperfusion model, our findings help provide a better understanding of the beneficial effects of early G-CSF treatment in cardiac patients.

In the mammalian mitochondrial respiration system, most electrons entering complex I go through complexes III and IV before being delivered to oxygen. Each complex helps to maintain the assembly and stabilization of the other complexes. Complex IV deficiency caused by mutation of cytochrome c oxidase 10 results in a marked decrease in the level of complex I (7). Genetic alterations leading to the loss of complex III cause the secondary loss of complex I (43). As these reports show, damage can easily spread to all of the electron transport complexes. However, the present study demonstrates that most of the changes in oxygen consumption occurred in complex IV (Fig. 7). The reciprocal action of each complex has been suggested to be more pronounced in rapidly dividing cells (7, 32). Due to terminal differentiation, cardiac myocytes almost completely lose their ability to divide after birth. Thus one potential explanation could be that the stabilization or the assembly of each complex, including complex IV, is more independent in cardiac myocytes. An investigation into why the changes are more prominent in complex IV or into how such changes affect the later phase of cardiac injury may provide a better understanding of cardiac cellular fate.

Our study also suggests that G-CSF could be useful as a cotherapy in chemotherapy regimens for cancer patients. Dox is widely used to treat various cancers, including leukemia (4), non-Hodgkin’s lymphoma (11), and different types of solid tumors such as breast (41), liver, and colon cancers. However, the cardiotoxicity of this drug has limited its clinical use (44, 45). Recently, Dox-induced cardiac damage was reported to involve a mitochondrial mechanism that was distinct from that implicated in tumor cell killing (26). Dox-induced increases in mitochondrial mass and number precede apoptosis in cancer.
cells but not in cardiac cells, which might explain why Dox is more toxic to cancer cells than to cardiac cells (26). Consistent with this, we did not observe any changes in mitochondrial number throughout the experiment in vitro (data not shown), whereas Dox impairment of mitochondrial morphology and function was dramatically improved by G-CSF (Figs. 5B, 6, and 7). Although clinicians should be attentive to the angio-
genic action of G-CSF (37), the present study suggests that G-CSF could be useful for controlling the early cardiotoxicity of Dox.

In summary, we demonstrated that G-CSF protects cardiomyocytes from mitochondrial damage both morphologically and functionally in the early phase of cardiac injury. The mitochondrial findings presented here are critical for our understanding of cardiac cellular fate. Further research into how G-CSF administration rescues cardiac mitochondria may provide a better understanding of the mechanisms underlying the progression of heart failure and offer additional insights into how to treat cardiac patients.

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