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ORIGINAL ARTICLE

Post-MI treatment with G-CSF and EPO-liposome with SLX repairs infarcted myocardium through EPCs mobilization and activation of prosurvival signals in rabbits

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Abstract

We investigated whether combination therapy of G-CSF and erythropoietin (EPO)-liposome with Siaryl Lewis X (SLX) is more cardioprotective than G-CSF or EPO-liposome with SLX alone. For the purpose of generating myocardial infarction (MI), rabbits underwent 30 minutes of coronary occlusion and 14 days of reperfusion. We administered saline (control group, i.v.,), G-CSF (G group, 10 μg/kg/day × 5 days, i.c., starting at 24 hours after reperfusion), EPO-liposome with SLX (LE group, i.v., 2500 IU/kg EPO containing liposome with SLX, immediately after reperfusion), and G-CSF + EPO-liposome with SLX (LE + G group) to the rabbits. The MI size was the smallest in the LE+G group (14.7 ± 0.8%), and smaller in the G group (22.4 ± 1.5%) and LE group (18.5 ± 1.1%) than in the control group (27.8 ± 1.5%). Compared with the control group, the cardiac function and remodeling of the G, LE, and LE + G groups were improved, and LE + G group tended to show the best improvement. The number of CD31-positive microvessels was the greatest in the LE + G group, greater in the G and LE groups than in the control group. Higher expressions of phosphorylated (p)-Akt and p-ERK were observed in the ischemic area of the LE and LE + G groups. The number of $CD34^+/CXCR4^+$ cells was significantly higher in the G and LE + G groups. The cardiac SDF-1 was more expressed in the G and LE + G groups. In conclusion, Post-MI combination therapy with G-CSF and EPO-liposome with SLX is more cardioprotective than G-CSF or EPO-liposome with SLX alone through EPCs mobilization, neovascularization, and activation of prosurvival signals.

KEYWORDS
EPC, erythropoietin, G-CSF, infarct size, liposome, signal transduction

Abbreviations: BM, bone marrow; CXCR4, C-X-C Chemokine receptor 4; −dP/dt, peak rate of fall in pressure; dP/dt, peak rate of pressure rise; EDd, end diastolic dimension; EF, ejection fraction; EPCs, endothelial progenitor cells; EPO, erythropoietin; ERK, extracellular signal-regulated protein kinase; ESd, end systolic dimension; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; FS, fractional shortening; G-CSF, granulocyte colony stimulating factor; LV, left ventricular; LVEDd, left ventricular end diastolic dimension; LVEDs left ventricular end sistolic dimension; MI, myocardial infarction; MMP-1, matrix metalloproteinase-1; p-Akt, phosphorylated Akt; PBS, phosphate buffered saline; PE, phycoerythrin; p-ERK, phosphorylated ERK; PM, particulate matter; SDF-1, stromal cell-derived factor 1; SLX, Siaryl Lewis X; Stat3, signal transducers and activator of transcription 3; TTC, triphenyl tetrazolium chloride; VEGF, vascular endothelial growth factor.

1 | INTRODUCTION

It has been reported that G-CSF mobilized multipotent progenitor cells from bone marrow into the peripheral blood, and contributed the reduction of the myocardial infarction (MI) size and the improvement of left ventricular (LV) function and remodeling.[1-4] At present, proposed mechanisms by which G-CSF causes cardioprotection are considered to be transdifferentiation of bone marrow (BM) progenitor cells into cardiac-lineage and vascular-lineage cells such as cardiomyocytes, vascular endothelial cells, vascular alpha-smooth muscle actin or myofibroblast,[2-4] the acceleration of healing process,[3] and the prevention of apoptotic cardiomyocytes.[1] On the other hand, it was previously reported that gelatin hydrogel-containing erythropoietin (EPO) patched to the heart reduced the size of MI and improved LV function and remodeling.[5] Additionally, we recently reported that postinfarct intravenous administration of an EPO-encapsulated nano-size liposome with Siaryl-Lewis X (SLX), a cardiotargeting EPO delivery system (a drug delivery system) repairs the infarcted myocardium and reduces the infarct size and improves LV function and remodeling by raising activity of prosurvival signals, antifibrotic and angiogenic effects.[6] Both G-CSF and EPO-encapsulated liposome with SLX were cardioprotective and repaired infarcted myocardium.[3,6] However, on the basis of animal experiments on G-CSF in acute MI, it has been reported that many clinical trials performed in AMI patients.[7-14] Although the safety of using G-CSF for AMI patients has been confirmed in these clinical trials, there have been controversies as to the effects of G-CSF, and therefore G-CSF has not yet become a standard therapy for acute MI. Furthermore, although many clinical trials on EPO in acute myocardial infarction have been performed, EPO had no clinical effect on heart function, reducing infarct size, cardiovascular events, or all-cause mortality in AMI patients.[15] Therefore, we expected that combination of G-CSF and EPO-liposome with SLX may be more cardioprotective than G-CSF alone or EPO-encapsulated liposome with SLX alone. Therefore, we investigated the effect of combination therapy with G-CSF and EPO-liposome with SLX on the MI size, LV function, and remodeling in a rabbit AMI model.

2 | MATERIALS AND METHODS

We anesthetized about 2.0 kg body weight (at 10 weeks of age) male Japanese white rabbits with an intravenous administration of ketamine (10 mg/kg) and zylazine (3 mg/kg), and administrated additional anesthetic when needed. We anesthetized the animals and then endotracheally intubated rabbits and managed breathing with room air supplemented with a low flow of oxygen using a mechanical ventilator (tidal volume 25-35 mL, respiratory rate 20-30/minutes) (Shimano, model SN-480-5, Tokyo, Japan). We performed blood gas analysis consistently, and adjusted the ventilator to keep the arterial oxygen within physiological range. We performed surgical procedures under sterile conditions. We cannulated the carotid artery to monitor peripheral arterial pressure. Then, we injected heparin (500 U/kg) into rabbits intravenously, and perfomed a thoracotomy from the left fourth intercostal space, cut open the pericardium and exposed the heart. We passed a 4-0 silk suture through the myocardium beneath the middle position of the large artery on the anterolateral surface of the LV. Then, we passed the ends of the silk suture through a vinyl tube, and occluded the coronary branch using a snare. In this way, we occluded coronary artery for 30 minutes. We confirmed myocardial infarction by ST-segment elevation on electrocardiogram and cyanosis of the myocardial surface. Then, we confirmed reperfusion by myocardial surface color recovery after releasing the snare. We used meloxicam as postoperative pain countermeasure.

The researchers who evaluated the results were not informed of the protocol and treatment. We randomly assigned the rabbits by sealed envelopes to groups for the experiments.

2.1 | Preparation of EPO-encapsulated liposomes with SLX

EPO-liposomes with SLX were prepared according to the previous method.[6] Briefly, we made the liposomes from a mixture of dipalmitoylphosphatidylcholine, cholesterol, dihexadecylphosphate, ganglioside, dipalmitoylphosphatidylethanolamine, and sodium cholate using the improved cholate dialysis method.[16,17] To purify EPO-encapsulated liposomes, we resuspended the lipid film with 10 mL of EPO (180 000 IU) solution and generated the transparent micelle by sonication. Then, we obtained the EPO-liposomes after ultrafiltration of micelle solution using a PM10 membrane (Millipore, MA, USA). Hydrophilization with Tris and SLX conjugation on the surface of liposomes were carried out as described previously.[18] Finally, 1 mL of EPO-encapsulated liposomes with SLX solution contained 5000 IU EPO. The final concentration of lipid of the liposomes encapsulating EPO was in the range from 2.5 to 3.1 mg/mL. We measured Liposome particle size using Zetasizer Nano-S90 (Malvern, UK) at 25°C and the size of mean particle was about 100 nm in diameter.

2.2 | Protocol

2.2.1 | Animals

Male Japanese white rabbits were 10 weeks old (about 2.0 Kg body weight), and were generated myocardial infarction. We bred rabbits under temperature adjustment (20-28°C) and 12 hours cycle light adjustment. The rabbits were free to drink water and eat meals. The animal house technicians cared for and checked the rabbits daily, as with additional cares and checks by the researcher. The rabbits experimented in the present study received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, by the US National Institutes of Health (NIH publication 85-23, revised 1996). The Ethical Committee of Gifu University Graduate School of Medicine, Gifu, Japan approved this research protocol (permission number: 23-9).

As the control group, we injected saline (1 mL) intravenously after reperfusion. Then as the LE group, we intravenously injected liposomes with SLX encapsulating 2500 IU/kg EPO immediately after reperfusion. As the G group, we subcutaneously injected 10 μg/kg of G-CSF starting at 24 hours after MI once a day for 5 days. And, as the LE + G group, we intravenously injected liposomes with SLX encapsulating 2500 IU/kg EPO immediately after reperfusion and subcutaneously injected 10 μg/kg of G-CSF starting at 24 hours after MI once a day for 5 days. We did not include the group of liposomes with SLX without EPO (liposome with SLX by itself) in this study because we have previously reported that liposome with SLX without EPO did not affect the infarct size in a rabbit AMI model.[6]

Rabbits were sacrificed on day 14 (n = 10 in each group) for the determination of MI size, and for the determination of prosurvival signals and CD31-positive microvessels (n = 7 in each group), and on day 2 for the determination of plasma SDF-1 levels and the expression of SDF-1 in the cardiac tissue (n = 5 in each group).

Pentobarbital sodium (60-70 mg/kg body weight) was intravenously injected at sacrifice of rabbits.

2.3 | Measurement of myocardial infarct size

We used the Evans blue triphenyl tetrazolium chloride (TTC) stain protocol to measure the infarct area in the risk area. At first, we removed hearts and put them on a Langendorff apparatus, and injected Evans blue dye from the aorta after occlusion of the coronary branch. We measured the weights of the whole heart and the left ventricles, and cut the left ventricles into seven transverse slices with short axis cross section. We weighed the slices and incubated them in a 1% solution of TTC for 10 minutes at 37°C to clarify the infarct area and photographed them. We traced the ischemic areas and the infarcted area on LV slices using an image analyzer connected to a light microscope (LUZEX-F, NIRECO, Tokyo) and multiplied by the weight of each slices, and expressed them as a percentage of the ischemic area or the LV. We fixed each slice in 10% buffered formalin for 4 hours and embedded them in paraffin, and cut them into 4-μm-thick sections using a microtome. We stained these sections using Masson trichrome.

2.4 | Physiological studies

We performed echocardiography (SSD2000, Aloka Co. Ltd.) on the day 14 post-MI, and measured EF, FS, and left ventricular end diastolic dimension (LVEDd) and left ventricular end sistolic dimension (LVEDs). We also measured blood pressure and heart rate using a catheter, Mikro-Cath Pressure Catheter (Millar Inc.) introduced into the carotid artery, and inserted it into the left ventricle to record ± dP/dt.

2.5 | FACS analysis

The number of circulating $CD34^+/CXCR4^+$ cells, an endothelial progenitor cell (EPC) in the peripheral blood was measured as $CD34^+$ and $CXCR4^+$ double positive cells on the day 7 after MI in each group. Briefly, a 100 μL aliquot of heparinized whole blood was subjected to incubation with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34 (Bio Rad Laboratories Inc, Hercules, CA) and Phycoerythrin (PE)- conjugated mouse monoclonal anti-human CXCR-4 (R&D System, Inc.) at 4°C for 30 minutes. RBCs were lysed by adding Lysing Solution (Becton Dickinson, Franklin Lakes, NJ). After the cells were washed with phosphate buffered saline (PBS), they were analyzed by $S3^{TM}$ Cell Sorter (488/561 nm) 145-1002 (Bio-Rad Laboratories, Inc.) using S3 ProSort Soft Ware.

2.6 | Plasma SDF-1 level

Plasma SDF-1 concentrations were measured using ELISA method (SDF-1α, Human, ELISA Kit, R&D Systems, Inc.) on the 2 days after MI in each group.

2.7 | Western blot analysis

We obtained cardiac tissue samples from the viable area in the ischemic area. We defined the viable area between infarct area and noninfarct area within the risk area as the "border area". Using lysates from the cardiac tissues on the day 14 after MI, we separated and transferred the proteins to membranes. We assessed the phosphorylation of Akt and extracellular signal-regulated protein kinase (ERK) using antibodies against Akt (Akt 1/2, 1:1000, Santa Cruz Biotechnology, Inc., Dallas, TX), phosphorylated (p)-Akt (Phospho-Akt Mouse mAb, 1:100, Cell Signaling Technology, Inc., Danvers, MA), ERK (p44/42 MAP Kinase Mouse mAb, 1:3000, Cell Signaling Technology, Inc.), p-ERK (Phospho-p44/42 MAP Kinase Mouse mAb, 1:1000, Cell Signaling Technology, Inc.) on the day 7 after MI. The SDF-1 was assessed using SDF-1 antibody (1:250 abcam) using lysates from cardiac tissues on the day 2 after MI. We visualized the blots using chemiluminescence (ECL, Amersham), and quantified the bands using densitometry. We used α-Tubulin (Santa Cruz Biotechnology, Inc.) served as the loading control.

2.8 | Immunohistochemistry

An indirect immunoperoxidase method was used as immunostaining method for LV sections on the day 14 post-MI. We used monoclonal mouse anti-human CD31, an endothelial cell marker (1:100 Dako) as the primary antibodies.

2.9 | Drugs used

G-CSF and erythropoietin (EPO) were purchased from Chugai Pharmaceutical Company.

2.10 | Statistical analysis

We presented all values as means ± SEM. We assessed differences among the control, G, LE, and LE + G groups by ANOVA combined with a Fisher's method (Stat View, J5.0 software). Value of $P < 0.05$

meant statistically significant, and values of $P < 0.01$ and $P < 0.001$ meant highly statistically significant.

3 | RESULTS

3.1 | Mortality and animal exclusion

We initially enrolled 124 rabbits in the four study groups, and finally 108 rabbits were included in this study. The rabbits underwent 30 minutes of coronary occlusion followed by reperfusion. Because of ventricular fibrillation during generating myocardial infarction, five of the rabbits died and were excluded; two (control group), one (LE group), one (G group), and one (LE + G group) (Table 1). During the course after acute MI, 11 rabbits died and were excluded; four (control group), two (LE group), three (G group), and two (LE + G group) (Table 1). There was no significant difference in the inclusion rate among the four groups. In the other AMI rabbits (n = 68) for western blot analysis or immunohistochemical analysis, there was no exclusion due to death

3.2 | MI size and histological analysis

Typical pictures of the Evans blue TTC staining of the LV cutting sections were shown in Figure 1A. No significant difference was observed in the area at risk of LV among the control (28.1 ± 1.1%), G (29.6 ± 0.8%), LE (31.8 ± 2.0%), and LE + G (29.6 ± 1.4) groups (Figure 1B, n = 10). The MI size within the area at risk (as a percentage) was the smallest in the LE + G group (14.7 ± 0.8%), and smaller in the G group (22.4 ± 1.5%) and LE group (18.5 ± 1.1%) compared with the control group (27.8 ± 1.5%) (Figure 1C, n = 10). The MI size within the area at risk (as a percentage) in the LE + G group was significantly smaller than in the G group or in the LE group.

3.3 | Physiological findings

No significant difference was observed in heart rate, systolic or diastolic blood pressures among the four groups on the day 14 post-MI (Figure 2A, n = 10). The LVESd and LVEDd increased, and the LVEF, LVFS, and +dP/dt and −dP/dt decreased in the control group (MI group) (Figure 2B, n = 10) compared with the Sham group. On the other hand, the LVESd and LVEDd were significantly smaller in the G, LE, and LE + G groups compared with the control group, while the LVEF, LVFS, and +dP/dt and −dP/dt were significantly greater in the G, LE, and LE + G groups than in the control group (Figure 2C, n = 10). Although there was no statistically significant difference among the groups, the LE + G group tended to show the best improvement of the LV function and remodeling among the groups.

3.4 | FACS analysis

We confirmed that the number of CD34+/CXCR4+ cells in the peripheral blood was significantly higher in the G group and LE + G group than in the control group or in the LE group 7 days after MI (Figure 3, n = 7).

TABLE 1 Mortality and animal exclusion

	Number	Death during the operation	Death during the course after AMI	Inclusion rate (%)
Control	33	2	4	27/32 (84%)
G	31	1	3	27/31 (87%)
LE	30	1	2	27/30 (90%)
LE + G	30	1	2	27/30 (90%)
Total number	124	5	11	108

One hundred and twenty-four rabbits were initially enrolled in the studies. As shown in the table, five died during the operation and 11 died during the course after MI, and they were excluded from the analyses. Thus, the experiments were completed in the remaining 108 rabbits, and the data from these animals were used for the analysis. There was no significant difference in the inclusion rate among the four groups.

3.5 | Plasma SDF-1 level and the expression of SDF-1 in the cardiac tissue

The plasma SDF-1 concentrations 48 hours after MI were significantly higher in the G group and LE + G group than in the LE group or in the control group (Figure 4A, n = 5). Western blot analysis revealed that the expression of SDF-1 in the border area of the MI was significantly higher in the LE + G group and G group than in the LE group or in the control group 48 hours after MI (Figure 4B, n = 5).

3.6 | Immunohistochemistry

On the day 14 after MI, we observed that the density of the CD31-positive microvessels representing capillary density in the infarct border area was significantly greater in the G, LE and LE + G groups than in the control group, and was greater in the LE + G group than in the G group or in the LE group (Figure 5, n = 7). However, no difference was observed in the densities of CD31-positive microvessels in the noninfarct and infarct areas among the four groups.

3.7 | Prosurvival signals

We observed that the expressions of p-Akt and p-ERK were significantly higher in the LE + G group and LE group than in the G group or in the control group on the day 14 after MI (Figure 6, n = 5).

4 | DISCUSSION

4.1 | Effect of G-CSF

It has previously been reported that post-MI treatment with G-CSF reduces the infarct size and improves LV function and remodeling through the acceleration of the healing process, myocardial tissue regeneration and prevention of cardiac apoptosis.[1-4] And it has been reported that manipulation of SDF-

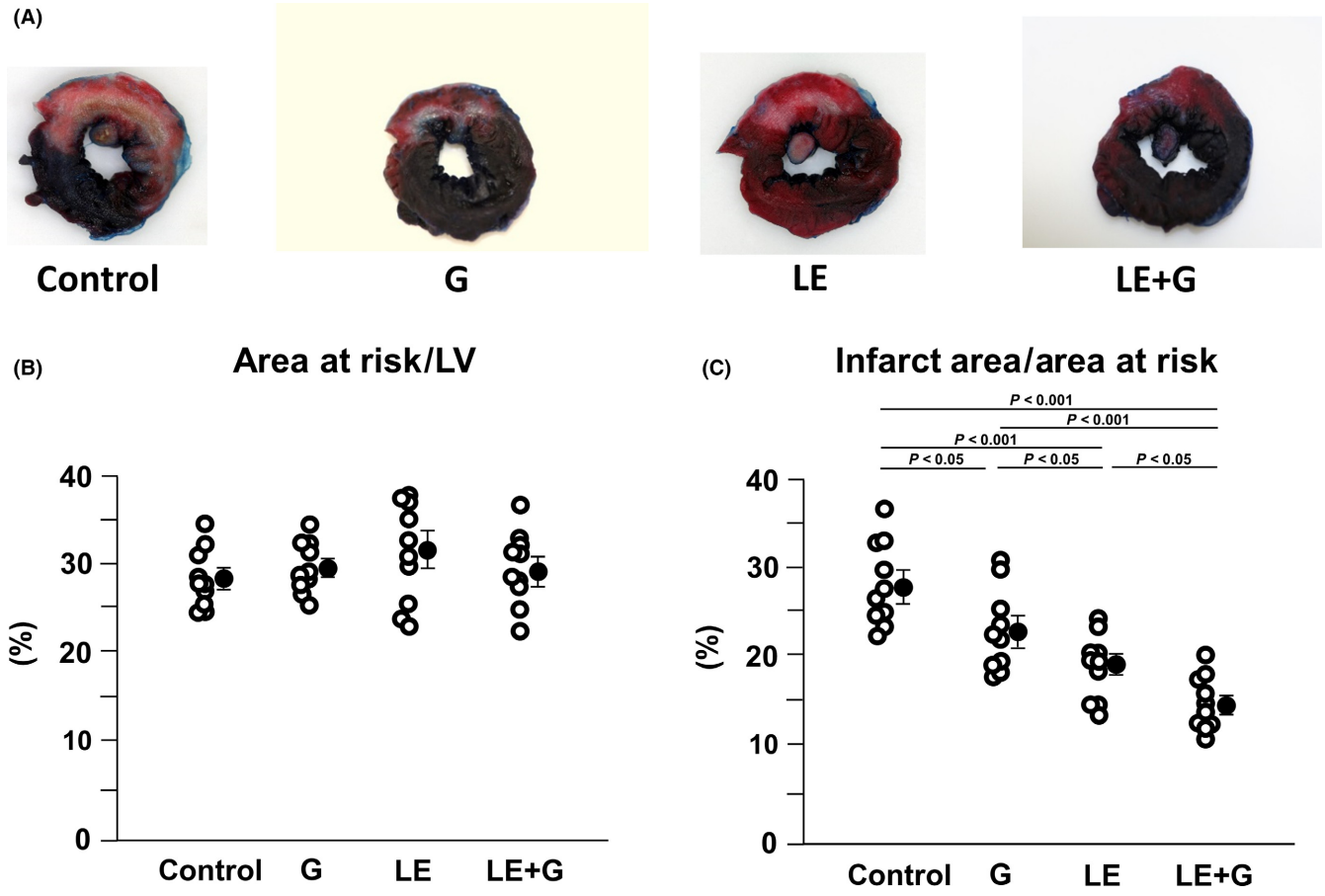

FIGURE 1 The myocardial infarct (MI) size. (A): Typical pictures of the triphenyl tetrazolium chloride (TTC) staining and Evans blue dye staining of cross sections of the left ventricular (LV) (B) The area at risk as a percentage of LV assessed by Evans blue dye staining (n = 10 in each group). (C) The MI size as a percentage of the area at risk assessed by TTC staining (n = 10 in each group)

1-CXCR4 interactions control the navigation of progenitors between the BM and blood to improve the outcome of clinical stem cell transplantation. In addition to these mechanisms, we found that postinfarct treatment with G-CSF increased the number of circulating $CD34^+/CXCR4^+$ cells as shown in the G and LE + G groups, suggesting that G-CSF mobilized $CD34^+/CXCR4^+$ cells, an endothelial progenitor cells (EPCs), into the peripheral blood from the bone marrow. G-CSF also increased the plasma levels of SDF-1 in the G and LE + G groups, suggesting that elevated plasma SDF-1 levels contributed to the mobilization of EPCs into the peripheral blood. As a matter of fact, it has previously been reported that SDF-1 contributes to the recruitment of bone marrow stem cells mobilized by G-CSF into the infarcted tissues.[19] We have previously reported that recruitment of $CXCR4^+$ cells into the infarcted myocardium by activation of the CXCR4/SDF-1 axis strongly affects to reduce the infarct size in the G-CSF-treated rabbits.[20] In the present study, elevated plasma SDF-1 (Figure 4A) and the upregulation of SDF-1 expression (Figure 4B) were observed in the infarct border area in the LE + G group and G group on the day 2 after MI. Therefore, G-CSF might have activated CXCR4/SDF-1 axis and contributed to the homing of $CD34^+/CXCR4^+$ cells into the infarcted border area and to reduce the infarct size in the LE + G group and G group.

4.2 | Effect of EPO-liposomes with SLX

We recently reported that liposomes with SLX can be used as a cardiac-targeting active drug delivery system.[6] SLX existing on the surface of liposomes binds to E-selectin and P-selectin expressed on the vascular endothelial cells in the infarcted myocardium, then the liposomes accumulate into the infarct areas of the myocardium.[6] We have already reported that EPO-encapsulated liposomes with SLX, but not liposomes with SLX without EPO (liposomes with SLX by itself), reduce the MI size and improve LV remodeling and function through activation of Akt, ERK, Stat3 and by exerting antifibrotic effect through activation of MMP-1 and Stat3 and by angiogenic effect through activation of vascular endothelial growth factor (VEGF).[6] The EPO-liposome with SLX did not upregulate the expression of SDF-1 in the infarct border area, did not increase the plasma level of SDF-1, nor increase the number of $CD34^{+/}CXCR4^+$ cells in the peripheral blood (Figure 4). This suggested that CXCR4/SDF-1 axis might not have contributed to the reduction in the MI size in the EPO group.

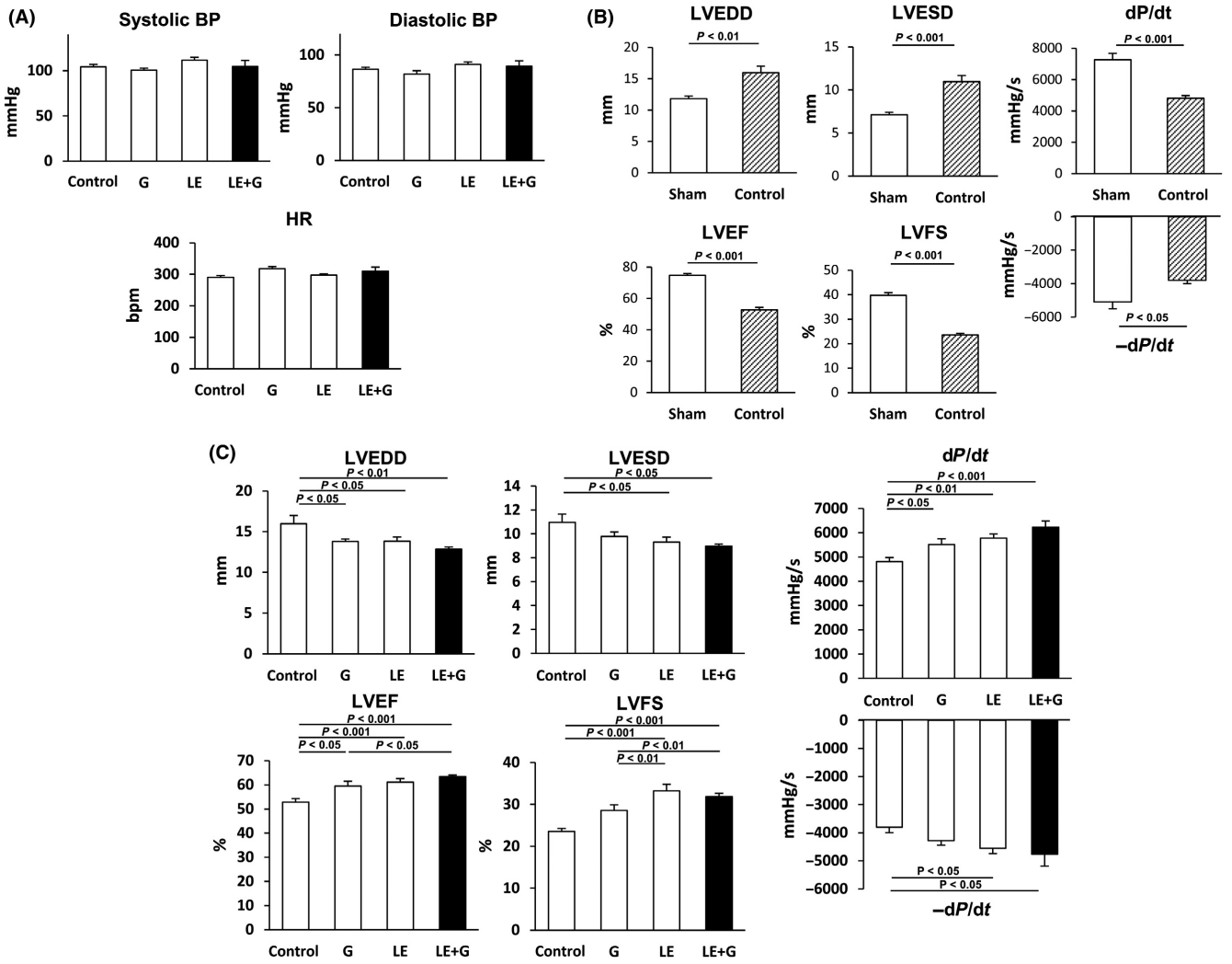

FIGURE 2 Hemodynamic parameters and cardiac function and remodeling. (A) Hemodynamic parameters of the heart rate, systolic blood pressure and diastolic blood pressure, and (B; Sham vs control group) (C; among the four groups) echocardiographic data of EF, FS, LVEDd and LVESd, and ±dP/dt obtained 14 days after MI. Note the improvement in left ventricular (LV) remodeling and function in the LE + G group, LE group and G group (n = 10 in each group. Data are presented as mean ± SE.) EF, ejection fraction; FS, fractional shortening

4.3 | Effect of combination therapy with G-CSF and EPO-liposome with SLX on the MI size, and LV function and remodeling

4.3.1 | The MI size

The MI size of the area at risk (as a percentage) was the smallest in the LE + G group (14.7 ± 0.8%) as compared to the other three groups; G group (22.4 ± 1.5%), LE group (18.5 ± 1.1%), and the control group (27.8 ± 1.5%) (Figure 1C). Because the MI size was significantly smaller in the G group or LE group than in the control group, and the MI size was similar between the G group and LE group, the striking reduction of the MI size in the LE + G group must be caused by the additive mechanisms of G-CSF and EPO-liposome with SLX.

4.3.2 | LV function and remodeling

Fourteen days after MI, as shown in Figure 3, EF, FS, +dP/dt and +dP/dt in the LE + G group were significantly greater than in the control group, but were not necessarily greater than in the LE group or in the G group. LVEDd and LVEDs in the LE + G group were significantly smaller than in the control group, but were not necessarily smaller than in the G group or LE group. These results suggest that combination therapy with G-CSF and EPO-liposome with SLX improves LV function and remodeling compared to the control group but does not necessarily improve LV function and remodeling statistically more effectively than in the G-CSF or EPO-liposome with SLX alone. However, despite the lack of statistical significance, LE + G group tended to show the best improvement of the LV function and remodeling among the groups.

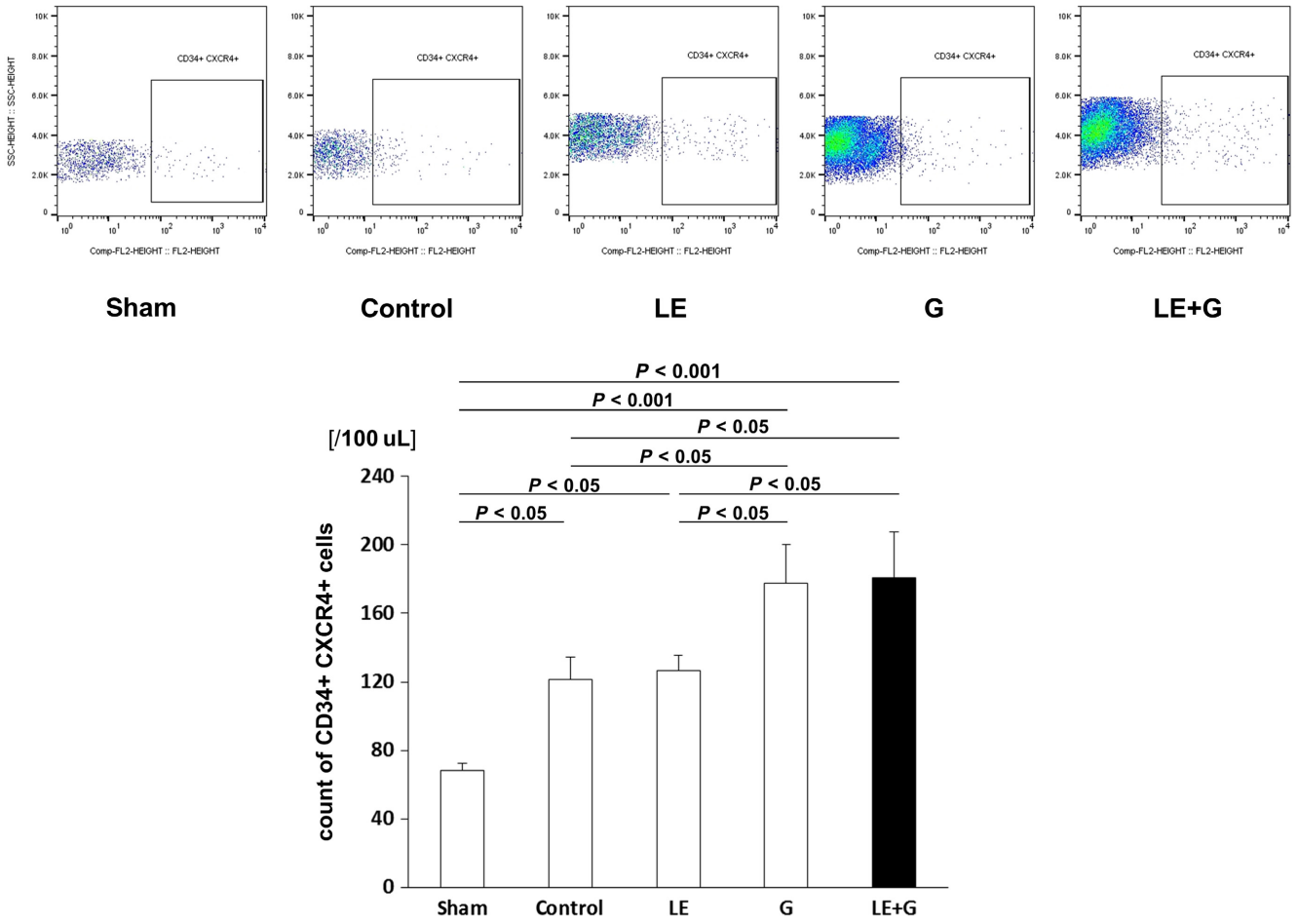

FIGURE 3 Fluorescence activated cell sorting (FACS) analysis of CD34+/CXCR4+ cells among peripheral blood 7 days after myocardial infarction (MI). (A) The FACS analysis showed that the CD34+/CXCR4+ cells (as percentages among total peripheral blood mononuclear cells) were significantly higher in the LE + G group and G group than in the LE group or on the control group (n = 7 in each group. Data are presented as mean ± SE)

4.4 | Mechanism by which combination of G-CSF and EPO-liposome with SLX remarkably reduced the MI size

In this study, consistent with our previous report,[6] EPO-liposome with SLX significantly reduced the MI size (Figure 1C). G-CSF also, consistent with our previous report,[3] reduced the MI size (Figure 1C). The new finding in this study is that LE + G group showed a smaller MI size than in the G group or in the LE group. It suggested that combination of G-CSF and EPO-liposome with SLX additively contributed to the remarkable reduction in the MI size (Figure 1C).

Our previous report using western blot analysis showed post-MI administration of EPO-liposomes with SLX significantly upregulated the expression of EPO receptors, p-Akt and p-ERK in the ischemic area of the myocardium.[6] This indicates that EPO delivered to the ischemic myocardium using liposomes stimulated EPO receptors and activated its downstream signals, Akt and ERK. In this study, the expressions of p-Akt and p-ERK were compared among the control, G, LE, and LE + G groups. As a result, the expressions of both p-Akt and p-ERK, prosurvival signals, were significantly upregulated in the LE group and LE + G group but not in the G group compared with those in the control group (Figure 6), suggesting that EPO-liposome with SLX significantly activated Akt and ERK but not G-CSF if any. It has been reported that the activation of Akt and ERK involves in cardioprotection by ischemic preconditioning, which leads to a decrease in the size of MI.[21-23] Therefore, in this study, EPO might have contributed to reduce the MI size through activation of Akt and ERK in the LE group and LE + G group.

It has been reported that G-CSF contributes to stem cell mobilization by decreasing bone marrow SDF-1 and upregulating CXCR4.[24] However, conversely, in this study, we observed upregulation of the expression of SDF-1 in the infarct border area in the G group and LE + G group (Figure 4B), and the number of $CD34^+/CXCR4^+$ cells in the peripheral blood was significantly increased in the LE + G group and G group (Figure 3). Therefore, G-CSF might have activated CXCR4/SDF-1 axis and contributed to the homing of $CD34^+/CXCR4^+$ cells into the ischemic border area and to the reduction in the MI size in the LE + G group and G group. In consideration of these mechanistic results mentioned

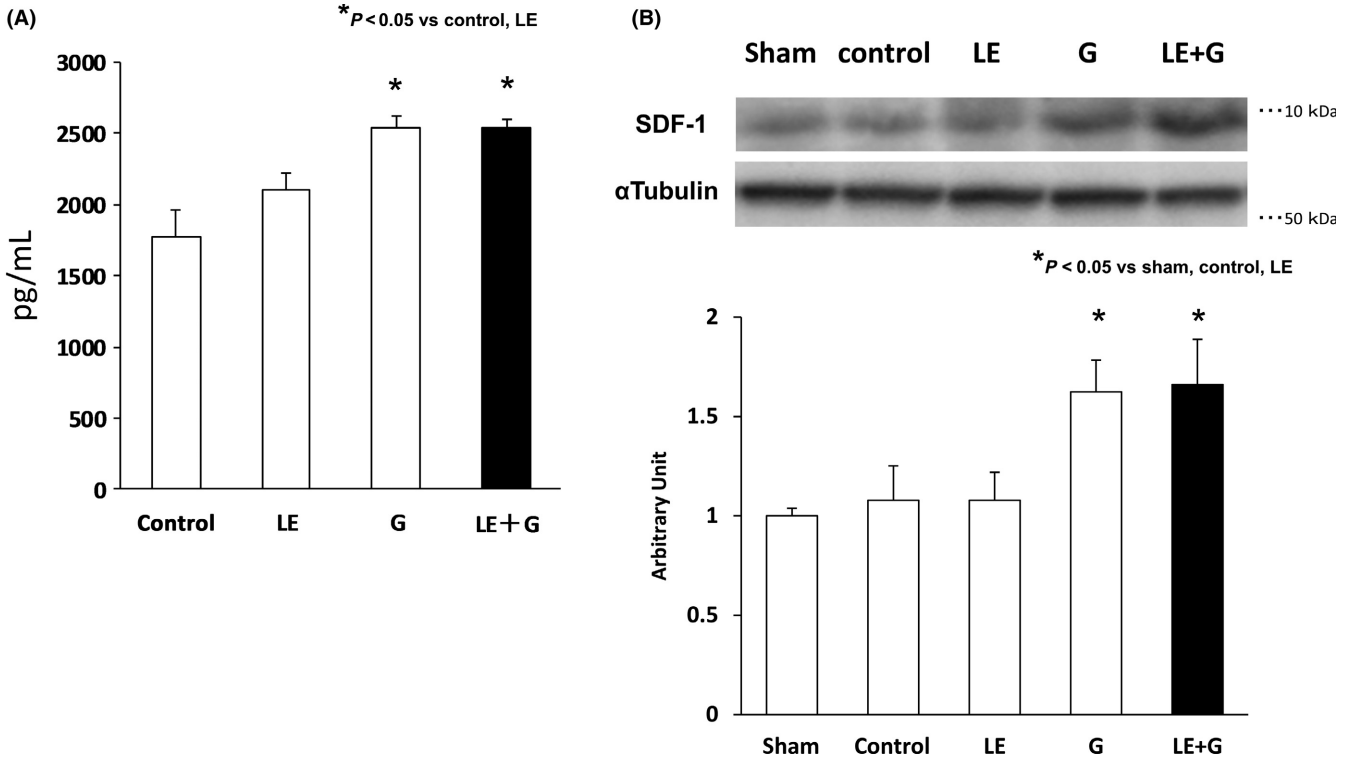

FIGURE 4 Plasma SDF-1 levels and western blot analysis on cardiac expression 2 days after MI. (A) The plasma SDF-1 levels were higher in the G group and LE + G group than in the LE group or in the control group (n = 5 in each group. Data are presented as mean ± SE.). * $P < 0.05$ vs control, LE group. (B) The upregulation of the expression of SDF-1 was observed in the infarct border area in the G group and LE + G group (n = 5 in each group. Data are presented as mean ± SE.) * $P < 0.05$ vs Sham, control, LE group

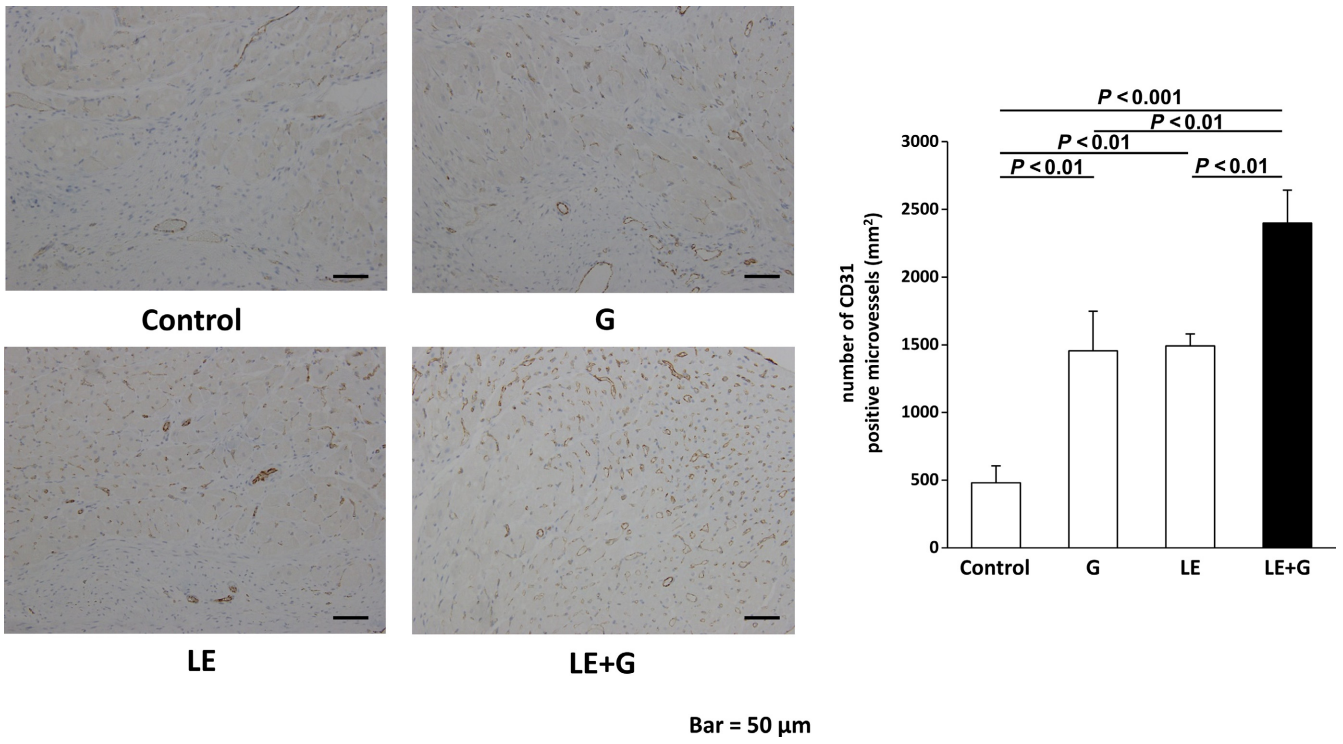

FIGURE 5 Immunohistochemistry on CD31. In the ischemic border area, the densities of the CD31-positive microvessels were significantly increased in the LE + G group, G group, and LE group compared with those in the control groups on 14 days after myocardial infarction. The number of the CD31-positive microvessels was the largest in the LE + G group than in the G group or in the LE group (n = 7 in each group. Data are presented as mean ± SE.). Bars = 50 μm

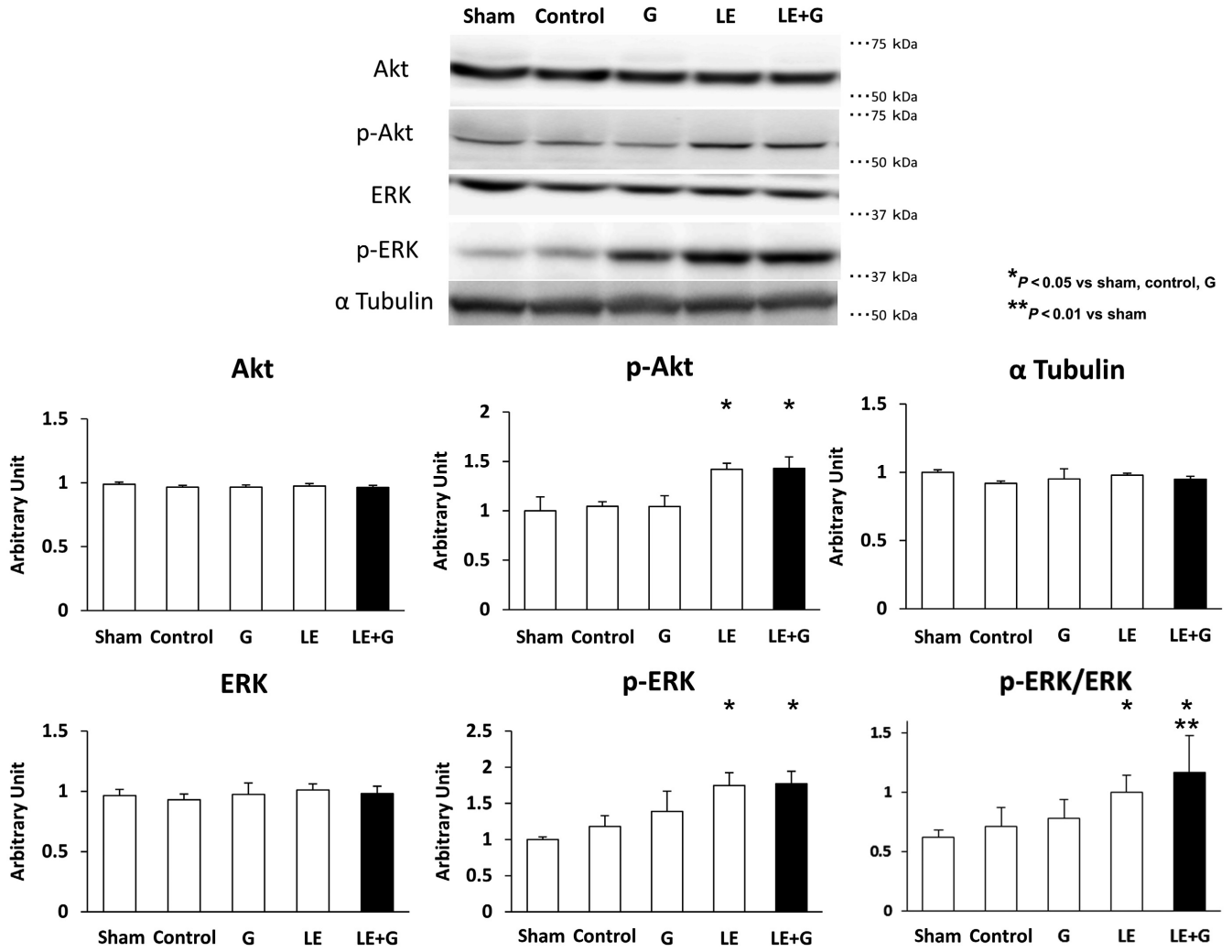

FIGURE 6 Western blot analysis of Akt, p-Akt, ERK, and p-ERK in the infarct border area 14 days after MI (n = 5 in each group. Data are presented as mean ± SE.) $*P < 0.05$ vs Sham, control, G group, $**P < 0.01$ vs Sham. ERK, extracellular signal-regulated protein kinase; MI, myocardial infarction

above, among the L-EPO + G group, L-EPO group, and G group, only the L-EPO + G group has common mechanisms; serum SDF-1 level was elevated; the expression of SDF-1 was upregulated; the number of $CD34^+/CXCR4^+$ cells in the peripheral blood was increased; and Akt and ERK were activated. These mechanisms contributed to the reduction in the MI size by combination therapy of G-CSF and EPO-liposome with SLX.

In addition to these mechanisms, neovascularization might have also been involved. EPO has also been reported to accelerate neovascularization.[25] The EPO receptor system has been reported to accelerate angiogenesis in response to hindlimb ischemia by upregulation of VEGF/VEGF receptor system in mice.[26] We previously reported that EPO upregulates the expression of VEGF and increases the density of CD31-positive microvessels in the infarct border area in a rabbit AMI model.[3,7] It has also been reported that G-CSF upregulates VEGF[20] and increases microvessels[3] in a rabbit AMI model. It is known that VEGF increases capillary density.[26,27] In this study, the density of CD31-positive microvessels was the greatest in the LE + G group and greater in the G and LE groups compared with the control group. Therefore, the greatest number of the microvessels in the border area in the LE + G group might have been due to the synergistical effect of EPO and G-CSF both of which causes neovascularization.

4.5 | Study limitation and clinical implication

In this study, cause and effect relationship between tissue injury and neovascularization or EPC mobilization, and between tissue injury and myocardial signaling was not examined. However, we focused on the effects of G-CSF, EPO-containing liposome with SLX or combination of G-CSF and EPO-containing liposome with SLX on the neovascularization, EPC mobilization and myocardial signaling, but not on the effect of tissue injury by itself in a rabbit AMI model. The standard therapy for human AMI is reperfusion therapy. However,

the combination therapy with G-CSF and EPO-containing liposomes with SLX in addition to reperfusion therapy would give an important suggestion for a new treatment of AMI. However, the safety of liposomes with SLX has not yet been examined clinically.

In conclusion, post-MI combination therapy with G-CSF and EPO-liposome with SLX is more cardioprotective than G-CSF or EPO-liposome with SLX alone through EPCs mobilization, neovascularization and activation of prosurvival signals.

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AUTHOR CONTRIBUTIONS

Study conception and design: Yoshihisa Yamada, Shinya Minatoguchi; Acquisition of data: Yoshihisa Yamada, Shingo Minatoguchi, Noriko Endo, Hiromitsu Kanamori, Atsushi Mikami; Analysis and interpretation of data: Yoshihisa Yamada, Shinya Minatoguchi, Masanori Kawasaki, Kazuhiko Nishigakil; Drafting of manuscript: Yoshihisa Yamada, Shinya Minatoguchi; Critical revision: Yoshihisa Yamada.

DISCLOSURE

None declared.

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