Cardiovasc Drugs Ther (2006) 20: 159–165
DOI 10.1007/s10557-006-8285-8

BASIC RESEARCH

Granulocyte Colony-Stimulating Factor Mediates Cardioprotection Against Ischemia/Reperfusion Injury via Phosphatidylinositol-3-Kinase/Akt Pathway in Canine Hearts

Hiroyuki Takahama · Tetsuo Minamino · Akio Hirata · Akiko Ogai · Hiroshi Asanuma · Masashi Fujita · Masakatsu Wakeno · Osamu Tsukamoto · Ken-ichiro Okada · Kazuo Komamura · Seiji Takashima · Yoshiro Shinozaki · Hidezo Mori · Naoki Mochizuki · Masafumi Kitakaze

Published online: 16 June 2006

Abstract
Purpose Recent studies suggest that G-CSF prevents cardiac remodeling following myocardial infarction (MI) likely through regeneration of the myocardium and coronary vessels. However, it remains unclear whether G-CSF administered at the onset of reperfusion prevents ischemia/reperfusion injury in the acute phase. We investigated acute effects of G-CSF on myocardial infarct size and the incidence of lethal arrhythmia and evaluated the involvement of the phosphatidylinositol-3 kinase (PI3K) in the *in vivo* canine models.
Methods In open-chest dogs, left anterior descending coronary artery (LAD) was occluded for 90 minutes followed by 6 hours of reperfusion. We intravenously administered G-CSF (0.33 μ/kg/min) for 30 minutes from the onset of reperfusion. Wortmannin, a PI3K inhibitor, was selectively administered into the LAD after the onset of reperfusion.
Results G-CSF significantly ($p < 0.05$) reduced myocardial infarct size (38.7±4.3% to 15.7±5.3%) and the incidence of ventricular fibrillation during reperfusion periods (50% to 0%) compared with the control. G-CSF enhanced Akt phospholylation in ischemic canine myocardium. Wortmannin blunted both the infarct size-limiting and anti-arrhythmic effects of G-CSF. G-CSF did not change myeloperoxidase activity, a marker of neutrophil accumulation, in the infarcted myocardium.
Conclusion An intravenous administration of G-CSF at the onset of reperfusion attenuates ischemia/reperfusion injury through PI3K/Akt pathway in the *in vivo* model. G-CSF administration can be a promising candidate for the adjunctive therapy for patients with acute myocardial infarction.

Takahama and Hirata contributed equally to this work.

H. Takahama · A. Ogai · H. Asanuma · M. Wakeno · K. Komamura · H. Mori · M. Kitakaze
Department of Cardiovascular Medicine,
National Cardiovascular Center,
Suita 565-8565, Osaka, Japan

H. Takahama · M. Wakeno · N. Mochizuki
Department of Structural Analysis,
National Cardiovascular Center,
Suita 565-8565, Osaka, Japan

H. Takahama · M. Wakeno · N. Mochizuki
Department of Bioregulatory Medicine,
Osaka University Graduate School of Medicine,
Suita 565-0871, Osaka, Japan

T. Minamino (✉) · A. Hirata · M. Fujita · O. Tsukamoto · K.-i. Okada · S. Takashima
Department of Cardiovascular Medicine,
Osaka University Graduate School of Medicine,
2-2 Yamadaoka, Suita 565-0871, Osaka, Japan
e-mail: minamino@medone.med.osaka-u.ac.jp

Y. Shinozaki
Department of Physiological Science,
Tokai University School of Medicine,
Isehara 259-1193, Kanagawa, Japan

Key words G-CSF · myocardial infarction · ischemia-reperfusion injury · ventricular fibrillation · phosphatidylinositol-3 kinase · Akt

Abbreviations

VF	ventricular fibrillation
G-CSF	granulocyte colony-stimulating factor
WTMN	wortmannin

Introduction

Granulocyte colony-stimulating factor (G-CSF), a 20-kDa glycoprotein, promotes the proliferation, survival and differentiation of hematopoietic cells [1]. Furthermore, G-CSF can mobilize hematopoietic stem cells from bone marrow [2, 3]. Thus, G-CSF is believed to improve cardiac remodeling after myocardial infarction (MI) through regeneration of the myocardium and angiogenesis [4, 5]. In addition to these effects of G-CSF, Komuro and colleagues clearly demonstrated that the high dose of G-CSF acutely reduces infarct size by preventing apoptosis in the isolated hearts [6]. However, it remains unclear whether clinically relevant dosages of G-CSF can reduce the infarct size in the in vivo model and, if so, it is not clear which downstream signaling pathway is involved in the acute cardioprotective effects of G-CSF. Furthermore, although lethal arrhythmias are a major cause of death in patients with acute myocardial infarction [7, 8], anti-arrhythmic effects of G-CSF have not been determined.

Thus, we investigated the acute effects of a clinical relevant dose of G-CSF on ischemia/reperfusion injury including both lethal arrhythmias and infarct size in canine hearts. We also examined a role of the PI3K/Akt pathway, a down stream of G-CSF receptors, in the cardioprotective effects of G-CSF. In the present study, we adopted ischemia/reperfusion protocols that have not been tested in previous studies [4, 5], because coronary revascularization has been established as a standard therapy to attenuate cardiac damage after MI.

Materials and methods

Materials

G-CSF was provided by Kirin brewery company (Tokyo, Japan). Recombinant human G-CSF can increase the number of white blood cells in dogs [9]. Wortmannin was obtained from Sigma (St. Louis, MO), and antibodies against Phospho-Akt and Akt were obtained from Cell signaling technologies (Beverly, MA).

Instrumentation

Twenty-nine beagle dogs (Kitayama Labes, Gifu, Japan) weighing 8 to 12 kg were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg), intubated and ventilated with room air mixed with oxygen (100% O_2 at flow rate of 1.0 to 1.5 l/min). Thoracotomy was done at the fifth left intercostal space, and the heart was suspended in a pericardial cradle. After intravenous administration of heparin (500 U/kg), the left anterior descending coronary artery (LAD) was cannulated for perfusion with blood from the left carotid artery through an extracorporeal bypass tube. This allows the selective infusion of drugs into the LAD-perfused areas through this bypass tube. The left atrium was catheterized for microsphere injection to measure myocardial collateral blood flow during ischemia as described previously [10]. Hydration was maintained by a slow normal saline infusion. Both systemic blood pressure (SBP) and heart rate (HR) were monitored continuously during the study. All procedures were performed in conformity with the Guide for the care and use of laboratory animals (NIH Publication No. 85–23, 1996 revision), and were approved by the *Osaka University Committee for Laboratory Animal Use*.

Experimental protocols

Protocol 1. Acute effects of G-CSF on infarct size and lethal arrhythmias in canine hearts

After hemodynamic stabilization, we intravenously administered either saline (Control group; $n = 9$) or G-CSF (0.33 μg/kg/min) (G-CSF group; $n = 6$) for 30 min following the onset of reperfusion. An intracoronary administration of wortmannin (WTMN), a PI3K inhibitor, was selectively administered into the LAD (1.5 μg/kg/min) for 60 min after the onset of reperfusion (GCSF + WTMN group, $n = 7$; WTMN group, $n = 7$) (Fig. 1). We have previously confirmed that the dose of wortmannin used prevents the phosphorylation of Akt in myocardium [10]. We measured infarct size and myocardial collateral blood flow during ischemia. In brief, infarct size was evaluated at the end of the protocol by Evans blue/TTC staining. Collateral blood flow during 90 min of ischemia was assessed by the non-radioactive microsphere method [10]. We also counted

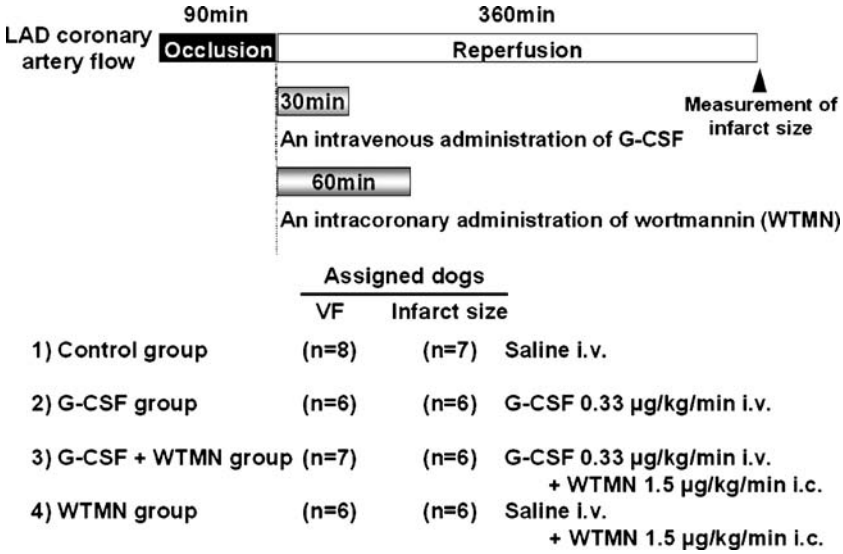

Fig. 1 Experimental protocols to assess myocardial infarct size and ventricular fibrillation (VF) in canine hearts. Myocardial infarct size was measured after 90 min of left anterior descending coronary artery (LAD) occlusion followed by 360 min of reperfusion. The incidence of VF was evaluated during reperfusion for 360 min. Intravenous administration of granulocyte colony-stimulating factor (G-CSF) was started at the onset of reperfusion and continued for 30 min. Intracoronary administration of wortmannin (WTMN) was started at the onset of reperfusion and continued for 60 min.

the incidence of VF during the 6 h reperfusion period (Fig. 1).

Finally, we measured myeloperoxidase (MPO) activity in LAD-perfused myocardium to check the accumulation of neutrophils in infarcted myocardium.

Protocol 2. Phosphorylation of Akt in ischemic myocardium

In this protocol, we used 11 dogs in Control group ($n = 3$), G-CSF group ($n = 4$), and G-CSF + WTMN group ($n = 4$). After 90 min of ischemia followed by 30 min of reperfusion, hearts were excised. The myocardial tissue in the ischemic zone, which was identified as the edge of the region showing necrosis, and non-ischemic zone were quickly placed into liquid nitrogen and stored at −80°C. Phosphorylation of Akt and total content of Akt were evaluated by immunoblotting as reported previously [10].

Immunoblotting

Immunoblotting was performed as described previously [11], and the immunoreactive bands were quantified by densitometry (Molecular Dynamics).

MPO activity

Several myocardial tissue samples were taken from the ischemic area in the dogs studied, frozen in liquid nitrogen and stored at −80°C until assay. The technical procedure has been described previously [12]. One unit of MPO activity was defined as that which degrades 1 μmol hydrogen peroxide per minute at 25°C.

Statistical analysis

Results are expressed as the mean ± SEM. Comparisons of the time course of the change in mean SBP and HR between groups were performed using two-way repeated measures analysis of variance (ANOVA). Comparisons of other data between groups were performed using one-way factional ANOVA. The Bonferroni-Holm procedure was used for correction of multiple comparisons [13]. The incidence of VF was compared using the χ^2-test and Fisher's exact probability test. A p value < 0.05 was considered to represent statistical significance.

Results

Criteria for exclusion

Since there was a negative correlation between myocardial collateral blood flow during ischemia and the incidence of VF [14, 15], it was important to assess myocardial collateral blood flow and exclude the dogs with high myocardial collateral blood flow. We excluded two dogs with excessive collateral blood flow (>15 ml/100 g/min) (Control group: 1, WTMN group: 1) among 29 dogs tested. Thus, 27 dogs were

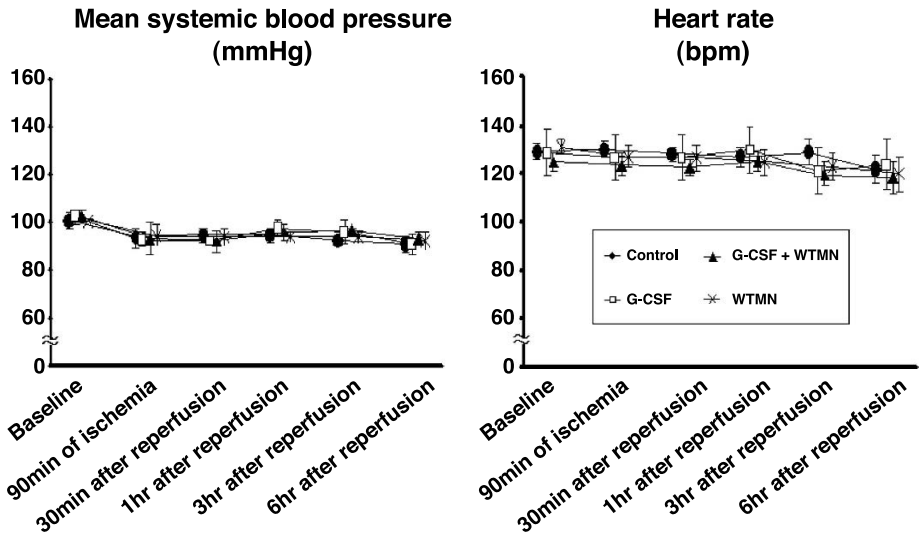

Fig. 2 The changes in mean systemic blood pressure (SBP) and heart rate (HR) during the experiment in groups tested. Neither SBP nor HR differed between the groups tested at baseline, 90 min of ischemia, at 30 min and 1, 3, and 6 h after reperfusion.

evaluated for VF analysis. Among these 27 dogs, we further excluded two dogs (Control group: 1, G-CSF + WTMN group: 1) from infarct size analysis that matched the exclusion criteria of lethal arrhythmia (more than two consecutive attempts required to convert VF with low-energy DC pulses applied directly to the heart) [10].

Effects of G-CSF on infarct size and VF during the reperfusion period

Throughout the study, neither SBP nor HR differed among the four groups (Fig. 2). The area at risk and myocardial collateral blood flow during myocardial ischemia were also comparable in the groups tested (Fig. 3). Figure 4 shows infarct size in the groups tested. G-CSF reduced ($p < 0.05$) infarct size compared with the control group. The intracoronary administration of wortmannin for 60 min after the onset of reperfusion abrogated the infarct size-limiting effects of G-CSF, although wortmannin alone did not affect infarct size.

G-CSF reduced ($p < 0.05$) the incidence of VF during the reperfusion period compared with the control group (Table 1). The antiarrhythmic effects of G-CSF were abolished by wortmannin.

Effect of G-CSF on MPO activity in infarcted myocardium

MPO activity in infarcted myocardium 6 h after reperfusion in G-CSF group did not differ from that in the control group. (10.0 ± 2.6 versus 10.7 ± 2.1 U/g protein).

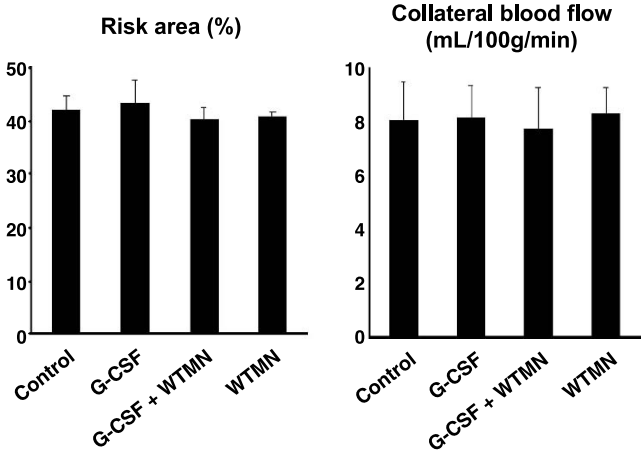

Fig. 3 Area at risk and myocardial collateral blood flow during ischemia in groups tested. Neither the area at risk nor myocardial collateral blood flow differed between the groups tested.

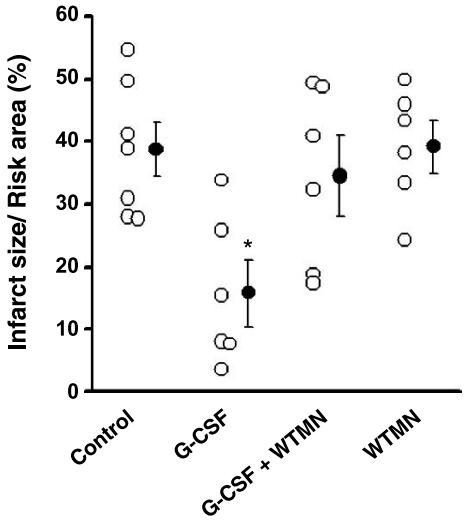

Fig. 4 Infarct size as a percentage of the area at risk in groups tested. Intravenous administration of G-CSF limited infarct size. The infarct-size limiting effect of G-CSF was blunted by the intracoronary administration of WTMN during reperfusion. *$p < 0.05$ vs. control group.

Table 1 Effects of G-CSF on the incidence of VF during reperfusion periods

Group	Incidence of VF (%)	
Control	50.0	(4/8)
G-CSF	0*	(0/6)
G-CSF + WTMN	42.9	(3/7)
WTMN	50.0	(3/6)

*$p < 0.05$ vs. control group

Effect of G-CSF on Akt phosphorylation in ischemic myocardium

G-CSF augmented Akt phosphrylation in the LAD-perfused myocardium. The increase in Akt phosphorylation was attenuated by wortmannin (Fig. 5).

Discussion

The present study demonstrated that administration of G-CSF following the onset of reperfusion limited infarct size in acute phase and reduced the incidence of lethal arrhythmia. The intracoronary administration of wortmannin abrogated these cardioprotective effects of G-CSF, suggesting that G-CSF mediated cardioprotection via the PI3K/Akt pathway. To our knowledge, this is the first study to reveal the acute effect of G-CSF against ischemia/reperfusion injury via the PI3K/Akt pathway in in vivo canine hearts.

Previous studies have reported that G-CSF improves cardiac remodeling after MI in the chronic ligation model of coronary artery [4, 5, 16]. It has been believed that G-CSF exerts cardioprotective effects through regeneration of myocardium and angiogenesis. Recently, Komuro and colleagues clearly demonstrated that the high dose of G-CSF limits infarct size in the acute phase in the isolated hearts [6]. To translate their remarkable findings into the clinical setting, we need to consider the dose of G-CSF and experimental models in their study. They used a perfusate containing 300 ng/ml G-CSF in the isolated heart model. This dose is relatively high compared with the dose used in clinical settings [17, 18]. In addition, effects of G-CSF on neutrophil function cannot be tested in the isolated heart model. In the present study, we demonstrated that a clinical relevant dose of G-CSF acutely limits infarct size in the in vivo model. In contrast with previous studies [4, 5, 16], we examined the effects of G-CSF in the ischemia/reperfusion model, because coronary revascularization is principally applied for patients with acute MI to attenuate ischemia/reperfusion injury. We found that G-CSF following the onset of reperfusion effectively limited infarct size. Our findings strongly support that G-CSF would be a promising candidate as an adjunctive therapy for patients with acute MI. Indeed, two recent publications by the FIRSTLINE-AMI trial clearly demonstrated that subcutaneous administration of G-CSF after percutaneous coronary intervention improved cardiac function and prevented cardiac remodeling [19, 20]. Considering our present data, the improvement of cardiac function by G-CSF in clinical studies will be due to limiting infarct size in the acute phase as well as preventing cardiac remodeling.

G-CSF can provoke multiple intracellular signal transductions including Jak/Stat, ERK and PI3K/Akt [16, 21]. Recently, we and others demonstrated that post-interventions which activate PI3K/Akt during the reperfusion protect against ischemia/reprfusion injury [10, 22]. Thus, we investigated a role of PI3K/Akt in G-CSF-mediated cardioprotection. WTMN significantly blunted the infarct size-limiting effects of G-CSF, and G-CSF enhanced Akt phosphorylation in the ischemic myocardium, indicating that G-CSF reduces infarct size via PI3K/Akt-dependent pathway. Further investigations will be needed to clarify the molecular target of PI3K/Akt and the role of other signals activated by G-CSF in this condition.

Although we demonstrated that G-CSF mediated cardioprotection, one small clinical study showed that G-CSF may induce coronary re-stenosis [23]. In contrast, other large-scale studies did not show that G-CSF induced coronary restenosis [19, 20]. Since there is still controversy about the restenosis effects of G-

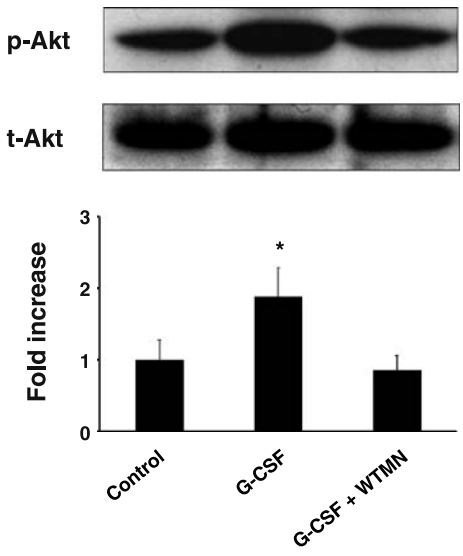

Fig. 5 Akt phosphorylation in LAD-perfused areas. G-CSF phosphorylated Akt in LAD-perfused myocardium. Akt phosphorylation by G-CSF was prevented by co-treatment with WTMN. Akt phosphorylation was normalized by total Akt. *$p < 0.05$ vs. control group.

CSF, this issue will be minimized by the concomitant use of a drug-eluting stent and G-CSF. Another possible adverse effect of G-CSF will be enhancement of neutrophil function. G-CSF appears not only to stimulate the formation of granulocyte colonies from bone marrow-derived precursors, but also to enhance the function of mature neutrophils [24] and elevates the number of white blood cells, which may predict adverse prognosis in the patients of acute MI [25]. Consistent with previous studies [26, 27], we also showed that G-CSF did not change MPO activity, a marker of neutrophil accumulation, in the infarcted myocardium. These findings suggest that G-CSF exerted cardioprotective effects independent of white blood cells. Although our findings suggest that the overall effect of G-CSF may be beneficial for ischemia/reperfused myocardium, we need to be cautious about these potential adverse effects of G-CSF.

Importantly, we clearly demonstrated that G-CSF reduced the incidence of VF during reperfusion via the PI3/Akt-dependent pathway. Since lethal arrhythmias are one of the major causes of death in patients with acute MI [8], the anti-arrhythmic effects of G-CSF have great clinical impact. We have previously demonstrated that another cytokine, erythropoietin, also reduced the incidence of lethal arrhythmia via the PI3/Akt pathway [10]. Although our findings suggest that the PI3K/Akt-dependent pathway will play an important role in the generation of lethal arrhythmias, further investigation will be needed to clarify the potential mechanism by which G-CSF exerts anti-arrhythmic effects. We need to consider whether G-CSF exerts anti-arrhythmic effects by the reduction of myocardial infarct size or by some other actions of G-CSF.

In conclusion, the intravenous administration of a clinically relevant dose of G-CSF will be a promising strategy to treat patients with acute MI. Further controlled studies will be warranted to check the safety and efficacy of G-CSF treatment in the acute phase after MI.

Acknowledgments We thank Yuko Okuda, and Yoko Nagamachi for their technical assistance.

References

1. Clark SC, Kamen R. The human hematopoietic colony-stimulating factors. Science 1987;236:1229–37.
2. To LB, Haylock DN, Simmons PJ, Juttner CA. The biology and clinical uses of blood stem cells. Blood 1997;89:2233–58.
3. Kronenwett R, Martin S, Haas R. The role of cytokines and adhesion molecules for mobilization of peripheral blood stem cells. Stem Cells 2000;18:320–30.
4. Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 2001;98:10344–9.
5. Ohtsuka M, Takano H, Zou Y, et al. Cytokine therapy prevents left ventricular remodeling and dysfunction after myocardial infarction through neovascularization. Faseb J 2004;18:851–3.
6. Harada M, Qin Y, Takano H, et al. G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. Nat Med 2005;11:305–11.
7. Nicod P, Gilpin E, Dittrich H, et al. Late clinical outcome in patients with early ventricular fibrillation after myocardial infarction. J Am Coll Cardiol 1988;11:464–70.
8. Rouleau JL, Talajic M, Sussex B, et al. Myocardial infarction patients in the 1990s—their risk factors, stratification and survival in Canada: the Canadian Assessment of Myocardial Infarction (CAMI) Study. J Am Coll Cardiol 1996;27:1119–27.
9. Lothrop CD Jr, Warren DJ, Souza LM, Jones JB, Moore MA. Correction of canine cyclic hematopoiesis with recombinant human granulocyte colony-stimulating factor. Blood 1988;72:1324–8.
10. Hirata A, Minamino T, Asanuma H, et al. Erythropoietin just before reperfusion reduces both lethal arrhythmias and infarct size via the phosphatidylinositol-3 kinase-dependent pathway in canine hearts. Cardiovasc Drugs Ther 2005;19:33–40.
11. Maruyama R, Takemura G, Aoyama T, et al. Dynamic process of apoptosis in adult rat cardiomyocytes analyzed using 48-hour videomicroscopy and electron microscopy: beating and rate are associated with the apoptotic process. Am J Pathol 2001;159:683–91.
12. Asanuma H, Kitakaze M, Funaya H, et al. Nifedipine limits infarct size via NO-dependent mechanisms in dogs. Basic Res Cardiol 2001;96:497–505.
13. Holm S. A simple sequentially rejective multiple test procedure. Scand J Statist 1979;6:65–70.
14. Hale SL, Lange R, Alker KJ, Kloner RA. Correlates of reperfusion ventricular fibrillation in dogs. Am J Cardiol 1984;53:1397–400.
15. Bolli R, Patel B. Factors that determine the occurrence of reperfusion arrhythmias. Am Heart J 1988;115:20–9.
16. Iwanaga K, Takano H, Ohtsuka M, et al. Effects of G-CSF on cardiac remodeling after acute myocardial infarction in swine. Biochem Biophys Res Commun 2004;325:1353–9.
17. Gabrilove JL, Jakubowski A, Fain K, et al. Phase I study of granulocyte colony-stimulating factor in patients with transitional cell carcinoma of the urothelium. J Clin Invest 1988;82:1454–61.
18. Bensinger WI, Clift RA, Anasetti C, et al. Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony stimulating factor. Stem Cells 1996;14:90–105.
19. Ince H, Petzsch M, Kleine HD, et al. Prevention of left ventricular remodeling with granulocyte colony-stimulating factor after acute myocardial infarction: final 1-year results of the Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony-Stimulating Factor (FIRSTLINE-AMI) Trial. Circulation 2005;112:I73–80.
20. Ince H, Petzsch M, Kleine HD, et al. Preservation from left ventricular remodeling by front-integrated revascularization and stem cell liberation in evolving acute myocardial infarction by use of granulocyte-colony-stimulating factor (FIRSTLINE-AMI). Circulation 2005;112:3097–106.
21. Avalos BR. Molecular analysis of the granulocyte colony-stimulating factor receptor. Blood 1996;88:761–77.

22. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Post-conditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 2004;95:230–2.
23. Kang HJ, Kim HS, Zhang SY, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 2004;363:751–6.
24. Weisbart RH, Golde DW, Clark SC, Wong GG, Gasson JC. Human granulocyte-macrophage colony-stimulating factor is a neutrophil activator. Nature 1985;314:361–3.
25. Barron HV, Cannon CP, Murphy SA, Braunwald E, Gibson CM. Association between white blood cell count, epicardial blood flow, myocardial perfusion, and clinical outcomes in the setting of acute myocardial infarction: a thrombolysis in myocardial infarction 10 substudy. Circulation 2000;102: 2329–34.
26. Adachi Y, Imagawa J, Suzuki Y, et al. G-CSF treatment increases side population cell infiltration after myocardial infarction in mice. J Mol Cell Cardiol 2004;36: 707–10.
27. Sugano Y, Anzai T, Yoshikawa T, et al. Granulocyte colony-stimulating factor attenuates early ventricular expansion after experimental myocardial infarction. Cardiovasc Res 2005;65:446–56.