activity is required for rhEPO to recover contractile dysfunction and to block apoptosis induced by myocardial ischemia-reperfusion in isolated hearts (*ex vivo*) [10]. However, it is not determined whether rhEPO just before reperfusion reduces infarct size via PI3 kinase-dependent pathway in the *in vivo* model.

In addition to myocardial cell death, myocardial ischemia-reperfusion triggers lethal arrhythmias [11]. It is believed that at least half of the deaths due to coronary artery disease are caused by a lethal arrhythmia [12]. Although high doses of rhEPO exert cardioprotective effects against ischemia/reperfusion injury in small animals [3–8], its effects on lethal arrhythmias remain unknown. If rhEPO reduces the incidence of ventricular fibrillation (VF) in the clinical setting, there would be additional advantage to use this drug in the realistic situation of acute myocardial infarction. Thus, in the present study, we examined dose-dependent effects of rhEPO administered just before reperfusion on myocardial infarct size and the incidence of VF in the *in vivo* canine model. We also evaluated whether any such effects were mediated via the PI3 kinase pathway.

Materials and Methods

Materials

Wortmannin was obtained from Sigma (St. Louis, MO) and Phospho-Akt and Akt antibodies were obtained from Cell Signaling Technologies (Beverly, MA). RhEPO was provided by Chugai Pharmaceutical Co., Ltd (Tokyo, Japan).

Instrumentation

Forty-eight beagle dogs (Kitayama Labes, Yoshiki Farm, Gifu, Japan) weighing 8 to 12 kg were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg), intubated, and ventilated with room air mixed with oxygen (100% O_2 at flow rate of 1.0 to 1.5 L/min). Thoracotomy was done at the left fifth intercostal space, and the heart was suspended by a pericardial cradle. After intravenous administration of heparin (500 U/kg), the left anterior descending coronary artery (LAD) was cannulated for perfusion with blood from the left carotid artery through an extracorporeal bypass tube. Coronary blood flow was measured with an electromagnetic flow probe attached to the bypass tube. We can selectively infuse drugs into LAD-perfused areas through this bypass tube. The left atrium was catheterized for microsphere injection to measure myocardial collateral blood flow during ischemia. Hydration was maintained by a slow normal saline infusion. The femoral artery was also cannulated to measure the mean systemic blood pressure (SBP). Both SBP and heart rate (HR) were monitored continuously during the study. 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.

Experimental protocols

Protocol 1. Long-term effects of rhEPO on hematometric parameters in dogs. To test the long-term effects of rhEPO on hematometric parameters, 100 IU/kg ($n = 5$) or 1,000 IU/kg ($n = 5$) of rhEPO was intravenously administered as a single injection. Blood was collected under pentobarbital (15 mg/kg) anesthesia before and 7, 14 days after rhEPO treatment. Hematometric parameters including hematocrit, white blood cell, and platelet counts were measured.

Protocol 2; Measurement of infarct size, coronary blood flow and myocardial collateral blood flow. After hemodynamic stabilization, we administered a low (100 IU/kg), or high (1,000 IU/kg) dose of rhEPO, or saline 10 min prior to reperfusion (n = 8–12 each) as a single intravenous injection (Fig. 1). To clarify whether rhEPO reduces myocardial infarct size through a PI3 kinase-dependent pathway, a PI3 kinase inhibitor, wortmannin, was selectively administered into the LAD (1.5 μg/kg/min) for 60 min after the onset of reperfusion. We have previously confirmed that the dose of wortmannin employed in this study is appropriate for blocking the phosphorylation of Akt in myocardium [13]. We measured infarct size and regional myocardial collateral blood flow during 90 min of ischemia as described previously [14]. In brief, infarct size was evaluated at the end of the protocol by Evans blue/TTC staining, while collateral blood flow was assessed by the non-radioactive microsphere method [14]. Coronary blood flow was monitored continuously during the study. To ensure that all of the animals included in the data analysis were healthy and were exposed to a similar extent of ischemia, the exclusion criteria reported previously for excessive myocardial collateral blood flow (>15 mL/100 g/min) and lethal arrhythmia (more than two consecutive attempts required to convert VF with low-energy DC pulses applied directly to the heart) were adopted [14].

Effects of rhEPO on VF during reperfusion period

In Protocol 2, we also evaluated the incidence of VF during the 6 h reperfusion period (Fig. 1). Since myocardial collateral blood flow during ischemia exhibited a negative correlation with the incidence of VF [15,16], the dogs with excessive collateral blood flow (>15 mL/100 g/min) were excluded from VF analysis.

Phosphorylation of Akt

We used 12 dogs for western blot in the control, low EPO, high EPO, and high EPO + WTMN groups ($n = 3$ each) in Protocol 2 (Fig. 1). After 90 min of ischemia followed by 6 h of reperfusion, hearts were excised and

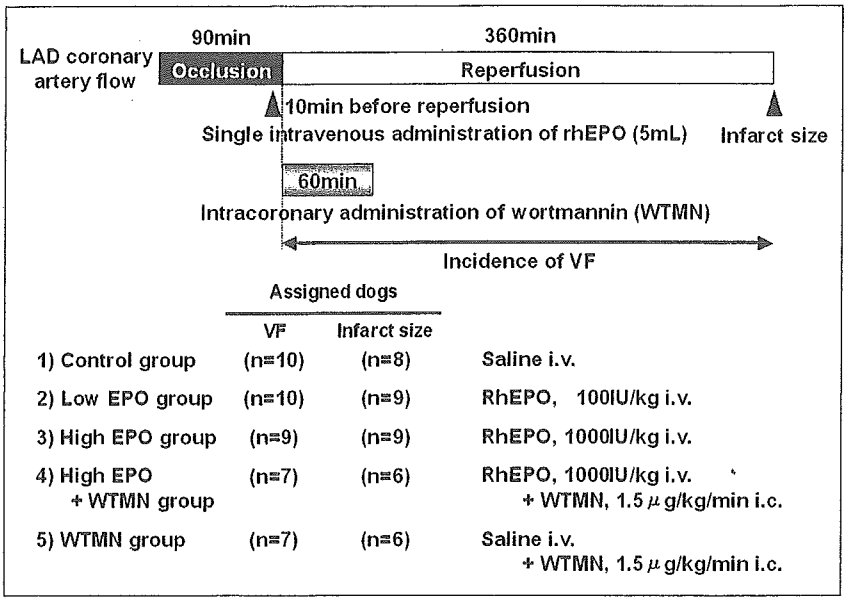

Fig. 1. *Experimental protocol for infarct size and VF.*

the myocardial tissue in the ischemic zone was quickly placed into liquid nitrogen and stored at −80°C. Phosphorylation of Akt and total content of Akt were evaluated as reported previously [13]. The immunoreactive bands were quantified by densitometry (Molecular Dynamics).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

In Protocol 2, the myocardial tissue samples were taken from the ischemic zone of dogs in the control, low EPO, high EPO, and high EPO + WTMN groups ($n = 3$ each). These were fixed in 10% buffered formalin, embedded in paraffin, and serially sectioned in the frontal plane at 5-μm thickness. Analysis by TUNEL method was performed according to the protocol supplied with the in situ apoptosis detection kit, the Apop Tag Peroxidase *In Situ* Apoptosis Detection Kit (CHEMICON International, USA). TUNEL-positive cell nuclei and total cell nuclei stained metylgreen were counted in 10–15 random high-power fields (×400), and the percentage of TUNEL-positive cell nuclei to total cell nuclei ($n =$ 1,000) were then calculated.

Statistical analysis

Statistical analysis was performed by one-way factional analysis of variance (ANOVA) with modified Bonferroni's post hoc test when the data were compared among groups. Time courses of the changes were compared by repeated measures ANOVA. The incidence of VF was compared using the χ^2-test and Fisher's exact probability test. Results were expressed as the mean ± SEM, with $p < 0.05$ considered significant.

Results

The long-term effects of rhEPO on hematometric parameters

The single administration of either 100 IU/kg or 1,000 IU/kg of rhEPO did not change any hematometric parameters including hematocrit, white blood cells, and platelet counts 7 or 14 days after rhEPO treatment (Table 1).

Table 1. *Long-term effects of rhEPO on hematometric parameters in dogs*

	EPO 100 IU/kg			EPO 1000 IU/kg		
Parameters	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Ht (%)	50.1 ± 0.8	51.1 ± 0.9	51.3 ± 1.0	51.5 ± 1.3	52.0 ± 1.0	53.1 ± 0.7
WBC ($10^3/\mu$L)	11.7 ± 1.3	12.1 ± 0.7	11.4 ± 0.6	11.5 ± 1.3	11.7 ± 0.7	12.0 ± 1.0
Platelet ($10^4/mm^3$)	33.7 ± 2.0	33.7 ± 1.8	33.3 ± 1.7	33.2 ± 1.9	34.0 ± 2.4	36.4 ± 3.4

Data are presented as Mean ± SEM. $n = 5$.

Abbreviations: rhEPO = recombinant human erythropoietin, Ht = hematocrit, WBC = white blood cell.

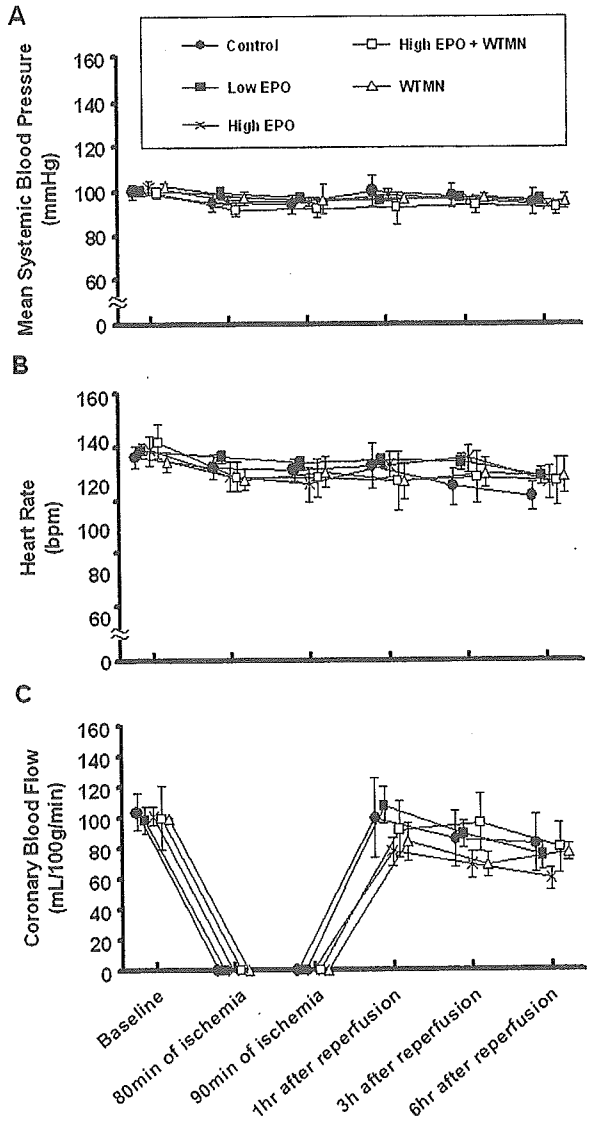

Fig. 2. The changes in mean systemic blood pressure, heart rate and coronary blood flow during the experiment in groups tested.

Effects of rhEPO on infarct size and VF during the reperfusion period

Since 5 of 48 dogs were excluded from analysis because of excessive collateral blood flow (>15 mL/100 g/min) (control: 1, low EPO: 2, high EPO: 1, high EPO + WTMN: 0, WTMN: 1), 43 dogs were evaluated for VF analysis. Among these 43 dogs, we excluded 5 dogs (control: 2, low EPO: 1, high EPO: 0, high EPO + WTMN: 1, WTMN: 1) that matched the exclusion criteria of lethal arrhythmia from infarct size analysis.

Throughout the study, neither SBP (Fig. 2A), nor HR (Fig. 2B), nor coronary blood flow (Fig. 2C) differed among the 5 groups. The area at risk (Fig. 3A) and myocardial collateral blood flow in the LAD region during myocardial ischemia (Fig. 3B) were also comparable in the groups tested.

Table 2. Effects of rhEPO on the incidence of VF during reperfusion periods

Group	Incidence of VF	
Control	50.0%	(5/10)
Low EPO	40.0%	(4/10)
High EPO	0%*	(0/9)
High EPO + WTMN	42.9%	(3/7)
WTMN	42.9%	(3/7)

*$p < 0.05$ vs. control group.
Abbreviations: VF = ventricular fibrillation, rhEPO = recombinant human erythropoietin, WTMN = wortmannin.

Figure 4 shows infarct size in the groups tested. A low or high dose of rhEPO significantly ($p < 0.05$) reduced the infarct size compared with that in the control group. Furthermore, a high dose of rhEPO reduced infarct size more than a low dose of rhEPO did. The intracoronary administration of wortmannin for 60 min after the onset of reperfusion abrogated the infarct-limiting effect of rhEPO, although wortmannin alone did not affect infarct size.

The high, but not low, dose of rhEPO significantly ($p < 0.05$) reduced the incidence of VF during the 6 h reperfusion period compared with the control. The anti-arrhythmic effects of rhEPO were abolished by wortmannin (Table 2).

Effects of rhEPO on Akt phosphorylation

After 90 min of ischemia followed by 6 h of reperfusion, the ratio of phosphorylated Akt to total Akt in the low and high EPO groups significantly ($p < 0.05$) increased compared with that in the control group. The increase in this ratio was completely abolished by the treatment with wortmannin (Fig. 5).

Effects of rhEPO on apoptosis

The ratio of TUNEL positive cells to total cells in the low and high EPO groups decreased compared with that in the control group. The reduction of TUNEL-positive cells by rhEPO was completely abolished by the treatment with wortmannin (Fig. 6).

Discussion

In this study, we demonstrated that a single intravenous administration of rhEPO just before reperfusion limited not only infarct size but also the incidence of VF. Moreover, our data suggest that the infarct size-limiting and anti-arrhythmic effects of rhEPO were through the PI3 kinase-dependent pathways in the *in vivo* canine hearts.

Important considerations towards clinical application of rhEPO are the timing and dose of its administration. The previous studies reported that rhEPO administered at the onset of reperfusion [7,8] as well

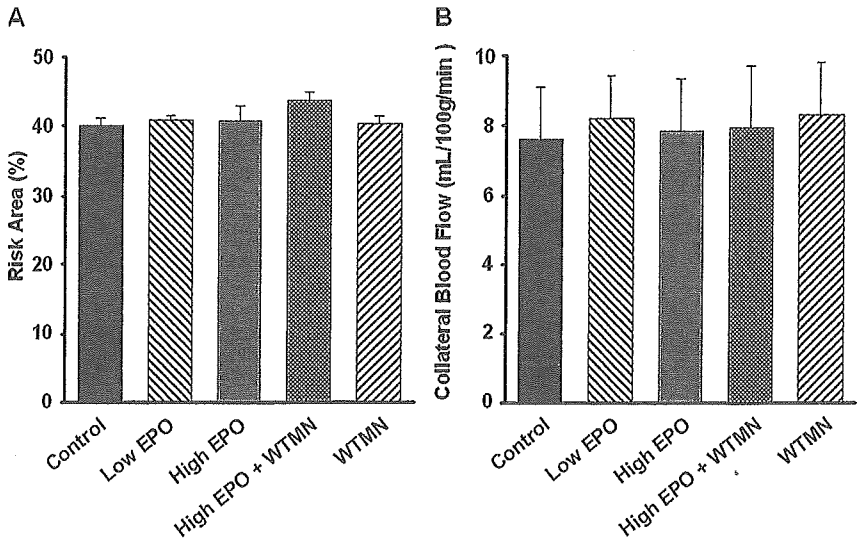

Fig. 3. *Area at risk and myocardial collateral blood flow during ischemia in groups tested.*

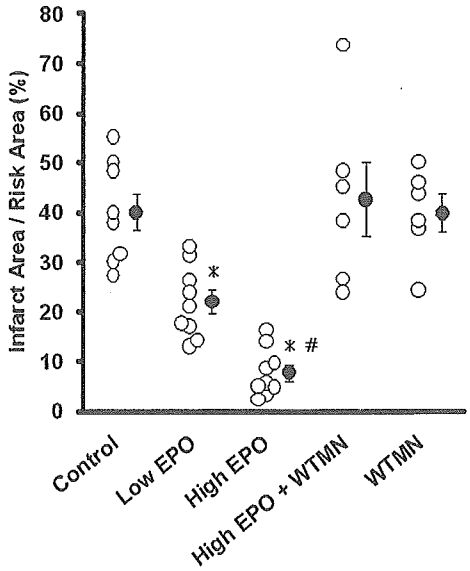

Fig. 4. *Infarct size in groups tested. *p < 0.05 vs. control group. #p < 0.05 vs. low EPO group. Open circles show the infarct size in each individual.*

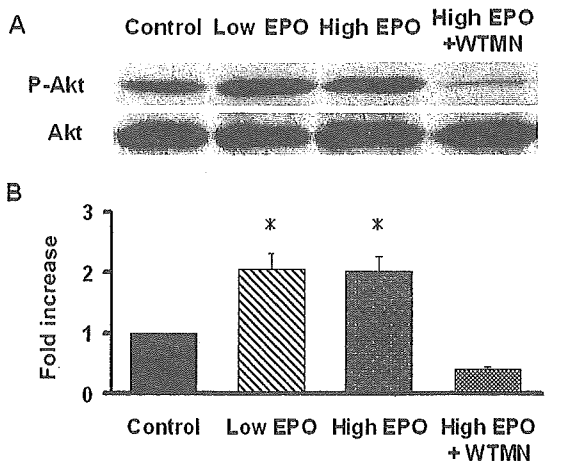

Fig. 5. *Phosphorylation of Akt in canine hearts. (A) Representative Western blot for phosphorylated and total Akt. (B) Densitometry graphs indicating fold expression over control for Akt. n = 3 each. *p < 0.05 vs. control group.*

as ischemia [7,8] reduces infarct size in rabbit and rat hearts. Consistent with these reports, we confirmed that rhEPO administered 10 min before reperfusion reduced myocardial infarct size in dogs. Our findings support the idea that in humans the adjunctive therapy with rhEPO treatment during coronary intervention would reduce myocardial infarct size.

The doses of rhEPO (1,000–5,000 IU/kg) administered in previous experimental studies [3–8] were nearly 10 times higher than those clinically used in anemic patients with chronic renal failure [9]. In the present study, we demonstrated that both 100 IU/kg and 1,000 IU/kg of rhEPO as a single administration significantly reduced myocardial infarct size, although a high dose of rhEPO significantly reduced infarct size more than a low dose of rhEPO did. This finding suggests that the clinically relevant dose of rhEPO used in patients with chronic renal failure can reduce myocardial infarct size. In the previous clinical studies, a high dose (33,000 IU once daily for the first 3 days) of intravenously administered rhEPO was well tolerated in patients with stroke and improved clinical outcome at 1 month [17]. On the other hand, a high dose (40,000–60,000 IU per week) of subcutaneously administered rhEPO, while not as a single injection,

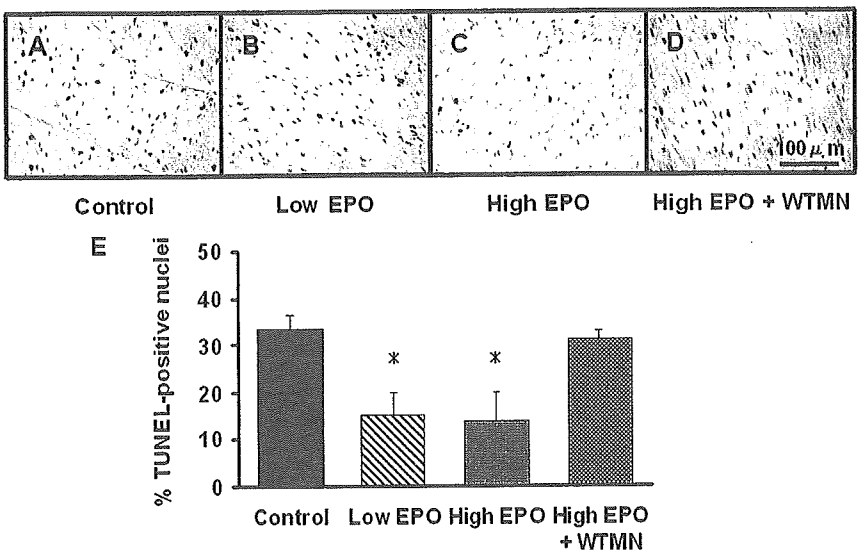

Fig. 6. *TUNEL staining in canine hearts after 90 min ischemia followed by 6 h of reperfusion. Representative examples of TUNEL-staining from canine hearts in the control (A), low EPO (B), high EPO (C), and high EPO + WTMN groups (D). (E) Quantitative data of the percentage of TUNEL-positive nuclei to total cell nuclei. *$p < 0.05$ vs. control group.*

increased the incidence of thrombotic events such as deep venous thrombosis or pulmonary embolisms in patients with breast cancer [18]. Furthermore, there are some reports that rhEPO increases the number of platelets in normal rats [19] and chronically hemodialyzed patients [20]. In the present study, we didn't find that either low or high dose of rhEPO, as a single injection, changed hematometric parameters. Although these findings suggest that a single administration of 1,000 IU/kg of rhEPO, that induced marked reduction of myocardial infarct size, could be used safely, we must be careful for the use of a high dose of rhEPO for the treatment of myocardial infarction.

Previous reports have shown that both phosphorylation of Akt and inhibition of apoptosis are associated with infarct size-limiting effects due to rhEPO [4,6–8]. Recently, it was reported that PI3 kinase activity is required for rhEPO to recover contractile dysfunction and to block apoptosis induced by myocardial ischemia-reperfusion in isolated hearts (*ex vivo*) [10]. Although the recovery of contractile function could be related to the reduction of infarct size, no evidence was presented that rhEPO reduced infarct size via the PI3 kinase-dependent pathway. In the present study we have demonstrated that the infarct size-limiting effect of rhEPO was blunted by the intracoronary administration of wortmannin in dogs. This is the first evidence showing that the infarct size-limiting effect of rhEPO is dependent on the PI3 kinase pathway in *in vivo* hearts.

In the present study, low and high doses of rhEPO equally increased phosphorylation of Akt and decreased equivalent number of TUNEL-positive cells in the ischemic myocardium of dogs. Either Akt phosphorylation or a decrease in the number of TUNEL-positive cells was prevented by the PI3 kinase inhibitor, wortmannin. This finding suggests that rhEPO prevents apoptotic cell death through PI3 kinase/Akt-dependent pathway in canine hearts. However, since the TUNEL method also detects single strand breaks occurring in the course of necrotic cell death [21], it is likely that rhEPO attenuates apoptotic and necrotic cell death. Indeed, if rhEPO only inhibits the apoptotic cell death, it may be difficult to explain the marked reduction of infarct size by rhEPO. Interestingly, the previous studies reported that the PI3 kinase activates not only Akt but also protein kinase C or mitogen-activated protein kinase in ischemia/reperfusion models [22–24], either of which mediates the cellular protection against necrotic process [25,26]. Furthermore, recent reports suggest that rhEPO can inhibit the release of free radicals from neutrophils [27] and act as a radical scavenger [28], both of which may reduce cardiac cell death after ischemia/reperfusion. Although further investigation will be needed, these characteristics of rhEPO may contribute to the reduction of necrotic as well as apoptotic cell death in ischemia/reperfused myocardium. In addition, since wortmannin inhibits not only PI3 kinase but also PI4 kinase and PI kinase related protein kinase, there is a limitation in using wortmannin as a specific inhibitor of PI3 kinase [29].

In clinical settings, ventricular arrhythmias are often observed in patients following reperfusion therapy and they can be life-threatening [30]. Importantly, the present study demonstrated that a high, but not a low dose of rhEPO prevented VF during reperfusion via the PI3 kinase-dependent pathway. Since low and high doses of rhEPO equally increased phosphorylation of Akt, it is unlikely that Akt is responsible for

the rhEPO-induced anti-arrhythmic effect. There are several possible mechanisms by which rhEPO exerts anti-arrhythmic effects via the PI3 kinase-dependent, but Akt-independent, pathway. First, under conditions of reperfusion, production of inositol-1,4,5-trisphospate (IP3) increases when phospholipase C (PLC) is activated through α-adrenoreceptors on the myocardial cell membrane [11]. This increase in IP3 activates IP3 receptors on the sarcoplasmic reticulum causing the release of Ca^{2+}. The increases in the intracellular Ca^{2+} levels caused by IP3 have been reported to initiate slow Ca^{2+} oscillations, which underlies the delayed afterdepolarizations that trigger many arrhythmias including VF [11,31]. PLC hydrolyzes phosphatidylinositol-4,5-bisphospate (PIP2) to produce IP3. Since PI3 kinase and PLC can act upon the common substrate, PIP2 [32], rhEPO may prevent lethal arrhythmia by activating the PI3 kinase pathway that results in the decrease in PIP2 levels, which will lead to prevent Ca^{2+} overload by IP3. Second, since oxygen-derived free radicals are involved in the generation of reperfusion arrhythmia [30,33,34], rhEPO may decrease reperfusion arrhythmia through the prevention of free radicals release from neutrophils or acting as a radical scavenger [27,28]. Finally, we need to consider that rhEPO exerts anti-arrhythmic effects by the reduction of myocardial infarct size.

In conclusion, our findings, when translated into clinical practice, may support the use of rhEPO as a cardioprotective agent in the treatment of patients with myocardial infarction.

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Microdialysis separately monitors myocardial interstitial myoglobin during ischemia and reperfusion

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Kitagawa, Hirotoshi, Toji Yamazaki, Tsuyoshi Akiyama, Masaru Sugimachi, Kenji Sunagawa, and Hidezo Mori. Microdialysis separately monitors myocardial interstitial myoglobin during ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 289: H924–H930, 2005. First published April 15, 2005; doi:10.1152/ajpheart.01207.2004.—Direct monitoring of myoglobin efflux during ischemia and reperfusion has been limited because of inherent sample collection problems in the ischemic region. Recently, the cardiac dialysis technique has offered a powerful method for monitoring myocardial interstitial levels of low-molecular-weight compounds in the cardiac ischemic region. In the present study, we extended the molecular target to high-molecular-weight compounds by use of microdialysis probes with a high-molecular-mass cutoff and monitored myocardial interstitial myoglobin levels. A dialysis probe was implanted in the left ventricular free wall in anesthetized rabbits. The main coronary artery was occluded for 60 or 120 min. We examined the effects of myocardial ischemia and reperfusion on myocardial interstitial myoglobin levels. Interstitial myoglobin increased within 15 min of ischemia and continued to increase during 120 min of ischemia, whereas blood myoglobin increased at 45 min of ischemia. Lactate and myoglobin in the interstitial space increased during the same period. At 60 min of ischemia, reperfusion markedly accelerated interstitial myoglobin release. The interstitial myoglobin level was fivefold higher at 0–15 min of reperfusion than at 60–75 min of coronary occlusion. The dialysis technique permits earlier detection of myoglobin release and separately monitors myoglobin release during ischemia and reperfusion. Myocardial interstitial myoglobin levels can serve as an index of myocardial injury evoked by ischemia or reperfusion.

infarction; interstitial space; membrane permeability

IT IS WELL KNOWN that certain proteins, including myoglobin, called serum cardiac markers, are released into the bloodstream in large quantities from necrotic cardiac muscle cells after myocardial infarction (20, 26, 43). However, because direct samples from the ischemic region are not readily obtainable, in situ studies on efflux of these proteins in the cardiac ischemic region have been limited (22). This problem of sample collection from the ischemic region remains unresolved. First, it is uncertain exactly when cardiac markers appear from injured myocardium. The appearance of cardiac markers indicates the turning point from reversible injury to irreversible damage (43). However, the first appearance of cardiac markers in the bloodstream is influenced by the slow transport of cardiac markers from the interstitial space into the bloodstream (20). Thus the detection of this appearance is of great value in understanding the pathophysiological events induced by myocardial ischemia. Second, recent experimental and clinical findings suggest that reperfusion itself seems to accelerate the release of cardiac markers (18, 37, 38). However, the extent to which reperfusion contributes to relative changes in their release is unclear. To determine myocardial injury evoked by reperfusion, more information is needed about the extent to which ischemia and reperfusion affect changes in the release of cardiac markers. Third, present methods used to measure infarct size require tissue analysis several hours after the ischemic event (8). Furthermore, histochemical analysis depends on the times of ischemia and reperfusion (23, 33). Concise, dissociated assessments of ischemia and reperfusion injury have been a frequent object of research.

In general, mobilization of cardiac markers from ischemic myocardium to the bloodstream has been divided into two different sequences: release from the myocardial cell to the interstitial space and transport from the interstitial space into the bloodstream (20). Therefore, if we examine the first process in in situ myocardium, we can discuss the pathophysiological changes during development of ischemic myocardial necrosis. However, little information is available on interstitial protein kinetics in the ischemic region (15). Examination of protein kinetics in the ischemic region has been limited to assessment of protein kinetics in the isolated Langendorff-perfused heart (28, 39). Recently, a cardiac dialysis technique has provided a powerful method for monitoring myocardial interstitial levels of low-molecular-weight compounds in the cardiac ischemic region (2, 6, 14, 31). Furthermore, this method is suitable for distinguishing between ischemia and reperfusion responses (32). By improving the microdialysis probes with a high-molecular-mass cutoff membrane, we have extended the molecular target to high-molecular-weight peptides and proteins and monitored myocardial interstitial protein levels.

In the present study, we chose myoglobin as one of the earliest biochemical markers in myocardial injury (4, 34). We applied the dialysis technique to the heart of anesthetized rabbits and investigated myocardial interstitial myoglobin levels during coronary occlusion and reperfusion. To address the above-mentioned issues, we compared the first appearance of myocardial interstitial myoglobin levels with that of low-molecular-weight metabolites (lactate and glycerol). Further-

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more, we compared the time course of myocardial interstitial myoglobin during reperfusion after ischemia with that of sustained ischemia and examined the changes in myoglobin release evoked by reperfusion. The results of the present study indicate that microdialysis is suitable for distinguishing between ischemia and reperfusion injury.

MATERIALS AND METHODS

Animal Preparation

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). All protocols were approved by the Animal Subjects Committee of the National Cardiovascular Center. Thirty adult male Japanese White rabbits (2.5–3.2 kg) were anesthetized with pentobarbital sodium (30–35 mg/kg iv). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 $mg \cdot kg^{-1} \cdot h^{-1}$). The rabbits were intubated and ventilated with room air mixed with oxygen. Body temperature was maintained at ~39°C with a heating pad and lamp. Heart rate (HR), mean arterial pressure (MAP), and electrocardiogram were monitored and recorded continuously. Heparin sodium (200 IU/kg) was first administered intravenously and then maintained with a continuous infusion (5–10 $IU \cdot kg^{-1} \cdot h^{-1}$) to prevent blood coagulation. With the animal in the lateral position, the fifth or sixth rib on the left side was partially removed to expose the heart. A small incision was made in the pericardium, and the dialysis probe was implanted in the region perfused by the left circumflex coronary artery (LCX) of the left ventricular wall. A snare was placed around the main branch of the LCX to act as the occluder for later coronary occlusion. To ensure that the sampling area was in the ischemic region, we examined the color and motion of the ventricular wall during a brief occlusion and confirmed that the dialysis probe was correctly located. To avoid a preconditioning effect, the duration of occlusion was limited to a few seconds.

Dialysis Technique

We designed a handmade long transverse dialysis probe (1). One end of a polyethylene tube (25 cm long, 0.5 mm OD, 0.2 mm ID) was dilated with a 27-gauge needle (0.4 mm OD). Each end of the dialysis fiber (8 mm long, 0.215 mm OD, 0.175 mm ID, 300 Å pore size; Evaflux type 5A, Kuraray Medical) was inserted into the polyethylene tube and glued. A fine guiding needle (25 mm long, 0.51 mm OD, 0.25 mm ID) was used for implantation of the dialysis probes. A guiding needle was connected to the dialysis probe with a stainless steel rod (5 mm long, 0.25 mm OD). At a perfusion speed of 5 μl/min, the in vitro recovery rate (RR) of myoglobin was 15 ± 0.6% (number of dialysis probes = 3). In vitro RR was defined as follows: $RR = (C_{in} - C_{out})/C_{in}$, where C_{in} and C_{out} are the concentrations of myoglobin in the perfusate and in the dialysate, respectively (19). For monitoring myocardial interstitial lactate and glycerol levels, we used a conventional dialysis fiber (PAN-1200, Asahi Chemical Japan) to detect low-molecular-weight compounds (1).

Dialysis probes were perfused with Ringer solution (in mM: 147.0 NaCl, 4.0 KCl, and 2.25 $CaCl_2$) at 5 μl/min using a microinjection pump (model CMA/100, Carnegie Medicine). Figure 1 shows the time course of dialysate myoglobin levels collected at 1-h intervals over a 4-h period after probe implantation. Dialysate myoglobin rapidly decreased to 261 ± 56 ng/ml at 2 h after probe implantation. Thereafter, it gradually decreased, reaching an almost steady level of 222 ± 37 ng/ml 4 h after probe implantation. On the basis of the results of this experiment, in the subsequent protocol, we discarded the first 120-min collections of dialysate and measured the dialysate myoglobin level twice at 30-min intervals. When dialysate myoglobin levels reached the steady level, we started the experimental protocol.

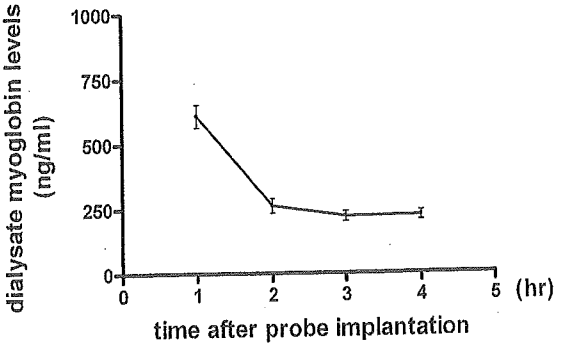

Fig. 1. Time course of dialysate myoglobin levels after probe implantation. Dialysate myoglobin levels decreased over the first 2 h and then reached an almost steady level. Values are means ± SE from 5 rabbits.

Sampling periods were 15 min (1 sampling volume = 75 μl) in control and during occlusion and reperfusion. Taking into consideration the dead space between the dialysis fiber and sample tube, we sampled the dialysate.

Dialysate myoglobin concentrations were measured as an index of myocardial interstitial myoglobin levels. Blood samples were obtained from the femoral artery. Using immunochemistry (Cardiac Reader, Roche Diagnostics), we measured the myoglobin levels (7). The detection limit of myoglobin was 30 ng/ml. The dialysate lactate and glycerol levels were measured by kinetic enzymatic analysis (CMA 600 analyzer, Carnegie Medicine) (30).

Experimental Protocols

After control sampling, we occluded the main branch of the LCX for 60 min and then released the occluder. We continuously sampled the dialysate from the ischemic region during 60 min of coronary occlusion and reperfusion.

Time course of dialysate lactate, glycerol, and myoglobin levels during myocardial ischemia. We compared the dialysate myoglobin levels with the blood myoglobin levels. After control sampling, we observed the time course of dialysate and blood myoglobin levels during 60 min of coronary occlusion. In addition, we measured simultaneously dialysate lactate and glycerol levels from the ischemic region in separate rabbits.

Time course of dialysate myoglobin levels during 60 min of reperfusion following 60 and 120 min of ischemia. Reperfusion modulates myocardial membrane damage and may accelerate dialysate myoglobin levels (18, 21, 37). We compared the time course of dialysate myoglobin during 60 min of reperfusion following 60 min of ischemia with that of 120 min of ischemia.

Time course of dialysate myoglobin levels during local administration of cyanide. To confirm whether the dialysate myoglobin level reflects myocardial damage evoked by ischemia or hypoxia, we tested the effect of local sodium cyanide (NaCN) administration on dialysate myoglobin levels. We collected a control dialysis sample and then replaced the perfusate with Ringer solution containing NaCN (30 mM), thereby locally administering NaCN for 60 min. We obtained four consecutive dialysate samples and measured the dialysate myoglobin levels.

At the end of each experiment, the rabbits were killed with an overdose of pentobarbital sodium, and the implant regions were checked to confirm that the dialysis probes had been implanted within the cardiac muscle.

Statistical Analysis

Dialysate lactate, glycerol, and myoglobin responses to coronary occlusion were statistically analyzed by one-way analysis of variance with repeated measures. When a statistically significant effect of

Table 1. *Changes in HR and MAP in coronary artery occlusion*

	120 min Coronary Occlusion								
	C	5	15	30	45	60	75	90	105
HR, beats/min	268±7	264±8	242±8*	250±3*	253±4*	252±4*	250±4*	251±3*	248±4*
MAP, mmHg	84±4	74±5*	69±5*	71±5*	68±5*	67±5*	66±4*	65±4*	64±5*
	60 min Occlusion - 60 min Reperfusion								
	C	5	15	30	45	R5	R15	R30	R45
HR, beats/min	274±9	261±6*	255±7*	254±6*	261±7*	256±5*	263±10*	264±12*	263±9*
MAP, mmHg	78±4	67±3*	65±2*	67±2*	65±2*	61±2*	60±3*†	61±2*	58±2*†

Values are mean ± SE. Data were obtained during control (C), after 5, 15, 30, 45, 60, 75, 90, and 105 min of coronary artery occlusion, and after 5, 15, 30, and 45 min of reperfusion (R). *$P < 0.05$ vs. control. †$P < 0.05$ vs. 45 min occlusion.

coronary occlusion was detected as a whole, the Newman-Keuls test was applied to determine which mean values differed significantly from each other (40). Statistical significance was defined as $P < 0.05$. Values are means ± SE.

RESULTS

Time Course of HR and MAP

Table 1 shows the time courses of HR and MAP during coronary occlusion and reperfusion. Coronary occlusion decreased HR and MAP. Reperfusion did not alter HR but temporarily decreased MAP.

Time Course of Dialysate Lactate, Glycerol, and Myoglobin Levels During Myocardial Ischemia

Coronary occlusion significantly altered dialysate myoglobin levels (Fig. 2). Dialysate myoglobin levels increased significantly from 168 ± 32 ng/ml in the control to 570 ± 107 ng/ml at 0–15 min of occlusion. During 60 min of coronary occlusion, dialysate myoglobin levels progressively increased and reached 2,583 ± 208 ng/ml at 45–60 min of occlusion. A significant increase in blood myoglobin occurred at 45–60 min of coronary occlusion. Dialysate lactate levels were 1.00 ± 0.21 mmol/l in the control and increased after coronary occlusion (Fig. 3). During 60 min of coronary occlusion, dialysate lactate levels markedly increased and reached 3.34 ± 0.50 mmol/l at 45–60 min of occlusion. During 60 min of coronary occlusion, dialysate glycerol levels also increased and reached 232 ± 33 μmol/l at 45–60 min of occlusion.

Time Course of Dialysate Myoglobin Levels During 60 min of Reperfusion Following 60 and 120 Minutes of Ischemia

There were no significant differences in the control dialysate myoglobin levels between the two groups (Fig. 4). During ischemia, the dialysate myoglobin levels progressively increased and reached 4,054 ± 659 ng/ml at 105–120 min of coronary occlusion. During 60 min of coronary occlusion, there were no statistically significant differences in the dialysate myoglobin levels between the two groups. After release of the occluder, the dialysate myoglobin levels markedly increased to 12,569 ± 2,347 ng/ml at 0–15 min of reperfusion. The dialysate myoglobin levels at 0–15 min of reperfusion were fivefold higher than those at 60–75 min of 120 min of coronary occlusion. Furthermore, these values were higher than peak levels during 120 min of coronary occlusion. The

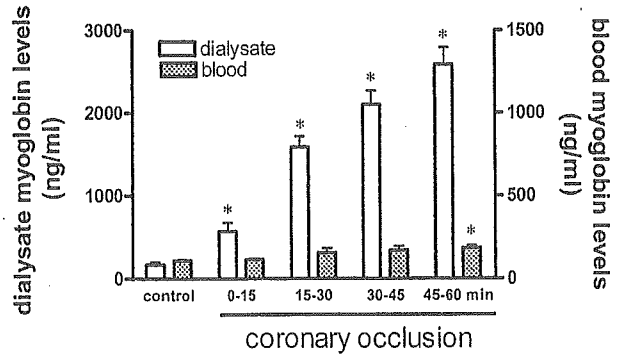

Fig. 2. Time courses of dialysate and blood myoglobin levels during 60 min of ischemia. Values are means ± SE. *$P < 0.05$ vs. control.

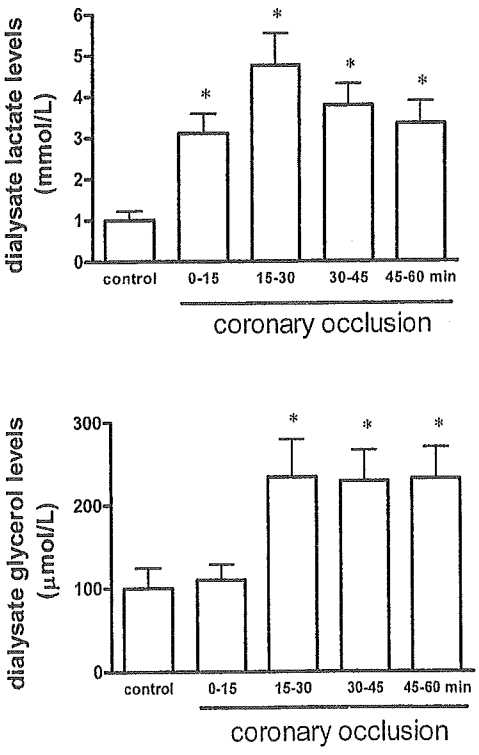

Fig. 3. Time courses of dialysate lactate (*top*) and glycerol (*bottom*) levels during 60 min of ischemia. Values are means ± SE. *$P < 0.05$ vs. control.

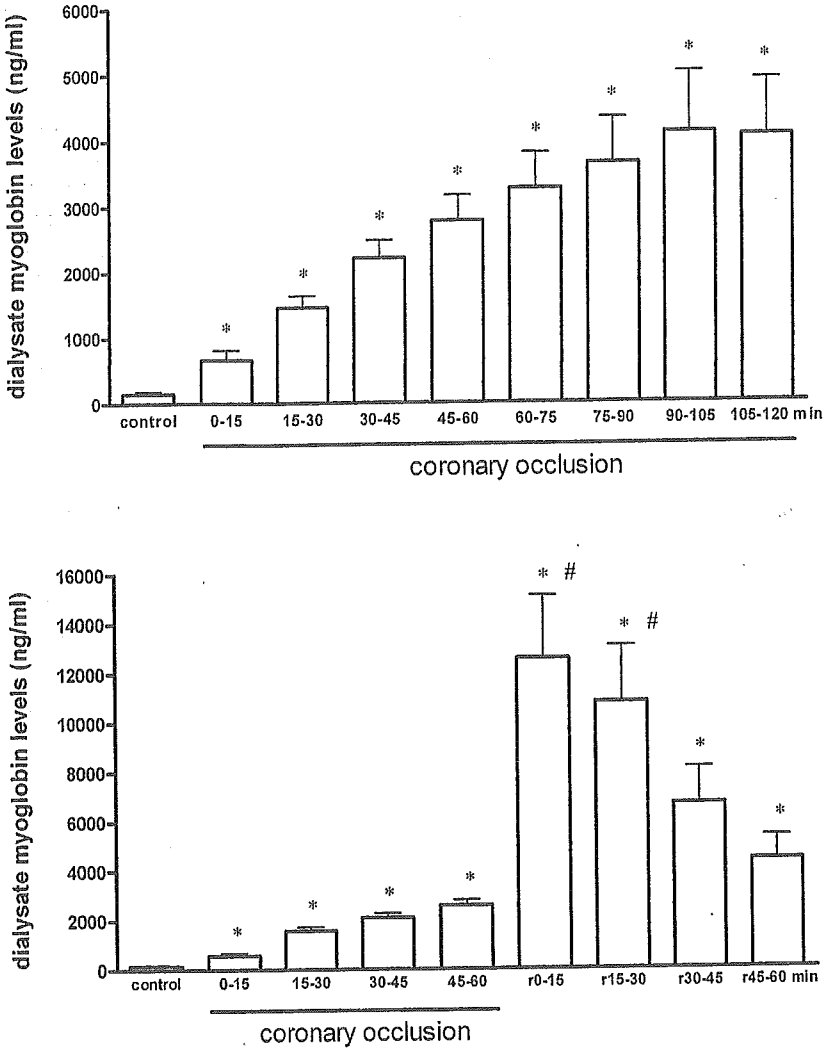

Fig. 4. Time courses of dialysate myoglobin levels during 120 min of ischemia (*top*) and 60 min of ischemia followed by 60 min of reperfusion (r, *bottom*). Values are means ± SE. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. 45–60 min of occlusion.

dialysate myoglobin levels gradually decreased and reached 4,391 ± 879 ng/ml at 45–60 of reperfusion. At 0–15 min of reperfusion, dialysate lactate and glycerol levels were 3.27 ± 0.61 mmol/l and 242 ± 37.7 μmol/l, respectively. Dialysate lactate and glycerol levels remained unchanged at 0–15 min of reperfusion.

Time Course of Dialysate Myoglobin Levels During Local Administration of NaCN

Local administration of NaCN increased the dialysate myoglobin levels (Fig. 5). This increase was statistically significant compared with the control level at all collection periods during NaCN administration, except at 0–15 min. The maximum myoglobin level was comparable to that observed during 60 min of ischemia.

DISCUSSION

Using the dialysis technique in the in vivo rabbit heart, we observed myocardial interstitial myoglobin levels during myocardial ischemia and reperfusion. Our data demonstrated myoglobin release in the early stage of cardiac ischemia and its enhancement by reperfusion. We discuss here the time course of myocardial myoglobin release during coronary occlusion and after reperfusion.

We show for the first time that myoglobin release increases within 15 min of ischemia and continues to increase during 60 min of ischemia. However, significant changes in the blood myoglobin level occurred at 45–60 min of coronary occlusion. Our data suggest a contrast between blood and dialysate

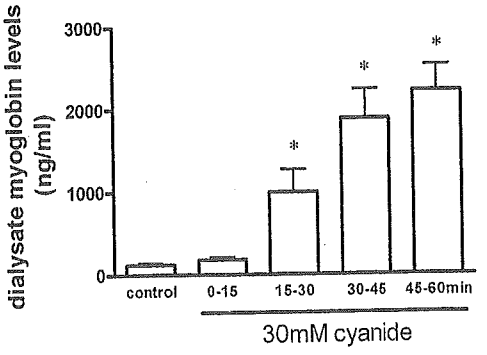

Fig. 5. Time course of dialysate myoglobin levels during local administration of sodium cyanide (30 mM). Values are means ± SE. $^{*}P < 0.05$ vs. control.

myoglobin levels during ischemia. The delay of the first appearance of myoglobin in the bloodstream is mainly due to the slow transport of myoglobin from the interstitial space into the bloodstream (20). Therefore, myoglobin concentration measured by cardiac microdialysis provides information regarding early release of cytosol protein into the interstitial space. Within the 15-min time resolution, this increase in myoglobin release was accompanied by increases in interstitial lactate. Dead space volume between the dialysis fiber and the sample microtube was identical for lactate, glycerol, and myoglobin. The currently accepted concept (20) is that leakage of anaerobic metabolites precedes macromolecular protein release during ischemia. Anaerobic metabolites accumulate and leak from the ischemic region within minutes via diffusion or transport (6, 12, 41). In contrast to low-molecular-weight metabolites, macromolecular proteins could be released into the interstitial space without cytosol accumulation of myoglobin, probably via bleb or altered permeability. Although sampling periods of 15 min are too long to enable us to distinguish the rate of release of lactate vs. myoglobin, our data at least suggest that cellular metabolic derangement is involved in membrane disruption for myoglobin release.

Myocardial injury caused by ischemia-reperfusion is associated with membrane phospholipid degradation, which is thought to underlie disruption of the cell membrane (27). Glycerol is an end product of membrane phospholipid degradation and has been used to study membrane phospholipid degradation after cerebral ischemia and seizures (12). In the present study, dialysate glycerol was examined as a potential marker for membrane phospholipid degradation in myocardial ischemia and reperfusion. We observed increases in dialysate glycerol levels during 15–60 min of ischemia but not during reperfusion. In general, phospholipid degradation is accentuated during reperfusion (27). Therefore, dialysate glycerol is not suitable as an index of membrane phospholipid degradation, and the release of glycerol from membrane phospholipid degradation might be too small to allow detection in blood-perfused heart.

Early change of cytosol myoglobin was detected by immunofluorescence after occlusion of the coronary artery (16, 25). Histochemical studies demonstrated that intracellular diffusion of cytosol myoglobin into the nuclei and mitochondria was evident as early as 0.5 h after coronary artery occlusion (17, 25). Our data demonstrate early loss of cytosol myoglobin into the interstitial space. Release of cytosol protein is caused by membrane damage via alteration of permeability or bleb formation. Blebs appeared on the cell surfaces, and the cell began to swell within 10–20 min of ATP depletion in a glia cell line or hepatocytes (13, 24). Furthermore, NMR spectroscopy suggested that sarcolemmal membranes are gradually permeabilized to large molecules by ischemia (3). These alterations of sarcolemmal membranes might be involved in early release of myoglobin during the myocardial ischemia. Our method offers extremely fast and sensitive analysis of membrane injury in myocardial ischemia that is not evident by histological or blood analysis. Quantitative assessment of interstitial myoglobin levels could be performed independently of reperfusion cell injury and could be helpful in devising various myocardial preservation treatments.

We show that reperfusion markedly accelerates myoglobin release in the ischemic region. The interstitial myoglobin levels at 0–15 min of reperfusion were fivefold higher than those at 60–75 min of 120 min of coronary occlusion. During the reperfusion period, interstitial accumulated myoglobin might be washed out into the bloodstream (37). Therefore, the amount of released myoglobin at reperfusion could be markedly greater than the changes in interstitial myoglobin concentrations at reperfusion. Release of cytosolic protein resulted from a disruption of a sarcolemmal bleb or an enhancement of membrane permeability (5, 29, 35). Either condition may gain relevance during the reperfusion period. Thus the release of myoglobin during the reperfusion seems to serve as an index of disrupted sarcolemmal membrane.

Although the exact mechanisms of accelerated myoglobin release cannot be determined from the present study, our data suggest that substances induced during reperfusion differ from those induced during ischemia. Reperfusion enhanced myoglobin release but did not accelerate lactate or glycerol release in the interstitial space, whereas ischemia accompanied macromolecular myoglobin release as well as anaerobic metabolite release. Furthermore, in the previous studies, neither catecholamine nor acetylcholine release was accelerated by reperfusion in ischemic cardiac sympathetic and parasympathetic nerve endings (2, 14). During reperfusion, surviving myocardial cells and nerve terminals quickly recover aerobic metabolism and take up these accumulated substances, whereas myocardial cells have no capability of myoglobin uptake via the sarcolemmal membrane, leading to continued myoglobin release via the disrupted membrane. Reperfusion may enhance membrane permeability (5). Further disruption of membrane blebs may cause rupture of the membrane (29, 35). Alternatively, in isolated perfused rats, leakage of cytoplasmic enzymes during reoxygenation is accelerated by cardiac revived beating, because the cell membrane becomes fragile during the preceding anoxia (36). In either condition, reperfusion-induced breakdown of membrane phospholipids contributes to an alteration of permeability or bleb formation (27). Disruption of the membrane phospholipid bilayer is likely to play a role in myoglobin release from the cyotosome into the interstitial spaces.

In the present study, we demonstrate that loss of cytosol myoglobin occurs during myocardial ischemia and reperfusion and might be involved in the outcome and pathophysiology of the ischemic heart. Loss of cytosol myoglobin may precede, at least in part, histological evidence of necrosis and occur in the remaining viable myocardium that is not necrotic (11). In vertebrate heart, myoglobin is involved in the transport of oxygen from the sarcolemma to the mitochondria (42). Recent studies from myoglobin knockout mice indicate that myoglobin contributes to the scavenging of bioactive nitric oxide (NO) or oxygen radicals during ischemia-reperfusion (9, 10). NO production and/or oxidant injury occur during the reperfusion period. In hearts lacking myoglobin, changes in NO and oxidative stress have a much larger impact on the maintenance of vascular tone and cardiac function (44). Similarly, in myoglobin knockout mice, loss of cytosol myoglobin may be involved in the delayed restoration of cardiac contractility in the postischemic region.

There are several limitations to the present study. First, with application of the dialysis technique to the heart, we had to perform this experiment as an acute surgical preparation. Probe implantation and/or surgical preparation might affect the con-

centration of myocardial interstitial myoglobin. To examine the effect of probe implantation and/or surgery, we performed the preliminary experiment on brief occlusion (3 min). Three minutes of coronary occlusion did not alter dialysate myoglobin levels. Furthermore, to confirm whether the dialysate myoglobin level reflects myocardial damage evoked by ischemia or hypoxia, we tested the effect of local NaCN administration on dialysate myoglobin levels: with NaCN, we found increases in dialysate myoglobin levels similar to the increase evoked by myocardial ischemia. Therefore, we believe that dialysate myoglobin levels reflect the release of myoglobin evoked by ischemia as well as by chemical hypoxia. The absolute myoglobin level might be affected by implantation and/or surgical preparation. However, it is possible to estimate myoglobin release from relative changes in myoglobin levels.

Second, in the present study, myocardial interstitial myoglobin levels during coronary occlusion and reperfusion were determined regionally. We implanted the dialysis probe in the midwall of the left ventricle. When the dialysis probe was implanted in the subendocardial zone, it is likely that subendocardial ischemia was much more severe than in the midwall, where the sampling was performed. Actually, subendocardial lactate was significantly greater than epicardial lactate during severe ischemia in the anesthetized dogs (6). Further studies are warranted concerning the influence of the ischemic area (subendocardial or marginal zone) on its myocardial interstitial myoglobin levels.

In summary, this microdialysis study in an ischemic animal model shows that coronary occlusion induced myoglobin release in minutes. Micromolecular metabolite (lactate) and macromolecular protein (myoglobin) increased during the first 15 min of ischemia. Reperfusion markedly enhanced myoglobin release without increases in lactate or glycerol levels. Elevation of myoglobin release represents an increase in sarcolemmal permeability or bleb formation during ischemia and reperfusion. Massive disruption of myocardial membrane occurs immediately after ischemia and is markedly accelerated by reperfusion. The dialysis technique permits more concise in vivo monitoring of myocardial membrane disruption during ischemia and reperfusion separately.

GRANTS

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Transplantation of Mesenchymal Stem Cells Improves Cardiac Function in a Rat Model of Dilated Cardiomyopathy

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Background—Pluripotent mesenchymal stem cells (MSCs) differentiate into a variety of cells, including cardiomyocytes and vascular endothelial cells. However, little information is available about the therapeutic potency of MSC transplantation in cases of dilated cardiomyopathy (DCM), an important cause of heart failure.

Methods and Results—We investigated whether transplanted MSCs induce myogenesis and angiogenesis and improve cardiac function in a rat model of DCM. MSCs were isolated from bone marrow aspirates of isogenic adult rats and expanded ex vivo. Cultured MSCs secreted large amounts of the angiogenic, antiapoptotic, and mitogenic factors vascular endothelial growth factor, hepatocyte growth factor, adrenomedullin, and insulin-like growth factor-1. Five weeks after immunization, MSCs or vehicle was injected into the myocardium. Some engrafted MSCs were positive for the cardiac markers desmin, cardiac troponin T, and connexin-43, whereas others formed vascular structures and were positive for von Willebrand factor or smooth muscle actin. Compared with vehicle injection, MSC transplantation significantly increased capillary density and decreased the collagen volume fraction in the myocardium, resulting in decreased left ventricular end-diastolic pressure (11 ± 1 versus 16 ± 1 mm Hg, $P<0.05$) and increased left ventricular maximum dP/dt (6767 ± 323 versus 5138 ± 280 mm Hg/s, $P<0.05$).

Conclusions—MSC transplantation improved cardiac function in a rat model of DCM, possibly through induction of myogenesis and angiogenesis, as well as by inhibition of myocardial fibrosis. The beneficial effects of MSCs might be mediated not only by their differentiation into cardiomyocytes and vascular cells but also by their ability to supply large amounts of angiogenic, antiapoptotic, and mitogenic factors. **(*Circulation*. 2005;112:1128-1135.)**

Key Words: myocytes ■ angiogenesis ■ heart failure ■ growth substances ■ transplantation

Despite advances in medical and surgical procedures, congestive heart failure remains a leading cause of cardiovascular morbidity and mortality.[1] Idiopathic dilated cardiomyopathy (DCM), a primary myocardial disease of unknown etiology characterized by a loss of cardiomyocytes and an increase in fibroblasts, is an important cause of heart failure.[2] Although myocyte mitosis and the presence of cardiac precursor cells in adult hearts have recently been reported,[3] the death of large numbers of cardiomyocytes results in the development of heart failure. Thus, restoring lost myocardium would be desirable for the treatment of DCM.

Mesenchymal stem cells (MSCs) are pluripotent, adult stem cells residing within the bone marrow microenvironment.[4] In contrast to their hematopoietic counterparts, MSCs are adherent and can be expanded in culture. MSCs can differentiate not only into osteoblasts, chondrocytes, neurons, and skeletal muscle cells but also into vascular endothelial cells[5] and cardiomyocytes.[6,7] In vitro, MSCs can be induced to differentiate into beating cardiomyocytes by 5-azacytidine treatment.[8] In vivo, MSCs directly injected into an infarcted heart have been shown to induce myocardial regeneration and improve cardiac function.[9] In addition, MSC implantation induces therapeutic angiogenesis in a rat model of hindlimb ischemia through vascular endothelial growth factor (VEGF) production by MSCs.[10,11] Myocardial blood flow abnormalities, even in the presence of angiographically normal coronary arteries, have been documented in patients with DCM.[12]

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These findings raise the possibility that transplanted MSCs have beneficial effects on myocardial structure and function via myogenesis and angiogenesis. However, little information is available about the therapeutic potential of MSCs for DCM.

A unique model of myocarditis in the rat has been created by immunization with porcine cardiac myosin,[13] which results in severe heart failure characterized by increased cardiac fibrosis and left ventricular (LV) dilation.[14] Thus, the late phase of this model can serve as a model of DCM.

The purpose of this study was to investigate the following topics: (1) whether transplantation of MSCs induces myogenesis and angiogenesis, decreases collagen deposition in the myocardium, and thereby improves cardiac function in a rat model of DCM and (2) whether the beneficial effects of MSCs are mediated by their differentiation into cardiomyocytes and vascular cells and/or by their supplying angiogenic, antiapoptotic, and mitogenic factors.

Methods

Expansion of Bone Marrow MSCs

MSC expansion was performed according to previously described methods.[4] In brief, we humanely killed male Lewis rats and harvested bone marrow by flushing their femoral and tibial cavities with phosphate-buffered saline (PBS). Bone marrow cells were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. A small number of cells developed visible symmetric colonies by days 5 to 7. Nonadherent hematopoietic cells were removed, and the medium was replaced. The adherent, spindle-shaped MSC population expanded to $>5\times10^7$ cells within $\approx$4 to 5 passages after the cells were first plated.

Flow Cytometry

Cultured MSCs were analyzed by fluorescence-activated cell sorting (FACS) (FACScan flow cytometer, Becton Dickinson). Cells were incubated with fluorescein isothiocyanate (FITC)–conjugated mouse monoclonal antibodies against rat CD31 (clone TLD-3A12, Becton Dickinson), CD34 (clone ICO-115, Santa Cruz), CD45 (clone OX-1, Becton Dickinson), CD90 (clone OX-7, Becton Dickinson), vimentin (clone V9, Dako), and smooth muscle actin (SMA; clone 1A4, Dako). FITC-conjugated hamster anti-rat CD29 monoclonal antibody (clone Ha2/5, Becton Dickinson) and rabbit anti-rat c-Kit polyclonal antibody (clone C-19, Santa Cruz) were used. Isotype-identical antibodies served as controls.

Model of DCM

Male Lewis rats weighing 220 to 250 g (Japan SLC Inc, Hamamatsu, Japan) were used in this study. These isogenic rats served as donors and recipients of MSCs to simulate autologous implantation. DCM was produced by inducing experimental myocarditis, as described previously.[13,14] In brief, 1 mg (0.1 mL) of porcine heart myosin (Sigma) was mixed with an equal volume of Freund's complete adjuvant (Sigma) and injected into a footpad on days 1 and 7. Five weeks after immunization, these rats served as a model of heart failure due to DCM.

MSC Transplantation

In a preliminary experiment, we performed dose-response studies to obtain the maximal effects of cell transplantation. Because the effect of 10^6 MSCs was modest, we used 5×10^6 MSCs for transplantation. Five weeks after immunization, we injected a total of 5×10^6 MSCs/100 μL PBS, or PBS alone, into the myocardium at 10 points. In brief, the LV was divided into 3 levels (basal, middle, and apical). The basal and middle levels were each subdivided into 4 segments, and the apical level was subdivided into 2 segments. Injection into each segment was performed with a 27-gauge needle. Sham rats received intramyocardial injections of 100 μL PBS. This protocol resulted in the creation of 3 groups: DCM rats given MSCs (MSC-treated DCM group, n=10); DCM rats given PBS (untreated DCM group, n=10); and sham rats given PBS (sham group, n=10). The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

Echocardiographic Studies

Echocardiographic studies were performed by an investigator, blinded to treatment allocation, at 5 weeks after immunization (before treatment) and 4 weeks after cell transplantation (after treatment). Two-dimensional, targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system equipped with a 7.5-MHz transducer (HP Sonos 5500, Hewlett-Packard).[15] LV dimensions were measured according to the American Society for Echocardiology leading-edge method from at least 3 consecutive cardiac cycles. Fractional shortening was calculated as (LVDd−LVDs)/LVDd×100, where LVDd=LV diastolic dimension and LVDs=LV systolic dimension.

Hemodynamic Studies

Hemodynamic studies were performed 4 weeks after cell transplantation. A 1.5F micromanometer-tipped catheter (Millar Instruments) was inserted into the right carotid artery for measurement of mean arterial pressure.[16] Next, the catheter was advanced into the LV for measurement of LV pressure. Hemodynamic variables were measured with a pressure transducer (model P23 ID, Gould) connected to a polygraph. After completion of these measurements, the left and right ventricles were excised and weighed.

Histological Examination

To detect fibrosis in cardiac muscle, the LV myocardium (n=5 from each group) was fixed in 10% formalin, cut transversely, embedded in paraffin, and stained with Masson's trichrome. Transverse sections were randomly obtained from the 3 levels (basal, middle, and apical), and 20 randomly selected fields per section (n=60 per animal) were analyzed. After each field was scanned and computerized with a digital image analyzer (WinRoof, Mitani Co), collagen volume fraction was calculated as the sum of all areas containing connective tissue divided by the total area of the image.[15]

To detect capillaries in the myocardium, samples of harvested muscle (n=5 each) were embedded in OCT compound (Miles Scientific), snap-frozen in LN_2, cut into transverse sections, and stained for alkaline phosphatase by an indoxyltetrazolium method. Transverse sections were randomly obtained from the 3 levels (basal, middle, and apical), and 5 randomly selected fields per section (n=15 per animal) were analyzed. The number of capillaries was counted by light microscopy at a magnification of ×200. The number of capillaries in each field was averaged and expressed as the number of capillary vessels. These morphometric studies were performed by 2 examiners who were blinded to treatment assignment.

Assessment of Cell Differentiation

Suspended MSCs were labeled with fluorescent dyes with use of a PKH26 red fluorescent cell linker kit (Sigma), as reported previously.[17] Fluorescence-labeled MSCs were injected into the myocardium 5 weeks after immunization. Rats (n=5) were humanely killed 4 weeks after cell transplantation. LV samples were embedded in OCT compound, snap-frozen in LN_2, and cut into sections. Immunofluorescence staining was performed with monoclonal mouse anti-cardiac troponin T (Novo), anti-desmin (Dako), anti-connexin-43 (Sigma), polyclonal rabbit anti–von Willebrand factor (Dako), and monoclonal mouse SMA (Dako). FITC-conjugated IgG antibody (BD Pharmingen) was used as a secondary antibody. To perform quantitative analysis of the magnitude of MSC differentiation into cardiomyocytes, heart cells from each rat (n=5) were isolated by incubation in balanced salt solution containing 0.06% collagenase type II (Worthington Biochemical Co), as reported previously.[18] PKH26/troponin T double-positive cells were detected by FACS.

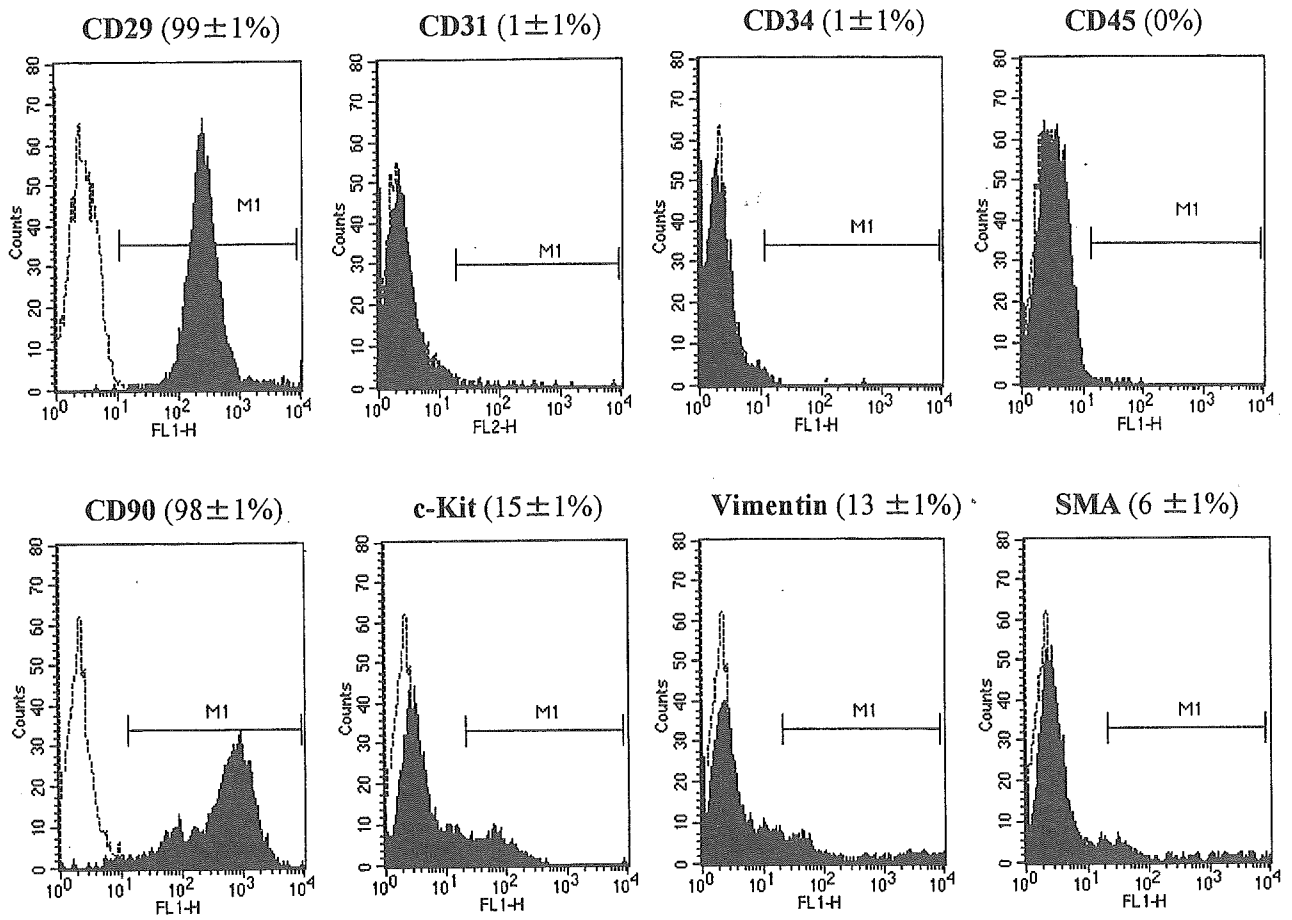

Figure 1. Flow-cytometric analysis of the adherent, spindle-shaped MSC population expanded to 4 to 5 passages. Most of the MSCs expressed CD29 and CD90, whereas they were negative for CD31, CD34, CD45, and SMA. Some of the cells were positive for c-Kit and vimentin.

Western Blot Analysis of Matrix Metalloproteinases

To identify the protein expression of matrix metalloproteinases (MMPs)-2 and -9, Western blotting was performed with rabbit polyclonal antibody raised against MMP-2 (Laboratory vision Co) and MMP-9 (Chemicon Co). The LV obtained from individual rats was used for comparison among the 3 groups (n=5 each). These samples were homogenized on ice in 0.1% Tween 20 homogenization buffer with a protease inhibitor. Then, 40 μg of protein was transferred into sample buffer, loaded on a 7.5% sodium dodecyl sulfate–polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane (Millipore Co). After being blocked for 120 minutes, the membrane was incubated with primary antibody at a dilution of 1:200. The membrane was incubated with peroxidase labeled with secondary antibody at a dilution of 1:1000. Positive protein bands were visualized with an ECL kit (Amersham) and measured by densitometry. Western blot analysis with a mouse polyclonal antibody raised against β-actin (Santa Cruz) was used as a protein loading control.

Assay for Angiogenic, Antiapoptotic, and Mitogenic Factors

To investigate whether MSCs produce angiogenic and growth factors, we measured VEGF, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and adrenomedullin (AM) levels in conditioned medium 24 hours after medium replacement. VEGF, HGF, and IGF-1 were measured by enzyme immunoassay (VEGF immunoassay, R&D Systems Inc; rat HGF enzyme immunoassay, Institute of Immunology Co, Ltd; and active rat IGF-1 enzyme immunoassay, Diagnostic Systems Laboratories, Inc). AM level was measured with a radioimmunoassay kit (Shionogi Co), as reported previously.[19] The amounts of these products produced by MSCs were compared with those produced by bone marrow–derived mononuclear cells (MNCs) because MNCs have commonly been used for regenerative therapy.[19–21] There was no significant difference in cell viability between MSCs and MNCs 24 hours after seeding (88±5% versus 85±4% by trypan blue solution). In vivo, circulating levels of VEGF, HGF, IGF-1, and AM were measured before and 24 hours after administration of MSCs or vehicle (n=6 from each group).

Statistical Analysis

Numerical values are expressed as mean±SEM unless otherwise indicated. Comparisons of parameters between 2 groups were made with unpaired Student *t* test. Comparisons of parameters among 3 groups were made with a 1-way ANOVA, followed by the Scheffe multiple-comparison test. Comparisons of changes in parameters among the 3 groups were made by a 2-way ANOVA for repeated measures, followed by the Scheffe multiple-comparison test. A value of $P<0.05$ was considered significant.

Results

Characterization of Cultured MSCs

Most cultured MSCs expressed CD29 and CD90 (Figure 1). In contrast, the majority of MSCs were negative for CD31, CD34, CD45, and SMA. Some of the MSCs expressed c-Kit and vimentin.

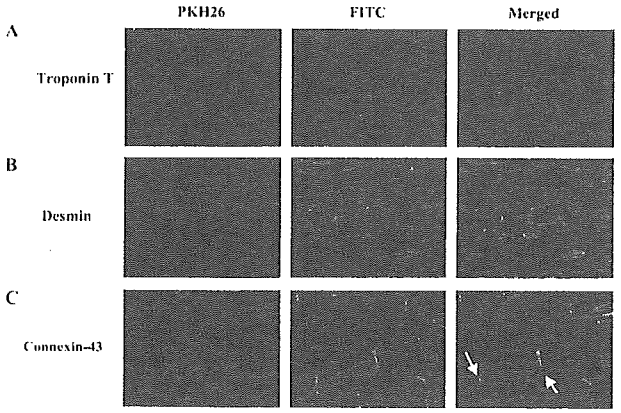

Figure 2. Differentiation of transplanted MSCs into cardiomyocytes. Transplanted MSCs were engrafted in the myocardium and stained for cardiac troponin T (A) and desmin (B). Engrafted MSCs also expressed connexin-43, a gap junction protein, at contact points with native cardiac myocytes (left arrow) and other transplanted cells (right arrow) (C). Magnification ×400.

Myogenesis and Angiogenesis Induced by MSCs

Red fluorescence–labeled MSCs were transplanted into the myocardium 5 weeks after immunization. Four weeks after transplantation, MSCs were engrafted into the myocardium (Figure 2). Immunofluorescence demonstrated that transplanted MSCs were positive for the cardiac markers cardiac troponin T and desmin (Figure 2). Transplanted MSCs also expressed connexin-43, a gap junction protein, at contact points with native cardiac myocytes as well as with MSCs. FACS analysis of isolated heart cells demonstrated that 8±1% of transplanted MSCs were double-positive for PKH26 and troponin T. These results suggest that a small number of transplanted MSCs can differentiate into cardiomyocytes.

Some transplanted MSCs formed vascular structures in the myocardium and were positive for von Willebrand factor (Figure 3A). Other MSCs were positive for SMA and participated in vessel formation as mural cells (Figure 3B). Alkaline phosphatase staining of the ischemic myocardium showed marked augmentation of neovascularization in the MSC-treated DCM group (Figures 4A–4C). Quantitative analysis demonstrated that capillary density was significantly

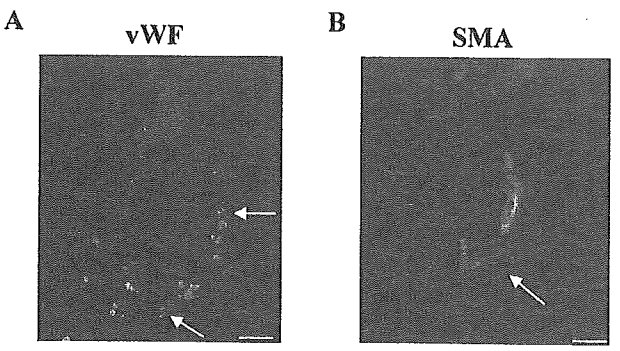

Figure 3. Differentiation of transplanted MSCs into vascular endothelial cells and smooth muscle cells. Some of the transplanted MSCs were positive for von Willebrand factor (vWF, A) and SMA (B) and formed vascular structures (A and B). Scale bars=10 μm.

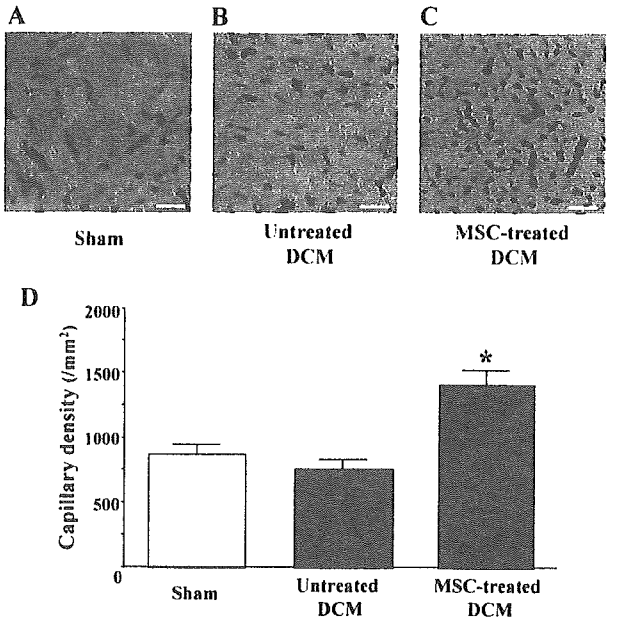

Figure 4. A–C, Representative samples of alkaline phosphatase staining of myocardium. Magnification, ×200. Scale bars=10 μm. D, Quantitative analysis of capillary density in the myocardium. Data are mean±SEM *P<0.05 vs untreated DCM group.

higher in the MSC-treated DCM group than in the untreated DCM group (Figure 4D).

Angiogenic, Antiapoptotic, and Mitogenic Factors Released From MSCs

After 24 hours of culture, MSCs secreted large amounts of angiogenic and antiapoptotic factors, including VEGF, HGF, and AM (Figure 5). Compared with MNCs that have commonly been used for regenerative therapy,[20–22] MSCs secreted 4-fold more VEGF and 5-fold more HGF. Similarly, MSCs secreted 6-fold more AM, an angiogenic and antiapoptotic peptide, compared with MNCs. MSCs also secreted a large amount, 10-fold greater than MNCs, of IGF-1, a growth hormone mediator for myocardial growth (Figure 5). Transplantation of MSCs significantly increased circulating VEGF (45.8±1.6 to 68.5±3.6 pg/mL, P<0.05), HGF (431.8±56.6 to 517.2±67.1 pg/mL, P<0.05), and AM (23.4±0.8 to 41.2±4.8 pg/mL, P<0.05) 24 hours after transplantation, although vehicle injection did not alter these parameters. Serum IGF-1 tended to increase after MSC transplantation (938.1±151.6 to 1063.5±116.9 pg/mL, P=NS), but this increase did not reach statistical significance.

Hemodynamic Effects of MSC Transplantation

Nine weeks after immunization, LV end-diastolic pressure showed a marked elevation in the untreated DCM group; this elevation was significantly attenuated in the MSC-treated DCM group (Figure 6A). LV maximum *dP/dt* was significantly lower in the untreated DCM group than in the sham group (Figure 6B). However, LV maximum *dP/dt* was significantly improved 4 weeks after MSC transplantation. There was no significant difference in heart rate or mean arterial pressure among the 3 groups (the Table). Echocardiographic studies demonstrated LV dysfunction and dilation

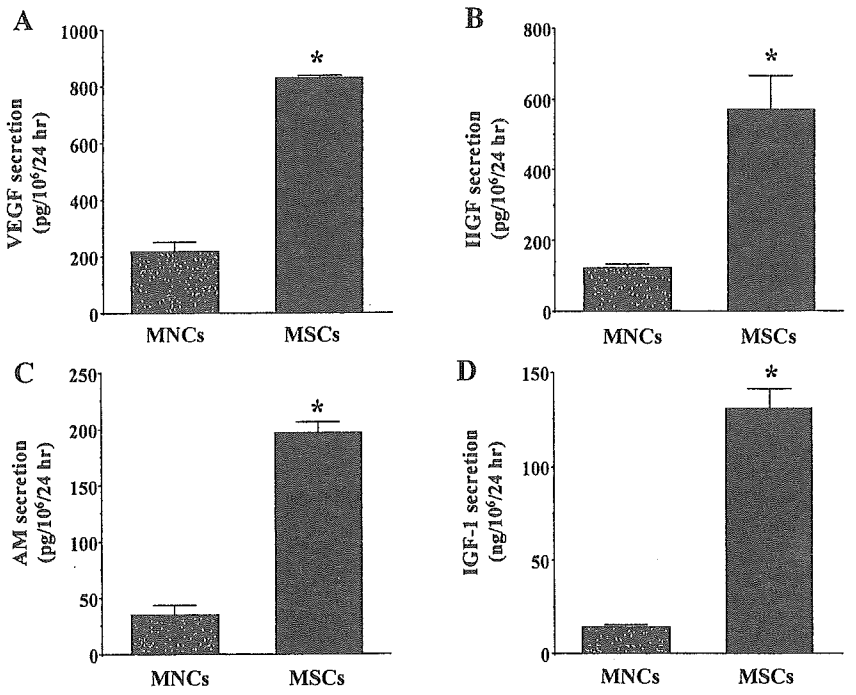

Figure 5. A–D, Angiogenic, antiapoptotic, and mitogenic factors produced by MSCs and bone marrow–derived MNCs). Compared with MNCs, MSCs secreted large amounts of VEGF, HGF, AM, and IGF-1. *$P<0.05$ vs MNCs.

in the untreated DCM group, as indicated by a decrease in percent fractional shortening and an increase in LV diastolic dimension (Figure 6C and 6D). However, MSC transplantation increased percent fractional shortening and inhibited the increase in LV diastolic dimension.

Reduction of Myocardial Fibrosis by MSC Transplantation

Masson's trichrome staining demonstrated modest myocardial fibrosis in the untreated DCM group (Figure 7A). However, MSC transplantation significantly attenuated the development of myocardial fibrosis. Quantitative analysis also demonstrated that the collagen volume fraction in the MSC-treated DCM group was significantly smaller than that in the untreated DCM group (Figure 7B). Western blot analysis showed that myocardial contents of MMP-2 and MMP-9 in the untreated DCM were significantly increased compared with those in the sham group (Figure 7C–E). However, the increases in MMP-2 and MMP-9 levels were attenuated by MSC transplantation, although the change in MMP-9 did not reach statistical significance.

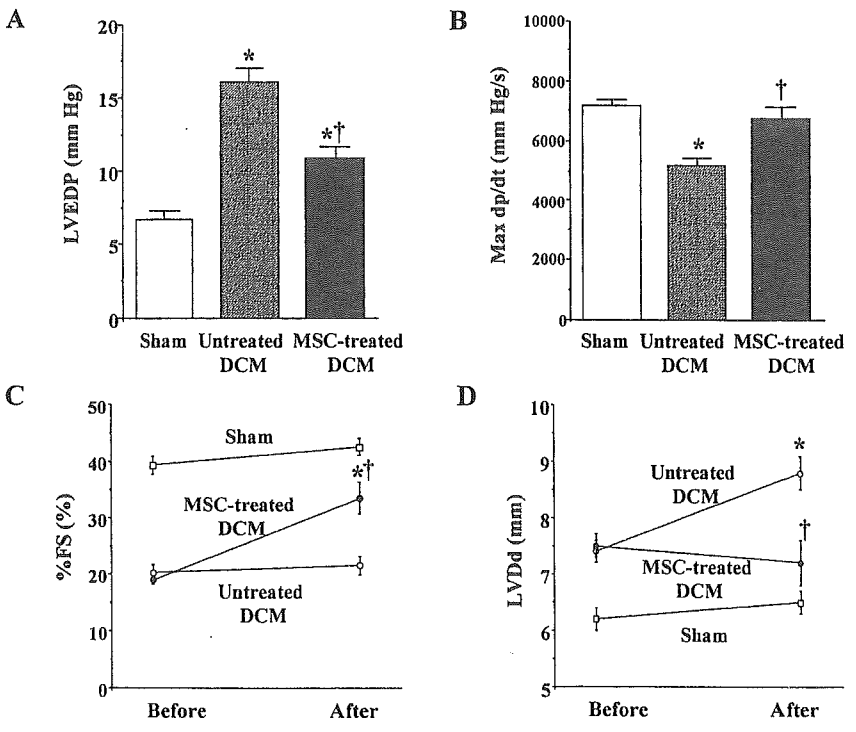

Figure 6. A and B, Effects of MSC transplantation on hemodynamic parameters. LVEDP indicates LV end-diastolic pressure; Max *dP/dt*, LV maximum *dP/dt*. Data are mean±SEM. *$P<0.05$ vs sham group; †$P<0.05$ vs untreated DCM group. C and D, Changes in echocardiographic parameters induced by MSC transplantation. %FS indicates LV fractional shortening. Data are mean±SEM *$P<0.05$ vs before transplantation; †$P<0.05$ vs the time-matched untreated DCM group.

Physiological Profiles of the 3 Experimental Groups

	Sham	Untreated DCM	MSC-Treated DCM
n	10	10	10
Body wt, g	421±8	372±4*	389±5*
LV wt/body wt, g/kg	1.91±0.05	2.18±0.06*	2.05±0.05
RV wt/body wt, g/kg	0.55±0.01	0.68±0.02*	0.60±0.03†
Heart rate, bpm	403±10	432±15	417±12
Mean arterial pressure, mm Hg	134±2	123±3	132±5

wt indicates weight; RV, right ventricle. Sham-operated rats were given vehicle only. The untreated DCM group included DCM rats treated with vehicle. The MSC-treated DCM group included DCM rats treated with MSCs. Data are mean±SEM.

*$P<0.05$ vs sham group; †$P<0.05$ vs untreated DCM group.

Discussion

In the present study, we have demonstrated the following effects of MSC transplantation in a rat model of DCM: (1) induction of myogenesis and angiogenesis; (2) differentiation of transplanted MSCs into cardiomyocytes, vascular endothelial cells, and smooth muscle cells; (3) secretion of large amounts of VEGF, HGF, AM, and IGF-1; (4) improvement of cardiac function and inhibition of ventricular remodeling; and (5) decrease in collagen volume fraction in the myocardium.

Earlier studies have shown that transplantation of MSCs improves cardiac function in experimental models of ischemic heart disease.[9,23] However, little information is available about the therapeutic potential of MSCs for chronic heart failure due to DCM. Previous studies have shown that porcine cardiac myosin-induced myocarditis progresses to a chronic phase resembling DCM.[13,14] Thus, we used this model 5 weeks after immunization as an example of experimental DCM.

In the present study, transplanted MSCs were engrafted into the myocardium in a rat model of DCM. Four weeks after transplantation, some of the engrafted MSCs were positively stained for cardiac troponin T and desmin. Transplanted MSCs also expressed connexin-43, a gap junction protein, at contact points with native cardiac myocytes as well as with MSCs. These results suggest that MSCs differentiate into cardiomyocytes in the myocardium and form connections with native cardiomyocytes in rats with DCM. Unlike earlier studies that have used a model of myocardial infarction,[7,9,23] we used a rat model of DCM to demonstrate the engraftment and cardiogenic differentiation of MSCs. Importantly, MSC transplantation improved cardiac function in these rats, as indicated by a significant decrease in LV end-diastolic pressure and an increase in LV dP/dt_{max}. Thus, the improvement in cardiac function may be a result of MSC-induced myocardial regeneration; however, further studies are necessary to investigate the mechanisms by which MSCs develop into cardiac myocyte–like cells.

Some of the transplanted MSCs were positive for a vascular endothelial cell marker and participated in vessel formation. MSC transplantation significantly increased capillary density in the myocardium. SMA staining revealed that MSCs differentiated into vascular smooth muscle cells, which play an important role in vessel maturation. Earlier studies have shown that transplantation of MNCs induces therapeutic angiogenesis in patients with limb ischemia or ischemic heart disease.[20–22] The angiogenic potential of MNCs is mediated at least in part by production by the cells of a variety of angiogenic factors.[24] Although MSCs have also been shown to produce VEGF,[10,25] there has been no study to compare their production between MSCs and MNCs. The present study demonstrated that MSCs secreted ≈4-fold more VEGF compared with MNCs. Furthermore, MSCs secreted large amounts of HGF and AM, potent angiogenic factors.[26–30] Taking these findings together, MSCs may contribute to neovascularization in the myocardium not only through their ability to generate capillary-like structures but also through growth factor–mediated paracrine regulation. Myocardial blood flow abnormalities have been documented in patients with heart failure caused by DCM.[12] Thus, it is possible that MSC-induced neovascularization contributes to improvement in cardiac function.

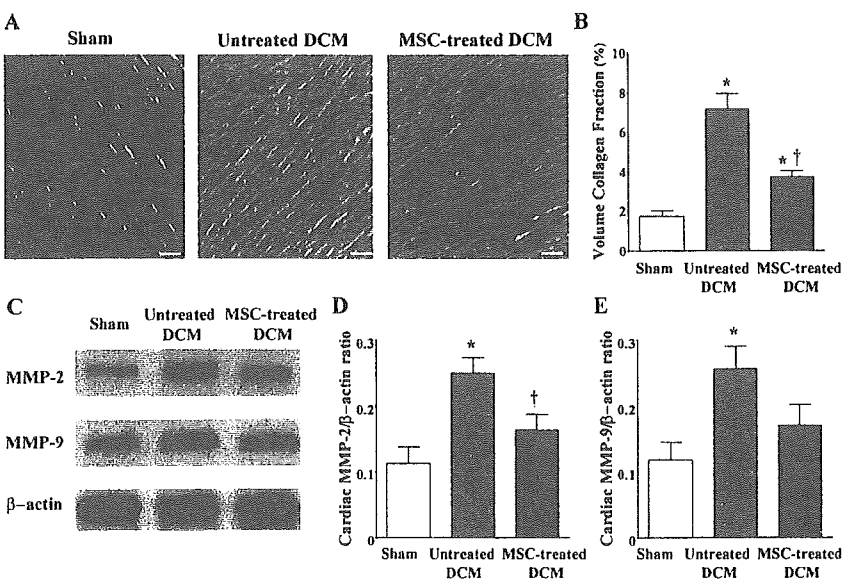

Figure 7. Effects of MSC transplantation on myocardial fibrosis. A, Photomicrographs show representative myocardial sections stained with Masson's trichrome. Scale bars=10 μm. B, Quantitative analysis demonstrated that the collagen volume fraction in the MSC-treated DCM group was significantly smaller than that in the untreated DCM group. C, Representative Western blots for MMPs-2 and -9 and β-actin in the heart. D and E, Quantitative analysis of cardiac tissue contents of MMP-2 and -9. Data are mean±SEM *$P<0.05$ vs sham group; †$P<0.05$ vs untreated DCM group.