Postinfarct active cardiac-targeted delivery of erythropoietin by liposomes with sialyl Lewis X repairs infarcted myocardium in rabbits

Yoshihisa Yamada,1 Hiroyuki Kobayashi,1 Masamitsu Iwasa,1 Shohei Sumi,1 Hiroaki Ushikoshi,1 Takuma Aoyama,1 Kazuhiko Nishigaki,1 Genzou Takemura,1 Takako Fujiwara,2 Hisayoshi Fujiwara,3 Makoto Kiso,4 and Shinya Minatoguchi1

1Department of Cardiology, Gifu University Graduate School of Medicine, Gifu, Japan; 2Kyoto Women’s University, Kyoto, Japan; 3Hyogo Prefectural Amagasaki Hospital, Hyogo, Japan; and 4Department of Applied Bio-Organic Chemistry, Gifu University, Gifu, Japan

Submitted 20 September 2012; accepted in final form 5 February 2013

Recent studies (4, 28) have suggested that erythropoietin (EPO) exerts a cardioprotective effect in cases of acute myocardial infarction (MI). With systemic administration, however, the beneficial effects in the context of MI are observed only when large doses of EPO are administered, which are frequently accompanied by polycythemia and, therefore, the potential for thromboembolic complications (2, 14). We (15) previously reported that a EPO-gelatin hydrogel patch applied intravenously immediately after MI. MI sizes and numbers of microvessels were assessed 14 days after MI. Confocal microscopy and electron microscopy showed the specific accumulation of liposomes with SLX in the infarcted myocardium. MI and cardiac fibrosis areas were significantly smaller in the L-EPO group than in the other groups. LV function and remodeling were improved in the L-EPO group. The number of CD31-positive microvessels was significantly greater in the L-EPO group than in the other groups. Higher expressions of EPO receptors, phosphorylated (p)Akt, pERK, pStat3, VEGF, Bcl-2, and promatrix metalloproteinase-1 were observed in the infarct area in the L-EPO group than in the other groups. EPO-encapsulated liposomes with SLX selectively accumulated in the infarct area, reduced MI size, and improved LV remodeling function. However, since this method needs a surgical approach, we developed a noninvasive cardiac-targeting drug delivery system to deliver EPO to the infarct zone. Thus, we aimed to determine whether post-MI treatment with EPO-encapsulated liposomes with sialyl-LewisX (SLX) reduces infarct size and improves LV remodeling and function and investigated the local molecular mechanisms underlying the beneficial effects of liposomal EPO.

**MATERIALS AND METHODS**

All rabbits used in this study received humane care in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine (Gifu, Japan).

Male Japanese White rabbits weighing ~2.5 kg were anesthetized with an intravenous injection of ketamine (10 mg/kg) and zylazine (3 mg/kg), and additional doses were given when required throughout the experiment. Once anesthetized, animals were then intubated and ventilated with room air supplemented with a low flow of oxygen using a mechanical ventilator (tidal volume: 25–35 ml, respiratory rate: 20–30 breaths/min, model SN-480-5, Shimano, Tokyo, Japan). Serial blood gas analysis was performed, and ventilatory conditions were adjusted to maintain arterial blood gas within the physiological range. Surgery was performed under sterile conditions. The carotid artery and jugular veins were cannulated to monitor peripheral arterial pressure and administer drugs, respectively. Thereafter, rabbits were systemically heparinized (500 U/kg), a thoracotomy was performed in the left fourth intercostal space, and the heart was exposed after incision of the pericardium. A 4.0 silk suture on a small curved needle was passed through the myocardium beneath the middle segment of the large arterial branch coursing down the middle segment of the anterolateral surface of the LV. Both ends of the silk suture were then passed through a small vinyl tube, and the coronary branch was occluded by pulling the suture, which was fixed by clamping the tube with a mosquito hemostat. Myocardial ischemia was induced for 30 min. Myocardial ischemia was confirmed by S-T segment elevation on the ECG and regional cyanosis of the myocardial surface. Reperfusion was confirmed by myocardial blush over the risk area after release of the suture.

**Preparation of liposomes with SLX, Cy5.5-encapsulated liposomes with SLX, and EPO-encapsulated liposomes with SLX**

Liposomes were made from a mixture of dipalmitoylphosphatidylcholine, cholesterol, dihexadecyolphosphate, ganglioside, dipalmitoylphosphatidylethanolamine, and sodium cholate using the improved cholate dialysis method (41, 42). Cy5.5-encapsulated liposomes, in which Cy5.5 was bound to human serum albumin, were prepared as previously described by Hirai et al. (12). To prepare EPO-encapsulated liposomes, the lipid film was resuspended with 10 ml EPO (180,000 IU) solution, and the transparent micelle was generated by sonication.

**Address for reprint requests and other correspondence:** S. Minatoguchi, Dept. of Cardiology, Gifu Univ. Graduate School of Medicine, Yanagido 1-1, Gifu 501-1194, Japan (e-mail: minatos@gifu-u.ac.jp).
EPO-encapsulated liposomes were obtained after ultrafiltration of the micelle solution using a PM10 membrane (Millipore). Hydropholization with Tris and SLX conjugation on the surface of liposomes were carried out as previously described (12). Finally, 5 ml of EPO-encapsulated liposomes with SLX solution contained 25,000 IU EPO (5,000 IU/ml). The final concentration of lipid in the liposomes encapsulating Cy5.5 or EPO was in the range of 2.5–3.1 mg/ml. Liposome particle size was measured using Zetasizer Nano-S90 (Malvern) at 25°C, and the mean particle size was ~100 nm in diameter.

Accumulation of liposomes in infarcted cardiac tissues. Rabbits underwent 30 min of coronary occlusion and reperfusion. Liposomes in which the fluorescent substance Cy5.5 were encapsulated (0.5 ml) were intravenously administered immediately after reperfusion. At 24 and 48 h and on 7 and 14 days after reperfusion, rabbits were euthanized under deep anesthesia, and their hearts were excised. Liposomes without SLX in which Cy5.5 was encapsulated were also intravenously administered immediately after reperfusion, and the heart was excised at 48 h of reperfusion. Hearts were immersed in iced PBS at <4°C immediately after death. Tissues (~3 × 3 × 2 mm each) obtained from the risk area, including the MI and from the nonrisk area of each heart, were included in OCT compound (Miles Scientific) and snap frozen in liquid nitrogen. OCT compound-embedded tissues were sectioned with 4 μm thickness using a cryostat for immunohistochemical analysis. Immunohistochemical staining was performed using Hoechst 33342 for nuclear staining with an indirect immunoperoxidase method; immunohistochemical stainings were performed using monoclonal anti-actin (α-sarcomeric) antibody (Sigma-Aldrich) at 1:500, which cross reacts with rabbit tissues. These were observed using confocal microscopy (LSM510 NLO, Zeiss, Tokyo, Japan).

Electron microscopy. To trace the liposomes at the subcellular level in vivo, we used recently developed SLX-containing liposomes encapsulated with colloidal gold (SLX-Lipo-Gold) (21). The mean particle size of SLX-Lipo-Gold was estimated to be 135 nm, and it contained colloidal gold particles with a diameter of 8 nm (21).

Rabbits underwent 30 min of coronary occlusion and reperfusion. SLX-Lipo-Gold (500 μl) was intravenously administered immediately after reperfusion. At 48 h post-MI, rabbits were euthanized under deep anesthesia, and their hearts were excised. Cardiac tissue was quickly cut into 1-mm cubes, immersion fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) overnight at 4°C, and postfixed in 1% buffered osmium tetroxide. Specimens were then dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections (90 nm) double stained with uranyl acetate and lead citrate were examined under an electron microscope (H-800, Hitachi, Tokyo, Japan).

Measurement of cardiac tissue EPO content. The amount of EPO in the cardiac tissue in the risk (infarct area and border area) and nonrisk areas at 48 h and 14 days after reperfusion was measured by ELISA (Mitsubishi Kagaku Bio-Chemical Laboratories, Tokyo, Japan).

Protocol. In total, 105 rabbits were initially enrolled in this study: 45 rabbits for infarct size measurements at 14 days after infarction, 40 rabbits for Western blot analysis or immunohistochemical analysis at various periods of time, and 20 rabbits for the detection of accumulation of liposomes in the infarcted cardiac tissues using the fluorescent substance Cy5.5 and colloidal gold and for measurements of cardiac tissue EPO contents. In the 4 study groups used to measure infarct size at 14 days after infarction, 45 rabbits were initially enrolled and were subjected to 30 min of coronary occlusion followed by reperfusion, as shown in Fig. 1. Of these animals, 5 rabbits were excluded because of death during the experiments due to ventricular fibrillation during 30-min ischemia: two rabbits in the group that received saline treatment (saline group), one rabbit in the group that received liposomes with SLX without EPO (L group), one rabbit in the group that received EPO-encapsulated liposomes without SLX (L-EPO without SLX group), and one rabbit in the group that received EPO-encapsulated liposomes with SLX (L-EPO group). The remaining 40 rabbits survived for 14 days for the measurement of infarct size. In the saline group, saline (0.5 ml) was intravenously injected immediately after reperfusion. In the L group, liposomes with SLX encapsulating no EPO were intravenously injected immediately after reperfusion. In the L-EPO group, liposomes with SLX encapsulating 2,500 IU/kg EPO were intravenously injected immediately after reperfusion. In the L-EPO group, liposomes without SLX encapsulating 2,500 IU/kg EPO were intravenously injected immediately after reperfusion. Rabbits were killed at 5 h and 2 and 14 days after MI (n = 10 rabbits/group).

Blood sampling. Blood samples (0.3 ml each) were collected from an ear vein before and 14 days after MI for peripheral blood cell counts using an automatic cell count analyzer (Sysmex XE-2100, Sysmex, Nagoya, Japan).

Physiological experiments. Echocardiography (SSD2000, Aloka) was performed on 14 days post-MI, and ejection fraction, fractional shortening, and LV end-diastolic and end-systolic dimensions were obtained. Arterial blood pressure and heart rate were also measured via a catheter introduced into the carotid artery. A micrometer-meter tipped catheter (SPR 407, Millar Instruments) was inserted into the LV to record +dP/dt.

MI size and histological analysis. To measure the risk area, excised hearts were mounted on a Langendorff apparatus, and Evans blue dye (4°C) was injected via the aorta for 1 min after reocclusion of the coronary branch. The LV was then weighed and sectioned into seven transverse slices parallel to the atrioventricular ring. Each slice was weighed, incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 10 min at 37°C to visualize the infarct area, and photographed. The areas of the ischemic regions and the infarcted myocardium were traced on each LV slice, multiplied by the weight of the slice, and expressed as a percentage of the risk region or the LV. Each slice was then fixed in 10% buffered formalin for 4 h, embedded in paraffin, and cut into 4-μm-thick sections with a microtome. These sections were stained with hematoxylin-eosin and Masson trichrome. Since it is generally accepted that the Evans blue dye-TTC method is not reliable to evaluate infarct size at time points after 72 h of reperfusion because of remodeling due to scar shrinkage within the infarct, we further assessed the infarct size histologically. For transversely sliced preparations with infarction at the level of papillary muscle, LV wall areas and Masson trichrome-positive areas were calculated using an image analyzer connected to a light microscope.
(LUZEX-F, NIRECO, Tokyo, Japan) and, the histological infarct size was then obtained by expressing the Masson trichrome-positive area as a percentage of LV wall area.

**Immunohistochemistry.** We used an indirect immunoperoxidase method for immunohistochemical staining of cardiac sections on day 14 post-MI. The primary antibody used was monoclonal mouse anti-human CD31, an endothelial cell marker (1:100 dilution, Dako), which cross reacts with rabbit tissues.

**Western blot analysis.** Cardiac tissue samples for Western blot analysis were taken from the viable area close to the infarcted area in the ischemic area. Western blot analysis was carried out using lysates from heart tissues at 5 h and 2 and 14 days post-MI. Proteins were separated, transferred to membranes using standard protocols, and then probed with antibodies against the EPO receptor (EPO) (E4644, Sigma), P-selectin (sc-6941, Santa Cruz Biotechnology), E-selectin (sc-6939, Santa Cruz Biotechnology), VEGF (RDI-VeGFabm-12, Fitzgerald), promatrix metalloproteinase-1 (pro-MMP-1; F-67, Daiichi Fine Chemical), tissue inhibitor of metalloproteinase-1 (TIMP-1; sc-6832, Santa Cruz Biotechnology), transforming growth factor-β (TGF-β; sc-65378, Santa Cruz Biotechnology), and Bcl-2 (sc-7382, Santa Cruz Biotechnology) served as the loading control. Blots were visualized by means of enhanced chemiluminescence (Amersham), and signals were quantified by densitometry. α-Tubulin (analyzed with an antibody from Santa Cruz Biotechnology) served as the loading control.

**Statistical analysis.** All values are presented as means ± SE. Differences among the saline, L, L-EPO without SLX, and L-EPO groups were assessed by ANOVA combined with a Scheffé’s method (Stat View, J5.0 software). P values of <0.05 were considered significant.

**RESULTS**

**Cardiac accumulation of liposomes.** We observed that the fluorescent substance Cy5.5 accumulated not in the noninfarct area but in the border area of the myocardium at 24 and 48 h and 7 and 14 days after MI (Fig. 2A). There was no accumulation of Cy5.5 in the control myocardium. Cy5.5 was not observed in the myocardium in which liposomes without SLX were administered (Fig. 2B).

**Electron microscopy.** We obtained detailed localization data of SLX-Lipo-Gold particles in the rabbit myocardium by examining colloidal gold signals in liposomes under an electron microscope. Colloidal gold particles were abundantly distributed in the cytoplasm of capillary endothelial cells and cardiomyocytes and in the extracellular space (Fig. 2C). In contrast, we could not find sufficient evidence of colloidal gold-containing liposomes without SLX (data not shown).

**EPO levels in the heart.** The level of EPO in the infarct border area was significantly higher in the L-EPO group than in the saline group at 48 h after infarction but not at 14 days after MI (Fig. 3).

**Peripheral blood cell count.** There were no differences among the groups in white blood cell, red blood cell, and thrombocyte counts or hematocrit before and 14 days after MI (Table 1).

**Physiological experiments.** There were no significant differences in heart rate or systolic and diastolic blood pressures among the three groups at 14 days post-MI (Fig. 4A). LV end-systolic dimension (saline group: 16.6 ± 3.1 mm, L group:...
15.8 ± 1.4 mm, and L-EPO group: 13.9 ± 1.7 mm) and LV end-diastolic dimension (saline group: 11.0 ± 2.2 mm, L group: 11.8 ± 0.5 mm, and L-EPO group: 9.3 ± 1.3 mm) were significantly smaller, whereas LV ejection fraction (saline group: 52.8 ± 4.9%, L group: 53.6 ± 3.7%, and L-EPO group: 61.2 ± 4.7%), fractional shortening (saline group: 23.7 ± 1.9%, L group: 26.1 ± 3.3%, and L-EPO group: 33.4 ± 4.9%), +dP/dt (saline group: 4,909.5 ± 530 mmHg/s, L group: 5,152.8 ± 259 mmHg/s, and L-EPO group: 5,763.9 ± 521 mmHg/s), and −dP/dt (saline group: −3,839.1 ± 651 mmHg/s, L group: −4,103.4 ± 366 mmHg/s, and L-EPO group: −4,539.3 ± 613 mmHg/s) were significantly larger in the L-EPO group than in the saline or L group (Fig. 4B).

MI size and histological analysis. On day 14 post-MI, there were no significant differences in the area at risk as a percentage of the LV among the saline (29.2 ± 4.3%), L (29.9 ± 6.0%), L-EPO without SLX (31.6 ± 6.2%), and L-EPO with SLX (31.8 ± 6.8%) groups (Fig. 5A,a). LV weights and volumes of nonrisk areas as a percentage of the LV were 4.7 ± 0.2, 4.4 ± 0.6, 4.3 ± 0.4, and 4.6 ± 0.7 g and 71.2 ± 4.6%, 70.1 ± 6.0%, 68.4 ± 2.4%, and 70.9 ± 4.7% in the saline, L, L-EPO without SLX, and L-EPO with SLX groups, respectively, and there were no significant differences among the groups. MI size as a percentage of the LV was significantly smaller in the L-EPO group (18.7 ± 3.7%) than in the saline (29.0 ± 5.4%), L (26.2 ± 3.3%), or L-EPO without SLX (24.6 ± 4.3%) groups (Fig. 5A,b). Histological assessment of MI size as a percentage of the LV as assessed by Masson trichrome staining was also significantly smaller in the L-EPO group (13.0 ± 6.4%) than in the saline (27.3 ± 8.4%), L (26.5 ± 4.8%), or L-EPO without SLX (19.3 ± 3.3%) groups (Fig. 5B).

Western blot analysis. Western blot analysis revealed that myocardial expressions of E-selectin and P-selectin were both markedly upregulated in the ischemic area of myocardium in the L and L-EPO groups compared with those in the saline group at 5 h after MI (Fig. 6A). Myocardial expression of EPORs was markedly stronger in the L-EPO group than in the saline or L groups on day 2 post-MI (Fig. 6B). In the ischemic area, pAkt, pERK, and pStat3 were significantly higher in hearts from the L-EPO group than in those from the saline or L groups on day 2 post-MI (Fig. 6B). Expression of pro-MMP-1, VEGF, and Bcl-2 was upregulated in hearts from the L-EPO group but not in the saline or L groups on day 2 post-MI (Fig. 6B). On day 14 post-MI, expression of EPOR, pAkt, pERK, pro-MMP-1, and VEGF, but not pStat3 or Bcl-2 (data are not shown), was significantly upregulated in the L-EPO group compared with the saline or L groups (Fig. 6C). There were no differences in the expression of TIMP-1 or TGF-β among the three groups.

Immunohistochemistry. On day 14 post-MI, the density of CD31-positive microvessels (capillary density) was clearly greater in the infarct border area in the L-EPO group than in the saline or L groups (Fig. 7). However, there were no differences in the densities of CD31-positive microvessels in the remote noninfarct area or in the infarct area among the three groups.

Table 1. Blood tests

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>14 Days After Myocardial Infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>White blood cells, number/μl</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline group</td>
<td>6,340 ± 213.5</td>
<td>8,720 ± 287.1</td>
</tr>
<tr>
<td>L group</td>
<td>6,580 ± 755.2</td>
<td>9,075 ± 143.6</td>
</tr>
<tr>
<td>L-EPO group</td>
<td>6,840 ± 679.4</td>
<td>8,460 ± 462.2</td>
</tr>
<tr>
<td><strong>Red blood cells, number/μl × 10⁴</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline group</td>
<td>618.4 ± 18.6</td>
<td>561.9 ± 20.7</td>
</tr>
<tr>
<td>L group</td>
<td>617.6 ± 4.0</td>
<td>607.5 ± 24.6</td>
</tr>
<tr>
<td>L-EPO group</td>
<td>610.9 ± 11.2</td>
<td>572.9 ± 21.3</td>
</tr>
<tr>
<td><strong>Hematocrit, %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline group</td>
<td>40.5 ± 1.1</td>
<td>37.3 ± 1.0</td>
</tr>
<tr>
<td>L group</td>
<td>41.0 ± 0.8</td>
<td>40.7 ± 2.3</td>
</tr>
<tr>
<td>L-EPO group</td>
<td>39.5 ± 0.8</td>
<td>36.9 ± 1.1</td>
</tr>
<tr>
<td><strong>Platelets, number/μl × 10⁴</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline group</td>
<td>20.5 ± 1.1</td>
<td>18.2 ± 1.4</td>
</tr>
<tr>
<td>L group</td>
<td>23.9 ± 1.4</td>
<td>18.8 ± 1.9</td>
</tr>
<tr>
<td>L-EPO group</td>
<td>22.4 ± 0.8</td>
<td>18.1 ± 2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. Shown are results from the following groups: saline treatment (saline group), liposomes with sialyl Lewis x (SLX) without erythropoietin (EPO) (L group), and EPO-encapsulated liposomes with SLX (L-EPO group). There were no differences among the groups in terms of white blood cell, red blood cell, hematocrit, and thrombocyte measurements made on days 0 and 14 after myocardial infarction.
DISCUSSION

Active targeting of EPO-encapsulated liposomes with SLX. Liposomes are usually used in cancer treatment for drug delivery to target specific organs to prevent the degradation of chemotherapy agents (6, 26, 35). These delivery systems deliver the drugs by controlling the distribution of liposomes by adjustment of particle size and the electric charge of the surface, i.e., a passive delivery system (7, 19, 37). In contrast, it has been reported that the specific recognition and binding between selectin and the sugar chain convey an active targeting ability to liposomes (40, 43). In the inflamed region, E-selectin and P-selectin are expressed on the cell surface of vascular endothelial cells. On the surface of leukocytes, the sugar chain called SLX is present and can bind with E-selectin and P-selectin, and leukocytes roll on the vascular endothelial cell and thus accumulate specifically in the inflamed region (3, 36). Since inflammation occurs in the MI region, the application of liposomes with SLX for MI will specifically and efficiently deliver substances to inflamed regions. As a matter of fact, in the present study, as shown in Fig. 6A, the expression of E-selectin and P-selectin was upregulated in the infarct area of the myocardium at 5 h after MI. Furthermore, as shown in Fig. 2, A and B, although Cy5.5-encapsulated liposomes without SLX did not accumulated in the infarct border area, Cy5.5-encapsulated liposomes with SLX accumulated in the infarct border areas of the myocardium as long as 14 days after MI. However, the accumulation of Cy5.5 in the infarcted myocardium only shows that EPO-encapsulated liposomes with SLX reached the infarcted myocardium and does not necessarily mean that EPO was released from the liposome as long as 14 days.
days after MI. As a matter of fact, the cardiac tissue level of EPO in the border area was significantly higher in the L-EPO group than in the saline group at 48 h after MI, but there were no differences in cardiac tissue levels of EPO between the groups 14 days after MI. This suggests that EPO will be gradually released from the liposome as long as 48 h but not as long as 14 days after MI.

The diameter of the liposomes was ~100 nm, and this size of liposomes can pass through microvessels. SLX on the surface of liposomes combines with E-selectin and P-selectin expressed on vascular endothelial cells in the infarct areas of myocardium; liposomes then accumulate into infarct areas of the myocardium. In the present study, electron microscopy revealed that colloidal gold particles were abundantly distributed in the cytoplasm of capillary endothelial cells and cardiomyocytes and in the extracellular space, as shown in Fig. 2C. These findings indicate that some liposomes may be incorporated by endothelial cells and are then extravasated and reach the myocardium. Furthermore, since the level of EPO in the MI border area was significantly higher in the L-EPO group than in the saline group, as shown in Fig. 3, our results suggest that liposomes with SLX can deliver EPO selectively to the local infarct areas of the myocardium. Furthermore, since the liposome with SLX is made of phospholipids, after incorporation into tissues, it is considered to eliminate through the metabolism system of lipids.

Fig. 5. A: comparison of myocardial infarct (MI) area at 14 days post-MI. a, Area at risk as a percentage of the LV as assessed by Evans blue dye. b, MI area as a percentage of the area at risk as assessed by 2,3,5-triphenyltetrazolium chloride method. *P < 0.05. B: areas of fibrosis stained positive with Masson trichrome staining at 14 days post-MI. *P < 0.05.
Fig. 6. A–C: Western blot analysis of myocardial E-selectin and P-selectin expression at 5 h (A) and EPO receptor (EPO-R), Akt, phosphorylated (p)Akt, ERK, pERK, Stat3, pStat3, VEGF, Bcl-2, pro-matrix metalloproteinase-1 (pro-MMP-1), tissue inhibitor of metalloproteinase (TIMP)-1, and transforming growth factor (TGF)-β expression on day 2 (B) and on day 14 (C) post-MI. *P < 0.05 vs. the saline, L, and L-EPO without SLX groups; **P < 0.05 vs. the L and L-EPO without SLX groups.
Effect on MI size and LV remodeling and function. There have been some reports (4, 8, 22, 27) showing that systemic pre- and postischemic treatment with EPO has a cardioprotective effect, such as a MI size-reducing effect in rats, and others (16, 24) have reported that EPO administered at reperfusion did not show any MI size-reducing effect in sheep and pigs. In rabbits, Parsa et al. (28) reported that EPO administered even at the time of reperfusion at doses of 1,000 and 5,000 IU/kg significantly reduced MI size, although cardiac function was not improved. However, we (15) have previously reported that EPO intravenously administered immediately after reperfusion at a dose of 1,500 IU/kg for 5 days (7,500 IU/kg in total) did not reduce MI size but that an EPO-gelatin hydrogel patch containing only 1,500 IU/kg EPO applied to the surface of the ischemic area of the heart (1/5 of the dose administered for systemic EPO treatment) significantly reduced MI size 14 days after MI. LV end-systolic and end-diastolic dimensions were significantly smaller, whereas LV ejection fraction, fractional shortening, and \( \frac{dP}{dt} \) were significantly larger in the EPO-gelatin hydrogel patch group than in the saline group (15). These data suggest that the application of a locally high concentration of EPO in the heart is needed to achieve a sufficient cardioprotective effect of EPO.

In the present study, 1 ml of liposome with SLX, which contains 5,000 IU EPO (~2,000 IU/kg EPO), administered intravenously immediately after reperfusion significantly reduced MI size compared with that in the saline, L, or L-EPO groups (Fig. 5). Since it has been reported that SLX itself has cardioprotective effects against ischemia and reperfusion injury and reduces myocardial infarct size (5, 33), we examined the effect of liposome with SLX (L group) on MI size. As a result, in the present study, liposomes with SLX without EPO did not significantly reduce MI size. LV ejection fraction, fractional shortening, and \( \pm \frac{dP}{dt} \) were significantly larger in the L-EPO group than in the saline or L groups. LV end-diastolic and LV end-systolic dimensions were significantly smaller in the L-EPO group than in the saline or L groups. These data suggest that post-MI treatment with EPO-encapsulated liposomes with SLX reduces MI size and improves LV remodeling and function. As a matter of fact, the infarct size-reducing effect of EPO-encapsulated liposomes with SLX was significantly greater than that of EPO-encapsulated liposomes without SLX. This may be explained by the results of a previous report (5) showing that the accumulation of Cy5.5-containing liposomes with sugar chain SLX to inflammation or tumor regions was significantly higher than control Cy5.5-containing liposomes without SLX at 24 and 48 h after administration. Therefore, EPO-encapsulated liposomes with SLX constitute an active cardiac-targeting noninvasive drug delivery system.

Expression of EPORs and their downstream signal transduction. It has been previously reported that EPORs are expressed in the heart (33). Our Western blot analysis confirmed those earlier findings and further showed that application of an EPO-encapsulated liposome with SLX significantly upregulated cardiac expression of EPORs (Fig. 6, A and B). We (12, 25, 38) have previously reported an upregulation of EPORs in the heart by treatment with EPO, which we again confirmed in the present study. Although the mechanism for the upregulation of EPORs is unknown, a recent study (17) has revealed a positive feedback loop between EPO and EPORs through GATA-1 activation.

Phosphatidylinositol 3-kinase/Akt, MAPK/ERK, and JAK-STAT are all known to be downstream mediators of EPO signaling in cardiac cells, both in vitro and in vivo (28). Moreover, activation of Akt, ERK, and Stat3, which mediate prosurvival signaling, was upregulated in ischemic areas of hearts in the L-EPO group but not in the saline or L groups. This suggests that EPO delivered by liposomes stimulated EPORs and their downstream signal transduction.

We observed significant phosphorylation of Stat3, Akt, and ERK in the ischemic myocardium on day 2 post-MI in the L-EPO group. The JAK-STAT pathway has been reported to play an important role in ischemic preconditioning, thereby...
TUNEL-positive myocytes among the groups (data are not
However, since there were no differences in the number of
sion of Bcl-2 may play a role in the early phase after MI.
expression of TIMP-1 or TGF-
L-EPO group than in the saline or L groups. The progression of
trichrome staining within the infarct area, were smaller in the
saline or L groups. The fibrotic areas, as assessed by Masson
dimensions were all smaller in the L-EPO group than in the
post-MI, as previously reported (15).

EPO has been reported to stimulate neovascularization (44). In
the present study, myocardial expression of VEGF was
upregulated and the density of CD31-positive microvessels
was increased in the L-EPO group. VEGF is known to increase
capillary density (11, 34), and the EPOR system reportedly
plays an important role in angiogenesis in response to hindlimb
ischemia through upregulation of the VEGF/VEGFR receptor
system in mice (31). In the present study, expression of Bcl-2
was upregulated in the ischemic area of the myocardium in the
L-EPO group on day 2 post-MI. This suggests that the expres-
sion of Bcl-2 may play a role in the early phase after MI.
However, since there were no differences in the number of
TUNEL-positive myocytes among the groups (data are not
shown), Bcl-2 may have acted as an antioxidant and protected
against necrosis (1).

On day 14 post-MI, MI size and LV systolic and diastolic
dimensions were all smaller in the L-EPO group than in the
saline or L groups. The fibrotic areas, as assessed by Masson
trichrome staining within the infarct area, were smaller in the
L-EPO group than in the saline or L groups. The progression of
tissue remodeling after MI depends on the balance between
antifibrotic and fibrotic factors. There were no differences in
the expression of TIMP-1 or TGF-β fibrotic factors among
three groups. However, expression of the collagenase MMP-1,
an antifibrotic factor, was upregulated in the infarct border
zone in the L-EPO group at 2 and 14 days post-MI. In post-MI
heart failure models in which MMPs are typically upregulated,
MMP inhibitors have been reported to exert beneficial effects
on both cardiac structure and function (9, 23, 29). On the other
hand, the volume of reactive granulation and/or scar tissue
after skin injury, burn, or surgery frequently becomes exces-
sive, resulting in hypertrophic scarring. In the heart, excessive
fibrosis accelerates cardiac remodeling and deteriorates cardiac
function, as seen in ischemic and dilated cardiomyopathies. In
such cases, an increase in MMP expression may exert a
protective effect via proteolysis of excessive collagen. Consis-
tent with this idea, a post-MI increase in MMP-1 induced by
hepatocyte growth factor exerted a beneficial effect on the
heart via its antifibrotic action (32), and an increase in MMP-1
induced by granulocyte colony-stimulating factor reduced the
size of fibrotic areas in MI hearts (20, 30). Furthermore, mice
with cardiomyocyte-restricted deletion of Stat3 are reportedly
susceptible to inflammation-induced heart damage and show a
dramatic increase in cardiac fibrosis (13). Thus, activation of
MMP-1 and Stat3 in the L-EPO group may be involved in the
reduction in myocardial fibrosis, resulting in improved LV
function.

Conclusions. EPO-encapsulated liposomes with SLX re-
duced MI size and improved LV remodeling and function
through the activation of Akt, ERK, and Stat3, by exerting
antifibrotic effects through the activation of MMP-1 and Stat3,
and by angiogenic effects through the activation of VEGF.
EPO-encapsulated liposomes with SLX may be a promising
strategy for active cardiac-targeting treatment of acute MI.

ACKNOWLEDGMENTS

The authors thank Akiko Tsujimoto and Tosie Otsubo for technical assis-
tance.

GRANTS

This work was supported by a grant from the Gifu University Graduate
School of Medicine (to S. Minatoguchi) and by a grant from Fukuda Founda-
tion for Medical Technology (to S. Minatoguchi).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Y.Y., T.F., M.K., and S.M. conception and design of
research; Y.Y., H.K., M.I., S.S., T.F., and M.K. performed experiments; Y.Y.,
M.I., S.S., H.U., T.A., K.N., and T.F. interpreted results of experiments; Y.Y.,
H.K., S.S., H.U., T.A., K.N., G.T., and H.F. prepared figures; Y.Y., G.T., and
H.F. drafted manuscript; Y.Y., G.T., H.F., and S.M. edited and revised
manuscript; S.M. approved final version of manuscript.

REFERENCES

1. Asahara T, Takahashi T, Masuda H, Kaika C, Chen D, Iwaguro H,
Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascular-
ization by mobilizing bone marrow-derived endothelial progenitor cells.
2. Besarab A, Bolton WK, Browne JK, Egrie JC, Nissenson AR, Okamoto
DM, Schwab SJ, Goodkin DA. The effects of norm as compared with
low hematocrit values in patients with cardiac disease who are
leucocytes adherence molecule-1: an inducible receptor for neutrophils
related to complement regulatory proteins and lectins. Science 243: 1160–
1165, 1989.
Cerami A, Brines M. Recombinant human erythropoietin protects the
myocardium from ischemia-reperfusion injury and promotes beneficial
5. Flynn DM, Buda AJ, Jeffords PR, Lefer DJ. A sialyl Lewis-x-containing
hydroxyethylamine reduces infarct size: role of selectins in myocardial reper-
6. Gabison A, Shmeeha H, Barenholz Y. Pharmacokinetics of pegylated
liposomal doxorubicin: view of animal and human studies. Clin Phar-
7. Gabizon A, Papahadjopoulos D. Liposome formation with prolonged
circulation time in blood and enhanced uptake by tumours. Proc Natl Acad
8. Hale SI, Setti C, Kloner RA. Administration of erythropoietin fails to
improve long-term healing or cardiac function after myocardial infarction
essential role of the antioxidant gene Bcl-2 in myocardial adaptation to
ischemia: an insight with antisense Bcl-2 therapy. Antioxid Redox Signal
10. Hausten DJ, Tsang A, Mocanu MM, Yellon DM. Ischemic precondi-
tioning protects by activating prosurvival kinases at reperfusion. Am J
Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S. Erythropoietin is a
potent physiologic stimulus for endothelial progenitor cell mobilization.
LIPOSOMAL ERYTHROPOIETIN PROTECTS THE HEART

H1133


