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G-CSF prevents the progression of atherosclerosis and neointimal formation in rabbits

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Abstract

Granulocyte colony-stimulating factor (G-CSF) prevents left ventricular remodeling after myocardial infarction, but its effect on atherosclerosis is unknown. We examined two kinds of rabbit atherosclerosis models. Myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHL-MI) rabbits were treated with G-CSF or saline for 7 days from 14 months old. The vascular injury models were created by inflating angioplasty balloon in the iliac artery of rabbits and were divided into G-CSF and saline group. G-CSF significantly reduced the stenosis score of coronary artery and lipid plaque area of thoracic aorta in WHHL-MI rabbits at 4 weeks after the treatment. In the vascular injury model, G-CSF significantly prevented an increase in neointima/media ratio at 4 weeks after the treatment. G-CSF accelerated the reendothelialization of denuded arteries, and the pretreatment with nitric oxide synthase inhibitor significantly inhibited it. These results suggest that G-CSF has a therapeutic potential for the progression of atherosclerosis.
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Granulocyte colony-stimulating factor (G-CSF) is a member of a group of glycoproteins called hematopoietic cytokines. G-CSF induces the release of hematopoietic stem cells and endothelial progenitor cells (EPCs) from bone marrow into the peripheral blood circulation. Moreover, it has been reported that G-CSF also has anti-inflammatory and anti-apoptotic effects [1]. Orlic et al. [2] have reported that pretreatment with G-CSF and stem cell factor improves cardiac dysfunction after AMI by accelerating regeneration of cardiac myocytes and vessels through mobilization of bone marrow stem cells. We and others have recently reported that G-CSF prevents left ventricular (LV) remodeling and dysfunction after acute myocardial infarction (AMI) [3–6] and chronic myocardial ischemia [7] in animal models [8]. We have demonstrated that G-CSF directly activates the Jak2/STAT3 pathway in cardiomyocytes, and has anti-apoptotic effects and angiogenic effects on post-MI hearts [6]. However, Kang et al. [9] reported that G-CSF treatment (10 μg/kg for 4 days before PCI) increased the rate of in-stent restenosis at the culprit lesion in patients with AMI or old MI who underwent elective PCI. Although the size of their clinical study was very small and the mechanism by which G-CSF accelerated restenosis was unclear, the result attracted much attention in the use of G-CSF for the patients with atherosclerosis.

There had been no suitable animal models that mimic spontaneous human coronary artery diseases. Although most MI animal models are produced by ligation of coronary artery, there is no atherosclerotic lesion in these models. In 1980, Watanabe et al. developed Watanabe heritable hyperlipidemic (WHHL) rabbits as a model for human familial hypercholeste rolemia and atherosclerosis [10–12]. Shiomi et al. [13] have developed a new WHHL strain with spontaneous MI by selective breeding of myocardial

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infarction-prone WHHL rabbit strain and named it as the WHHL-MI rabbit. This strain has high serum levels of low-density lipoprotein (LDL)-cholesterol and shows typical coronary atheromatous plaques similar to those of human. The WHHL-MI rabbit shows the high incidence of fatal MI at ages 11–35 months [13].

Many patients with AMI are currently treated with PCI and restenosis by intimal hyperplasia is the biggest problem at present [14–16]. To use G-CSF for preventing LV remodeling after AMI, it is critical to know whether G-CSF modulates intimal hyperplasia after PCI. In the present study, we investigated the effects of G-CSF on the development of atherosclerosis using WHHL-MI rabbits and on intimal formation using the balloon injury model of rabbits.

Materials and methods

Animals. To examine the effects of G-CSF on atherosclerosis, we used two rabbit models, WHHL-MI and balloon injury model. Female WHHL-MI rabbits were bred in Institute for Experimental Animals, Kobe University School of Medicine, Kobe, Japan. We fed 1% cholesterol diet to Japanese white rabbit (purchased from Takasugi laboratory animal) for balloon injury model. All protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

G-CSF treatment and analysis in WHHL-MI rabbits. Female WHHL-MI rabbits weighing 3.0–3.5 kg were randomized into two groups. (1) G-CSF group ($n = 7$); injected subcutaneously with recombinant human G-CSF (rhG-CSF, 100 μg/kg/day, Kirin Brewery Co., Ltd., Tokyo, Japan), (2) control group ($n = 7$); injected subcutaneously with saline as the same volume as G-CSF, beginning at 14 months old and continued daily for 7 days because the coronary stenosis of WHHL-MI rabbits was significantly increased during 11–35 months of age [13].

The numbers of circulating white blood cell (WBC), red blood cell (RBC), platelet (Plt), total cholesterol (TC), triglyceride (TG), LDL-cholesterol (LDL-C), activated partial thromboplastin time (APTT), and prothrombin time (PT) were measured before and at 7 days after treatment in both animal models.

At 28 days after G-CSF treatment, WHHL-MI rabbits were sacrificed, and hearts and thoracic aorta were removed and fixed with 10% formaldehyde as reported previously [17]. Coronary atherosclerosis was examined in the right coronary artery (RCA), the left anterior descending artery (LAD), and the left circumflex artery (LCX). The coronary arteries were divided into blocks, and the blocks were embedded in paraffin. The blocks were sectioned at 1 mm intervals and 8-μm-thick cross sections from each paraffin block were cut from each ostium for 10 mm long as described previously [17].

A total of 10 sections from each block were examined. Sections of the hearts were stained with hematoxylin and eosin to analyze stenosis score. The coronary stenosis score was graded as described previously [17]. The degree of atherosclerosis was evaluated by the percent of lesion area in the surface area of intima (the surface area of lesions/the surface area of the whole intima) and by the score of stenosis in the coronary arteries [17]. The coronary stenosis score was graded as follows: no lesion, 0 points; stenosis of 10% or less narrowing, 1 point; stenosis of 10–20% narrowing, 2 points; stenosis of 20–30% narrowing, 3 points; stenosis of 30–40% narrowing, 4 points; stenosis of 40–50% narrowing, 5 points; stenosis of 50–60% narrowing, 6 points; stenosis of 60–70% narrowing, 7 points; stenosis of 70–80% narrowing, 8 points; stenosis of 80–90% narrowing, 9 points; stenosis over 90% narrowing, 10 points. These points were summed for each section, and the total points for each rabbit were also counted. To examine the cellular composition of atherosclerotic lesion, the sections were stained immunohistochemically with monoclonal antibodies against smooth muscle α-actin (1A4, Dako Cytomation) and rabbit macrophages (RAM11, Dako Cytomation), reacted with avidin–conjugated peroxidase (Dako Cytomation), and visualized with 3,3′-diaminobenzidine (Dako Cytomation). Numbers of smooth muscle cells and macrophages were counted by light microscopy and quantitatively estimated with a color image analyzer (Scion Image 1.62). Lesional components were quantitatively evaluated with the color image analyzer at a magnification of 400×, and the lesion area and the area at each lesional component were measured as described previously [11,18,19]. The descending aorta of WHHL-MI rabbit was stained with Sudan IV to calculate the atherosclerotic lesions [20]. The immunohistochemical analyses were performed in a blind fashion by two researchers.

G-CSF treatment and analysis in balloon injury model rabbits. Male Japanese white rabbits weighing 2.5–3.0 kg were used to create the iliac artery balloon injury model [15]. Rabbits were fed with 1% cholesterol diet from 2 weeks before the balloon injury to the time of sacrifice. Before angioplasty, the right common carotid artery was cannulated with 6-F sheath, and 0.014-in. guidewire was passed down the descending aorta into the common iliac artery. The bifurcation of the aorta into the common iliac arteries was used as a landmark. Noncompliant balloon angioplasty catheter (3.0/20 mm) was advanced over the guidewire into the proximal common iliac artery under fluoroscopic guidance. Each animal received 500 U of heparin intravenously before balloon inflation. The balloon injury was achieved by three inflations of each of 30-s duration to obtain a maximal balloon to artery ratio of 1.5–1. The balloon was deflated for 30 s between inflations. Rabbits were injected subcutaneously with G-CSF (G-CSF group, 100 μg/kg/day) or saline (control group) from next day of the operation for 7 days. After 1 week (each group, $n = 5$) and 4 weeks (each group, $n = 10$), rabbits were sacrificed and iliac arteries were perfused and fixed with 10% formaldehyde removed and cut into 8 μm-thickness cross sections as reported previously [15]. Sections of the artery were stained with hematoxylin and eosin to analyze the area of neointima and media. To elucidate whether G-CSF accelerates reendothelialization through mobilization of EPCs, we pretreated rabbits with a nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 15 mg/kg/day), from 3 days before balloon injury to 1 week (each group, $n = 5$). To examine the cellular composition of atherosclerotic lesion, the sections were stained immunohistochemically with monoclonal antibodies against rabbit macrophage (RAM11, Dako Cytomation), CD31 (JC70A, Dako Cytomation), and neutrophil (MCA805, Serotech), reacted with avidin-conjugated peroxidase (Dako Cytomation), and visualized with 3,3′-diaminobenzidine (Dako Cytomation). The ratio of reendothelialization was calculated as the ratio of the surface covered by CD31-positive cells to the total luminal surface.

Statistical analysis. Numerical values are presented as means ± SEM. Comparisons of parameters between two groups were made with unpaired Student's t test. A probability value of $P < 0.05$ was considered to be statistically significant.

Results and discussion

Effects of G-CSF on plasma lipid content

Throughout this study, there was no AMI or death in both groups of WHHL-MI rabbits. In the WHHL-MI rabbit model, there were no significant differences in serum levels of TC, LDL-C, and TG between control group and G-CSF group (Table 1). There were also no significant differences in APTT and PT between control group and G-CSF group in both models. Although the numbers of RBC and Plt were not different, the number of WBC at 7 days after treatment was significantly higher in G-CSF group than control group (G-CSF group, $41{,}600 \pm 8450/mm^3$ vs control group, $10{,}300 \pm 1240/mm^3$, $P < 0.05$) (Table 1). The degree of increase in

Table 1
Effects of G-CSF on complete blood count, coagulation time, and biochemical parameters in WHHL-MI rabbits

	WBC (/mm^3)	RBC ($\times 10^4$/mm^3)	Plt ($\times 10^4$/mm^3)	APTT (s)	PT (s)	TC (mg/dl)	LDL-C (mg/dl)	TG (mg/dl)
Control ($n = 7$)								
Pretreatment	8300 ± 240	658 ± 106	41.5 ± 12.6	62.3 ± 24.2	9.6 ± 2.6	509 ± 102	423 ± 102	46 ± 12
1 week	10,300 ± 1240	634 ± 133	48.6 ± 18.6	72.5 ± 25.6	10.1 ± 1.9	530 ± 87	445 ± 77	53 ± 9
4 weeks	9200 ± 580	632 ± 133	52.3 ± 19.5	89.3 ± 36.3	8.9 ± 3.2	527 ± 76	420 ± 80	49 ± 11
G-CSF ($n = 7$)								
Pretreatment	8300 ± 240	658 ± 113	52.3 ± 19.8	89.6 ± 25.2	10.2 ± 2.1	598 ± 133	417 ± 144	50 ± 15
1 week	41,600 ± 8450[a]	685 ± 103	49.5 ± 24.2	104.2 ± 56.3	8.5 ± 2.7	579 ± 116	432 ± 117	47 ± 14
4 weeks	9400 ± 680	690 ± 156	56.6 ± 28.5	78.5 ± 45.3	8.1 ± 2.3	521 ± 266	450 ± 96	62 ± 13

Results are expressed as means ± SEM.
[a] $P < 0.05$ vs control group.

Table 2
Effects of G-CSF on complete blood count, coagulation time, and biochemical parameters in balloon injury model

	WBC (/mm^3)	RBC ($\times 10^4$/mm^3)	Plt ($\times 10^4$/mm^3)	APTT (s)	PT (s)	TC (mg/dl)	LDL-C (mg/dl)	TG (mg/dl)
Control ($n = 10$)								
Preoperation	11,300 ± 1700	623 ± 103	51.0 ± 19.5	55.6 ± 35.3	12.6 ± 10.8	951 ± 467	392 ± 164	40 ± 59
1 week	26,800 ± 9400[a]	538 ± 126	62.2 ± 20.5	74.0 ± 33.7	8.1 ± 1.9	1699 ± 587	637 ± 147	74 ± 33
4 weeks	14,400 ± 3600	609 ± 98	53.5 ± 11.8	140.0 ± 56.8	9.0 ± 2.7	2435 ± 990	735 ± 279	111 ± 99
G-CSF ($n = 10$)								
Preoperation	8900 ± 1600	598 ± 98	60.2 ± 21.1	131.2 ± 35.3	9.0 ± 1.1	1193 ± 420	385 ± 100	28 ± 4
1 week	66,800 ± 23,600[a,b]	485 ± 83	36.3 ± 20.7	104.7 ± 30.3	7.5 ± 1.6	1951 ± 315	715 ± 384	104 ± 41
4 weeks	10,100 ± 200	586 ± 93	47.9 ± 18.5	61.3 ± 36.3	8.1 ± 0.1	2275 ± 234	593 ± 110	184 ± 86

Results are expressed as means ± SEM.
[a] $P < 0.05$ vs preoperation.
[b] $P < 0.05$ vs control group.

WBC in this model was similar to that in human treated with 10 μg/kg/day of G-CSF.

In balloon injury model, there were no significant differences in serum levels of TC, LDL-C, and TG between control group and G-CSF group (Table 2). There were also no significant differences in APTT and PT between control group and G-CSF group in both models. The numbers of RBC and Plt were not different between two groups. The number of WBC was increased at 7 days after treatment in control group (1 week, 26,800 ± 9400/mm^3 vs preoperation, 11,300 ± 1700/mm^3, $P < 0.05$) and in G-CSF group (1 week, 66,800 ± 23,600/mm^3 vs preoperation, 8900 ± 1600/mm^3, $P < 0.05$) (Table 2).

Effects of G-CSF on coronary atherosclerotic lesions in WHHL-MI rabbits

To investigate the effect of G-CSF on the progression of the atherosclerosis of coronary artery in WHHL-MI rabbit, we examined the coronary artery and the thoracic aorta. Fig. 1 shows the coronary stenosis in G-CSF group and control group of WHHL-MI rabbits. There was a marked difference in the stenosis score of all coronary arteries including RCA, LAD, and LCX of WHHL-MI rabbits between the G-CSF group and control group (Figs. 1A and B). The coronary stenosis score of the G-CSF group was much lower (8.9 ± 7.0) than that of control group (22.6 ± 8.0) in LCX (Fig. 1B). Since the stenosis of LCX was more prominent among three coronary arteries in this model [18], we examined the composition of smooth muscle cells, macrophages, extracellular lipid deposits and collagen in the coronary atherosclerotic lesion of the LCX in the presence or absence of G-CSF treatment. There was no significant difference in the percent area of each composition in the plaque between G-CSF group and control group (G-CSF group: smooth muscle cells, 40.4 ± 11.2%; macrophages, 11.4 ± 8.9%; extracellular lipid deposits, 4.4 ± 4.7%; collagen, 39.2 ± 10.9%, control group: smooth muscle cells, 45.3 ± 14.4%; macrophages, 13.5 ± 9.3%; extracellular lipid deposits, 6.5 ± 4.7%; collagen, 38.3 ± 2.2%) (Fig. 1C). The average of the most severe stenosis of G-CSF group was significantly low (48.5 ± 28.5%) compared with that of control group (72.7 ± 24.5%) in LCX. The area of lipid plaque detected by Sudan IV staining of thoracic aorta was significantly smaller in G-CSF group than control group (G-CSF group, 34.4 ± 18.0% vs control group, 49.6 ± 22.2%, $P < 0.05$) (Figs. 2A and B).

It has been reported that intraplaque neovascularization facilitates the progression and rupture of plaque [21]. Although we previously reported that G-CSF prevents LV remodeling and dysfunction after AMI through neovascularization in the ischemic region, G-CSF did not induce neovascularization in atherosclerotic plaque of WHHL-MI rabbits (data not shown). The treatment with G-CSF inhibited the progression of atherosclerosis in coronary artery and aorta at 4 weeks after the treatment. The role of G-CSF on

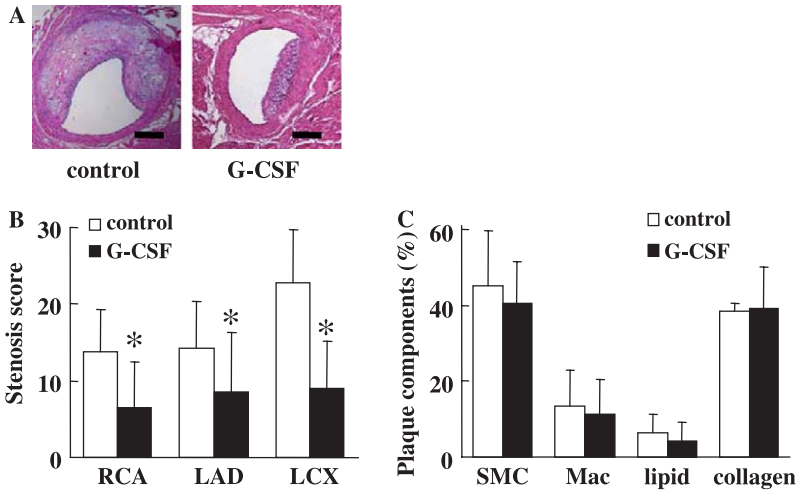

Fig. 1. Effects of G-CSF on the coronary atherosclerosis of WHHL-MI rabbits. The representative atherosclerotic lesion in LCX (A), coronary stenosis score (B), and plaque components (C) in WHHL-MI rabbits. $n = 7$ in each group. RCA, right coronary artery; LAD, left anterior descending artery; LCX, left circumflex artery; SMC, smooth muscle cells; Mac, macrophages. Results are expressed as means ± SEM. Scale bars indicate 200 μm. $^{*}P < 0.05$ vs control.

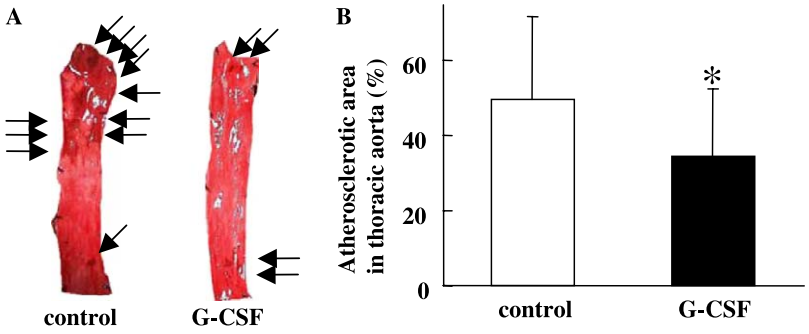

Fig. 2. Effects of G-CSF on the atherosclerosis of aorta of WHHL-MI rabbits. The representative atherosclerotic lesion of aorta (A) and the ratio of atherosclerotic area to thoracic aorta area in WHHL-MI rabbits (B). Arrows indicate atherosclerotic legions. Results are expressed as means ± SEM. $n = 7$ in each group. $^{*}P < 0.05$ vs control.

coronary atherosclerosis is thought to be the prevention of the progression of atherosclerosis but not the regression since age-dependent atherosclerotic change would be very progressive during 14–15 months old (coronary stenosis score at 14 months old: RCA, 6.8 ± 6.8; LAD, 7.0 ± 6.7; LCX, 7.8 ± 5.4; $n = 4$). Since the persistent inflammatory response has been reported to be involved in the progression of atherosclerosis [22–24], the anti-inflammatory effect [1,25] of G-CSF may block the vicious circle of the progression of atherosclerosis in those models. There was no significant difference in the percent area of smooth muscle cells, macrophages, extracellular lipid deposits, and collagen in the plaque between G-CSF group and control group. It remains to be determined how G-CSF prevents the progression of atherosclerosis in WHHL-MI rabbits.

Effects of G-CSF on neointimal formation after vascular injury and reendothelialization in balloon injured rabbit artery

The restenosis after balloon angioplasty is mainly caused by neointimal hyperplasia. In our balloon injury model, neointima volume of injured arteries after 4 weeks was significantly higher than that of contralateral artery (Fig. 3B). The treatment with G-CSF strongly prevented the neointimal formation in injured arteries compared with control group (G-CSF group, 0.56 ± 0.14 mm^2 vs control group, 0.84 ± 0.13 mm^2, $P < 0.05$) (Figs. 3A and B). The neointima/media ratio was significantly smaller in G-CSF group than control group (G-CSF group, 0.91 ± 0.22 vs control group, 1.24 ± 0.21, $P < 0.05$) (Fig. 3C).

The treatment with G-CSF did not influence the infiltration of macrophages in the plaque area of balloon injury (1 week; G-CSF group, 3.8 ± 0.6% vs control group, 3.5 ± 0.5%, 4 weeks; G-CSF group, 5.1 ± 1.1% vs control group, 6.7 ± 1.1%) (Figs. 3D and E). The number of infiltrated neutrophils at 1 week was more observed in G-CSF group than control group (G-CSF group, 0.18 ± 0.04% vs control group, 0.01 ± 0.01%, $P < 0.05$), although there was no difference in infiltration of neutrophils in the plaque area between two groups at 4 weeks (Fig. 3F).

The ratio of reendothelialization was significantly accelerated by the treatment with G-CSF at both 1 week and 4 weeks (1 week; G-CSF group, 62.8 ± 2.2% vs control

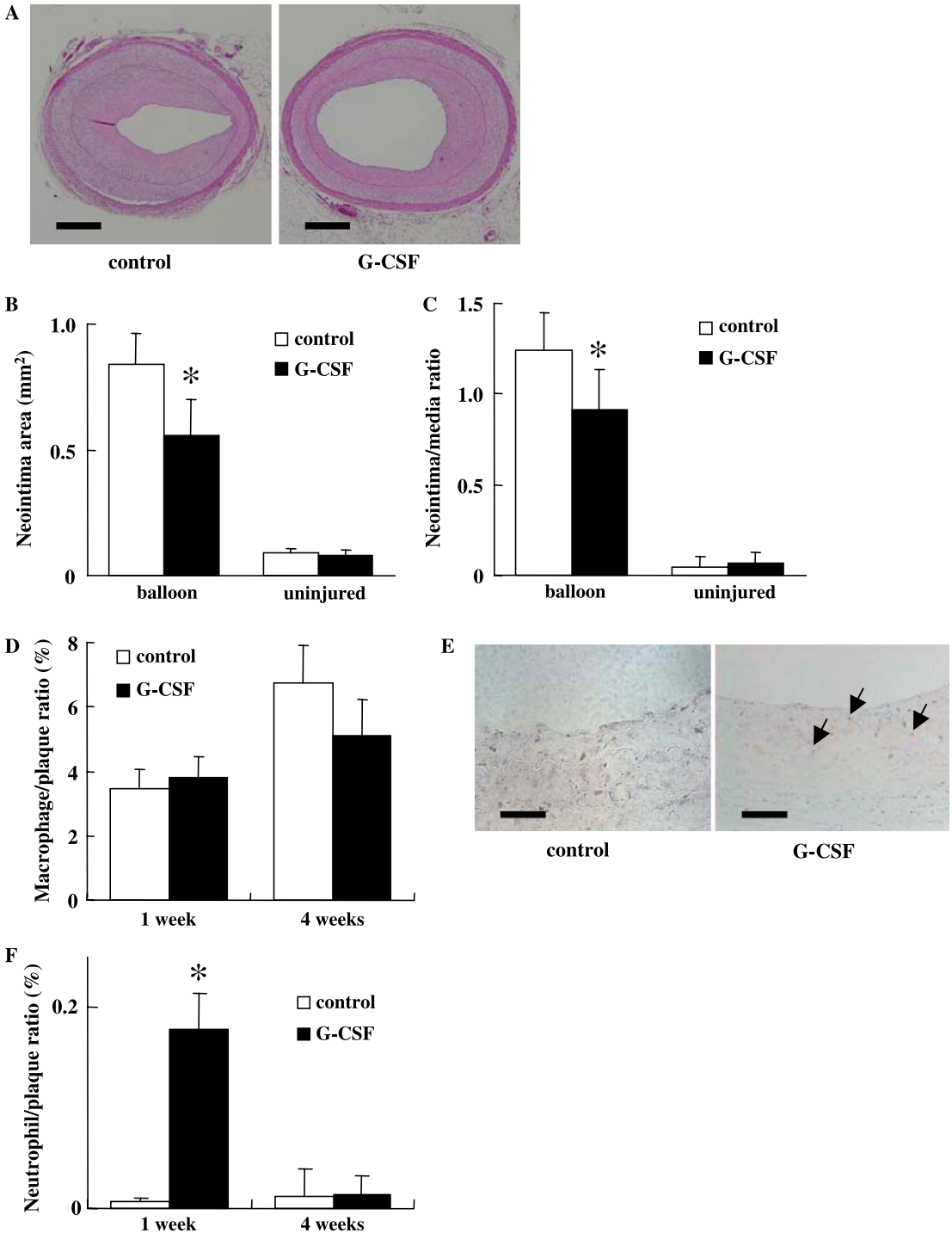

Fig. 3. Effects of G-CSF on the atherosclerosis of balloon injured artery. The representative balloon injury-induced neointimal formation (A), neointima area (B), and neointima/media ratio of rabbit iliac arteries (C) at 4 weeks ($n = 10$ in each group). The percentage of infiltrated macrophages at 1 week ($n = 5$ in each group) and 4 weeks ($n = 10$ in each group) (D) after balloon injury in rabbit iliac arteries. The representative photograph of neutrophils infiltrated in neointima of control rabbit and G-CSF-treated rabbit (E). The percentage of infiltrated neutrophils at 1 week after balloon injury in rabbit iliac artery (F). Arrows indicate the infiltrated neutrophils. Scale bars indicate 50 μm. Results are expressed as means ± SEM. $^{*}P < 0.05$ vs control.

group, 46.7 ± 2.9%, $P < 0.05$, 4 weeks; G-CSF group, 79.6 ± 3.7% vs control group, 58.9 ± 8.5%, $P < 0.05$) (Figs. 4A and B). The pretreatment with L-NAME significantly inhibited G-CSF-induced reendothelialization (G-CSF group, 41.8 ± 9.1% vs control group, 36.5 ± 7.6%) (Fig. 4B).

These results suggest that G-CSF also prevented the progression of the neointimal formation in rabbit vascular injury model. Vascular injury-induced denudation of endothelial cells (ECs) leads to elevated vascular tone, migration and infiltration of smooth muscle cells and activation of cytokine network, resulting in the

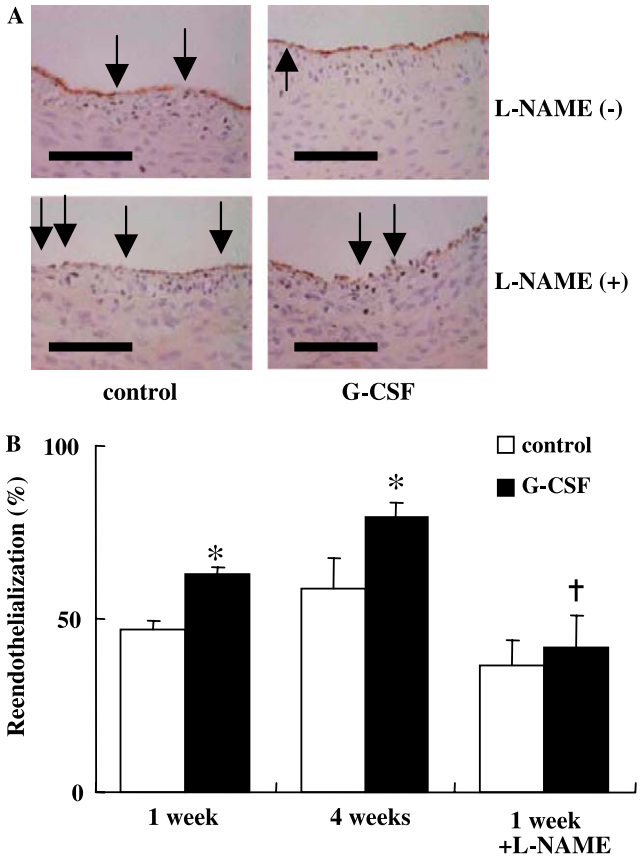

Fig. 4. Effects of G-CSF on reendothelialization. (A) The representative photographs of CD31-positive cells in injured arteries at 1 week after balloon injury in the absence or presence of L-NAME ($n = 5$ in each group). Arrows indicate the absence region of endothelium. Scale bars indicate 100 μm. (B) The percentage of CD31-positive cell coverage in injured arteries at 1 and 4 weeks after balloon injury. Results are expressed as means ± SEM. $^{*}P < 0.05$ vs control. $^{\dagger}P < 0.05$ vs G-CSF group at 1 week without pretreatment with L-NAME.

neointimal formation [15]. EPCs have been recently reported to enhance reendothelialization and reduce neointimal formation after carotid artery injury in mice and rabbits [26,27]. G-CSF treatment has been reported to increase the number of circulating EPCs [28]. Since nitric oxide plays a critical role in the mobilization of EPCs from bone marrow [29,30], we examined using a NOS inhibitor, L-NAME, whether the mobilization of EPCs is involved in the G-CSF-induced reendothelialization. The pretreatment with L-NAME significantly inhibited the G-CSF-induced reendothelialization in balloon injured artery, suggesting that the recruitment and mobilization of EPCs from bone marrow are involved in the beneficial effects of G-CSF on balloon-injured stenosis. The other mechanisms including anti-inflammatory effect, anti-apoptotic effect, and modification of extracellular matrix may be also involved in the effects of G-CSF. Recent clinical studies have reported that G-CSF therapy does not induce the progression of atherosclerosis in the patients with coronary artery disease [31–35].

Concluding remarks

In the present study, we demonstrated using two kinds of rabbit models of atherosclerosis that the treatment with G-CSF ameliorates the progression of atherosclerosis. The treatment with G-CSF inhibited the progression of atherosclerosis and neointimal formation. These results suggest that G-CSF has a therapeutic potential for the progression of atherosclerosis. Randomized clinical trials to evaluate the feasibility and safety of G-CSF on coronary artery diseases are warranted.

Acknowledgments

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