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Intravenous injection of phagocytes transfected ex vivo with FGF4 DNA/ biodegradable gelatin complex promotes angiogenesis in a rat myocardial ischemia/reperfusion injury model

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■ **Abstract** Conventional gene therapies still present difficulties due to poor tissue-targeting, invasiveness of delivery, method, or the use of viral vectors. To establish the feasibility of using non-virally ex vivo transfected phagocytes to promote angiogenesis in ischemic myocardium, gene-transfection into isolated phagocytes was performed by culture with positively charged gelatin impregnated with plasmid DNA. A high rate of gene transfection was achieved in rat macrophages and human monocytes, but not in mouse fibroblasts. The efficiency was $68 \pm 11\%$ in rat macrophages and $78 \pm 8\%$ in human monocytes. Intravenously injected phagocytes accumulated predominantly in ischemic tissue ($13 \pm 8\%$) and spleen ($84 \pm 6\%$), but negligibly in other organs in rodents. The efficiency of accumulation in the target ischemic tissue reached more than 86% on direct local tissue injection. In a rat model of myocardial ischemia-reperfusion, intravenous injection of fibroblast growth factor 4 (FGF4)-gene-transfected macrophages significantly increased regional blood flow in the ischemic myocardium ($78 \pm 7.1\%$ in terms of flow ratio of ischemic/non-ischemic myocardium) compared with intravenous administration of saline ($36 \pm 11\%$) or non-transfected macrophages ($42 \pm 12\%$), or intramuscular administration of naked DNA encoding FGF4 ($75 \pm 18\%$). Enhanced angiogenesis in the ischemic tissue we confirmed histologically. Similarly, intravenous injection of FGF4-gene-transfected monocytes enhanced regional blood flow in an ischemic hindlimb model in mice ($93 \pm 22\%$), being superior to the three other treatments described above (38 ± 12, 39 ± 15, and $55 \pm 12\%$, respectively).

Phagocytes transfected ex vivo with FGF4 DNA/gelatin promoted angiogenesis. This approach might have potential for non-viral angiogenic gene therapy.

■ **Key words** angiogenesis – cells – gene therapy – growth substances – ischemia

Abbreviations and acronyms

ANOVA = analysis of variance
FGF4 = fibroblast growth factor-4
GFP   = green fluorescent protein
pI   = isoelectric point

Introduction

Conventional gene therapies still require improvement with regard to transfection efficiency and safety [1, 2], as well as tissue targeting [3], despite recent advances. Achievement of a high transfection rate often requires a viral vector, but the safety of the viruses has not yet been

established [4–6]. Conventional non-viral vectors seem to be inferior to viral ones in transfection efficiency, except for nucleofection [7, 8]. Conventional gene therapy using a viral vector can induce inflammation in the gene-transduced tissue [9]. Moreover, in vivo gene-delivery to the localized target tissue usually necessitates invasive approaches. For example, direct gene-transfection to cardiomyocytes requires surgical operation [10] or cardiac catheterization [11, 12]. On the other hand, ex vivo gene-transfection is less invasive, but tissue-targeting by intravenous injection is difficult to achieve [3].

Macrophages accumulate in ischemic tissue based on the mechanism of immune response (chemotaxis) [13]. This suggests that intravenous transplantation of macrophages may target the ischemic tissue in vivo. Tabata et al. previously reported that gelatin particles are phagocytized by macrophages [14, 15]. The isoelectric point (pI) of gelatin can be changed by modification of its residues, and positively charged gelatin can be impregnated with negatively charged substances [16] such as nucleic acid [17]. Thus, gelatin may be suitable as a vector for transfecting phagocytes ex vivo.

We describe here a study aimed at examining the feasibility of a new concept for less invasive, cell-based gene therapy, by means of ex vivo gene transfection into isolated phagocytes (macrophages and monocytes) using a non-viral vector, gelatin, followed by intravenous injection of the transfected phagocytes. The present method has significant advantages over conventional cell-based gene delivery [18, 19], in that the intravenously injected cells (phagocytes) not only produce protein from the transfected gene, but have a tissue-targeting ability.

Methods

This study was performed in accordance with the Guideline of Tokai University School of Medicine on Animal Use, which conforms to the NIH Guide for the Care and Use of Laboratory Animals (DHEW publication No. (NIH) 86-23, Revised 1985, Offices of Science and Health Reports, DRR/NIH, Bethesda, MD 20205).

■ Animals

A total of 121 Fisher rats (male, 10 weeks old, Clea Japan Inc., Tokyo) and 61 nude SCID mice (male, 6 weeks old, Shizuoka Animal Center, Shizuoka, Japan) were used. Rats were anesthetized by inhalation of diethyl ether for harvesting macrophages and with isofluren (1.5–3%) for thoracotomy, after which they were mechanically ventilated with a mixture of oxygen and nitrous oxide. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg).

A model of myocardial ischemia-reperfusion injury

was prepared in 41 rats. The remaining 80 rats were used for collecting activated macrophages. The heart was exposed via thoracotomy, and the proximal left anterior descending coronary artery was ligated [20] for 180 min, followed by reperfusion. A model of hindlimb ischemia was prepared in 61 mice. The left femoral artery was ligated and resected [21].

■ Cells

Macrophages were obtained from 80 rats. Thioglycolate (4%, 8 ml) was injected into the peritoneal cavity, and after 4 days, peritoneal macrophages were collected [22]. Monocytes were obtained from peripheral blood of healthy volunteers. Leukocyte-rich plasma was obtained by dextran 500 sedimentation and layered onto Nycoprep 1.068 (Nycomed, Birmingham, UK). The monocyte-containing layer was aspirated, washed twice and allowed to adhere to the dish for 90 minutes. Fibroblasts (NIH 3T3, Invitrogen Corporation, Carlsbad, CA) were also used. The cells were resuspended in RPMI 1640 medium (Sigma) containing 5% heat-inactivated fetal calf serum and cultured for 7–14 days. The cell viability and type were determined by trypan blue exclusion and by immunostaining using anti-macrophage antibody up to 14 days.

■ Genes and vector

Complementary DNA (cDNA) of green fluorescent protein (GFP), Renilla luciferase or human hst1/FGF4 (FGF4) [17] was inserted into the expression vector pRC/CMV (Invitrogen Corporation, Carlsbad, CA) and the constructs were designated as pRC/CMV-GFP, pRC/CMV-luciferase and pRC/CMV-HST1-10, respectively. Preparation and purification of the plasmid from cultures of pRC/CMV-GFP-, pRC/CMV-luciferase-, or pRC/CMV-HST1-10-transformed *Escherichia coli* were performed by equilibrium centrifugation in cesium chloride-ethidium bromide gradients.

Gelatin was prepared from porcine skin [14]. After swelling in water the gelatin particles used in this study were spheroids with a diameter of approximately 5–30 μm, water content of 95%, and pI of 11. Gelatin (2 mg) was incubated with 50 μg of the plasmid for 7 days at 4 °C to make a gelatin-DNA complex [14].

■ Experimental protocols

Ex vivo gene transfection Macrophages, monocytes, and fibroblasts (1×10^6) were cultured with the gelatin-DNA complex (2 mg of gelatin plus 50 μg of DNA) for 14 days on a culture dish (100 mm in diameter). Gene ex-

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pression of GFP was evaluated by fluorescence microscopy and fluorescence-activated cell sorting. Luciferase activity in the cell lysate was evaluated with a photon counter system after cell lysis [23].

Organ distribution of phagocytes injected intravenously and directly into ischemic muscle To examine tissue-targeting by intravenous injection of transfected phagocytes, the distribution of the cells into organs was evaluated by immunohistochemistry. In the rat model of myocardial ischemia-reperfusion injury, the GFP-gene-transfected macrophages (1.0×10^6 each) were injected into the superficial dorsal vein of the penis at the initiation of reperfusion (n = 7 and 5, respectively). In the mouse model of hindlimb ischemia, the GFP-gene-transfected monocytes (1.0×10^6) were injected into the caudal vein 14 days after induction of ischemia (n = 5). To examine the tissue-targeting by direct local injection of transfected phagocytes, the distribution of the cells into organs was also evaluated. In the rat model of myocardial ischemia-reperfusion injury (n = 7) and the mouse model of hindlimb ischemia (n = 5), the same numbers of transfected macrophages and monocytes were directly injected into ischemic myocardium and ischemic skeletal muscle, respectively. Tissue samples were obtained 24 hours after cell administration. Each tissue was homogenized and cytospin was performed. Immunohistochemical analysis was done with anti-GFP antibody (CLONTECH, USA. GFP-monoclonal antibody). GFP positive macrophages were counted in each tissue and expressed as a percentage of total GFP-positive cells.

Amelioration of ischemia by intravenous injection of angiogenic gene-transfected phagocytes The angiogenic effect of intravenously injected FGF4-gene-transfected phagocytes on the ischemia models was evaluated. In the rat model of myocardial ischemia-reperfusion injury, FGF4-gene-transfected macrophages (n = 5), non-transfected macrophages (1.0×10^6 each) (n = 5), or saline (n = 5) were injected into the superficial dorsal vein of the penis, or naked FGF4-DNA (50 µg) was injected directly into the ischemic myocardium (n = 5), at the initiation of reperfusion. Fourteen days after the cell administration, blood flows in the ischemic and non-ischemic regions in the heart were evaluated with a noncontact laser Doppler flowmeter (FLO-N1, Omegawave Corporation). Then, tissue samples were obtained and histological analysis was performed. In a mouse model of hindlimb ischemia, just after induction of ischemia, FGF4-gene-transfected monocytes (n = 15), non-transfected monocytes (n = 8) (1.0×10^6 each), or saline (n = 10) were injected into the caudal vein, or naked FGF4-DNA (50 µg) was injected directly into the ischemic muscle (n = 12). Fourteen days after induction of ischemia, blood flows in the limbs were evaluated with the noncontact laser Doppler flowmeter (FLO-N1, Omegawave Corporation).

Histology

Ten micrometer sections were cut from formalin-fixed, paraffin-embedded tissue. Two sections were used for H.E. staining and azan staining, and eight sections were used for immunohistochemical staining. Immunohistochemical staining was performed by an indirect immunoperoxidase method. Anti-GFP antibody, anti-Mac1 antibody (BMA Biomedicals Ag, Switzerland), and anti-CD31 antibody (Serotec, UK) were used as primary antibodies. Mac1-antigen is specific to macrophages/monocytes. Anti-Ig, peroxidase-linked species-specific F(ab')2 fragments (Amersham Pharmacia Biotech UK Ltd., UK), were used as a secondary antibody. Double staining was performed with alkaline staining and peroxidase staining. The vessel density stained with von Willebrand factor-antibody was calculated by morphometric assessment in one 16 randomly selected fields of each heart and expressed as number/mm^2.

Statistical analysis

Data are presented as mean values ± SD. Differences were assessed by using ANOVA (analysis of variance) with the Scheffe's multiple comparisons test. A value of P < 0.05 was considered statistically significant.

Results

Ex vivo gene transfection

We studied whether genes could be transfected into isolated rat macrophages, human monocytes, and mouse fibroblasts ex vivo by using gelatin. Transfection of the GFP gene into isolated rat macrophages (Figs. 1A and B) and human monocytes (Figs. 1C and D), but not into mouse fibroblasts (data not shown), was achieved by culture with gelatin-DNA complex for 14 days. The gene transfection efficiency into rat macrophages was 68 ± 11 % (30 experiments, Fig. 2A) and that into human monocytes was 78 ± 8 % (30 experiments) as determined with a fluorescence activated cell sorter. Sequential analysis after luciferase-gene transfection into rat macrophages revealed high expression after 14 days of culture (Fig. 2B).
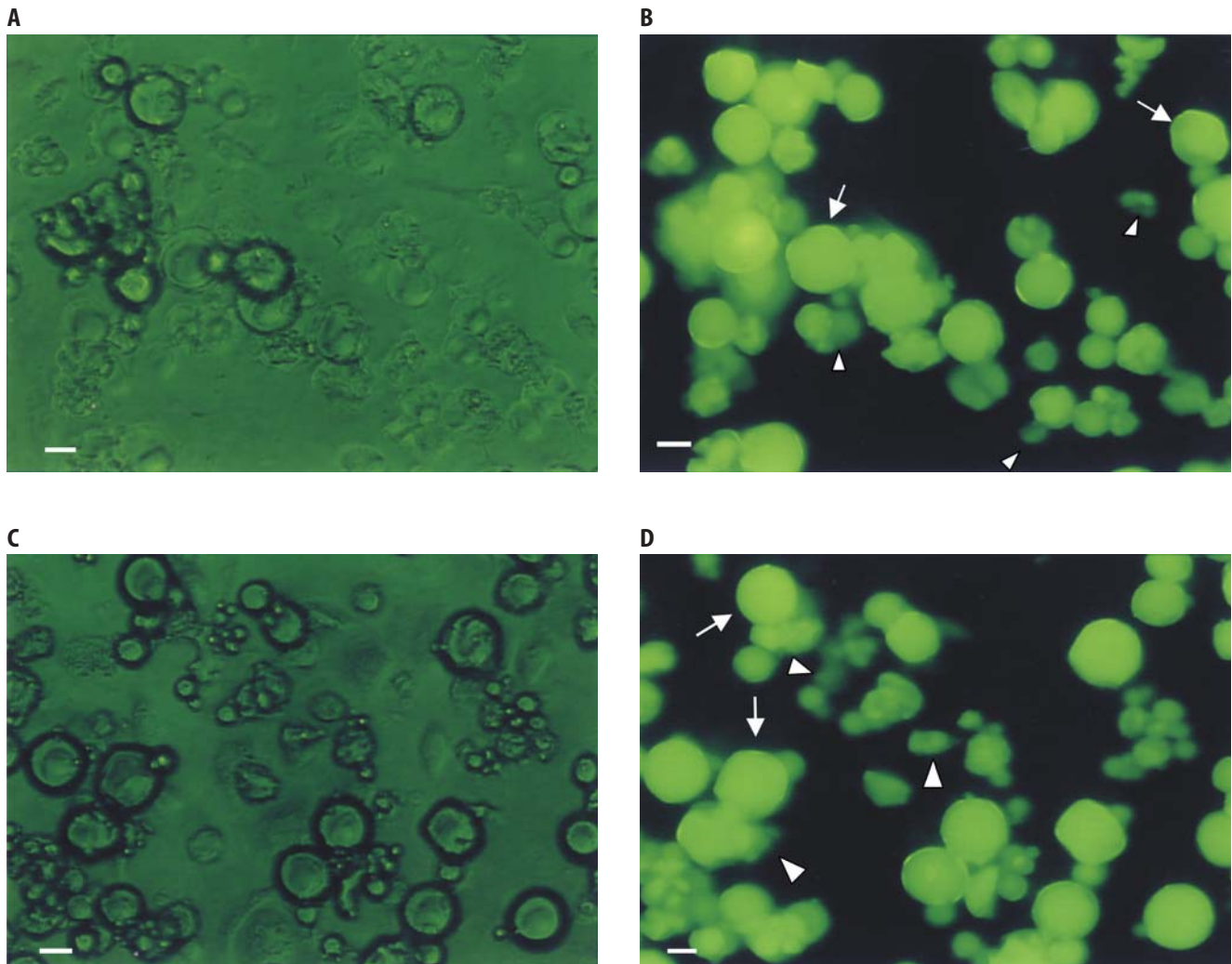
Fig. 1 Fluorescent presentation of ex vivo gene transfection with gelatin-DNA complex in macrophages/monocytes as well as fibroblasts. Rat macrophages (**A** and **B**) and human monocytes (**C** and **D**) were cultured with gelatin-GFP-gene complex for 14 days. Transmittance microscopic images (**A** 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 ($98 \pm 4\%$) ($n = 7$, 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 $13 \pm 6\%$ ($n = 7$) (non-ischemic rats $0 \pm 0\%$, $n = 7$, Table 1). The incorpo-

rated 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 ($13 \pm 7\%$, $n = 7$, 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, $86 \pm 10\%$ and $88 \pm 6\%$ of the cells remained in the target tissue in the two models. Thirteen and 11% of phagocytes in-
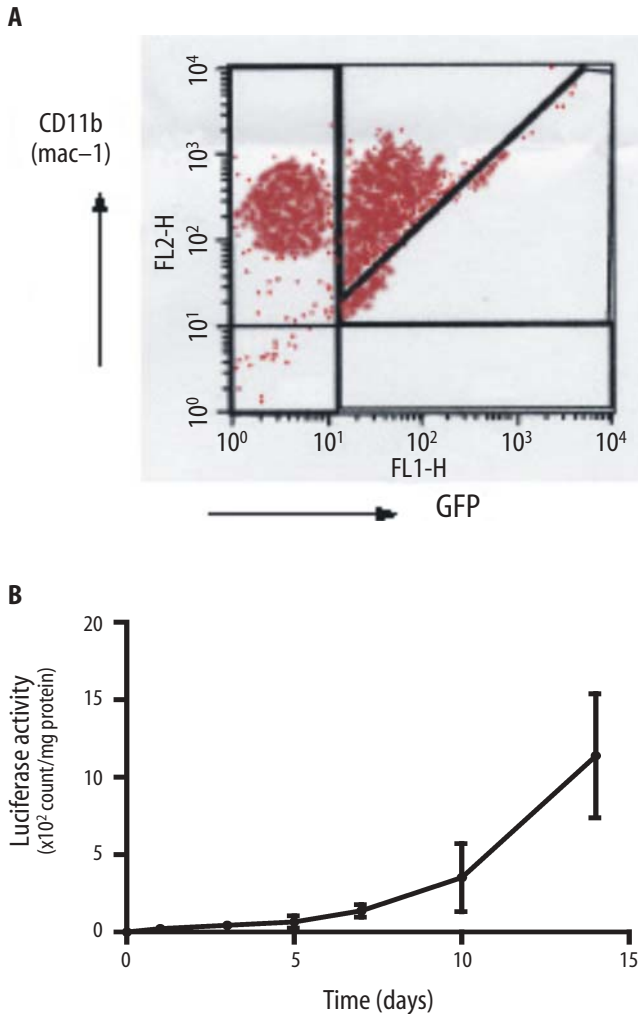
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A

B

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 $\pm$ SD. The number of experiments is shown in parentheses

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 $\pm$ SD). *i.v.* intravenous injection into the vein; *i.m.* direct injection into the jeopardized muscle

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 ($78 \pm 7.1\%$, n = 8, in terms of flow ratio of

ischemic/non-ischemic myocardium) compared with the other three treatments (P < 0.05, ANOVA), that is, intravenous administration of saline ($35 \pm 10\%$, n = 8), intramuscular administration of naked DNA encoding FGF4 ($50\,\mu g$, direct intramyocardial injection after thoracotomy) ($58 \pm 5.3\%$, n = 8), and intravenous administration of the same number of non-transfected macrophages ($42 \pm 12\%$, 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 ($93 \pm 22\%$ 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 $39 \pm 15\%$, 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 ($68 \pm 11\%$) 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-
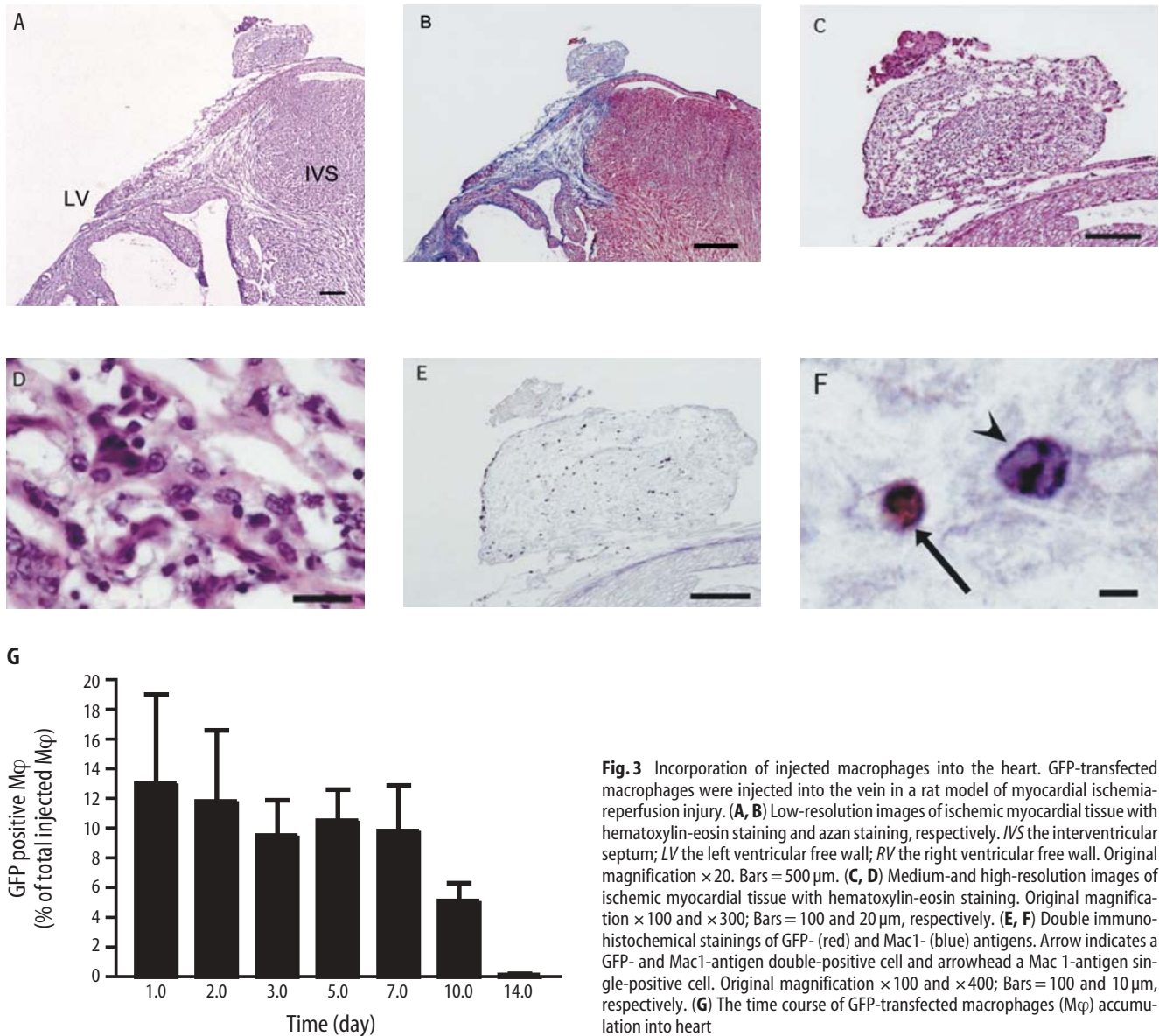
Fig. 3 Incorporation of injected macrophages into the heart. GFP-transfected macrophages were injected into the vein in a rat model of myocardial ischemia-reperfusion injury. (**A, B**) Low-resolution images of ischemic myocardial tissue with hematoxylin-eosin staining and azan staining, respectively. *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 immuno-histochemical 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
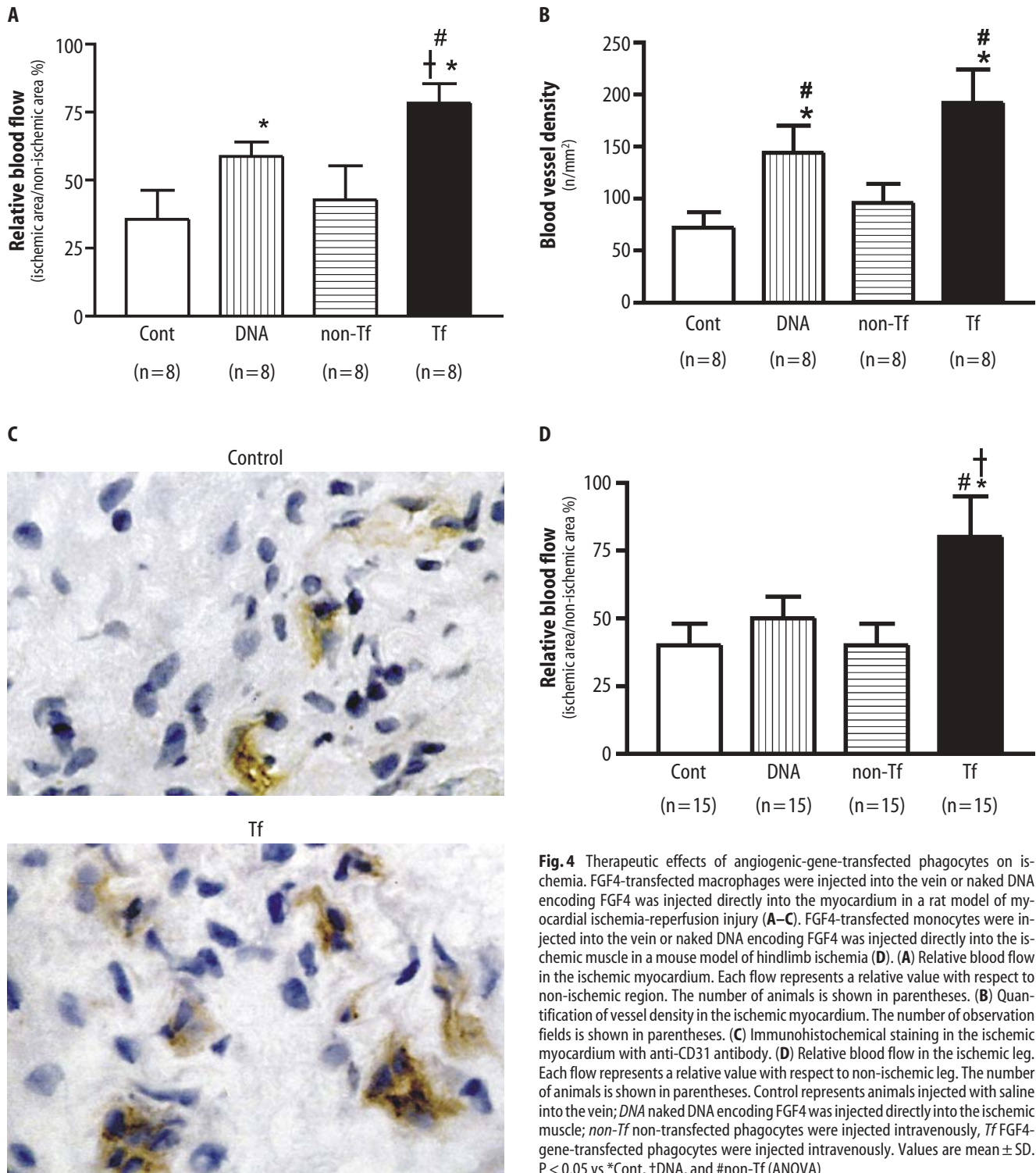
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A

B

C

Control

Tf

D

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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