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Less-invasive method for microsphere delivery to coronary circulation and evaluation of myocardial ischemic change in small animals

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We developed a less-invasive method for semi-selective administration of drugs into coronary arteries in small animals. With this method, we created microvascular myocardial ischemia in rabbits by microsphere injection. A 4F catheter was inserted into the left ventricle via the right common carotid artery and a balloon catheter into the descending thoracic aorta. Microspheres were administered into the left ventricle with temporary occlusion of the descending aorta and carotid arteries. In these conditions, regional blood flow in the heart was 10.8-times as much as in the kidney. Seventeen days after microsphere injection, the contractile function of the heart muscle deteriorated and the left ventricular endodiastolic pressure was increased. Patchy NADH-fluorescence was observed all over the left ventricular myocardium. Myocardial lactate concentration was higher than the normal standard animals. Histological analysis revealed that microscopic patchy necrosis was noted only in the myocardium but not in other organs. Semi-selective delivery of recombinant adenovirus expressing lacZ using the same method induced a gene expression in the heart. Thus, a unique model for microvascular myocardial ischemia was created by semi-selective delivery of microspheres into the coronary artery without special technique or equipment. The present model is also applicable to semi-selective gene transfer to the heart.

Key words: microsphere, myocardial ischemia, blood flow, gene transfer, heart failure

INTRODUCTION

Selective drug delivery into the coronary artery is very important for physiological experiments of the heart. Selective coronary catheterization makes this possible, but the method is available only in large animals such as dogs or pigs, but difficult in small animals such as rats and rabbits. Thus for small animals, thoracotomy is usually necessary. One study showed under thoracotomy drugs were administered via a fine catheter which was directly inserted into the left ventricle and injection was performed while the isolated ascending aorta was clamped in rats [3]. This method is very invasive. In middle size animals such as rabbits, coronary catheterization can be possible but requires radiofluoroscopy and specific catheter techniques [4]. Thus it is important 1) to develop a less-invasive method for semi-selective administration of drugs into the coronary artery without special equipment or techniques and 2) to find whether or not microsphere injection using the method can induce microvascular myocardial ischemia as a simulation of microvascular angina when microspheres are administered instead of drugs.

Microvascular angina is a clinical entity which is refined as chest pain with normal coronary arteriogram and its cause is suggested to be microvascular dysfunction [1, 12]. Ischemia caused by microsphere injection into the coronary artery may mimic this disease. In this study, after developing a less-invasive semi-selective coronary injection method, we examined pathophysiology of microsphere-induced ischemia in acute and chronic phases to show that this method can be an animal model of "microvascular angina". Additionally we tested whether this method can be applicable to transfer gene to the heart.

MATERIALS AND METHODS

Semi-selective administration of microspheres into the coronary artery in rabbits

This investigation was performed in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. Fifteen Japanese white rabbits weighing 3.68 ± 0.11 kg (Tokyo Laboratory Animal Science Co., Ltd., Tokyo) were used. All procedures were performed under general anesthesia, i.e., after intravenous administration of sodium pentobarbital (30 mg/kg). In 11 rabbits, the tip of a 4F catheter was positioned in the left ventricle via the right common carotid artery to record left ventricular pressure and to administer reagents. The tip of a 4F balloon catheter (Baxter Inc., Irvine, USA) was positioned in the thoracic descending aorta via the left femoral artery to obstruct the aortic blood flow by balloon inflation. A 3F catheter was placed in the right femoral artery distal to the balloon to record aortic pressure and to obtain reference blood samples for measurement of regional blood flow. During balloon inflation, we confirmed that femoral arterial pressure was $<$20 mmHg (Fig. 1). The left common carotid artery was also exposed to allow temporary occlusion. Microspheres were injected via

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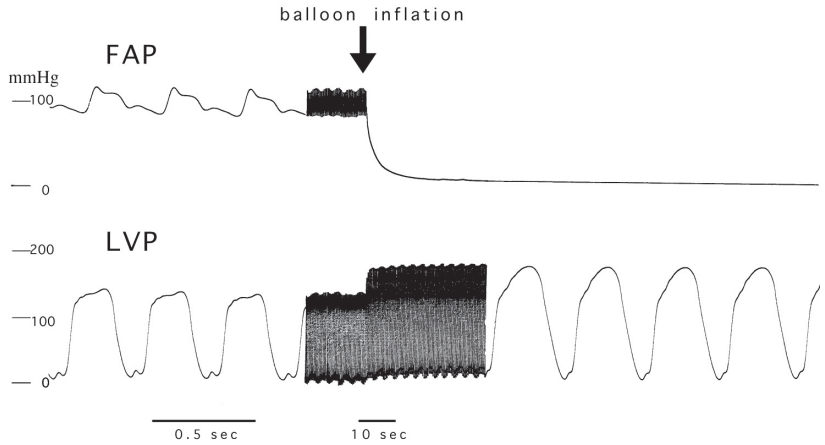

Fig. 1 Representative recordings of blood pressure in the left ventricle (LVP, lower trace) and in the femoral artery (FAP, upper trace). An arrow shows the balloon inflation of the catheter in the descending thoracic aorta.

the catheter in the left ventricle while the balloon in the descending aorta was inflated and the left common carotid artery was temporary occluded.

Methods of microsphere injection and tissue flow measurement

The microspheres (15 μm in diameter, Sekisui Plastic, Japan) [5, 6, 13] were suspended in 0.05% sodium dodecyl sulfate at a concentration of 5.0×10^5/ml. Four kinds of microspheres were prepared, which were labeled with Barium, Indium, Iodine and Cerium. Indium-labeled microspheres were injected semi-selectively into the coronary artery to create myocardial ischemia and Barium, Iodine and Cerium microspheres were used to measure blood flows.

Blood flows in the major organs (the heart, the brain, the kidney, and the liver) were measured by Barium- and Iodine-labeled microspheres (1.25×10^5/kg) respectively before and after the Indium-labeled microsphere injection. Seventeen days after coronary injection of microspheres, Cerium-labeled microsphere (1.25×10^5/kg) was injected to measure blood flow in the major organs to evaluate conditions in the chronic phase.

After the animals had been killed, their major organs were removed by dissection, and the four segments (subepicardium and subendocardium in the left ventricular free wall, the interventricular septum, and the right ventricular free wall) were removed from the heart. The X-ray fluorescence of each heavy element was measured to calculate regional blood flow [10, 13].

Protocol 1: Evaluation of ischemic changes in the heart after microsphere injection

To create myocardial ischemia, Indium-labeled microspheres (2.8×10^5/kg) were administered "semi-selectively to the coronary artery" in 3 doses at 10 min intervals in 5 rabbits. The administration of microspheres was terminated within 40 sec, but balloon inflation and temporary occlusion of the left common carotid artery were maintained for 70 sec after the start of administration.

Heart rate, left ventricular pressure, and femoral artery pressure were continuously measured throughout the procedure. Left ventricular ejection fraction was also measured using echocardiogram. Development of ischemia was evaluated by left ventricular ejection fraction in an acute phase and 17 days after the injection of microspheres as a chronic phase.

Lactate content [9] and NADH-fluorescence [8] in the left ventricle were also evaluated after the myocardium was frozen quickly in liquid nitrogen. Histological analysis was also performed: Two investigators blinded to treatment graded histological evidence of infarction in each tissue section stained with AZAN and hematoxylin-eosin on a scale of 0 to 3 as follows: 0 = none; 1 = mild (less than 5% of the section surface); 2 = moderate (more than 5%, up to 20% of the section surface); 3 = severe (more than 20% of the section surface) [13]. The scoring was done separately on subepicardium and subendocardium.

Protocol 2: Gene delivery to the heart

To expand the applicability of the "semi-selective administration" to the gene therapy, recombinant adenovirus expressing lacZ (Adex-lacZ, 6×10^9 plaque-forming units) was administered in 2 rabbits. In order to make the gene therapy to be relevant, the microvascular ischemia was produced by injecting Indium-labeled microspheres as the same method of protocol 1. Forty minutes after completion of the Indium-microsphere injection, adenovirus-containing saline (2 ml) was injected semi-selectively. Expression of lacZ was determined 4 days after transfection by X-Gal histostaining by the methods of Ueno *et al.* [14].

Adex-lacZ was a replication-defective E1- and E3-adenoviral vector [7] expressing ß-galactosidase under a CA promoter comprising a cytomegalovirus enhancer and chicken ß-actin promoter [11], and was prepared by in vitro homologous recombination in a 293-cell assay system [14]. The desired recombinant adenovirus was purified by ultracentrifugation through a $CsCl_2$ gradient followed by extensive dialysis. Contamination of wild-type adenovirus was excluded by PCR designed for E1 amplification. The titer of the virus stock was assessed by a plaque-formation assay using the 293-cell system.

Table 1 Blood flows in various organs during semi-selective injection

Heart	Brain	Liver	Kidney
10.8 ± 3.6	1.6 ± 1.0	0.2 ± 0.1	1

Numbers are relative values of Indium microsphere counts per gram. As reference blood sampling from femoral artery could not be done due to balloon inflation, absolute flow calculation was not possible.

Table 2 Cardiac indices

	Baseline	Selective phase	Acute phase	Chronic phase
Ejection fraction (%)	68 ± 3	ND	48 ± 8 †	52 ± 9 †
Heart rate (/min)	239 ± 36	223 ± 37	217 ± 43	252 ± 18
LVSP (mmHg)	138 ± 10	163 ± 13 † ‡	126 ± 11	149 ± 22
LVEDP (mmHg)	5.2 ± 1.8	19.8 ± 4.11*	6.0 ± 2.2	10.0 ± 3.7 †
mFAP (mmHg)	114 ± 9	16 ± 41*	100 ± 5	119 ± 19

The above-described indices were measured immediately before, during, immediately after and 17 days after Indium-labeled microsphere injection (Baseline, Selective phase, Acute phase, and Chronic phase, respectively).
LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; mFAP, mean femoral artery pressure. ND, not done.
* $p < 0.05$ vs. baseline, acute phase, and chronic phase; † $p < 0.05$ vs. baseline; ‡ $p < 0.05$ vs. acute phase.

Table 3 Regional blood flow (ml/g/min) in the heart

	Baseline	Acute phase	Chronic phase
Whole Heart	2.58 ± 0.87	2.01 ± 0.63	2.23 ± 0.65
Epi	2.40 ± 0.84	1.92 ± 0.58	2.15 ± 0.58
Endo	2.83 ± 0.92	2.13 ± 0.79	2.32 ± 0.87
Sep	2.98 ± 1.88	2.14 ± 0.56	1.90 ± 0.54
RV	2.07 ± 1.03	2.12 ± 1.09	1.86 ± 0.92

Indium-labeled microspheres were injected into the coronary arteries in 5 rabbits to induce myocardial ischemia. The above-described indices were measured immediately before, after, and 17 days after the microsphere injection (Baseline, Acute phase, and Chronic phase). These were not statistically significant.
Epi, subepicardium in the left ventricular free wall; Endo, subendocardium in the left ventricular free wall; Sep, interventricular septum; RV, right ventricular free wall.

Statistical analysis

Data are presented as mean values ± SD. Differences between means were assessed using paired t test, unpaired t test, Wilcoxon signed rank sum test, or ANOVA for repeated measurements with the Scheffe F-test when applicable. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Semi-selectivity to the coronary artery

Representative recordings of blood pressure in the left ventricle and in the femoral artery are shown in Fig. 1. Femoral arterial pressure was under 20 mmHg during balloon inflation in the descending thoracic aorta. Regional blood flows in various organs during semi-selective injection are shown in table 1. There was nearly 10-times difference in blood flow between heart and other organs; indicating the injection was almost selective to the heart. The presence of 1.6/gram in brain appears to be delivered via vertebral arteries.

Evaluation of ischemic changes in the heart after microsphere injection

Immediately after the semi-selective injection of Indium-microspheres into the coronary arteries, the contractile function of the heart muscle deteriorated as manifested in ejection fraction (68 ± 3 to 48 ± 8%, $p < 0.05$, ANOVA) (Table 2). Left ventricular end-diastolic pressure (LVEDP) was increased from 5.2 ± 1.8 to 6.0 ± 2.2 mmHg. Blood flow to the heart muscle was reduced from 2.58 ± 0.87 to 2.01 ± 0.63 ml/g/min (Table 3). But these were not statistically significant. There was no difference in the blood flows among the myocardial regions. The number of microsphere used in this study (2.8×10^5/kg) was shown to be adequate to produce mild degree microsphere ischemia without severe cardiac failure.

Seventeen days after ischemia induction, body weight had significantly decreased (3.72 ± 0.12 to 3.59 ± 0.18 kg, $p < 0.05$, paired t-test). Deterioration of the contractile indices (ejection fraction and LVEDP) occurred in

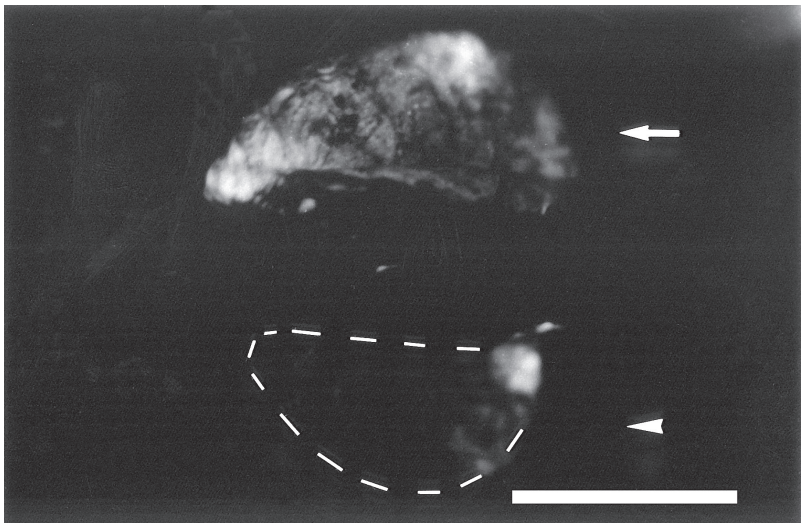

Fig. 2 NADH-fluorescence of left ventricular myocardium in chronic phase. Patchy myocardial ischemia throughout the myocardium is seen in an ischemic rabbit (arrow). The arrowhead shows myocardium obtained from a control rabbit. Original magnification ×1; bar =30 mm.

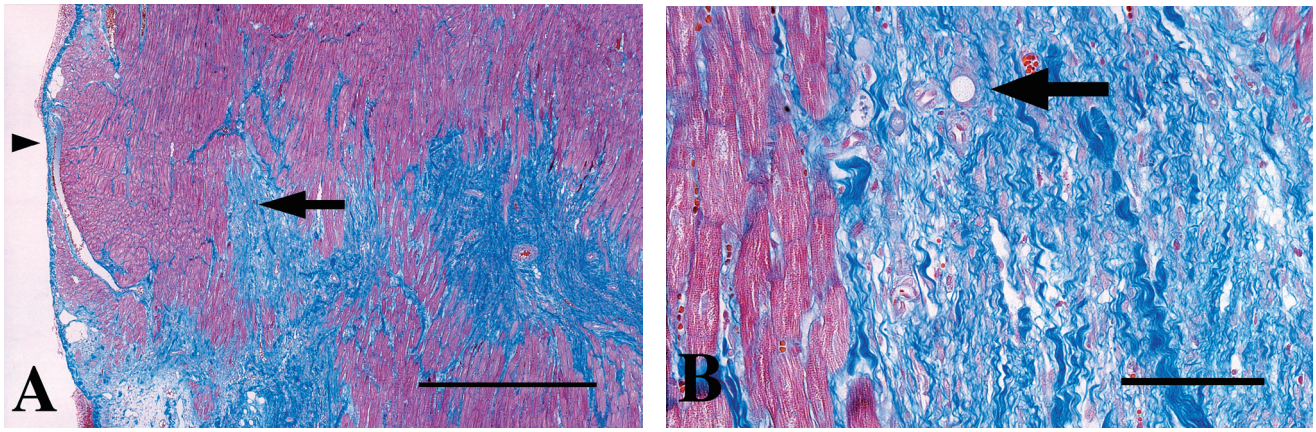

Fig. 3 AZAN staining of the myocardium in chronic phase. Seventeen days after semi-selective injection of microspheres, the tissue was stained. Blue area indicates necrotic areas. An arrow depicts microsphere located in the center of necrosis. An arrowhead depicts epicardium of the left ventricular free wall. (**A**) Original magnification ×10; bar = 1 mm. (**B**) Original magnification ×50; bar = 0.1 mm.

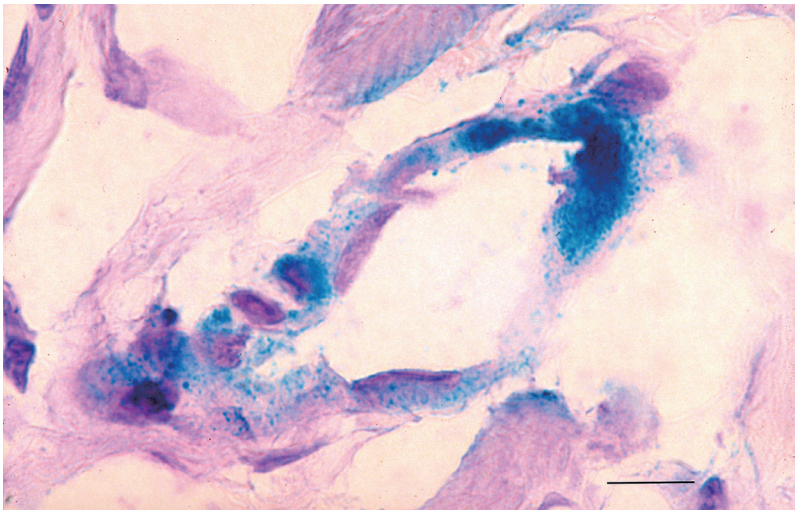

Fig. 4 β-Galactosidase expression in the endothelial cells of the coronary artery after administration of Adex-lacZ into the coronary artery. Original magnification ×350; bar = 20 μm.

the acute phase, and it was sustained (Table 2). Patchy NADH-fluorescence was observed all over the left ventricular myocardium, while myocardium of the control rabbits did not show any fluorescence area (Fig. 2). Myocardial lactate content was significantly higher in the ischemic rabbits than in 4 normal standards (1.12 ± 0.53 vs. 0.59 ± 0.05 mg/g, $p < 0.05$, unpaired t-test). Histological analysis revealed multiple small patchy infarctions surrounded by non-infarcted myocardium throughout the heart (Fig. 3). Microspheres were found in some infarction areas (arrow in Fig. 3A and 3B). The score of infarction area was higher in the subendocardial region of the left ventricular free wall than in the subepicardial muscle ($P = 0.05$, Wilcoxon signed rank sum test). No histological evidence of ischemia was found in other major organs (brain, kidney, and liver).

Gene delivery to the heart

In the 2 ischemic rabbits in which Adex-lacZ was semi-selectively administered to the coronary artery, β-galactosidase was expressed in the endothelial cells of the coronary artery on day 4 after administration (Fig. 4). No β-galactosidase expression was observed in the kidney and the liver.

DISCUSSION

This study showed that 1) left ventricular injection of microspheres with transient occlusion of descending aorta and carotid arteries enables selectively deliver microspheres to coronary arteries in small animals, 2) microspheres of 2.8×10^5/kg with a diameter of 15 μm induce mild microvascular ischemia in both acute and chronic phases, and 3) this method is applicable to selective gene delivery to the heart.

The present method (balloon inflation in the aorta and occlusion of bilateral common carotid arteries) was simple and easy, and did not require fluoroscopic catheterization. Regional blood flow in the heart was 10.8-times as much as in the kidney by this method; indicating that semi-selective injection to the heart was accomplished. Blood flow to the abdominal organs and lower extremities was substantially interrupted during the balloon inflation but the brain appeared to be supplied by vertebral arteries although common carotid arteries were occluded. During balloon inflation, femoral arterial pressure was always < 20 mmHg. Although in this method we had to ligate one side of the common carotid artery and bilateral femoral arteries to insert catheters, all rabbits did not show any abnormal movements during the observation period.

In preliminary experiments, various numbers of microspheres were tried and we found that the present dose of microspheres (2.8×10^5/kg) was enough to create cardiac ischemia without leading to severe heart failure of death [13].

The reason why the reduction of coronary flow after injection is mild is that reserve of coronary circulation is very large and coronary vessels can dilate as much as 5 times when necessary [2, 5]. This means oxygen supply to the heart is almost sufficient at rest. However at chronic phase, patchy ischemia was evident as shown by NADH-fluorescence [8] and elevated lactate content in the myocardium. This evidence of chronic ischemia indicates that reduction of coronary reserve induced ischemia while rabbits were allowed to move freely for 17 days. Moreover, aniline blue-stained areas were also scattered with AZAN staining. This indicates the presence of patchy necrosis besides ischemia since necrotic tissues were replaced with collagen fibers which are stained blue with AZAN staining.

The degree of ischemia was supposed to be higher in subendocardium than in subepicardium, because the patchy necrosis was more potent in subendocardium than in subepicardium. This is in agreement with higher oxygen consumption, lower oxygen supply by the squeezing of vessels in the subendocardium than in the subepicardium during systole.

Mild reduction of left ventricular ejection fraction both at acute and chronic phases appears to be consistent with the mild degree of ischemia. The microsphere plugging of the coronary arterial system induced microvascular myocardial ischemia. In this model, the source of myocardial ischemia is in the peripheral coronary arteries. Pathophysiological manifestation of this model appears to be an exact animal model of one type of ischemic heart disease called “microvascular angina”. “Microvascular angina” is refined as angina-like chest pain with a normal coronary arteriogram, and microvascular dysfunction or spasm is the cause of the disease [1, 12]. Therefore this model is useful to study this clinical entity.

Semi-selective administration of Adex-lacZ to the heart induced gene expression only in the heart but not in the kidney and the liver; suggesting the method is applicable as gene delivery to the heart in small animals.

In conclusions, we developed a chronic microvascular ischemia model by semi-selective injection of microspheres into the coronary artery in rabbits without special equipment or techniques. With this method, it is probably safe and easy to deliver drugs into coronary arteries even in smaller animal such as rats.

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