Basic Research in Cardiology
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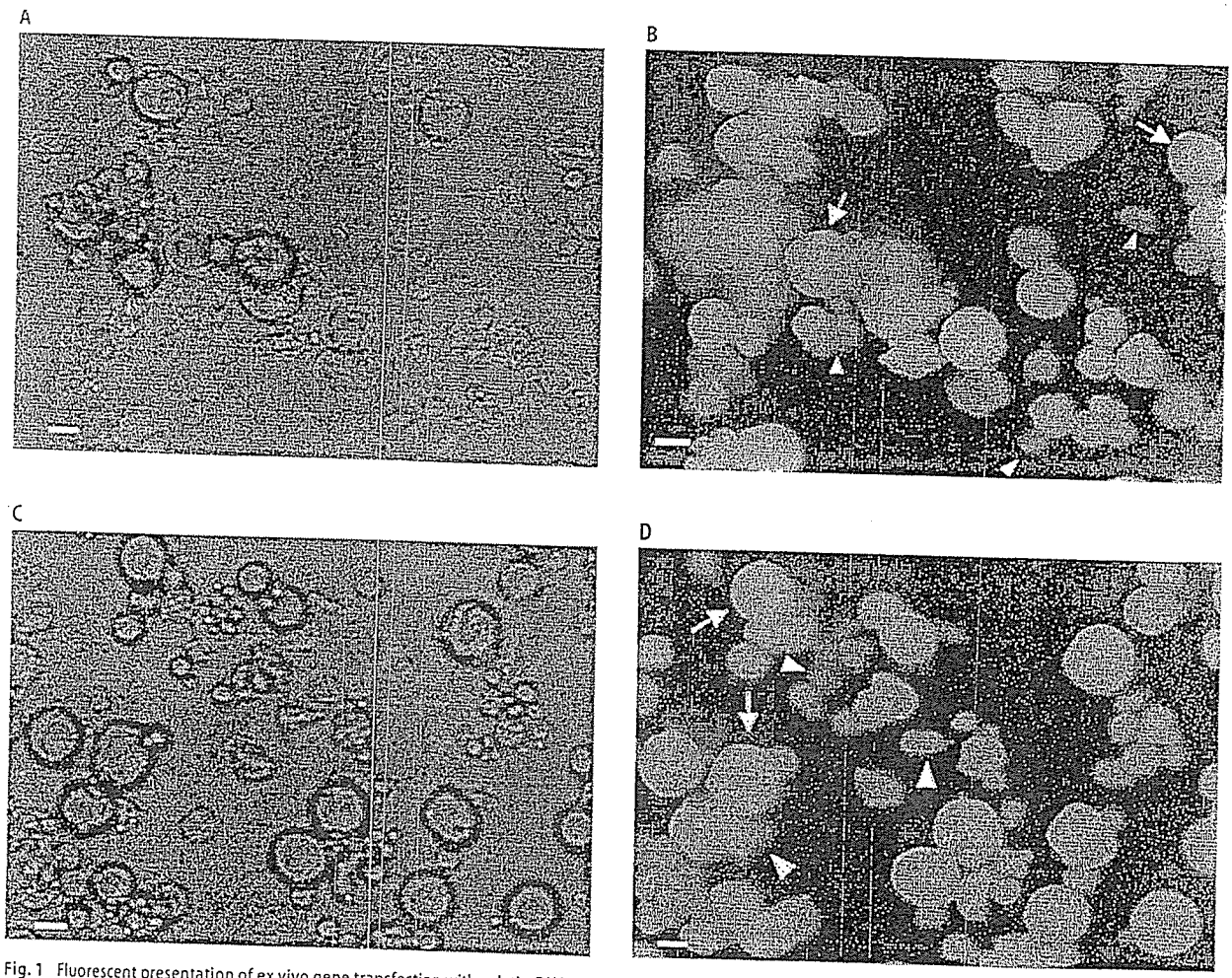

Fig. 1 Fluorescent presentation of ex vivo gene transfection with gelatin-DNA complex in macrophages/monocytes as well as fibroblasts. Rat macrophages (A and B) and human monocytes (C and D) were cultured with gelatin-GFP-gene complex for 14 days. Transmittance microscopic images (A and C) and fluorescence images (B and D) of the cells are shown. Macrophages (B) and monocytes (D) show fluorescence due to GFP. Arrowheads indicate GFP-expressing cells. Arrows indicate gelatin particles themselves. Bars = 20 μm

Organ distribution of phagocytes injected intravenously or directly into ischemic muscle

We studied quantitatively whether intravenously injected luciferase-gene-transfected phagocytes could target ischemic tissues (the third and fifth columns from the left in Table 1). In non-ischemic rats, the injected macrophages were recognized almost exclusively in the spleen (98 ± 4 %) (n = 7, the second column in Table 1). In non-ischemic mice, similar results were observed (n = 7, data not shown). In a rat with myocardial ischemia-reperfusion injury, some of the intravenously injected macrophages were incorporated into the heart (the third column in Table 1). The incorporation into the post-ischemic pericardium amounted to 13 ± 6 % (n = 7) (non-ischemic rats 0 ± 0 %, n = 7, Table 1). The incorporated cells expressed GFP (Fig. 3). Fibrosis with inflammatory infiltrates was recognized in the anterior wall of the left ventricle, extending to the interventricular septum (Figs. 3A and B). These infiltrates were mainly polymorphonuclear leukocytes and macrophages (Figs. 3C and D). Approximately 20 % of the macrophages showed GFP-positivity in this area (Figs. 3E and F). Similar tissue-targeting by intravenously injected monocytes was confirmed in a mouse model with hindlimb ischemia (13 ± 7 %, n = 7, the fifth column in Table 1). Furthermore, we studied whether local intramuscular injection increased the degree of tissue targeting (the fourth and sixth columns from the left in Table 1). After direct injection of phagocytes into ischemic muscle, 86 ± 10 % and 88 ± 6 % of the cells remained in the target tissue in the two models. Thirteen and 11 % of phagocytes in-

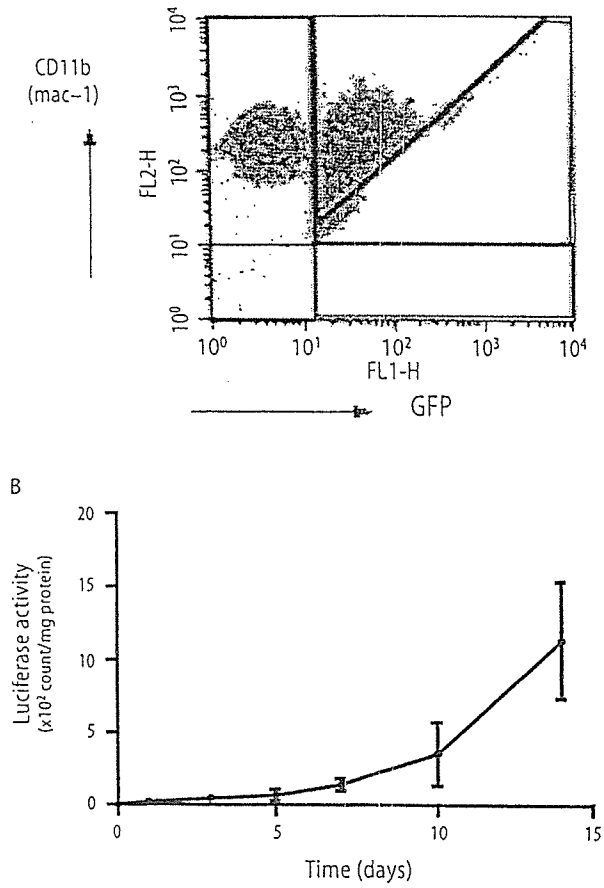

Fig. 2 Quantitative assessment of gene transfection into rat macrophages. (A) Fluorescence-activated cell sorting analysis of transfected macrophages done on day 14 of culture with reference to GFP-positive and Mac1-positive cells. (B) Sequential changes of luciferase activity in cultured macrophages in the presence of luciferase-gene-gelatin complex. Values are mean ± SD. The number of experiments is shown in parentheses

jected into the cardiac or hindlimb muscle migrated to the spleen. In the other organs, accumulation of phagocytes were negligible.

Amelioration of ischemia by intravenously injected angiogenic-gene-transfected phagocytes

In the rat model with myocardial ischemia-reperfusion injury, we studied the angiogenic effect of intravenously injected macrophages transfected with fibroblast growth factor 4 (FGF4) gene by using gelatin. Intravenous injection of these macrophages (1.0×10^6) significantly increased the regional blood flow in the ischemic myocardium (78 ± 7.1 %, n = 8, in terms of flow ratio of

Table 1 Organ distribution of phagocytes injected into the vein and into local tissue

Organ	Normal i.v. (7 rats)	Myocardial injury i.v. (7 rats)	Myocardial injury i.m. (7 rats)	Hindlimb ischemia i.v. (7 mice)	Hindlimb ischemia i.m. (7 mice)
Heart	0 ± 0	13 ± 6	86 ± 10	0 ± 0	0 ± 0
Hindlimb muscle	0 ± 0	0 ± 0	0 ± 0	13 ± 7	88 ± 6
Spleen	98 ± 4	84 ± 6	13 ± 10	84 ± 6	11 ± 6
Lung	1 ± 2	1 ± 1	1 ± 2	1 ± 2	1 ± 1
Liver	1 ± 2	1 ± 1	1 ± 1	1 ± 2	1 ± 1
Brain	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Kidney	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Intestine	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Each value shows a distribution ratio (%) into organs of transfected macrophages/monocytes (mean ± SD). *i.v.* intravenous injection into the vein; *i.m.* direct injection into the jeopardized muscle

ischemic/non-ischemic myocardium) compared with the other three treatments ($P < 0.05$, ANOVA), that is, intravenous administration of saline (35 ± 10 %, n = 8), intramuscular administration of naked DNA encoding FGF4 (50 μg, direct intramyocardial injection after thoracotomy) (58 ± 5.3 %, n = 8), and intravenous administration of the same number of non-transfected macrophages (42 ± 12 %, n = 8) (Fig. 4A). Histological analyses revealed angiogenesis in the ischemic tissue after the administration of transfected cells (Figs. 4B and C). Similar results were observed in the mouse model with hindlimb ischemia. Intravenous injection of FGF4-gene-transfected monocytes (1.0×10^6) enhanced regional blood flow in the ischemic leg (Fig. 4D). The increase of blood flow in the mice with transfected monocytes (93 ± 22 % in terms of flow ratio of ischemic/non-ischemic leg) was significantly larger than those obtained with the other three treatments described above (38 ± 12, 55 ± 12, and 39 ± 15 %, $P < 0.05$, ANOVA). Neither lymph node swelling in any part of the body nor pathologic change in the spleen or lung, such as angioma or abnormal immune response, was found in any of the animals.

Discussion

The advantages of the present method are as follows. First, genes can easily be transfected into phagocytes (macrophages/monocytes). In preliminary experiments, we found that genes can also be transfected into endothelial progenitor cells [25]. Compared with other transfection method, the transfection efficiency was high (68 ± 11 %) and it is not necessary to use a potentially hazardous viral vector [2, 26, 32]. Second, the phagocytes can target the pathologic tissues by chemotaxis even after intravenous injection, and higher tar-

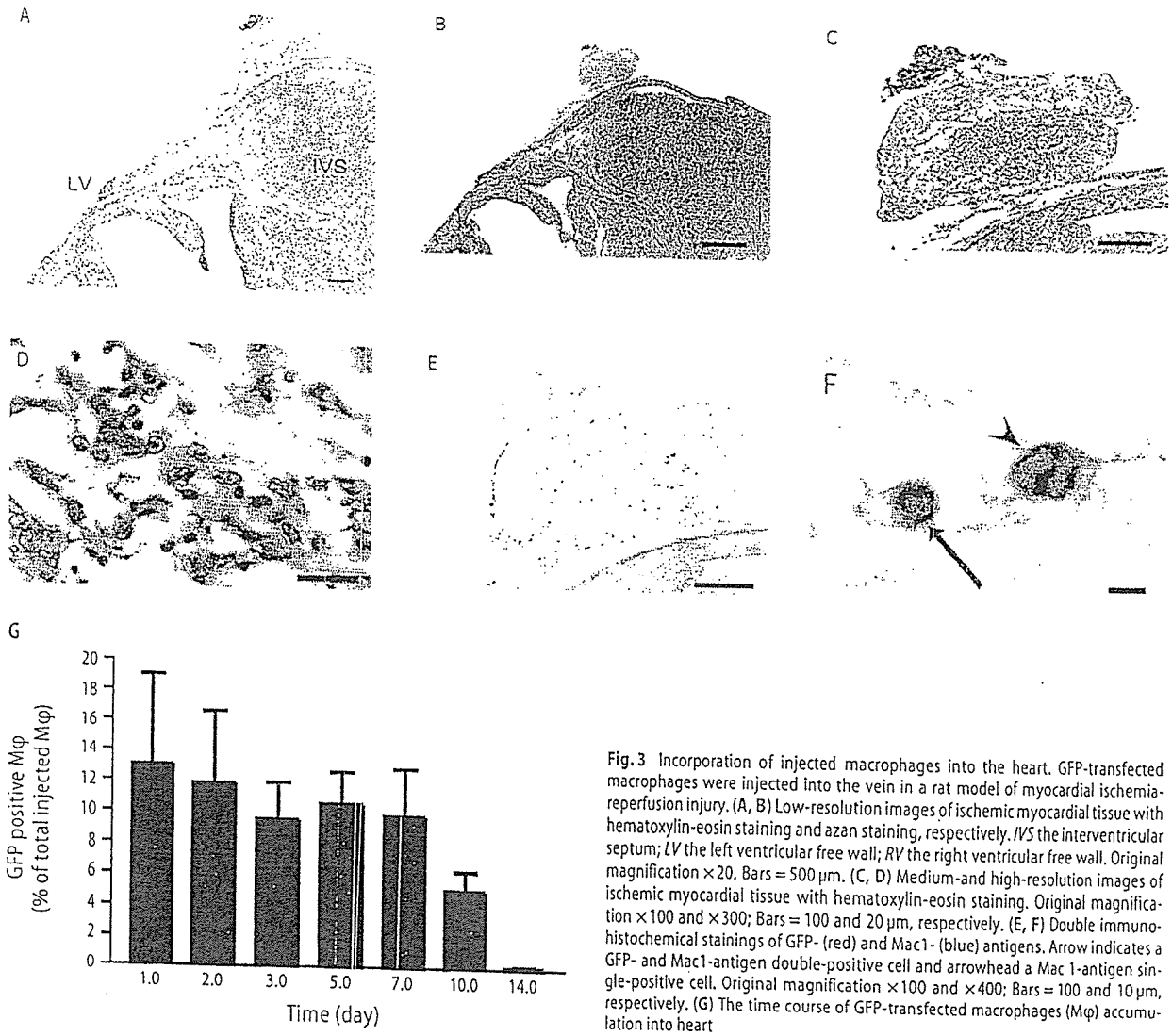

Fig. 3 Incorporation of injected macrophages into the heart. GFP-transfected macrophages were injected into the vein in a rat model of myocardial ischemia-reperfusion injury. (A, B) Low-resolution images of ischemic myocardial tissue with hematoxylin-eosin staining and azan staining, respectively. *IVS* the interventricular septum; *LV* the left ventricular free wall; *RV* the right ventricular free wall. Original magnification ×20. Bars = 500 μm. (C, D) Medium-and high-resolution images of ischemic myocardial tissue with hematoxylin-eosin staining. Original magnification ×100 and ×300; Bars = 100 and 20 μm, respectively. (E, F) Double immunohistochemical stainings of GFP- (red) and Mac1- (blue) antigens. Arrow indicates a GFP- and Mac1-antigen double-positive cell and arrowhead a Mac 1-antigen single-positive cell. Original magnification ×100 and ×400; Bars = 100 and 10 μm, respectively. (G) The time course of GFP-transfected macrophages (Mφ) accumulation into heart

geting is available if they are administered locally. The injection is repeatable. We confirmed that the angiogenic gene-transfected phagocytes enhanced angiogenesis after ischemia-reperfusion injury in rat heart and ameliorated ischemia in a mouse hindlimb model.

The injected phagocytes migrated into pathologic tissues, presumably in response to the release of cytokines such as monocyte chemoattractant protein 1 by injured endothelial cells [27]. Adhesion molecules such as P-selectin [28] are probably involved in the recruitment of phagocytes to the vessel wall. The injected phagocytes also migrated to the spleen, but no pathologic change was found in the spleen.

The present method has several advantages over conventional methods of cell-based gene therapy such as fibroblast-based and smooth muscle cell-based approaches [18, 19, 33, 34]. For example, monocytes do not aggregate in vessels, while fibroblasts or smooth muscle cells cannot be injected intravenously because of aggregation. The transfected phagocytes not only synthesize protein from the transfected gene, but also are partially targeted to the impaired tissue. In addition, the transfection rate was better than those of methods such as lipofection, viral vectors and electroporation [26, 29]. The newly developed technique of nucleofection has a transfection efficiency of 40–70 % [30], which is similar to that of our method, but our procedure is easier to use [30, 31]. Further, the therapeutic effect obtained here was superior to that of conventional gene therapy which we reported previously, i.e., intramuscular injection of

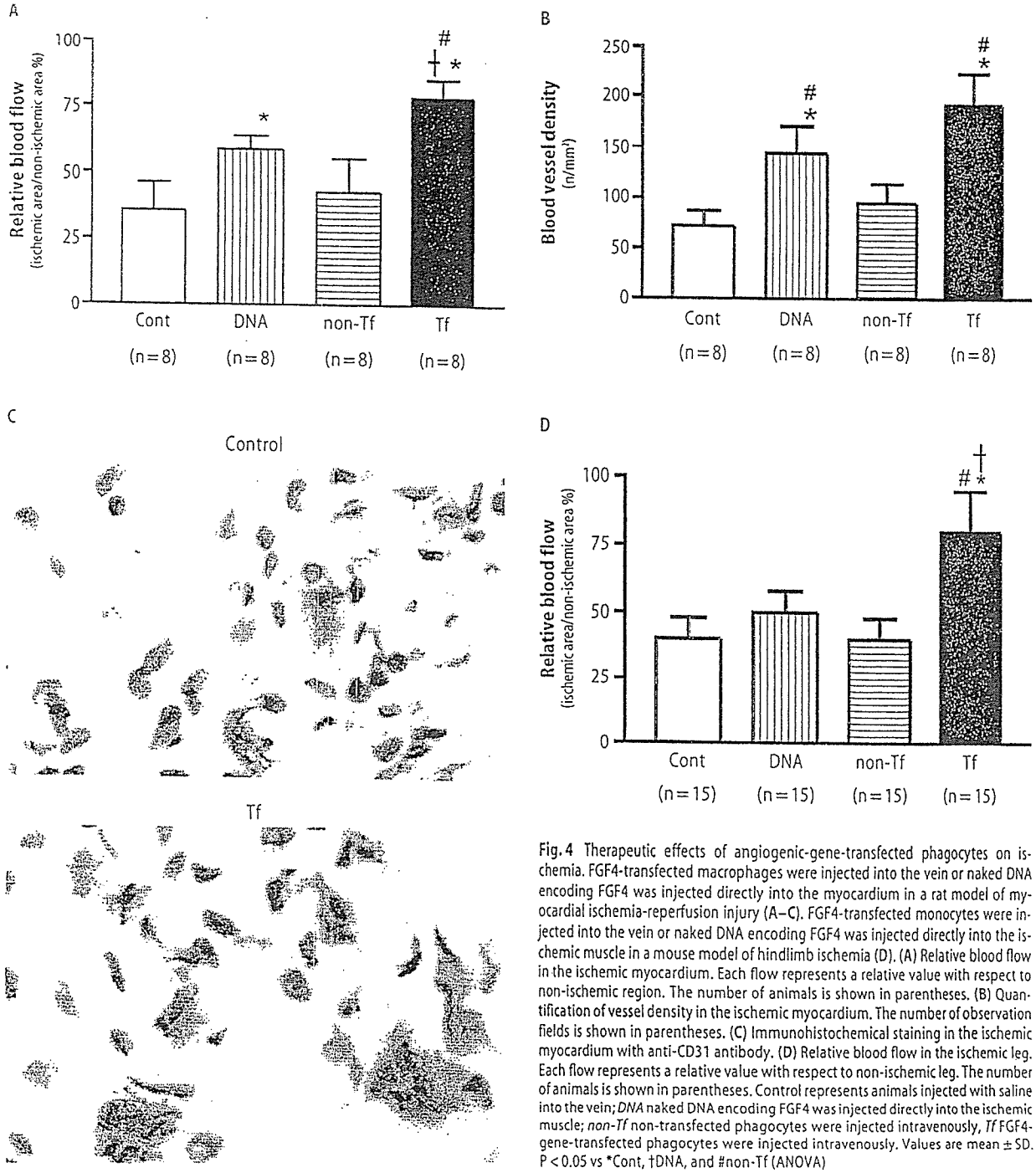

Fig. 4 Therapeutic effects of angiogenic-gene-transfected phagocytes on ischemia. FGF4-transfected macrophages were injected into the vein or naked DNA encoding FGF4 was injected directly into the myocardium in a rat model of myocardial ischemia-reperfusion injury (A–C). FGF4-transfected monocytes were injected into the vein or naked DNA encoding FGF4 was injected directly into the ischemic muscle in a mouse model of hindlimb ischemia (D). (A) Relative blood flow in the ischemic myocardium. Each flow represents a relative value with respect to non-ischemic region. The number of animals is shown in parentheses. (B) Quantification of vessel density in the ischemic myocardium. The number of observation fields is shown in parentheses. (C) Immunohistochemical staining in the ischemic myocardium with anti-CD31 antibody. (D) Relative blood flow in the ischemic leg. Each flow represents a relative value with respect to non-ischemic leg. The number of animals is shown in parentheses. Control represents animals injected with saline into the vein; *DNA* naked DNA encoding FGF4 was injected directly into the ischemic muscle; *non-Tf* non-transfected phagocytes were injected intravenously, *Tf* FGF4-gene-transfected phagocytes were injected intravenously. Values are mean ± SD. $P < 0.05$ vs *Cont, †DNA, and #non-Tf (ANOVA)

naked DNA, in ischemia models of heart and leg [17]. The major disadvantage of our method is the cell preparation time of 2 weeks before therapy can be started, and further work is needed to speed up this process.

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Search for appropriate experimental methods to create stable hind-limb ischemia in mouse

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Objective: Stable animal models for refractory peripheral arterial disease are not established. A standardized animal model of hind-limb ischemia is required upon searching effective treatment for this condition. The aim of the study is to verify previously used hind-limb ischemia models to find a standard method.
Methods: Using Balb/ca mice six various methods of inducing hind-limb ischemia were applied and two weeks after operation degree of ischemic damage were examined. Six methods include V group, A group, AV group, A-strip group, AV-strip group and Prox-A group (refer the text).
Results: Degree of ischemia was evaluated macroscopically by judging toes, foot, knee, and total hind-limb necrosis. We found that severity of damage was markedly different among different methods. Furthermore the severity of necrosis was not uniform even in the same method group.
Conclusions: The A-strip group in which the femoral artery from the bifurcation of the deep femoral artery to the saphenous artery was stripped appears to be suitable as a stable severe ischemia model. The A group in which the femoral artery were cut just below the bifurcation of the deep femoral artery appears to be suitable as a chronic mild ischemia model.

Key words: angiogenesis, animal model, blood vessels, femoral artery, hind-limb ischemia

INTRODUCTION

Refractory peripheral arterial disease is becoming an important therapeutic target since its incidence has been markedly increasing due to the increase in aged population and patients with diabetes mellitus. Consequently various therapeutic approaches including angiogenic treatment with growth factors or cell transplantation have been attempted using mouse hind-limb ischemia models [1-4]. Scrutinizing these studies we found that experimental methods to create hind-limb ischemia are not standardized. Method of occluding artery varies from ligation, or cutting, to excision of the artery. The targeted artery varies also from the iliac artery [1], the femoral artery [2-4], or the femoral with saphenous artery [5-9]. In some cases, both the femoral artery and vein were occluded. Strangling of the thigh itself was attempted in some studies [10, 11]. As the severity of ischemic damage cannot be uniform in different experimental methods, the comparison of effect among various therapies becomes very difficult.

Another important problem in previously used animal models is lack of data on blood flow when hind-limb is lost. In case of severe ischemic damage in mouse necrosis and loss of hind-limb often occur within three days but in patients of peripheral artery disease ischemia is chronic and acute necrosis is seldom seen. In these patients the improvement of blood flow is one of the key indices in determining effective treatments. Therefore it is mandatory to have an animal model in which chronic hind-limb ischemia is present but necrosis seldom occurs and sequential evaluation of blood flows is possible in order to evaluate various therapies.

In the present study, firstly we examined six methods for inducing hind-limb ischemia in Balb/ca mice and evaluated the severity of ischemic change to search for a stable severe ischemia model. Secondly we selected three methods with mild ischemic changes among six methods which do not produce severe necrosis and examined degree of ischemia by measuring CPK release, muscle weight, and histological changes to find an appropriate mild ischemia model.

MATERIALS AND METHODS

Animals

Seventy-five male Balb/ca mice (12 weeks old, 20 to 30 g, Japan Clea Inc, Ishibe) were used. All operations and measurements were performed under general anesthesia (1.0 to 1.5% isoflurane, 60% dinitrous monoxide, and 40% oxygen). The operation was performed, by only one investigator (T.G.), under a microscope (Konan Operation Microscope 707, Konan Keeler Co. LTD, Japan). To create ischemia, the vessels were cut or resected after ligation of the stumps with sterilized 6-0 silk suture (Azwel Inc, Osaka). The investigation conforms with The Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

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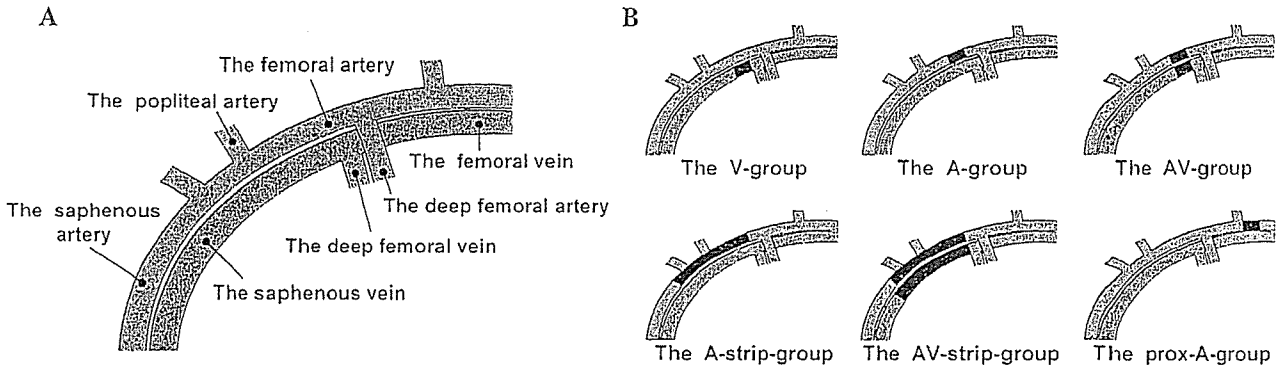

Fig. 1 (A) Schematic illustration of vascular anatomy in the mouse lower limb. (B) Schematic illustrations of surgical procedures in the six groups. The black bar indicates the cut or stripped sites of the vessels.

EXPERIMENTAL PROTOCOLS

1. The six hind limb ischemia models (n = 60)

The following six types of ischemia were created in the right lower limbs of 60 mice (schematic illustrations are presented in Fig. 1). These included, (1) cutting the femoral vein at the distal site of the bifurcation of the deep femoral vein (V-group, n = 10), (2) cutting the femoral artery just below the bifurcation of the deep femoral artery (A-group, n = 10), (3) cutting both the femoral artery and vein (AV-group, n = 10), (4) resection of the femoral artery from the distal site of the bifurcation of the deep femoral artery to the saphenous artery (A-strip-group, n = 10). By disecting the femoral artery as was shown in the Fig. 1B, branches including the popliteal artery were also obstracted and retrograde flows from these branches were completely avoidable. (5) resecting both the femoral artery and vein from the distal site of the bifurcation of the deep femoral artery to the saphenous artery (AV-strip-group, n = 10), and (6) cutting the femoral artery at the proximal site of the bifurcation of the deep femoral artery (Prox-A-group, n = 10).

2. Macroscopic evaluation of ischemic severity

Two weeks after the operation, the ischemic limb was macroscopically evaluated by using graded morphological scales for necrotic area; grade 0: absence of necrosis, grade I: necrosis limiting to toes (toes loss), grade II: necrosis extending to a dorsum pedis (foot loss), grade III: necrosis extending to a crus (knee loss), grade IV: necrosis extending to a thigh (total hind-limb loss).

3. Blood flow measurement

Calf blood flows on both sides were measured below a patella with a noncontact laser Doppler flowmeter (FLO-N1, Omegawave Corporation, Tokyo) before the operation, just after the operation, and two weeks post operatively, and were expressed as the ratio of the flow in the ischemic limb to that in the normal limb.

4. CPK release, muscle weights and histological evaluation in three mild ischemia groups

In additional mice of V-, A-, and AV-groups (n = 5 each) blood samples were obtained from the orbital plexus before the operation and 1, 2, and 7 days thereafter and concentrations of creatine phosphokinase (CPK) were measured. At two weeks after the operation, the animals were sacrificed under an overdose of sodium pentobarbital and the anterotibial, gastrocnemius, and soleus muscles were dissected out and weighed. Histological analysis (HE staining) was performed in each muscle [12].

Table 1 Degree of Ischemic Damage in 6 Groups.

Group \ Grade	0	I	II	III	IV
V group	10				
A group	7	3			
AV group	5	5			
A-strip-group	1	9			
AV-strip-group		6	3	1	
Prox-A-group		3			7

0: no change, I: toes necrosis, II: foot necrosis,
III: knee necrosis, IV: total necrosis

RESULTS

1) Severity of ischemic change in the six groups

Two weeks after the operation necrotic changes were macroscopically evaluated (Table 1). In the V-group, no macroscopic change was observed throughout the experimental period. In other 5 groups tissue necrosis appeared at either toes, dorsum pedis, crus or thigh. The position of necrosis was not uniform even in one group. Loss of total hind-limb was observed only in the Prox-A-group (7/10 mice). The order of necrosis severity among groups were Prox-A-group, AV-strip-group, A-strip-group, AV-group and A-group. The necrosis occurred as early as three days after the operation when it happens.

2) Changes in calf blood flow in the six groups

Just after the operation, calf blood flows were decreased to 30-35% of the pre-operative value without significant differences among the six groups except for the V-group (V-group: 98.5 ± 1.7, A-group: 34.1 ± 12.8, AV-group, 34.4 ± 9.9, A-strip-group: 31.4 ± 3.6, AV-strip-group: 32.9 ± 5.0 and Prox-A-group: 31.3 ±

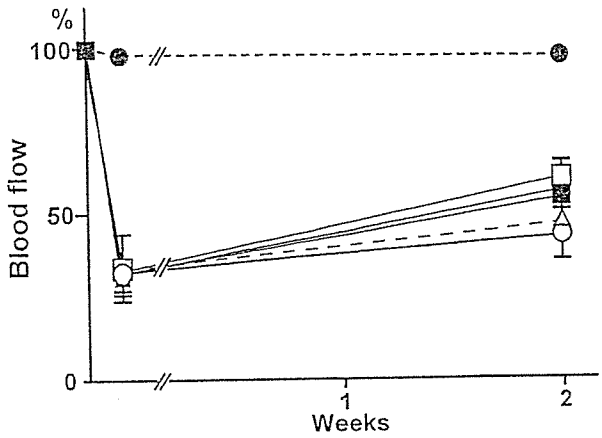

Fig. 2 Blood flow changes in 6 groups. Blood flow is express as a ratio of that in the ischemic limb to that in the opposite limb. V-group: closed circles, A-group: open circles, AV-group: open triangles, A-strip-group: closed triangles, AV-strip group: open squares, Prox-A-group: closed squares. Measurement of blood flows was available only in intact hind-limb or in stable data in case of toes necrosis.

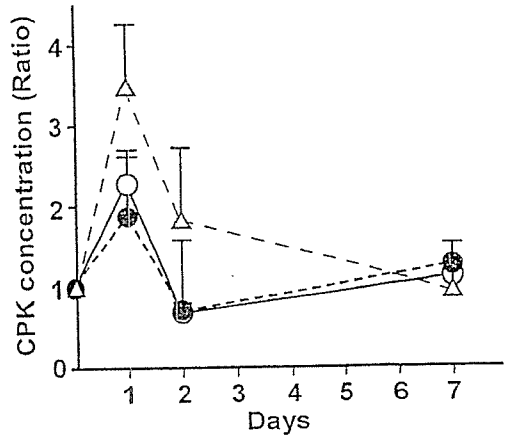

Fig. 3 Concentrations of serum creatine phosphokinase (CPK) in three groups. The concentration is expressed as the value relative to that before the operation. V-group: closed circles, A-group: open circles, AV-group: open triangles.

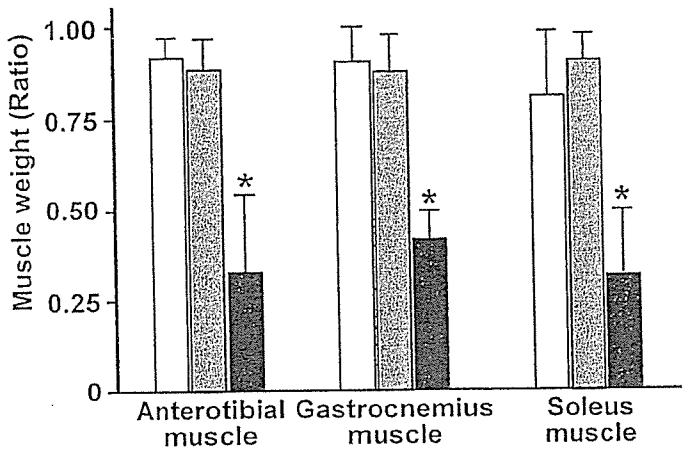

Fig. 4 Muscle weights in the ischemic limb at 14 days after the operation. The weights of the three muscles are expressed as the value relative to those of the same muscles in the opposite limb. V-group: open bar, A-group: grey bar, AV-group: black bar. ($p<0.05$ vs A- and V-groups, ANOVA).

6.5%, respectively, Fig. 2). Two weeks after the operation, calf blood flows had recovered to 45-55% of the pre-operative value (V-group: 97.8 ± 1.2, A-group: 44.7 ± 8.9, AV-group, 48.0 ± 9.0, A-strip-group: 54.0 ± 6.2, AV-strip-group: 57.0 ± 7.3 and Prox-A-group: 59.8 ± 5.1, respectively). The blood flow measurement was applicable only in mice in which calf was well preserved. We found difficulty to obtain stable calf blood flow in some mice with toes necrosis even though their calf was preserved and unstable measurements were omitted. Namely blood flow could be measured in 10 mice in the V group, 10 mice in the A group, 8 mice in the AV-group, 8 mice in the A-strip-group, 7 mice in the AV-strip-group, 3 mice in the Prox-A-group. At two weeks there were no significant differences among the six groups except for the V-group.

3) CPK release, muscle weights and histological evaluation in three mild ischemia groups

As we found that ischemic changes were too severe in A-strips, AV-strip and Prox-A-groups, we selected V-, A- and AV-groups as mild ischemia models. Blood CPK concentrations were significantly elevated one day after the operation in V-, A- and AV-groups ($p<0.05$ vs pre-operation, ANOVA, Fig. 3). Two days after the operation, CPK values had returned to baseline in the A- and V-groups but were still high in the AV-group ($p<0.05$ vs pre-operation, ANOVA). Seven days after the operation, CPK values had returned to baseline in all groups.

Two weeks after the operation, weight loss in the calf muscles was observed only in the AV-group. The values relative to those of the same muscles in the opposite limb were anterotibial: 0.32 ± 0.22, gastrocnemius: 0.42 ± 0.08, and soleus muscles: 0.32 ± 1.8 (Fig. 4). HE staining of the gastrocnemius muscle in the V-group disclosed that there was not obvious changes in the V-group compared with that in the normal limb (Figs. 5A and B). On the other hand, in the A- and AV-groups, microscopic features of necrotic changes such as cell size inhomogeneity, cellular wall degeneration, denucleation, and edema were observed (Figs. 5C

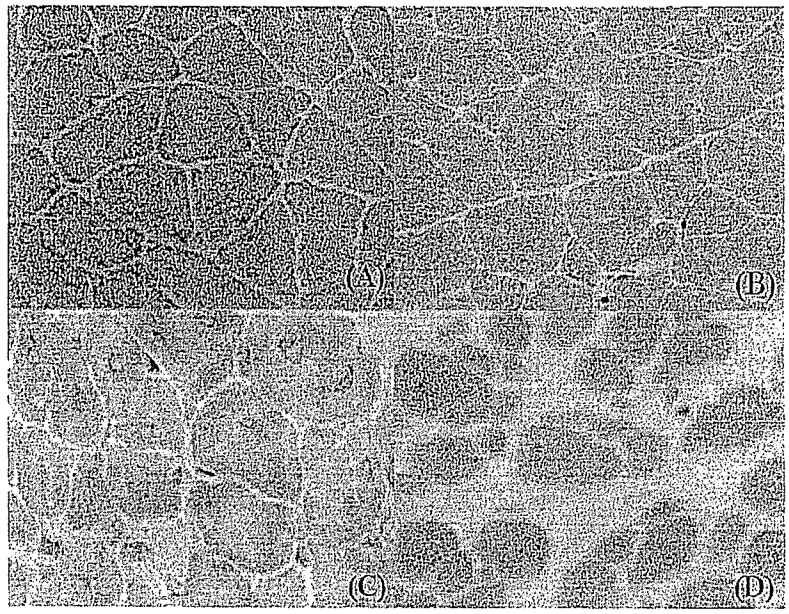

Fig. 5 There was not obvious changes in the V-group compared with that in the normal limb. In the A- and AV-groups, microscopic features of necrotic changes such as cell size inhomogeneity, cellular wall degeneration, denucleation, and edema were observed.

and D). These changes were more severe in the AV-group than in the A-group.

DISCUSSION

The mouse hind-limb has a well-developed innate collateral system and is shown to have remarkably high resistance to ischemia, e.g., a simple ligation of the femoral artery is known to produce no serve ischemic change [1]. This is because the hind-limb would be nourished by collateral circulation via the deep femoral artery and other branches (Fig. 1). As was shown in the Fig. 1A, there are several small branches including the popliteal artery and simple ligation of the femoral artery (A-group) results in obstruction of forward flow, but collateral flows via the deep femoral artery from these branches can retrogradely enter the distal portion of the femoral artery. In contrast, when the site of ligation is proximal, the deep femoral arterial flow will be also interrupted. Similarly in the A-strip group collateral flows from branches cannot enter the distal portion of the femoral artery. When site of ligation is proximal or includes several branches, collateral flow reduces and ischemic change becomes severe. However degree of ischemic damage would not be the same even in the site of ligation because richness and course of collateral vessels differ markedly among individual animals.

As was expected, this study revealed that the degree of ischemic damage varies markedly in six models of mouse hind-limb ischemia. Another important finding of this study is that even in the one model the ischemic damages were not uniform ranging from toes necrosis to knee necrosis as such in the AV-strip-group or from toes necrosis to total hind-limb necrosis in the Prox-A-group (Table 1). Only the A-strip-group showed relatively uniform ischemic damage: 9 toes necrosis and one no change.

On examining effect of various therapies including angiogenic treatments with growth factors or cell transplantation we have to use stable and uniform ischemic damage model, but previous studies used various degree of ischemic damage [1-9]. In this study we showed that A-strip method appears to be most appropriate as a mild ischemia model. The Prox-A method can be utilized when very severe ischemic damage model is necessary.

Regarding mild ischemia model simple ligation of the vein without treatment of the artery (V-group in protocol 2, Fig. 5B) produced only very weak edematous change and this is not an ischemic model. However, obstruction of venous return appears to have additional effects because damage became more serious in the AV-group than that in the A-group (Table 1 and Fig. 5). For example, edematous changes were seen by the histological analysis in the AV-group, and the AV-group showed muscle weight reduction in all anterotibial, gastrocnemius and soleus muscles (Fig. 5). Since venous obstruction is not associated with peripheral arterial disease, pathology under AV-group cannot be consistent with patients suffering from peripheral arterial disease. Although blood flow measurements were possible in both A- and AV-groups, severity of ischemic damage was relatively stable in the A-group, which resembles to the degree of ischemia seen in patients (7 mice showed no necrosis and three had toes necrosis). Taken together, the A-group appears to be the most suitable as a chronic mild ischemia model.

In conclusion, the relatively severe stable ischemia model is created by stripping the femoral artery from the distal site of the bifurcation of the deep femoral artery to the saphenous artery and the mild ischemic model should be made by cutting the femoral artery

just below the bifurcation of the deep femoral artery.

ACKNOWLEDGMENTS

The authors wish to thank Ms. Y Shinozaki for technical assistance. This work was supported by Grants-in-Aid for Scientific Research (15390066, 15659285, 16790761) from the MECSST; The Science Frontier Program of MECSST; Industrial Technology Research Grant Program in '03 from NEDO of Japan; The Research Grants for Cardiovascular Disease (H16C-6), Health and Labour Sciences Research Grants (nano-001, genome-005 and Saisei-003) from the MHLW; the Promotion Fundamental Studies in Health Science of the OPSR, Japan; Tokyo University of Agriculture Soken-Project Research Aid.

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PRECLINICAL STUDY

Erythropoietin Enhances Neovascularization of Ischemic Myocardium and Improves Left Ventricular Dysfunction After Myocardial Infarction in Dogs

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OBJECTIVES We investigated the effects of erythropoietin (EPO) on neovascularization and cardiac function after myocardial infarction (MI).

BACKGROUND Erythropoietin exerts antiapoptotic effects and mobilizes endothelial progenitor cells (EPCs).

METHODS We intravenously administered EPO (1,000 IU/kg) immediately [EPO(0) group], 6 h [EPO(6h) group], or 1 week [EPO(1wk) group] after the permanent ligation of the coronary artery in dogs. Control animals received saline immediately after the ligation.

RESULTS The infarct size 6 h after MI was significantly smaller in the EPO(0) group than in the control group (61.5 ± 6.0% vs. 22.9 ± 2.2%). One week after MI, the circulating CD34-positive mononuclear cell numbers in both the EPO(0) and the EPO(6h) groups were significantly higher than in the control group. In the ischemic region, the capillary density and myocardial blood flow 4 weeks after MI was significantly higher in both the EPO(0) and the EPO(6h) groups than in the control group. Four weeks after MI, left ventricular (LV) ejection fraction in the EPO(6h) (48.6 ± 1.9%) group was significantly higher than that in either the control (41.9 ± 0.9%) or the EPO(1wk) (42.6 ± 1.2%) group but significantly lower than that in the EPO(0) group (56.1 ± 2.3%). The LV end-diastolic pressure 4 weeks after MI in both the EPO(0) and the EPO(6h) groups was significantly lower than either the control or the EPO(1wk) group. Hematologic parameters did not differ among the groups.

CONCLUSIONS In addition to its acute infarct size-limiting effect, EPO enhances neovascularization, likely via EPC mobilization, and improves cardiac dysfunction in the chronic phase, although it has time-window limitations. (J Am Coll Cardiol 2006;48:176–84) © 2006 by the American College of Cardiology Foundation

Erythropoietin (EPO) is a cytokine that promotes proliferation and differentiation of erythroid precursor cells (1) and is widely used for the treatment of anemia in patients with chronic renal failure (2). Erythropoietin can also exert antiapoptotic and radical scavenger effects on nonerythroid cells (3,4). Indeed, we and others showed that an administration of EPO before or shortly after the onset of ischemia

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reduced myocardial infarct size and improved cardiac function in acute phases (5–8). Another interesting nonerythroid function of EPO is the promotion of endothelial progenitor cell (EPC) mobilization in animals and humans (9–11), which may enhance neovascularization of ischemic areas (12,13). We hypothesized that EPO increases blood supply to ischemic regions through promoting neovascularization and improves cardiac dysfunction after ischemic insult. Thus, the goal of this study was to characterize the effects of EPO on neovascularization and cardiac function after myocardial infarction (MI) in the chronic phase.

METHODS

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.

Instrumentation. Forty-seven beagle dogs (Kitayama Labes, Yoshiki Farm Gifu, Japan), weighing 8 to 12 kg were used in these experiments. After an intravenous injection of sodium pentobarbital (15 mg/kg), the dogs were intubated and ventilated. General anesthesia was maintained with 0.5% to 2.0% inhaled isoflurane. After baseline echocardiography and hemodynamic assessment, minimal thoracot-

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Abbreviations and Acronyms

ABP	= arterial mean blood pressure
Dil-ac-LDL	= 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein
EPC	= endothelial progenitor cell
EPO	= erythropoietin
HR	= heart rate
LAD	= left anterior descending coronary artery
LCX	= left circumflex coronary artery
LV	= left ventricle/ventricular
LVEDD	= left ventricular end-diastolic dimension
LVEDP	= left ventricular end-diastolic pressure
MBF	= myocardial blood flow
MI	= myocardial infarction
MNC	= mononuclear cell
UEA-I	= *Ulex europaeus* agglutinin I
VEGF	= vascular endothelial growth factor

omy was performed, and then the left anterior descending coronary artery (LAD) was ligated just distal to the first diagonal branch. To ensure that all animals included in the data analysis were exposed to a similar extent of ischemia, animals with excessive myocardial collateral blood flow (>15 ml/100 g/min) were excluded from study as previously described (14).

Experimental protocols. ACUTE EFFECTS OF EPO ON MYOCARDIAL INFARCT SIZE. Either a single dose of EPO (1,000 IU/kg; 5 ml) (n = 6) or the same volume of saline (n = 6) was administered intravenously immediately after the LAD ligation. Regional myocardial blood flow (MBF), area at risk, and infarct size at 6 h after the LAD ligation were determined as described previously (Fig. 1) (14). Recombinant human EPO was provided by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Recombinant human EPO is effective for correcting anemia in the beagle dog (15).

EFFECTS OF IMMEDIATE OR DELAYED TREATMENT WITH EPO ON NEOVASCULARIZATION AND CARDIAC FUNCTION. A single dose of EPO (1,000 IU/kg; 5 ml) was administered intravenously immediately [EPO(0) group, n = 8], 6 h [EPO(6h) group, n = 8], or 1 week [EPO(1wk) group, n = 7] after the LAD ligation. Control animals received the same volume of saline (control group, n = 8) immediately after the LAD ligation.

Hematologic parameters. Blood was sampled from a peripheral vein under pentobarbital (15 mg/kg) anesthesia at the time points indicated in Figure 2. Hematologic parameters, including hematocrit, white blood cell count, and platelet count, were measured.

Cytokine measurements. Plasma levels of vascular endothelial growth factor (VEGF) were measured by enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, Minnesota). The detection limit of the assays was 9 pg/ml. The reliability of this assay in dogs has already been reported previously (16).

Quantification of CD34-positive mononuclear cells. The circulating CD34-positive mononuclear cells (CD34+MNCs) were quantified at the time points indicated in Figure 2. In brief, peripheral white blood cells were stained with a phycoerythrin-conjugated anticanine CD34 monoclonal antibody (BD Pharmingen, San Diego, California). Samples were then subjected to a two-dimensional side-scatter-fluorescence dot plot analysis (FACScan, Becton-Dickinson, Tokyo, Japan). After appropriate gating of

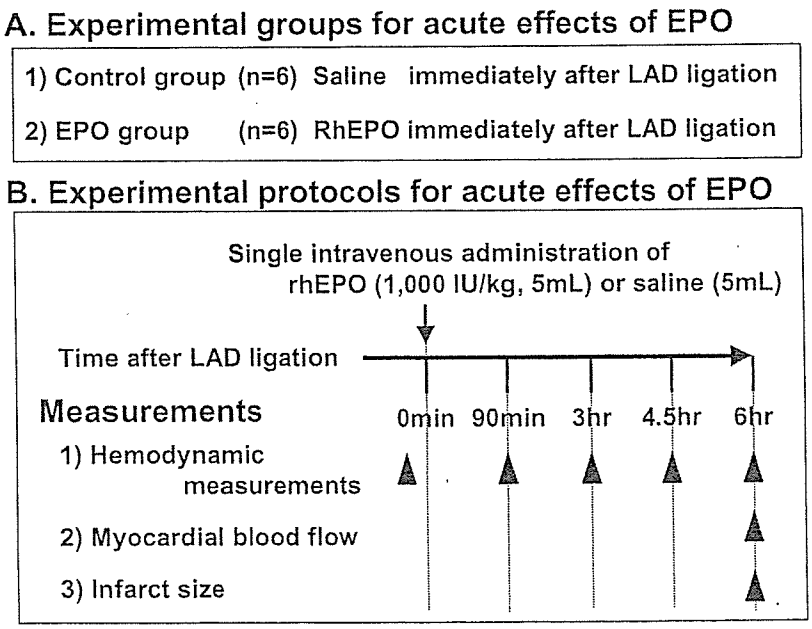

Figure 1. Experimental protocols to investigate acute effects of erythropoietin (EPO) on myocardial infarct size. LAD = left anterior descending coronary artery; RhEPO = recombinant human erythropoietin.

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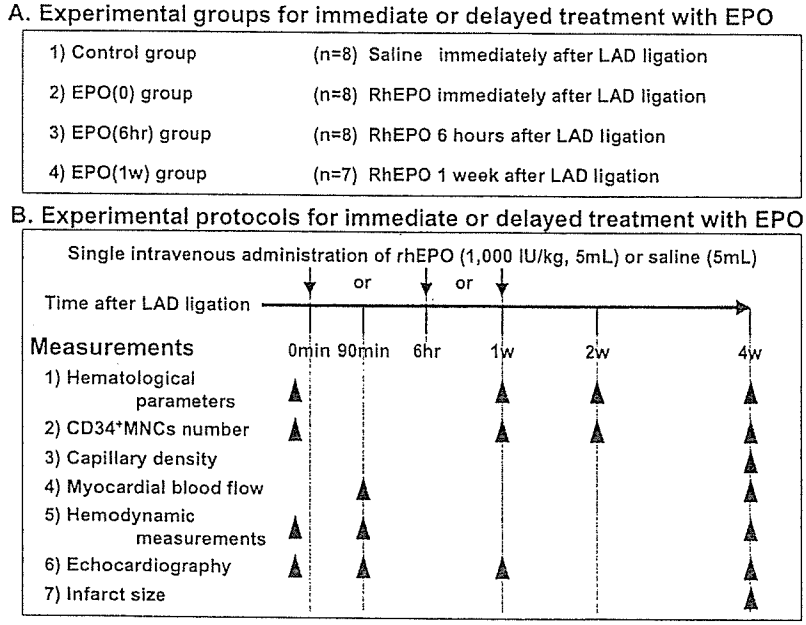

Figure 2. Experimental protocols to investigate effects of immediate or delayed treatment with erythropoietin (EPO) on neovascularization and cardiac function. CD34+MNC = CD34-positive mononuclear cell; other abbreviations as in Figure 1.

MNCs, the number of CD34+MNCs with low cytoplasmic granularity (low sideward scatter) was quantified and expressed as the number of cells per 1-μl blood sample.

In vitro MNC culture assay. Circulating MNCs were isolated from blood (10 ml) of dogs at baseline and 1 week after MI in the control and EPO(0) groups (n = 4 each) by Ficoll density-gradient centrifugation. After MNCs (107 per well) were plated in Medium 199 (Gibco, Grand Island, New York) supplemented with 20% fetal calf serum and antibiotics on human fibronectin-coated six-well dishes. After 7 days in culture, adherent cells were stained for the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-ac-LDL) (Biomedical Technologies, Stoughton, Massachusetts) and the binding of fluorescein isothiocyanate-labeled *Ulex europaeus* agglutinin I (UEA-I) (Vector Laboratories, Peterborough, England). Double-staining cells were quantified by examining five random microscopic fields (×200 power) (10,11).

Histologic assessments. Four weeks after MI, myocardial tissue was sampled from both ischemic (LAD) and non-ischemic (left circumflex coronary artery [LCX]) regions in each group. The tissues in the ischemic region were identified as the edge of the region showing necrosis. These samples were then fixed in 10% buffered formalin, embedded in paraffin, and serially sectioned in the frontal plane at 5-μm thickness. Endothelial cells were immunohistologically stained using rabbit antihuman von Willebrand factor antibody (Dako, Kyoto, Japan) and the Envision+/HRP Kit (Dako) (17). The peroxidase was visualized by incubation with 3,3'-diaminobenzidine, followed by incubation with a DAB-enhancing solution (Dako). We counted the numbers of capillaries and cardiomyocytes in 20 random high-power fields (×400 power), and then calculated the average capillary density and capillary-to-myocyte ratio (18).

Measurements of regional MBF. Regional MBF was determined as described previously (19). Nonradioactive microspheres (Sekisui Plastic Co., Tokyo, Japan) made of inert plastic were labeled with bromine or niobium. Microspheres were administered at 90 min and 4 weeks after MI. The MBF in the LAD region was calculated according to the following formula: time flow = (tissue count) × (reference flow)/(reference count), and was expressed in ml/g wet weight/min.

Hemodynamic measurements. Hemodynamic parameters, such as arterial mean blood pressure (ABP), heart rate (HR), and left ventricular end-diastolic pressure (LVEDP), were measured at the time points indicated in Figure 2. A 5-F sidearm sheath (Radifocus, Terumo, Tokyo, Japan) was placed in the right femoral artery for hemodynamic measurements. A 4-F pigtail catheter (Outlook, Terumo) was placed in the LV for measurement of LVEDP and was connected to a pressure transducer (model DX-200, Nihon Kohden, Tokyo, Japan). The ABP and HR were monitored via the 5-F sidearm sheath.

Echocardiography. Cardiac function was assessed by echocardiography (Sonos 5500, S4-probe, 2-4 MHz, Philips, Bothell, Washington) at the time points indicated in Figure 2. Short-axis views were recorded at the level of midpapillary muscles, and two-dimensional and M-mode views were recorded at the same level. Measurements of left ventricular end-diastolic dimension (LVEDD) and LV ejection fraction were obtained from M-mode views. All measurements were made by one observer, who was blinded with respect to the identity of the tracings.

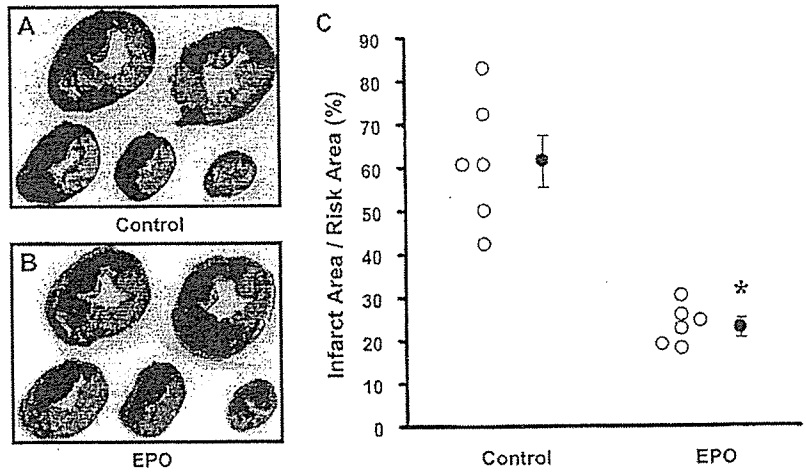

Figure 3. Representative left ventricular cross sections at 6 h after myocardial infarction (MI) in dogs with (B) and without (A) erythropoietin (EPO) treatment. (C) Infarct size at 6 h after MI. *p < 0.05 vs. the control group. Open circles = infarct size in each animal.

Infarct size 4 weeks after MI. Myocardial infarct area was determined at the end of the protocol by triphenyltetrazolium chloride staining as described previously (14). Infarct size was expressed as a percentage of the total LV area.

Statistical analysis. Results are expressed as the mean ± standard error of the mean. Comparisons of the time course of the change between groups were performed using two-way repeated measures analysis of variance. Comparisons of other data between groups were performed using one-way factional analysis of variance. If statistical significance was found for a group, a time effect, or a group-by-time interaction, further comparisons were made with paired *t* tests between all possible pairs of four groups at individual time points. The Bonferroni-Holm procedure was used for correction of multiple comparisons. A p value <0.05 was considered to represent statistical significance (20).

RESULTS

Exclusion. Four dogs [acute effects protocol; control: 1, EPO: 0, delayed treatment effects protocol; control: 1, EPO(0): 1, EPO(6h): 0, EPO(1wk): 1] were excluded from analysis because of excessive regional MBF (>15 ml/100 g/min). Thus, 12 and 31 dogs in acute and delayed EPO treatment protocols, respectively, were included.

Acute effects of EPO on infarct size. Myocardial infarct size was significantly smaller in animals receiving EPO compared with those that received saline, but there was no significant difference in regional MBF (9.0 ± 1.0 ml/100 g/min vs. 8.5 ± 1.2 ml/100 g/min) or area at risk (42.9 ± 2.3% vs. 42.3 ± 0.9%) when comparing the two groups (Fig. 3).

Effects of EPO on hematologic parameters. The average change in hematologic parameters was not different when comparing the three EPO-treated groups and the control group over the 4-week experimental protocol (Table 1).

Plasma VEGF levels. Table 2 shows the time course of changes in plasma VEGF level after MI. The plasma VEGF level was significantly and comparably elevated in both control and EPO(0) groups, peaking on 6 h after MI, and returned to baseline at 1 week after MI.

Circulating CD34+MNCs and in vitro cultured MNCs. Figure 4A shows the time course of changes in circulating CD34+MNC number in the different groups. One week after MI, the number of circulating CD34+MNCs increased in all groups. Furthermore, the number of circulating CD34+MNCs at 1 week after MI was higher in the EPO(0) and EPO(6h) groups than in either control or EPO(1wk) group. Two weeks after MI, the number of CD34+MNCs in the control group returned to the baseline. By contrast, the number of CD34+MNCs in the EPO(0) and EPO(6h) groups also decreased but still remained higher than those in either the control or

Table 1. Time Course of Changes in Hematologic Parameters

Parameters	Baseline	1 Week	2 Weeks	4 Weeks
Hematocrit (%)				
Control	52.9 ± 1.7	47.0 ± 1.6	48.9 ± 2.3	53.1 ± 1.8
EPO(0)	52.4 ± 1.1	48.2 ± 1.2	47.9 ± 1.4	53.4 ± 0.7
EPO(6h)	51.5 ± 1.6	49.3 ± 1.6	51.4 ± 1.1	51.3 ± 2.3
EPO(1wk)	48.9 ± 1.0	46.4 ± 1.1	49.4 ± 0.5	50.1 ± 1.0
WBC ($10^3/\mu l$)				
Control	13.8 ± 0.4	15.4 ± 1.4	15.3 ± 0.9	13.5 ± 0.9
EPO(0)	12.6 ± 0.6	14.0 ± 1.1	14.4 ± 0.3	12.3 ± 1.4
EPO(6h)	12.6 ± 0.8	15.6 ± 1.1	13.9 ± 1.0	12.0 ± 0.8
EPO(1wk)	13.1 ± 0.8	14.8 ± 1.2	13.3 ± 0.4	12.9 ± 0.8
Platelet ($10^4/mm^3$)				
Control	27.3 ± 2.0	26.5 ± 1.9	28.4 ± 1.2	26.2 ± 2.0
EPO(0)	28.5 ± 2.0	26.8 ± 4.3	27.0 ± 3.4	28.2 ± 1.8
EPO(6h)	26.9 ± 0.9	27.0 ± 1.4	26.1 ± 1.8	26.1 ± 1.5

Data are presented as mean ± SEM (n = 7 to 8).
EPO = erythropoietin; WBC = white blood cell.

Table 2. Time Course of Changes in Plasma VEGF Levels

Groups	n	Baseline	6 Hours	1 Week	2 Weeks
VEGF (pg/ml)					
Control	4	<9.0	22.5 ± 3.3*	<9.0	<9.0
EPO(0)	4	<9.0	21.6 ± 5.0*	<9.0	<9.0

Data are presented as mean ± SEM. *p < 0.05 vs. baseline.
EPO = erythropoietin; VEGF = vascular endothelial growth factor.

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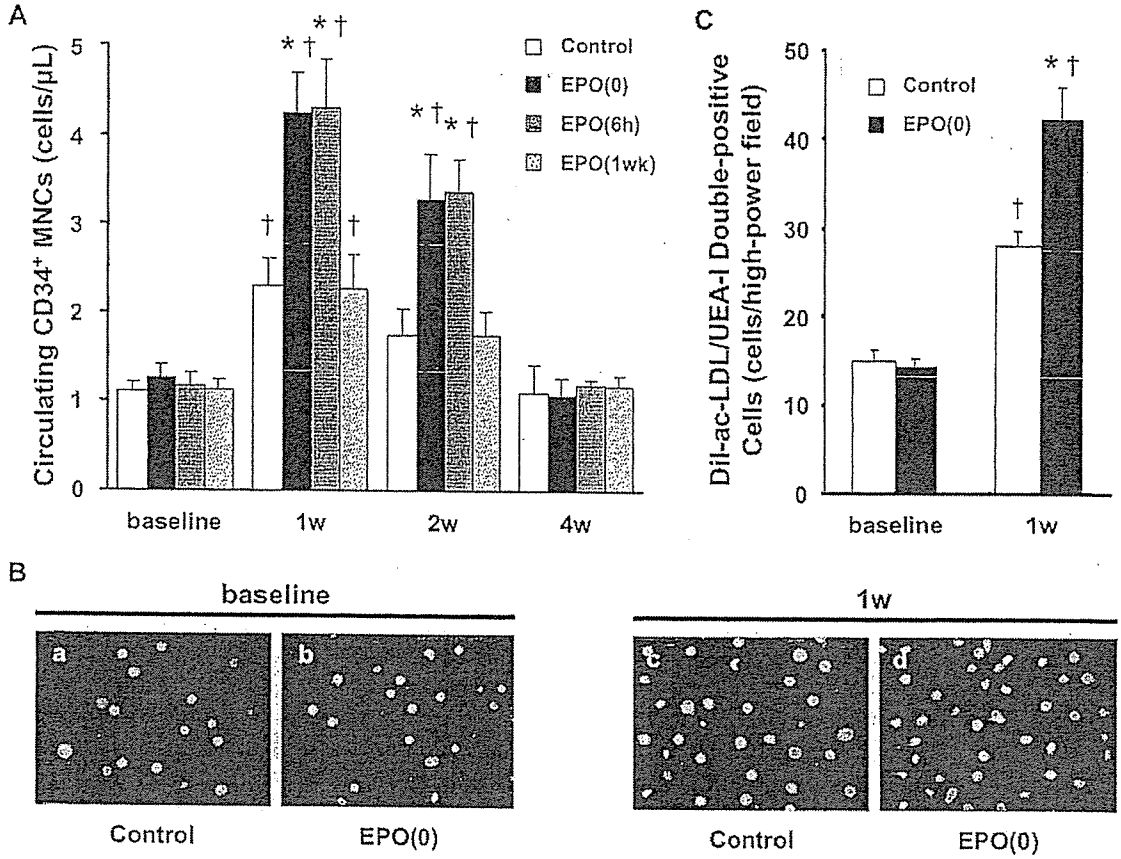

Figure 4. (A) Time course of changes in circulating CD34+MNC count after left anterior descending coronary artery (LAD) ligation in different experimental groups. (B) Representative images of double-stained cultured cells (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein [Dil-ac-LDL] and *Ulex europaeus* agglutinin I [UEA-I]) at baseline (a, b) and 1 week after LAD ligation (c, d) from dogs with and without erythropoietin (EPO) treatment immediately after LAD ligation. (C) Quantitative analysis of endothelial progenitor cell culture assay. *$p < 0.05$ vs. the control group. †$p < 0.05$ vs. baseline.

EPO(1wk) group. Furthermore, the administration of EPO 1 week after the LAD ligation did not affect the number of CD34+MNCs at any given time point.

In the culture assay of MNCs, the number of Dil-ac-LDL/UEA-I double-positive cells obtained from blood 1 week after MI increased compared with that at baseline in both control and EPO(0) groups. Importantly, the double-positive cell number obtained from blood 1 week after MI in the EPO(0) group was significantly higher than in the control group (Figs. 4B and 4C).

Capillary density and regional MBF. Figure 5A shows the representative immunohistologic findings in the non-ischemic (panels a to d) and ischemic (panels e to h) regions at 4 weeks after MI. In the nonischemic region, there was no difference in the capillary density and capillary-to-myocyte ratio when comparing groups. In the ischemic region, the capillary-to-myocyte ratio as well as capillary density was significantly higher in the EPO(0) and EPO(6h) groups, but not in the EPO(1wk) group, than in the control group (Figs. 5B to 5C).

Figure 6 shows the changes in regional MBF in the ischemic regions in different experimental groups. There was no significant difference in MBF at 90 min when comparing experimental groups. At 4 weeks after MI, MBF was more increased in the EPO(0) and EPO(6h) groups, but not in the EPO(1wk) group, than in the control group.

Effects of immediate or delayed EPO treatment on cardiac function and infarct size. Throughout the experimental protocols, there was no difference in either ABP or HR when comparing the groups (Table 3).

Figure 7 shows the time course of changes in LVEF (panel A), LVEDD (panel B), and LVEDP (panel C) in different experimental groups. There were no significant differences in baseline LVEF, LVEDD, and LVEDP when comparing the groups.

Ninety minutes, 1 week, and 4 weeks after MI, LVEF was higher in the EPO(0) group than in the other groups. Ninety minutes and 1 week after MI, there was no difference in LVEF when comparing the EPO(6h) group and the control group. When comparing the time points of 1 week and 4 weeks after MI, LVEF decreased in the control and the EPO(1wk) groups but not in the EPO(6h) group. One and 4 weeks after MI, LVEDD was lower in the EPO(0) group than in the other groups. When comparing the time points of 1 week and 4 weeks after MI, LVEDD increased in the control and EPO(1wk) groups but not in the EPO(6h) group. Ninety minutes after MI, LVEDP was lower in the

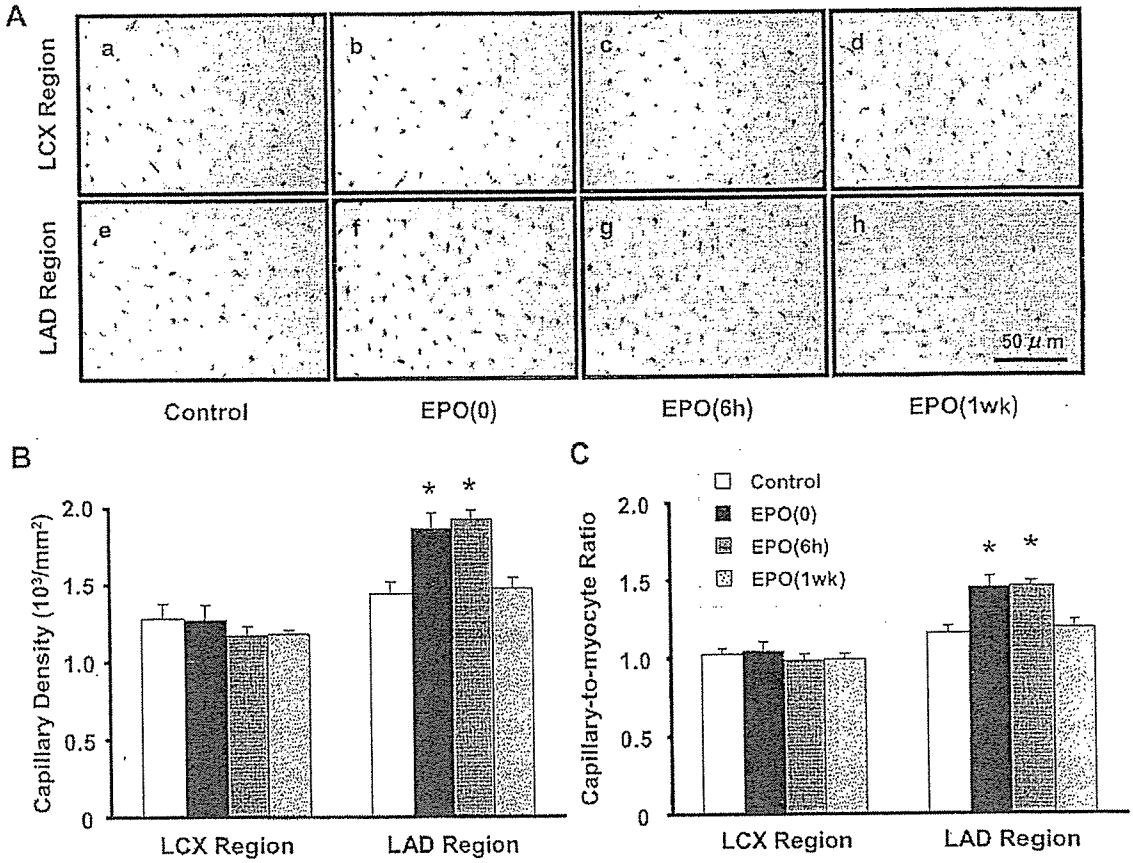

Figure 5. (A) Representative immunohistologic staining with an antibody against von Willebrand factor in nonischemic (left circumflex coronary artery [LCX]) (a, b, c, d) and ischemic (left anterior descending coronary artery [LAD]) (e, f, g, h) regions in different experimental groups. Capillary density (B) and capillary-to-myocyte ratio (C) of nonischemic (LCX) and ischemic (LAD) regions in different experimental groups. *$p < 0.05$ versus the control group. Abbreviations as in Figure 1.

EPO(0) group than in the other groups. Four weeks after MI, LVEDP was lower in the EPO(0) and EPO(6h) groups than in either the control or the EPO(1wk) group.

Myocardial infarct size 4 weeks after MI was smaller in the EPO(0) group than in the control group, although EPO treatment, initiated 6 h and 1 week after MI, did not reduce infarct size (Fig. 7D).

DISCUSSION

The present study showed that EPO administered 6 h after LAD ligation increased circulating CD34+MNCs, capillary density, MBF in the ischemic region, and prevented the worsening of cardiac function without reducing infarct size. The EPO enhances neovascularization, likely via EPC mobilization, and improves cardiac dysfunction in the chronic phase, although EPO has time-window limitations.

We showed that the EPO treatment immediately after the LAD ligation reduced infarct size, which is consistent with observations of previous reports (5–8). Because the infarct size-limiting effects of EPO appear rapidly, the nonerythroid effects of EPO, such as antiapoptosis and radical scavenging (4–8), may contribute to the reduction of infarct size.

Recent reports have shown that circulating CD34+MNC count correlated with EPC number in MNCs culture assay, and both increased at 1 to 2 weeks after EPO administration in animals and humans (9–11). In the culture assay, the number of Dil-ac-LDL/UEA-I double-positive cells obtained from blood at baseline did not differ between the two groups. The number of double-positive cells obtained from blood at 1 week after MI significantly increased compared with that at baseline in the control and EPO(0) groups. Further, the double-positive cell number obtained from blood in the EPO(0) group was higher than in the control group. These findings suggest that EPO augments increases in the number of MNCs that can differentiate into Dil-ac-LDL/UEA-I double-positive cells, an indicator of endothelial cells. Increases in the number of both CD34-positive cells and Dil-ac-LDL/UEA-I double-positive cells strongly suggest that EPO promotes EPC mobilization. The number of CD34+MNCs increased 1 week after MI in the canine model, which is consistent with observations from studies of patients with acute MI (21,22). Furthermore, the number of CD34+MNCs was higher in the EPO(0) and EPO(6h) groups than in the control group. This finding suggests that a single dose of EPO was effective in increasing the number of circulating EPCs after MI. Interestingly,

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EPO administered 1 week after MI failed to produce the identical effect, suggesting that EPO has a time window for promotion of EPC mobilization. We found that plasma VEGF levels were elevated, peaking at 6 h after MI and returned to the baseline 1 week after MI. The EPO did not affect plasma VEGF levels. Because both VEGF and EPO are known to promote EPC mobilization in experimental conditions and are independent predictors for the number of circulating EPCs in patients with coronary heart disease (9–11,23), they may additionally or synergistically contribute to EPC mobilization. Thus, it is likely that EPO alone, at least at the dose used in the present study, might not be enough to promote CD34+MNC mobilization 1 week after MI when VEGF returns to the baseline. Although we only investigated the low dose of EPO to consider the clinical implication, it is possible that high doses of EPO would show the different results. Further investigations are needed to clarify the mechanism of EPO-stimulated EPC mobilization.

The present study also showed that EPO increased capillary-to-myocyte ratio corrected for LV hypertrophy as well as capillary density in the EPO(0) and EPO(6h) groups, suggesting that EPO promotes the neovascularization in the ischemic region. Investigators have also reported that EPO enhances neovascularization in the ischemic region in the hind-limb occlusion model (9). As suggested in the present study, EPO may enhance neovascularization via EPC mobilization. Indeed, bone marrow-derived EPCs incorporate into foci of neovascularization at the border zone of MI (12,13), and administration of ex vivo-expanded EPCs resulted in increased myocardial neovascularization (24,25). In a rat stroke model, Wang et al. (26) showed that EPO treatment, initiated 24 h after MI, enhances angiogenesis. In addition, van der Meer et al. (27) showed that capillary density was increased in the rat post-MI model even when EPO was administered 3 weeks after MI. In contrast, we showed that EPO administered 1 week after MI failed to increase capillary density. The possible explanation for this discrepancy is attributable to the different doses of EPO used. In the studies by Wang et al. (26) (5,000 IU/kg for 7 days) and van der Meer et al. (27) (8,000 IU/kg every 3 weeks), relatively high doses of EPO were administered. In contrast, in the present study, a relatively low dose (1,000 IU/kg) of EPO was administered with a single injection, and the reason for this dose in the present study is for the possible translation of our results to clinical settings more easily (6), because 8,000 or 5,000 IU/kg EPO may cause side effects. On the other hand, we noticed that a higher dose of EPO would increase capillary density and improve the cardiac function even by the late administration of EPO for clinical use.

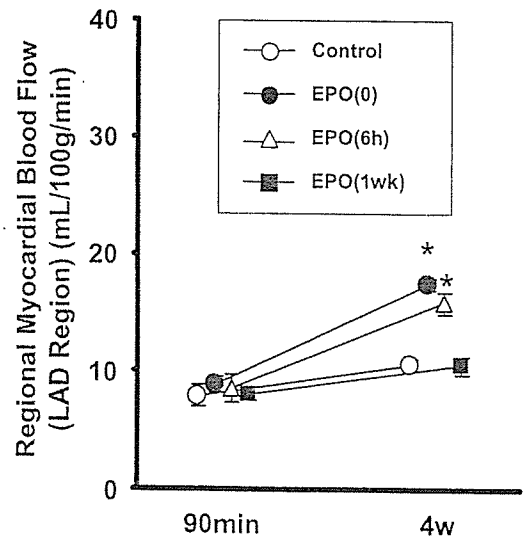

Figure 6. Regional myocardial blood flow in the ischemic (left anterior descending coronary artery [LAD]) region 90 min and 4 weeks after myocardial infarction in different experimental groups. *$p < 0.05$ versus the control group. EPO = erythropoietin.

Table 3. Time Course of Changes in Hemodynamic Parameters

Parameters	Baseline	90 Min	4 Weeks
ABP (mm Hg)			
Control	99 ± 3	101 ± 3	103 ± 2
EPO(0)	102 ± 3	99 ± 3	102 ± 2
EPO(6h)	101 ± 1	98 ± 2	101 ± 1
EPO(1wk)	102 ± 2	102 ± 3	103 ± 2
HR (per min)			
Control	131 ± 6	135 ± 6	129 ± 6
EPO(0)	128 ± 6	131 ± 3	131 ± 5
EPO(6h)	130 ± 7	135 ± 7	126 ± 6
EPO(1wk)	128 ± 6	128 ± 3	126 ± 6

Data are presented as mean ± SEM (n = 7 to 8).
ABP = arterial mean blood pressure; EPO = erythropoietin; HR = heart rate.

In the present study, MBF in the ischemic region was increased in both the EPO(0) and the EPO(6h) groups. Because neovascularization was also enhanced in these groups, increased MBF may occur secondary to the enhanced neovascularization.

The present study also showed that an administration of EPO immediately after the LAD ligation improved cardiac function at 90 min after MI, likely because of infarct size reduction, and subsequently prevented the development of cardiac dysfunction in the chronic phase. Because the previous reports showed that myocardial necrosis progresses within 6 h after the onset of MI (28,29), EPO was administered at time points of 6 h and later after LAD ligation to determine whether its activity is directed toward the acute phase of MI or the chronic phase of cardiac dysfunction. One week after MI, LVEF, LVEDD, or LVEDP was similar among the EPO(6h), EPO(1wk), and control groups. However, EPO administered 6 h, but not 1 week, after the LAD ligation improved cardiac dysfunction 4 weeks after MI when compared with the control group. Because we did not find any difference in infarct size at 4 weeks after MI between the EPO(6h) and the EPO(1wk) groups, the improvement of cardiac function in the EPO(6h) group was not attributable to the reduction of infarct size, but to the increased blood flow to the ischemic regions.

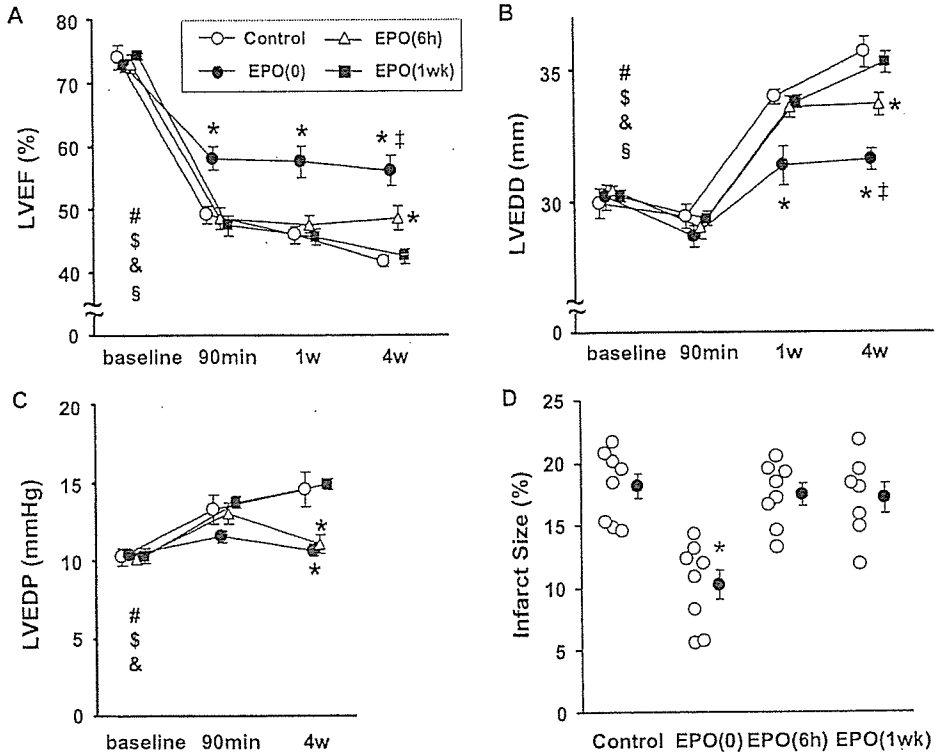

Figure 7. The time course of changes in left ventricular ejection fraction (LVEF) (A), left ventricular end-diastolic dimension (LVEDD) (B), and left ventricular end-diastolic pressure (LVEDP) (C) in different experimental groups. Statistically significant ($p < 0.05$) group-by-time interactions (analysis of variance for repeated measurements) are indicated by the following: # = all groups; $ = control × EPO(0) group; & = control × EPO(6h) group; § = EPO(0) × EPO(6h) group. (D) Infarct size at 4 weeks after myocardial infarction in different experimental groups. Open circles = infarct size in each animal. *$p < 0.05$ versus the control group. EPO = erythropoietin.

In conclusion, in addition to its acute effect on infarct size reduction, EPO may exert chronic cardioprotective effects through neovascularization and may be a useful adjunct for the treatment of patients with myocardial infarction.

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Vagal stimulation suppresses ischemia-induced myocardial interstitial norepinephrine release

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Abstract

Although electrical vagal stimulation exerts beneficial effects on the ischemic heart such as an antiarrhythmic effect, whether it modulates norepinephrine (NE) and acetylcholine (ACh) releases in the ischemic myocardium remains unknown. To clarify the neural modulation in the ischemic region during vagal stimulation, we examined ischemia-induced NE and ACh releases in anesthetized and vagotomized cats. In a control group (VX, $n=8$), occlusion of the left anterior descending coronary artery increased myocardial interstitial NE level from 0.46±0.09 to 83.2±17.6 nM at 30–45 min of ischemia (mean±SE). Vagal stimulation at 5 Hz (VS, $n=8$) decreased heart rate by approximately 80 beats/min during the ischemic period and suppressed the NE release to 24.4±10.6 nM ($P<0.05$ from the VX group). Fixed-rate ventricular pacing (VSP, $n=8$) abolished this vagally mediated suppression of ischemia-induced NE release. The vagal stimulation augmented ischemia-induced ACh release at 0–15 min of ischemia (VX: 11.1±2.1 vs. VS: 20.7±3.9 nM, $P<0.05$). In the VSP group, the ACh release was not augmented. In conclusion, vagal stimulation suppressed the ischemia-induced NE release and augmented the initial increase in the ACh level. These modulations of NE and ACh levels in the ischemic myocardium may contribute to the beneficial effects of vagal stimulation on the heart during acute myocardial ischemia.

Keywords: Acetylcholine; Coronary occlusion; Ventricular pacing

Introduction

Acute myocardial ischemia disrupts normal neural regulation of the heart (Armour, 1999). During prolonged ischemia, myocardial interstitial norepinephrine (NE) and acetylcholine (ACh) levels are increased in the ischemic region via local releasing mechanisms independent of efferent autonomic activities (Schömig et al., 1987; Lameris et al., 2000; Kawada et al., 2000, 2001). The excess NE release is thought to aggravate ischemic injury to the myocardium (Schömig et al., 1987). On the other hand, vagal stimulation exerts antiarrhythmic effects in the early phase of acute myocardial ischemia (Rosenshtraukh et al., 1994; Vanoli et al., 1991). A recent study from our laboratory demonstrated that vagal stimulation improved the survival rate of chronic heart failure after myocardial infarction in rats (Li et al., 2004), suggesting a long-term ameliorative effect of direct neural interventions against certain heart diseases.

With respect to electrical stimulation of the vagus, whether it alters myocardial interstitial NE and ACh levels in the ischemic region during acute myocardial ischemia remains unknown. To test the hypothesis that vagal stimulation increases the ACh level and suppresses the NE level in the ischemic region, we measured myocardial interstitial NE and ACh levels during acute myocardial ischemia in anesthetized cats using a cardiac microdialysis technique (Akiyama et al., 1991, 1994; Yamazaki et al., 1997). Effects of vagal stimulation were examined with or without fixed-rate ventricular pacing.

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