receptor subtypes, endothelin receptor type A (ET_AR) and type B (ET_BR). ET_AR is mainly located on vascular smooth muscle cells and mediates vasoconstriction, and ET_BR is mainly located on endothelial cells and mediates vasodilation. It has been shown that ET-1 and its receptors are involved in the pathophysiology of pulmonary arterial hypertension, [11] but the correlation between ET-1 and its receptors and the pulmonary vascular pathogenesis of the failed Fontan has not been clearly elucidated. Therefore, the aim of this study is to clarify the expressions of ET-1, ET_AR, and ET_BR in the pulmonary arteries of failed Fontan patients using immunohistochemical and quantitative real-time polymerase chain reaction (PCR) analyses.

2. Methods

2.1. Study population

The Fontan procedure has been performed since 1973 at the Osaka University Hospital and Osaka Medical Center for Maternal and Child Health, Osaka, Japan. Until 2004, 33 patients had died after the Fontan operation, from whom 10 autopsy lung tissues were analysed. Their Fontan procedure was performed between 1982 and 2000. As for normal controls, we analysed 4 age-matched autopsy lung tissues from patients without any cardiovascular and pulmonary disease: 2 patients who died from brain tumour, 1 patient from meningitis, and 1 patient from necrotic enteritis. The mean ages of Fontan patients and normal controls (12.2 ± 6.1 vs. 9.8 ± 1.5) were not significantly different.

We analysed their clinical courses and haemodynamic data including age at the Fontan procedure, previous palliative operations, type of the Fontan procedure, cause of death, mean pulmonary artery pressure, transpulmonary pressure gradient, and estimated pulmonary vascular resistance before and after the Fontan procedure from their clinical records.

Two patients died early after the Fontan operation; 1 died from severe bacterial infection despite a successful operation and another patient failed to establish the Fontan circulation and was included in the failing Fontan group (the pulmonary arterial pressure was elevated immediately after the operation, and oxygen saturation and cardiac output were severely decreased). During the follow-up, 3 patients died because of failure of the Fontan circulation, which included 1 case of Fontan takedown due to uncontrollable protein-losing enteropathy and 2 cases of low cardiac output, cyanosis, and impaired daily activity (WHO functional class 3 or 4) with high pulmonary vascular resistance. Three other patients died of severe ventricular dysfunction; 2 had severe atrioventricular valve regurgitation and 1 had low cardiac output and brain infarction. The other 2 patients died suddenly probably because of arrhythmia. We divided these 10 Fontan patients into three groups based on the cause of death. Three patients in the 'heart failure (HF) group had severe ventricular dysfunction with atrioventricular valve regurgitation and did not have high pulmonary vascular resistance (transpulmonary pressure gradient ≤ 5 mm Hg). Four patients in the 'failed Fontan (FF)' group had severe low cardiac output or uncontrollable protein-losing enteropathy with high pulmonary vascular resistance (transpulmonary pressure gradient ≥ 6 mm Hg). The other three patients in the 'sudden or infectious death (SD + INF)' group died from arrhythmia or infection, who had established and functioning Fontan circulation. We compared these three groups (FF, HF, and SD + INF) to normal controls (NC).

2.2. Histomorphometrical study

The lung autopsy specimens were fixed in 10% buffered formalin solution and embedded in paraffin. Serial sections (4 μm thick) were stained by the Elastica van Gieson method. The percent wall thickness of the pulmonary arteries was calculated as follows: percent wall thickness = (2 × Wall thickness / External diameter) × 100. The size of pulmonary arteries was categorised into 2 groups: proximal arteries (>200 μm) and intra-acinar arteries (50–200 μm). The wall thickness of capillary arteries could not be analysed because internal and external elastic laminae were not distinguishable in such small arteries. At least 5 arteries were measured in each diameter group and compared to normal controls.

2.3. Immunohistochemical study

Four-micrometer-thick serial paraffin-embedded sections were immunostained with anti-ET-1, (Phoenix Pharmaceuticals, Inc. Burlingame, CA, USA), anti-ET_AR, and anti-ET_BR (Immuno-Biological Technologies, Gunma, Japan) and with anti-α-smooth muscle cell actin (αSMA; Sigma-Aldrich, St. Louis, MO, USA) to evaluate the proliferation of vascular smooth muscle-derived cells. Tissue sections were deparaffinised in xylene, rehydrated through graded ethanol to water, and heated for 10 min in buffered citrate at pH 6. Slides were incubated in 0.3% hydrogen peroxide to block endogeneous peroxidase activity, rinsed in phosphate-buffered saline, and incubated for 30 min at room temperature with DAKO® protein blocker. Then, slides were incubated for 16 h at 4 °C in a humidified box with the primary antibodies anti-ET-1 (diluted 1:100), anti-ET_AR (1:100), and anti-ET_BR (1:100). Then, sections were incubated for 30 min with secondary antibodies (1:100) and incubated with horseradish peroxidase for 30 min. Finally, sections were stained with diaminobenzidine (Sigma-Aldrich) and counterstained with Mayer's haematoxylin. The sections were photographed with a cooled CCD camera (VB-7010, KEYENCE, Osaka, Japan) and analysed using Image J software (http://www.rsb.info.nih.gov/ij/) to evaluate the mean optical density of each stained pulmonary artery, including both endothelial cells and smooth muscle cells. The size of pulmonary arteries was categorised into three groups: proximal arteries (>200 μm), intra-acinar arteries (50–200 μm), and capillaries (<50 μm). The optical density was calibrated using the Kodak No. 3 Calibrated Step Tablet following the manufacturer's instructions (http://www.rsb.info.nih.gov/ij/docs/examples/calibration/). All slides were stained at the same time and incubated for the same duration; staining was done three times for each primary antibody for each tissue. At least 7 arteries in each size range were analysed and compared to normal controls.

2.4. Quantitative real-time PCR

Total RNA was purified from 10-μm-thick paraffin-embedded sections of lung tissues by using an RNeasy FFPE Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. An equal amount of total RNA (1 μg) was used for reverse transcription using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Inc., Foster City, CA, USA). Quantitative PCR was conducted in a total volume of 20 μL using LightCycler ST300 (Roche Diagnostics, Basel, Switzerland) and LightCycler FastStart DNA MasterPLUS SYBR-Green I Kit (Roche Diagnositics). PCR cycles were 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 20 s. Human ET-1, ET_AR, and ET_BR were amplified with the following primers: ET-1 forward, 5′-TCTCTGCTGTTTGTGGCTTG-3′; reverse, 5′-GAGCTCAGCGCCTAAGACTG-3′; ET_AR forward, 5′-GCGCTCTTAGTGTTGACCAGGT-3′; reverse, 5′-GAATCCCAATTCCCTGAACA-3′; ET_BR forward, 5′-ATCGTCATTGACATCCCTATCA-3′; reverse, 5′-GCTTACACATCTCAGCTCCAAA-3′; and β-actin forward, 5′-CCAACCGCGAGAAGATGA-3′; reverse, 5′-CCAGAGGCGTACAGGGATAG-3′. The levels of mRNA expression were normalised to that of human β-actin.

2.5. Statistical analysis

Results were expressed as mean ± standard error. Statistical analyses were performed by using Turkey–Kramer multiple comparison test. Statistical significance was defined as $P<0.05$.

3. Results

3.1. Patient profiles and haemodynamic data

The clinical characteristics and haemodynamic data of the Fontan patients are shown in Table 1. All patients underwent cardiac catheterisation before the Fontan procedure and were considered as acceptable candidates because of low pulmonary vascular resistance and preserved ventricular function. The median age of the patients at the time of the Fontan procedure was 6.3 (3–16) years, and the median duration from the Fontan operation to death was 4.9 (0–11) years. The mean pulmonary arterial pressure and pulmonary vascular resistance of all patients before the Fontan procedure were 12.4 ± 0.9 mm Hg and 1.5 ± 0.2 U m^2, and those after the Fontan procedure were 15.7 ± 1.6 mm Hg and 3.0 ± 0.3 U m^2, respectively.

The mean pulmonary artery pressures before the Fontan procedure were 12.0 ± 1.1 mm Hg (FF group), 15.0 ± 1.7 mm Hg (HF group), and 10.3 ± 0.3 mm Hg (SD + INF group), and those after the Fontan procedure were 17.8 ± 2.3, 13.0 ± 1.2, and 12.0 ± 2.5 mm Hg, respectively. The mean transpulmonary pressure gradients before the Fontan procedure were 5.5 ± 0.3 mm Hg (FF group), 5.0 ± 0.6 mm Hg (HF group), and 6.0 ± 0.6 mm Hg (SD + INF group), and those after the Fontan procedure, 11.5 ± 2.1, 4.0 ± 0.6, and 4.5 ± 0.4 mm Hg, respectively.

3.2. Histomorphometricalal study

The percent wall thickness of proximal arteries (>200 μm) and intra-acinar arteries (50–200 μm) was significantly increased in the FF group when compared with normal controls and the SD + INF group (Fig. 1A). In contrast, the percent wall thickness in the HF and SD + INF groups was not significantly different from normal controls.

The small muscular arteries from the patients with failed Fontan circulation showed not only medial hypertrophy but also severe intimal thickening (Fig. 1B). Immunostaining for αSMA showed massive proliferation of vascular smooth muscle cells in both proximal and distal arteries (Fig. 1C). Such medial and intimal thickening

Table 1
Characteristics of the patients died after the Fontan procedure.

Patient no.	Sex	Diagnosis	Previous operation	Age at Fontan (y)	Type of Fontan	Age of death (y)	Cause of death	mPAP (mm Hg)		TPG (mm Hg)		PVR (U m^2)	
								Pre	Post	Pre	Post	Pre	Post
1	F	SV	APS	9	APC	20	HF	12	11	6	3	1.0	2.3
2	M	SV	BCPS	16	LT	18	FF	13	26	5	14	1.6	3.8
3	F	PAIVS	APS	8	APC	13	FF	12	16	6	7	0.9	3.6
4	M	SV	APS	8	APC	15	FF	9	13	5	9	1.2	3.8
5	F	DORV	APS	3	LT	13	HF	15	22	4	5	3.0	3.1
6	M	SV	BCPS	3	LT	4	Infection	10	N.A.	6	5	2.0	N.A.
7	M	SV	PAB	3	LT	3	FF	14	18	6	16	1.4	N.A.
8	F	DORV	BCPS	2	ECC	3	HF	18	13	5	4	2.2	1.9
9	F	TA	APS	7	APC	16	SD	11	N.A.	5	N.A.	1.0	N.A.
10	M	SV	APS	4	ECC	7	SD	10	9	7	5	0.8	2.6

SV, single ventricle. PAIVS, pulmonary atresia with intact ventricular septum. DORV, double outlet of right ventricle. TA, tricuspid atresia. APS, aorto-pulmonary shunt. BCPS, bidirectional cavo-pulmonary shunt. PAB, pulmonary artery banding. APC, atrio-pulmonary connection. LT, lateral tunnel. ECC, extra-cardiac conduit. FF, failed Fontan. HF, heart failure. SD, sudden death. mPAP, mean pulmonary arterial pressure. TPG, transpulmonary pressure gradient. PVR, pulmonary vascular resistance. N.A., not available.

can be seen in patients with pulmonary arterial hypertension; this vascular remodelling can increase the pulmonary vascular resistance, resulting in the failure of the Fontan circulation and death.

3.3. Immunohistochemical study

In the small muscular arteries, strong positive immunostaining for ET-1 was present in both endothelial cells and vascular smooth muscle cells in the failed Fontan patients, whereas in normal controls, only very weak staining was observed (Fig. 2A). We quantified the optical density of ET-1 immunostaining by computational analysis, which revealed that the ET-1 expression levels of the failed Fontan patients were significantly higher than those of normal controls in arteries of each size range, and those of the HF group were also significantly increased in the capillary vessels (>50 μm). In the intra-acinar vessels (50–200 μm), the FF group showed significantly higher ET-1 expression than the SD + INF group. The SD + INF group did not show a significant difference from normal controls (Fig. 2B).

ET_AR was weakly expressed in the vascular smooth muscle cells of normal controls. In contrast, it was strongly expressed in both endothelial and smooth muscle cells in the FF group (Fig. 2C). The computational optical density analysis demonstrated significantly higher expression levels of ET_AR in the FF and HF groups than in normal controls in the small arteries, whereas there was no significant difference between the SD + INF group and normal controls (Fig. 2D). ET_BR immunolabelling was also low in normal controls and mainly observed in endothelial cells, whereas in the FF group, ET_BR was strongly expressed especially in vascular smooth muscle cells (Fig. 2E). The expression levels of ET_BR were also significantly higher in the FF group than in normal controls in arteries of each size range. The HF and SD + INF groups were not significantly different from normal controls (Fig. 2F). Moreover, we examined the expression of ET_BR in the media and found significantly stronger expression in the FF and HF groups compared to normal controls (Fig. 2G). Taken together, these results demonstrated that ET-1 expression was elevated in the pulmonary arteries of the failed Fontan patients and that both ET_AR and ET_BR were overexpressed in the failed Fontan patients, but not in the non-failed Fontan patients, indicating that the endothelin system could contribute to the pathogenesis of the failed Fontan circulation.

3.4. Quantitative real-time PCR study

To elucidate whether the mRNA expression levels of ET-1 and its receptors were also elevated, we extracted RNA from the whole lung tissues of patients and normal controls and performed real-time PCR analysis. The expression level of ET-1 mRNA in the FF group was about 2-fold higher than that in normal controls. Similarly, the expression levels of ET_AR and ET_BR were significantly higher in the FF group than in normal controls (Fig. 3). As for ET-1 and ET_BR, the HF groups also showed significantly higher expression compared with normal

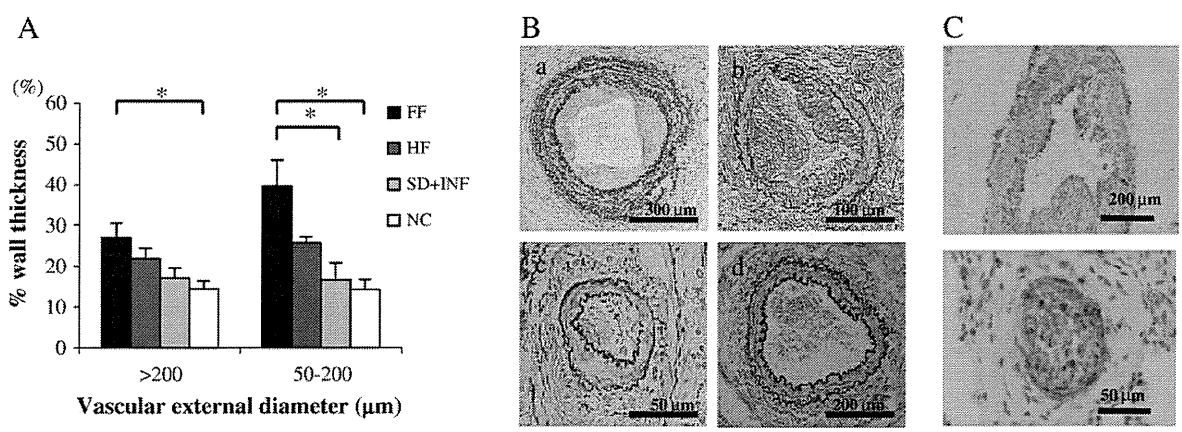

Fig. 1. Morphological analysis of the pulmonary arteries. A: The percent wall thickness of the pulmonary arteries of patients who died after the Fontan procedure and normal controls (NC). Patients who had undergone the Fontan procedure were divided into 3 groups based on the cause of death; failed Fontan group (FF), heart failure group (HF) and sudden or infectious death (non-failed Fontan) group (SD + INF). * $P<0.05$. B: Elastica van Gieson stain of the pulmonary arteries of the failed Fontan patients showed severe intimal and medial hypertrophy (a, b and c) as compared to normal controls (d). C: Immunostain of alpha-smooth muscle cell actin showed that proliferation of the vascular smooth muscle cells contributed to intimal and medial hypertrophy of the failed Fontan patients.

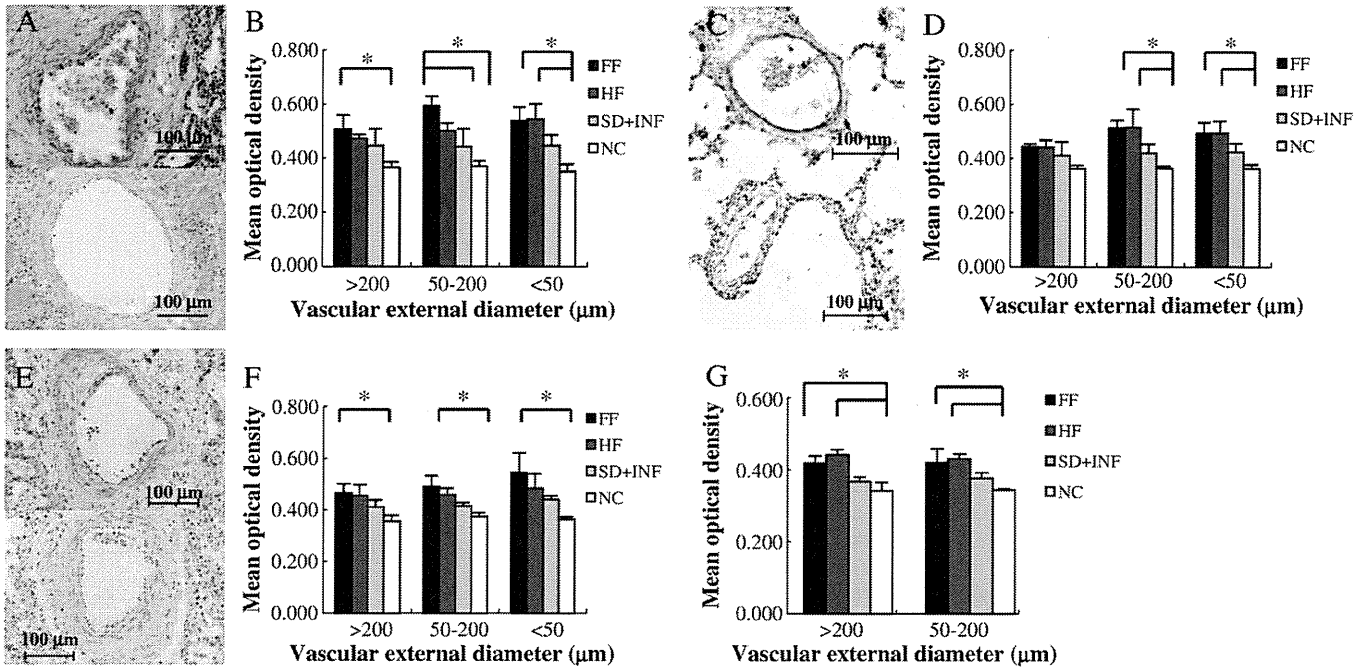

Fig. 2. Immunohistochemical analyses of ET-1 and its receptors in the pulmonary arteries. A: Immunostain of anti-ET-1 in the failed Fontan patients (upper panel) and normal controls (lower panel). Both endothelium and media were strongly stained in the failed Fontan patients. B: The result of the computational optical density analysis of ET-1 immunostain. C: Immunostain of anti-ET_AR in the failed Fontan patients (upper panel) and the normal controls (lower panel). In normal controls, endothelium was weakly stained, whereas in the failed Fontan patients, both endothelium and media were strongly stained. D: The result of the optical density analysis of ET_AR immunostain. E: Immunostain of anti-ET_BR in the failed Fontan patients (upper panel) and the normal controls lower panel). In the failed Fontan patients, media was strongly stained as compared to normal controls, indicating ET_BR overexpression in the vascular smooth muscle cells. F: The result of the optical density analysis of ET_BR immunostain. F: The result of the optical density analysis of ET_BR immunostain limiting to media. FF, failed Fontan group; HF, heart failure group; SD + INF, sudden or infectious death group and NC, normal controls. * $P<0.05$. Scale bars: 100 μm.

controls. These results were almost consistent with the immunohistochemical findings shown above.

4. Discussion

The Fontan procedure is currently the standard treatment for complex congenital heart defects, for which biventricular repair is not feasible. Although the outcome of the Fontan procedure has been satisfactory, some patients still present with a failing Fontan circulation. The Fontan circulation depends on various factors such as pulmonary vascular resistance, systemic ventricular function, atrioventricular valve regurgitation, thrombosis, and arrhythmias. Among these factors, pulmonary vascular resistance is the most critical for a successful Fontan circulation [5,6]. Pulmonary vascular resistance is associated with various vasoactive factors such as ET-1, prostacyclins, and nitric oxide. ET-1 is a potent vasoconstrictive substance and has a mitogenic activity in vascular smooth muscle cells and promotes vascular remodelling, as previously demonstrated in the pathogenesis of pulmonary arterial hypertension [11]. As for the relationship of the Fontan circulation and ET-1, Yamagishi et al. and Hiramatsu et al. demonstrated that plasma ET-1 level was elevated after the Fontan procedure, which was positively correlated to central venous pressure [9,10]. In this study, we revealed that ET-1 expression in the pulmonary endothelium and smooth muscle cells was increased in the failed Fontan patients, using immunohistochemical and quantitative real-time PCR analyses. On the other hand, Lévy et al., who performed histomorphological and immunohistochemical analyses of the pulmonary arteries of the lung biopsy specimens from patients who underwent the Fontan procedure, showed that the distal pulmonary arteries of poor-outcome Fontan patients displayed significant muscle extension without an increase in ET-1 expression [12]. This discrepancy could be explained by the difference in the timing when the lung tissues were obtained. They mainly used biopsy specimens which were taken at the time of the Fontan operation. Our immunohistochemical study showed that the ET-1 expression of the SD + INF group, which had an almost well-adapted Fontan circulation, exhibited no significant difference from that of normal controls, indicating that ET-1 expression might be significantly increased only when the Fontan circulation is failing.

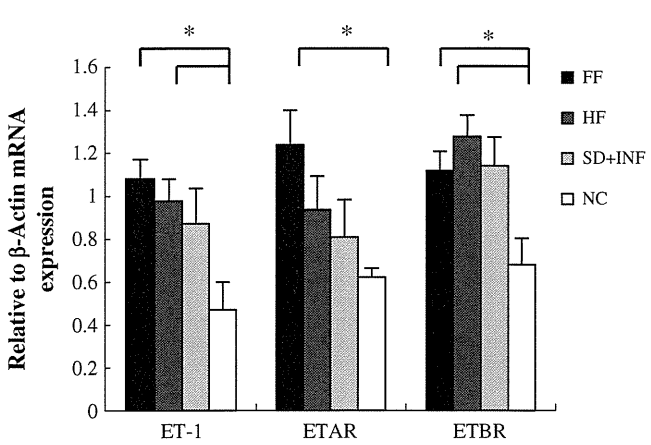

Fig. 3. The real-time PCR analysis of ET-1, ET_AR and ET_BR in the lung tissues. ET-1 expression in the failed Fontan patients is almost 2-fold higher than in the normal controls. ET_AR and ET_BR showed about 1.5-fold higher expression. FF, failed Fontan group; HF, heart failure group; SD + INF, sudden or infectious death group and NC, normal controls. * $P<0.05$.

ET-1 acts through 2 types of receptors, ET_AR and ET_BR. Under normal physiological conditions, ET_AR, located on smooth muscle cells and fibroblasts, plays a vasoconstrictive role, whereas ET_BR, predominantly located on endothelial cells, plays a vasodilative role. The expression of endothelin receptors in the pulmonary arteries of

patients with Fontan circulation has not been elucidated until now. In this study, we demonstrated for the first time that the expression levels of ET_AR and ET_BR were significantly elevated in the pulmonary arteries of failed Fontan patients. High ET-1 and ET_AR expression in the pulmonary arteries can contribute to vasoconstriction and vascular remodelling with proliferation of smooth muscle cells [13]. αSMA immunostaining of intra-acinar arteries of the failed Fontan patients in this study showed significant proliferation of vascular smooth muscle cells in the media and intima. Interestingly, the expression level of ET_BR was also elevated in the pulmonary arteries of the failed Fontan patients. Under normal physiological conditions, activation of ET_BR has a vasodilative role by releasing potent vasodilators, such as nitric oxide and prostacyclin. However, recent data suggested that ET_AR and ET_BR under pathological conditions formed heterodimers on vascular smooth muscle cells, and ET_BR mediated vasoconstriction similarly to ET_AR [14]. Moreover, another animal study demonstrated that increasing pulmonary flow induced ET_BR upregulation in pulmonary smooth muscle cells, and ET_BR mediated vascular constriction [15]. Correspondingly, in our study, the overexpression of ET_BR was significantly noticeable especially in the vascular smooth muscle cells of the failed Fontan patients. Therefore, our results indicate that high ET_BR expression on smooth muscle cells may contribute to the deterioration of the Fontan circulation through vascular constriction and vascular remodelling.

It is well established that endothelin receptor antagonists such as bosentan, sitaxentan, and ambrisentan improve the clinical symptoms and haemodynamic status of various types of pulmonary arterial hypertension [16–19]. Recently, the beneficial effects of pulmonary vasodilators administered to Fontan candidate patients or failing Fontan patients have also been reported [20–24]. For the Fontan candidate patients, low pulmonary vascular resistance is the most crucial factor in predicting the success of the Fontan operation; therefore, high pulmonary vascular resistance (>3 Wu m^2) is considered a contraindication to the Fontan surgery. Moreover, even after a successful Fontan operation, some patients may still present with various complications, including protein-losing enteropathy, plastic bronchitis, chronic heart failure, and pulmonary embolism, which are at least partially attributed to high central venous pressure and high pulmonary vascular resistance. Our findings showed that the expression levels of both ET-1 and its receptors were elevated in the pulmonary arteries of the failed Fontan patients. This gives a rationale for the treatment of patients with failing Fontan circulation with endothelin receptor antagonists. Clinical studies are needed to assess the clinical benefits of endothelin receptor antagonism in candidate patients for the Fontan procedure and also for the failing Fontan patients.

5. Limitations

Limitations of this study include (1) the small number of patients; (2) the use of immunohistochemistry, which is not a quantitative analysis; and (3) the extraction of total RNA from whole lung tissues and not from pulmonary arteries only. However, the study describes the potential role of ET-1 in Fontan patients and leads the way to further studies in this growing group of patients.

6. Conclusion

The pulmonary arteries of the patients with failing Fontan circulation exhibited significant medial hypertrophy with proliferation of vascular smooth muscle cells, and the expressions of ET-1, ET_AR, and ET_BR were significantly increased. Overexpression of ET-1 and its receptors in the pulmonary arteries can deteriorate the Fontan circulation owing to their vasoconstriction and vascular remodelling effects. This study suggests a histopathological rationale for the potential benefits of endothelin receptor antagonists in patients with failing Fontan circulation.

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Conflict of interest

Professor Maurice Beghetti has served on advisory boards/consulting for Pfizer, Actelion Pharmaceuticals, Bayer Schering, Encysive, GlaxoSmithKline, INO Therapeutics, Eli Lilly, and Mondobiotech and has received lecture fees from Actelion Pharmaceuticals, Encysive, Pfizer and Bayer Schering.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [25].

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ORIGINAL ARTICLE
Cardiovascular Surgery

Coronary Artery Bypass Grafting in Hemodialysis-Dependent Patients

– Analysis of Japan Adult Cardiovascular Surgery Database –

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Background: Perioperative risk during coronary artery bypass grafting (CABG) is reportedly high in patients with chronic renal disease. We aimed to determine postoperative mortality and morbidity and identify the perioperative risk factors of mortality during CABG in hemodialysis (HD)-dependent patients.

Methods and Results: From the Japan Adult Cardiovascular Surgery Database, we compared 1,300 HD-dependent chronic renal failure patients with 18,387 non-HD patients who all underwent isolated CABG between January 2005 and December 2008. The operative mortality and mortality, including major morbidity, was 4.8% vs. 1.4% and 23.1% vs. 13.7% in the HD and non-HD groups, respectively. Preoperative predictors of operative mortality included age, chronic obstructive pulmonary disease, peripheral arterial disease, congestive heart failure, arrhythmia, preoperative inotropic agent requirement, New York Heart Association class IV, urgent or emergency operation, poor left ventricular function, aortic valve regurgitation (>2), and mitral valve regurgitation (>3). Postoperative predictors of operative mortality included stroke, infection, prolonged ventilation, pneumonia, heart block, and gastrointestinal complications.

Conclusions: Compared with non-HD patients, CABG in HD patients was associated with high mortality and morbidity rates. An appropriate surgical strategy and careful perioperative assessment and management for prevention of respiratory and gastrointestinal complications might contribute to improved clinical outcomes after CABG in these patients. (*Circ J* 2012; **76:** 1115–1120)

Key Words: Coronary artery bypass grafting; Hemodialysis; Risk factor

Coronary artery disease (CAD) frequently occurs in patients with chronic renal failure (CRF) and is a major cause of mortality and morbidity in these patients.[1] CRF patients with CAD often need myocardial revascularization, and of the revascularization techniques, coronary artery bypass grafting (CABG) has been reported as having satisfactory survival rates in patients with kidney disease.[2,3] CABG has also been shown to yield better overall and angina-free survival than does percutaneous coronary intervention (PCI).[4–6] However, the operative mortality and morbidity are reportedly high compared with those of non-hemodialysis (HD) patients in both the short- and long-term. The hospital death rate after isolated CABG in HD-dependent patients was reported to be approximately 10%, which was higher than that for PCI.[7,8]

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Therefore, an appropriate surgical strategy and perioperative medical treatment based on the identification of perioperative risk factors would lead to an improvement in the clinical outcomes of these surgical procedures. For the past few decades, various studies have reported the clinical outcome of cardiac surgery in CRF patients, but almost all have been from single centers or consist of less than 200 patients.[1] Moreover, we found few previous large-scale studies that focused on isolated CABG and included multivariate analysis of perioperative risk factors of operative mortality.[8]

Therefore, in the present study we examined 19,687 isolated CABG patients, including 1,300 HD-dependent patients,

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Table 1. Baseline Characteristics of Patients Undergoing CABG

	Non-HD (n=18,387)	HD (n=1,300)	P value
Age, years			
≤60	17.9	28.3	<0.0001
61–65	13.8	19.0	
66–70	18.6	19.3	
71–75	22.0	17.9	
76–80	18.7	11.6	
≥81	9.0	3.8	
Mean age, years	68.7±9.4	65.4±9.2	<0.0001
BSA	1.64±0.39	1.59±0.16	0.001
Male (%)	77.3	78.7	0.246
History of smoking (%)	53.0	46.2	<0.0001
Current smoking (%)	20.0	16.7	0.004
DM (%)	48.0	65.7	<0.0001
DM requiring medication (%)	40.7	56.9	<0.0001
Serum creatinine (mg/dl)	1.01±1.1	8.61±25.2	<0.0001
Hyperlipidemia (%)	57.0	36.5	<0.0001
Hypertension (%)	73.3	83.1	<0.0001
History of cerebrovascular event (%)	13.5	17.7	<0.0001
Recent (<2 weeks) cerebrovascular event (%)	0.6	0.8	0.295
History of infective endocarditis (%)	0.1	0.2	0.342
Chronic lung disease (moderate, severe) (%)	1.5	1.4	0.650
Extracardiac arterial disease (%)	15.4	27.0	<0.0001
Peripheral arterial disease (%)	14.3	25.4	<0.0001
Thoracic aortic disease (%)	2.1	2.2	0.699
Mental disorder (%)	3.1	4.5	0.006
History of coronary intervention (%)	25.1	28.1	0.015
Previous MI (%)	34.7	31.1	0.008
Congestive heart failure (2 weeks) (%)	13.7	22.8	<0.0001
Angina (%)	88.2	89.9	0.056
Unstable (%)	28.8	34.7	<0.0001
Cardiogenic shock (%)	4.3	6.6	0.0001
Arrhythmia (%)	7.4	9.7	0.003
Inotropic agents requirement (%)	3.5	4.9	0.010
Reoperation (%)	2.2	2.3	0.875
Urgent operation (%)	11.8	13.7	0.044
Emergency operation (%)	6.9	8.7	0.017
BMI mean	24.0±29.7	22.7±14.0	0.003
>26 (%)	21.5	12.0	<0.0001
>30 (%)	3.7	2.2	0.004
NYHA class			
NA (%)	14.1	10.1	<0.0001
I (%)	26.3	23.1	0.011
II (%)	36.2	35.2	0.465
III (%)	14.1	17.8	0.0003
III or IV (%)	23.0	30.8	<0.0001
No. of diseased vessels (%)			
1	4.4	4.9	0.381
2	24.8	23.9	0.465
3	69.4	70.2	0.531
Ejection fraction (%)			
>60	48.9	34.1	<0.0001
30–60	44.4	55.4	<0.0001
<30	6.2	9.8	<0.0001
AS (%)	1.7	6.5	<0.0001
MS (%)	0.3	1.2	<0.0001
AVR (>2) (%)	5.6	5.7	0.845
MVR2 (>2) (%)	11.5	20.7	<0.0001
TVR2 (>2) (%)	5.4	8.7	<0.0001
AVR3 (>3) (%)	0.6	0.7	0.768
MVR3 (>3) (%)	1.4	4.4	<0.0001
TVR3 (>3) (%)	0.6	1.3	0.002

CABG, coronary artery bypass grafting; HD, hemodialysis; BSA, body surface area; DM, diabetes mellitus; MI, myocardial infarction; BMI, body mass index; NYHA, New York Heart Association; AS, aortic stenosis; MS, mitral stenosis; AVR, aortic valve regurgitation; MVR, mitral valve regurgitation; TVR; tricuspid valve regurgitation.

from between 2005 and 2008 in the Japan Adult Cardiovascular Surgery Database (JACVSD) to determine the contemporary clinical outcome of isolated CABG and to determine the risks for perioperative death following CABG in patients with HD-dependent CRF. We then discuss the appropriate surgical strategy for and the perioperative medical management of such patients.

Methods

Study Population

The JACVSD was initiated in 2000 to estimate surgical outcomes after cardiovascular procedures in many centers throughout Japan. The JACVSD adult cardiovascular division currently captures clinical information from nearly half of all Japanese hospitals performing cardiovascular surgery. The data collection form has a total of 255 variables (definitions are available online at http://www.jacvsd.umin.jp), and these are almost identical to those in the Society of Thoracic Surgeons (STS) National Database (definitions are available online at http://sts.org). The JACVSD has developed software for a web-based data collection system through which the data manager of each participating hospital electronically submits the data to the central office. Although participation in the JACVSD is voluntary, data completeness is a high priority. Accuracy of submitted data is maintained by data audit achieved by monthly visits by administrative office members to the participating hospital to check data against clinical records. Validity of data is further confirmed by an independent comparison of the volume of cardiac surgery at a particular hospital entered in the JACVSD with that reported to the Japanese Association for Thoracic Surgery annual survey.[9]

We examined cases of isolated CABG between January 1, 2005 and December 31, 2008. JACVSD records that had been obtained without the patient's informed consent were excluded from this analysis. Records with missing or out of range age, sex, or 30-day status (see Endpoints section below) were also excluded. After data cleaning, the population for this risk model analysis consisted of 1,300 HD-dependent patients and 18,387 non-HD-dependent patients who underwent cardiovascular procedures at 167 participating sites throughout Japan.

Endpoints

The primary outcome measure of the JACVSD was 30-day operative mortality, which was defined exactly the same as the 30-day operative mortality in the STS National Database. The 30-day operative mortality included any patient who died during the index hospitalization, regardless of the length of hospital stay, and any patient who died after being discharged from hospital within 30 days of the operation. Operative mortality also included any patients who died after 31 days during the hospital stay in addition to patients included in the 30-day mortality. Using a definition from previous studies,[10,11] major morbidity was defined as any of the following 5 postoperative in-hospital complications: stroke, reoperation for any reason, need for mechanical ventilation for more than 24 h after surgery, renal failure, or deep sternal wound infection.

Statistical Analysis

We examined differences between 2 groups (isolated CABG with and without HD) using bivariate tests: Fisher's exact test and the chi-square test for categorical covariates, and the unpaired t-test or Wilcoxon rank sum test for continuous covariates. To develop risk models of isolated CABG with HD, we conducted multivariate stepwise logistic regression analysis for each outcome. Stability of the model was checked every time a variable was eliminated. When all statistically non-significant variables ($P<0.10$) had been eliminated from the model, "goodness-of-fit" was evaluated and the area under the receiver-operating characteristic curve was used to assess how well the model could discriminate between patients who lived from those who had died. To investigate the relationship between postoperative complications and operative death in HD patients, we conducted multivariate stepwise logistic regression analysis for operative mortality. Complications such as cardiac arrest and multisystem failure were excluded from this analysis because they are highly associated with operative death.

Results

Patient Demographics

Baseline characteristics of the study population are summarized in Table 1. Patients in the HD group were significantly younger (65.4±9.2 vs. 68.7±9.4 years) and had less body surface area (1.59 vs. 1.64 m^2) than the non-HD patients. As expected, the HD-dependent patients had a significantly greater degree of baseline comorbidity than did non-HD patients. Patients in the HD group were more likely to have a history of diabetes (56.9% vs. 40.7%), hypertension (83.1% vs. 73.3%), and peripheral vascular disease (27.0% vs. 15.4%). A higher rate of current congestive heart failure (22.8% vs. 13.7%) with a lower ejection fraction and lower New York Heart Association (NYHA) status was observed in the HD group. As for valvular disease, aortic stenosis (6.5% vs. 1.7%), mitral valve regurgitation (MVR) (>2) (20.7% vs. 11.5 %), and tricuspid valve regurgitation (>2) (8.7% vs. 5.4%) were more common in HD patients. In both the HD and non-HD groups, off-pump surgery was performed approximately twice as often as on-pump surgery, and the off-pump ratio did not differ between groups. Transfusion was required more often in the HD group. Bilateral internal mammary artery usage in the HD group was less frequent than in the non-HD group (22.8% vs. 31.4%) (Table 2).

Postoperative Outcomes

In-hospital outcomes are summarized in Table 3. The 30-day mortality was 4.8% vs. 1.4% and the operative mortality was 7.8% vs. 2.1% in the HD and non-HD groups, respectively. Both the 30-day and operative mortalities in HD patients were approximately 3-fold more frequent than in non-HD patients. Operative mortality with a major complication was more frequent in the HD group (23.1% vs. 13.7%).

Table 2. Intraoperative Characteristics

	Non-HD CABG (n=18,387)	HD CABG (n=1,300)	P value
On-pump surgery (%)	37.3	35.6	0.230
Transfusion (%)	52.5	87.8	<0.0001
Bilateral IMA usage (%)	31.4	22.8	<0.0001
Single IMA usage (%)	61.7	68.7	<0.0001

IMA, internal mammary artery. Other abbreviations see in Table 1.

Table 3. Mortality and Morbidity

	Non-HD CABG (n=18,387)	HD CABG (n=1,300)	P value
30-day mortality (%)	1.4	4.8	<0.0001
Operative mortality (%)	2.1	7.8	<0.0001
Operative mortality+major complication (%)	13.7	23.1	<0.0001
Reoperation (any reason)	5.4	6.6	0.067
Infection			
Deep sternum	1.8	2.6	0.026
Thoracotomy	0.5	1.1	0.007
Leg	1.9	4.5	<0.0001
Urinary	0.8	0.6	0.402
Septicemia	1.0	2.7	<0.0001
Prolonged ventilation	6.6	9.4	<0.0001
Pneumonia	2.4	4.4	<0.0001
Pulmonary embolism	0.2	0.2	0.989
Stroke	1.5	1.6	0.636
TIA	1.3	2.4	0.001
Coma	0.6	1.1	0.017
Paraparesis	0.3	0.4	0.871
Atrial fibrillation	13.2	14.9	0.076
Heart block requiring pacemaker	0.5	0.8	0.159
Cardiac arrest	1.0	2.8	<0.0001
Reoperation for bleeding	1.8	3.0	0.003
Anticoagulant complication	0.3	0.5	0.084
Tamponade requiring drainage	1.0	1.4	0.132
Gastrointestinal complication	1.6	3.9	<0.0001
Multisystem failure	0.9	2.6	<0.0001
Dissection aorta	0.1	0	0.378
Dissection iliac	0.02	0.1	0.138
Limb ischemia	0.2	0.7	0.001
Re-admission	1.9	2.5	0.193
ICU stay >8 days	5.7	11.2	<0.0001

TIA, transient ischemic attack; ICU, intensive care unit. Other abbreviations see in Table 1.

Multivariate Predictors of In-Hospital Death

Multivariate predictors of operative mortality are summarized in Table 4. Predictors of operative mortality included

Table 4. Multivariate Preoperative Predictors of Operative Mortality of CABG for HD Patients

Characteristic	RR (95%CI)	P value
Age	1.38 (1.164–1.604)	<0.0001
Chronic pulmonary disease (moderate severe)	5.52 (1.786–17.033)	0.003
Extracardiac arterial disease	1.86 (1.15–3.01)	0.011
Congestive heart failure	1.77 (1.06–2.957)	0.029
Arrhythmia	1.84 (1.02–3.325)	0.043
Preoperative inotropic agent	2.46 (1.204–5.024)	0.014
NYHA class IV	1.99 (1.1–3.599)	0.023
Urgent operation	2.02 (1.085–3.752)	0.027
Emergency operation	2.27 (1.177–4.372)	0.014
Ejection fraction <30%	2.06 (1.125–3.787)	0.019
AVR ≥2	3.98 (1.987–7.979)	<0.0001
MVR ≥3	2.32 (1.094–4.913)	0.028

RR, relative risk; CI, confidence interval. Other abbreviations see in Table 1.

Table 5. Multivariate Postoperative Predictors of Operative Mortality of CABG for HD Patients

	OR	CI	P value
Stroke	9.85	3.1–30.8	<0.0001
Infection	6.72	2.6–17.7	<0.0001
Prolonged ventilation	3.82	2.1–7.0	<0.0001
Pneumonia	13.15	6.3–27.4	<0.0001
Gastrointestinal complication	5.43	2.3–12.7	<0.0001
Heart block	12.46	2.4–64	0.003

OR, odds ratio. Other abbreviations see in Tables 1,4.

age (odds ratio [OR]=1.38, P<0.0001), chronic obstructive pulmonary disease (COPD) (OR=5.52, P=0.003), peripheral arterial disease (OR=1.86, P=0.011), congestive heart failure (OR=1.77, P=0.029), arrhythmia (OR=1.84, P=0.043), preoperative inotropic agent requirement (OR=2.46, P=0.014), NYHA class IV (OR=1.99, P=0.023), urgent operation (OR=2.02, P=0.027), emergency operation (OR=2.27, P=0.014), ejection fraction <30% (OR=2.06, P=0.019), aortic valve regurgitation (AVR) (>2) (OR=3.98, P<0.0001), and MVR (>3) (OR=2.32, P=0.028).

Relationship Between Operative Mortality and Postoperative Complications

Results are summarized in Table 5. Among the complications observed relatively often (incidence >3%), prolonged ventilation (OR=3.82), pneumonia (OR=13.15), infection (OR=6.72), and gastrointestinal complications (OR=5.43) were significant factors in operative mortality.

Discussion

We investigated the clinical outcomes and risk factors of operative mortality and morbidity in patients with (n=1,300) and without (n=18,387) HD who underwent isolated CABG. The study data was extracted from the JACVSD, and is one of the largest comparative series of post-CABG outcomes in such patients.[8,12]

The operative mortality of HD patients after isolated CABG in this study was 7.8%, which was similar to previous studies that reported an operative mortality of approximately 10%.[8,12] As previously reported, HD patients have more preoperative comorbidities. Compared with other reports, the rates of emergency operation, male sex, shock state, and off-pump CABG tended to be high in this study, and those of congestive heart failure and chronic lung disease tended to be low. Age, hypertension, NYHA status, and prevalence of valvular disease were comparable. The postoperative morbidity rate of the HD group was higher than that in the non-HD group. Major postoperative morbidity (stroke, prolonged ventilation, deep sternal infection, renal failure and reoperation for any reason) were also comparable with those in the reports from the STS database, which included 7,152 dialysis patients.[8] Besides the major complications, the prevalence of leg infection, pneumonia, transient ischemic attack, cardiac arrest, gastrointestinal complications, multisystem failure, and limb ischemia in HD patients was significantly higher than in non-HD patients.

A series of studies have reported early and late outcomes of CABG with and without valve operations in CRF patients.[13–16] In those studies, several risk factors were reported for mortality after cardiac surgery in HD-dependent patients. Many reports have found a low ejection fraction to be an independent risk factor,[17–20] which was consistent with the findings of the present study. However, we found no previous large-scale studies that focused on isolated CABG and included a multivariate analysis of risk factors for hospital mortality. As a large-scale report that focused on isolated CABG, Cooper et al demonstrated that the glomerular filtration rate was a powerful predictor of operative morbidity after isolated CABG in 7,152 HD patients.[8] Charytan et al analyzed 77,323 non-HD and 635 HD patients who underwent CABG that included valve surgery. They demonstrated that HD-dependence, congestive heart failure, valvular heart disease, valve surgery, female sex, age, pathological weight loss, chronic lung disease, neurological disorders, admission for myocardial infarction, and liver disease were adjusted risks for perioperative mortality.[12]

Regarding valvular disease, we also demonstrated that AVR (>2) and MVR (>3) were independent risk factors for operative mortality after isolated CABG. The question then arose regarding whether valve operation should be performed simultaneously with CABG when moderate AVR or MVR was complicated. Horst et al reported that the risk for perioperative death associated with CABG combined with valve operation was approximately 10-fold that for isolated CABG.[1] Charytan et al also demonstrated concomitant valve surgery as a perioperative risk factor.[12] the surgical management of moderate, chronic ischemic MVR combined with CABG is still controversial.[21,22] Combined mitral valve surgery has been reported to be significantly associated with a lower residual grade of MVR compared with CABG alone. On the other hand, it has been reported that CABG alone was able to reduce the MVR grade in 40% of patients.[23] From the postoperative NYHA status perspective, the effect of mitral valve surgery is also controversial.[23,24] As for late mortality, a meta-analysis of 2,479 ischemic MVR patients showed that mitral valve surgery did not have advantages for late mortality compared with CABG alone.[24] As for aortic valve disease, most surgeons would not perform concomitant aortic valve surgery in HD patients with AVR=2. However, concomitant aortic valve surgery might be taken into consideration in some cases complicated by AVR >3. In the present study, the cohort of AVR >3 was very small (non-HD group: <100 patients, HD group: <10 patients.) Therefore, it was very difficult to investigate whether AVR >3 is a risk factor or not for postoperative mortality in our multivariate analysis.

In summary, concomitant surgery should be performed with consideration of the "risks and benefits" of additional valve surgery, and further study is necessary.

Relatively few large-series reports have documented the detailed incidence of postoperative morbidity after isolated CABG in HD-dependent patients.[8,12] Compared with non-HD patients, the incidence of major postoperative complications was high in HD-dependent patients in the present study, as previously reported. Postoperative complications could be considered to be closely associated with higher mortality. The higher incidence of these complications in HD-dependent patients might partly explain their poor clinical outcomes. To improve the clinical outcome of isolated CABG in HD, it seems important to prevent these complications. The complications that occurred at a relatively high incidence (>3%) in the present study were infection, prolonged ventilation, pneumonia, atrial fibrillation, reoperation for bleeding, and gastrointestinal complications. Among these, infection, prolonged ventilation, pneumonia, and gastrointestinal complications were significant independent risk factors for operative mortality.

Several studies have reported that higher mortality rates are associated with infection in HD-dependent patients undergoing cardiac surgery. Takami et al reported that following cardiac surgery in their 245 HD patients, almost half of the cases of hospital death were related to infection.[25] Akman et al demonstrated that infection was an independent postoperative risk factor for mortality after CABG in HD-dependent patients, and suggested the importance of early diagnosis of infection for both early recovery and shorter hospitalization in the postoperative period.[7]

Mangi et al reported that the incidence of gastrointestinal complications requiring surgical repair after cardiac and vascular surgery was 0.53% (46/8,709).[26] Of these, mesenteric ischemia comprised 67% and two-thirds of these patients died. To prevent gastrointestinal complications, especially mesenteric ischemia, preoperative abdominal screening, bowel preparations, and postoperative volume control might be important in isolated CABG for HD-dependent patients.

To prevent postoperative pulmonary complications, preoperative risk stratification and a risk-reduction strategy seem important. As for postoperative respiratory complications, COPD, the prevalence of which is higher by 10–12% among cardiovascular surgical candidates compared with aged-matched populations, might be one of the most important risk factors.[27] Several advances in surgery and anesthetic care have been shown to be particularly beneficial for COPD patients. Compared with standard operations, minimally invasive procedures produce less tissue damage and, in turn, attenuated neurohumoral and inflammatory responses.[27] Off-pump bypass is considered to be a minimally invasive surgery in coronary artery operations. Patients with FEV_1 (1 s to forced vital capacity) less than the lower limit of normal have better outcomes after off-pump bypass compared with those post-CABG.[28]

Conclusions

Compared with non-HD patients, CABG in HD patients was associated with high mortality and morbidity rates in the present study. An appropriate surgical strategy and careful perioperative assessment and management for prevention of respiratory and gastrointestinal complications might contribute to improvements in clinical outcomes after CABG in HD-dependent patients.

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Case Report

Failed Depiction of Patent Bypass Graft Due to Presence of Large Lateral Costal Artery

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We report a rare case of failed depiction of a patent right internal thoracic artery (RITA) to left anterior descending artery (LAD) bypass on 64-slice multidetector row computed tomographic (MDCT) angiography due to the presence of a large lateral costal artery. A 66-year-old male with acute coronary syndrome due to triple vessel disease underwent urgent coronary artery bypass grafting, in which bilateral ITA and saphenous vein grafts were used. Postoperative MDCT angiography showed an occluded RITA-LAD bypass, which was eventually shown to be patent by angiography. Angiography also revealed a large lateral costal artery that was considered to affect the flow to the LAD. Thus, coil embolization of the branch was attempted. However, it was abandoned because the patient suffered from severe back and intercostal pain during balloon occlusion of the lateral costal artery. Postoperative MDCT angiography is not always accurate for the assessment of graft patency in patients with large ITA side branches. In addition, embolization is not always possible because occlusion of this large branch may cause severe pain when its size becomes quite large.

Keywords: CABG, computed tomography, surgery, complications, anatomy

Introduction

Multidetector computed tomography (MDCT) has been widely used as a noninvasive assessment of postoperative graft patency after coronary artery bypass grafting (CABG).[1] We experienced a rare situation of a failed depiction of a patent right internal thoracic artery (RITA)-left anterior descending artery (LAD) graft by MDCT, which was considered to be due to the steal phenomenon by a large lateral costal artery on coronary angiography. Furthermore, although we attempted transcatheter occlusion of this large side branch as previously reported,[2,3] it could not be completed because of severe pain during balloon occlusion of the branch. This rare case provides important information regarding large costal arteries, which may affect postoperative bypass graft evaluation and cannot always be occluded because of severe pain.

Case Report

A 66-year-old male with unstable angina due to severe triple vessel disease was transferred to our hospital and underwent urgent CABG. He underwent RITA-LAD anastomosis, a left internal thoracic artery to high lateral branch anastomosis, and saphenous vein graft (SVG) anastomosis to the diagonal branch and distal circumflex artery and to the posterior descending and posterolateral branches of the right coronary artery. His postoperative course was uneventful, and he did not experience recurrent angina. However, routine MDCT performed 8 days

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after the operation revealed occlusion of the RITA-LAD graft (**Fig. 1**). Because the blood supply to the LAD was crucial for this patient, we decided to perform coronary angiography for further investigation.

On angiography, the RITA-LAD graft was found to be patent, but had low flow; in addition, a large intercostal side branch was present. The diameter of the branch was larger than that of the RITA, and it coursed as far as the seventh intercostal space to provide a blood supply to each intercostal artery (**Fig. 2A**). Thus, we considered that the failed depiction of the RITA-LAD graft was due to the steal phenomenon by this large branch. Although the patient was asymptomatic, we attempted occlusion of the branch using a coil embolization technique to prevent future complications involving the LAD.

During the procedure, the steal phenomenon was confirmed by test occlusion of the branch using a balloon catheter (**Fig. 2B**). However, the patient began to complain of severe pain along the chest wall immediately after the test balloon occlusion of the branch. The pain disappeared when the balloon was deflated. This phenomenon occurred each time we attempted to occlude the branch. Thus, we abandoned our attempt to close the large costal artery, and instead performed stent intervention to the LAD to protect its flow through the native coronary artery. The patient was discharged and remained symptom-free 12 months after the operation.

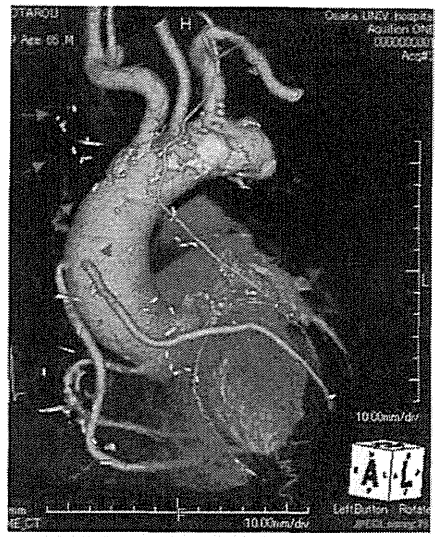

Fig. 1 A 64-slice multidetector-row computed tomographic angiograph reveals the occluded right internal thoracic artery to left anterior descending artery bypass graft (arrows).

Discussion

The presence of a large lateral costal artery of the internal thoracic artery is reported in 10% to 20% of the population.[4] The size of the branch varies depending on the length of the intercostal artery. The incidence of a very large lateral costal artery that reaches beyond the sixth intercostal space, as in our case, has been reported in only 2% of lateral costal artery cases.[5] Although several reports have revealed recurrent angina due to a large side branch of the internal thoracic artery,[2,3] there is continued controversy regarding whether this large branch can cause ischemia by stealing blood flow from the branch to the LAD.[3] This case revealed two important factors regarding this branch: the steal phenomenon and the risk of occlusion.

With recent improvement in the temporal and spatial resolution of CT, noninvasive assessment of bypass graft patency by MDCT can provide reliable information, and the diagnostic accuracy has been improving.[1] At our institute, MDCT is used as a routine evaluation modality for graft patency after CABG when there is no contraindication. This case involved a rare situation of failed depiction of RITA-LAD graft anastomosis due to the presence of a large lateral costal artery. MDCT could not detect this large branch, and the diagnosis was "occlusion," which was incorrect. To the best of our knowledge, this is the first MDCT case of failed depiction of a patent RITA-LAD graft due to the steal phenomenon by a large lateral costal artery. In this case, the patient did not complain of angina symptoms after the operation, and assessment by coronary angiography was able to provide us more accurate information. This may be a limitation of assessment by MDCT.

The effect of large side branches on internal thoracic artery to LAD bypass grafts is unknown. Some reports maintain that internal thoracic artery steal is very unlikely because the left coronary system is perfused in diastole while the chest wall artery system is perfused in systole[3]; others support the concept of the steal phenomenon, considering ligation of the side branch as the appropriate treatment.[2] In this case, LAD was not well depicted by MDCT, and the RITA-LAD flow was very slow on coronary angiography. Although the patient did not show any symptoms, we believed that it would be better for him to establish enough flow to the LAD. Occlusion of large lateral costal arteries has been widely reported, and almost all of the treatments have been suc-

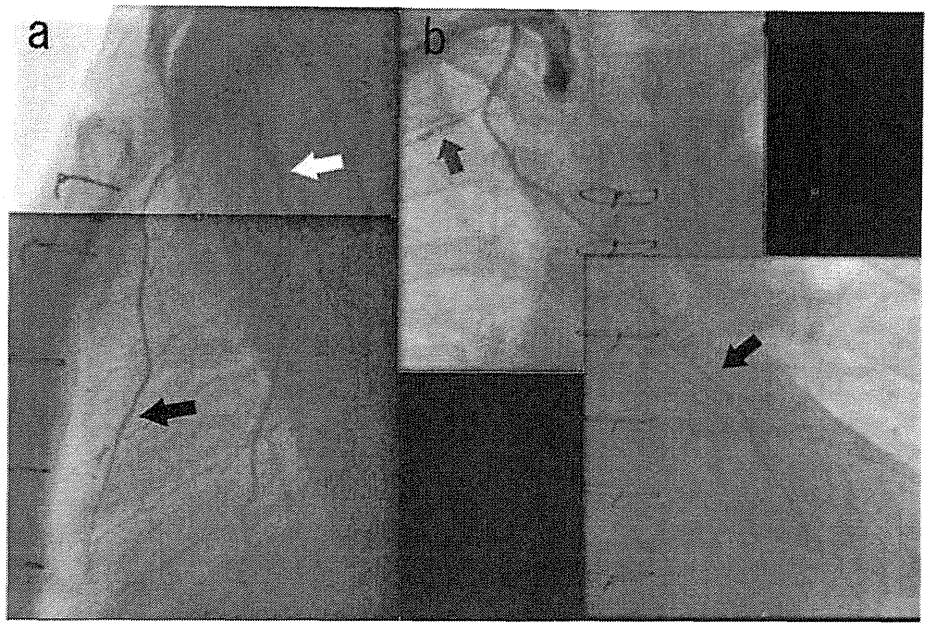

Fig. 2 (**A**) Coronary angiography showed the large costal artery from the right internal thoracic artery (white arrow). The flow toward the left anterior descending artery was slow (black arrow). (**B**) The flow from the right internal thoracic artery to the left anterior descending artery was well depicted (black arrow) during balloon occlusion of the large costal artery (gray arrow).

cessful without any problems.[2,3] Our case is the first report of a rare complication of occlusion of a large side branch. The patient suffered from severe, intolerable pain along the chest wall during test occlusion using a balloon. We must be aware of the potential for this complication when attempting to occlude a large lateral costal branch. Although it is rare to encounter a large lateral costal artery such as this, it is important to consider the possibility of its presence. Of course, long-term follow-up was needed in our case for further evaluation of the outcome of this anastomosis.

Conclusion

In conclusion, in the presence of a large lateral costal artery, care should be taken in the evaluation of ITA graft patency by MDCT. When occlusion of this artery is needed, we must consider that occlusion is not always possible because of severe pain.

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Erythropoietin, progenitor cells and restenosis. A critique of Stein et al.

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Dear Sirs,

In the recently published work by Stein et al., "Erythropoietin-induced progenitor cell mobilisation in patients with acute ST-segment-elevation myocardial infarction and restenosis" (2), the authors presented the measurements of target lesions that were analysed using quantitative coronary angiography (QCA) for patients from the Regeneration of Vital Myocardium in ST-Segment Elevation Myocardial Infarction by Erythropoietin (REVIVAL-3) study (1, 2).

In the REVIVAL-3 study, patients with ST-segment elevation myocardial infarction (STEMI) undergoing percutaneous coronary intervention (PCI) were randomly assigned to receive a high-dose of epoetin beta (EPO) (n=68) or a placebo (n=70). In a post-hoc analysis, Stein et al. investigated the effects of EPO on the target lesions following stent implantation using QCA. The authors showed that the segment diameter stenosis at six months was significantly increased in patients receiving EPO than in those receiving a placebo (32 ± 19% vs. 26 ± 14%, p=0.046), and that there was a trend towards a higher incidence of revascularisation of the infarct-related artery in the EPO group than in the placebo group (p=0.08). Therefore, the authors concluded that EPO administration was associated with an increased segmental diameter stenosis and increased target lesion revascularisation. However, readers may need to interpret the data with caution for the following reasons.

(i) Late lumen loss is often used as an efficacy end-point in clinical studies to evaluate restenosis of the target lesion because this parameter indicates the absolute loss of the minimum lumen diameter of the target lesion from the time of the stenting procedure through the follow-up period. In the REVIVAL-3 trial, the authors showed that the late lumen loss at six months tended to be lower in the EPO group than in the placebo group (2.1 ± 0.7 vs. 2.3 ± 0.6 mm, p=0.070). Consistently, two small clinical studies using low doses of EPO also demonstrated that late lumen loss did not differ between the control group and the EPO treated group; however, Stein et al. did not discuss this issue in the present study (3, 4). Because the segment diameter stenosis at six months was significantly increased in the EPO group than in the placebo group and because late lumen loss tended to be lower in the EPO group than in the placebo group, the segment diameter stenosis immediately after the completion of the stenting procedure is likely to be higher in the EPO group than in the placebo group. If this is the case, the increased segment diameter stenosis at the six-month follow-up in the EPO group could be attributable to increased residual stenosis immediately after the completion of the stenting procedure rather than to an additional increase in diameter stenosis by EPO. We would like the authors to confirm the value of late lumen loss and to list the angiographic parameters at baseline, immediately after the completion of the stenting procedure and at follow-up in their Table 2.

(ii) The authors may need to verify the results in their Table 2. The minimal lumen diameter and diameter stenosis are usually described as the mean ± standard deviation. Binary restenosis should be expressed as a number (%). In addition, to avoid treatment bias, target lesion revascularisation may need to be pre-specified as either ischaemia driven or clinically driven. Otherwise, binary restenosis may be a better measure of the actual clinical impact of EPO on neointimal proliferation.

Further studies assessing the administration of EPO to patients suffering from myocardial infarction are needed because inconsistent results have been obtained (2–6). The use of appropriate parameters and interpretation may contribute to a better understanding of how EPO affects neointimal proliferation in patients with STEMI.

Conflicts of interest

None declared.

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ORIGINAL ARTICLE

A novel synthetic derivative of human erythropoietin designed to bind to glycosaminoglycans

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Abstract

To synthesize long-acting and antiangiogenic erythropoietin to be clinically applied for treatment of patients with solid tumors, we synthesized a hybrid molecule of human erythropoietin added onto the C-terminus with a heparin-binding motif of human PLGF-2 to develop a novel derivative of long-acting and antiangiogenic erythropoietin: heparin-binding erythropoietin (HEPO), and studied the characteristics of this novel erythropoietin derivative. HEPO cDNA was synthesized, expressed in insect cells, and the protein was purified using a heparin-sepharose affinity column. The erythropoietic and angiogenic effects of the partially purified protein were analyzed *in vitro* and *in vivo*. The erythropoietic activity of the protein was equivalent to natural EPO *in vitro*. *In vivo* administration of the protein to mice revealed its long-acting erythropoietic activity as expected. Administration of the protein inhibited angiogenesis in a mouse limb ischemia model. In conclusion, the heparin-binding motif of PLGF-2 may act as, so to speak, a superendostatin. This novel long-acting erythropoietin derivative may have an advantage to inhibit tumor growth while preserving hematopoietic and tissue-protective effects.

Keywords: Protein engineering, angiogenesis, erythropoietin, PLGF-2, heparin-binding proteins

Introduction

Erythropoietin (EPO) is produced in the kidney in response to hypoxia and/or anemia through interaction between the hypoxia-inducible factor (HIF)-system and the GATA-system to stimulate erythropoiesis in bone marrow,[1] while constitutional erythropoiesis is maintained by endogenous EPO production in bone marrow.[2] The paracrine system of EPO exists in not only bone marrow but also in the central nervous and cardiovascular systems, and EPO shows tissue-protective effects on these organs.[3,4] The EPO receptor (EPOR) and cytokine receptor common β-chain (βc: CD131) belong to the type I cytokine receptor family. EPOR homoreceptors are expressed in erythroid cells, and EPOR/βc heteroreceptors are expressed in other organs.[5]

Human EPO is a glycoprotein and consists of 165 amino acid residues, 3 *N*-glycans, and an *O*-glycan. Most hematopoietic cytokines have very little or no heparin-binding properties, thus showing almost no tissue affinity to be released from secretion organs to work as an endocrine messenger in distant organs via blood flow. Although the frequency of arginine (R) and lysine (K) is relatively high in the helix D of human EPO (Figure 1), EPO does not express heparin-binding affinity presumably because of its settled position in the helix structure. On the other hand, VEGF/PDGF family members and chemokines show a high heparin-binding property via heparin-binding motifs (HBM) in the C-terminal random coil to construct a concentration gradation around cells secreting the factors to maintain

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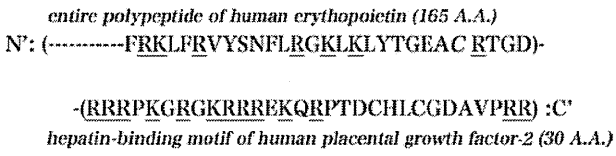

Figure 1. Peptide sequence of heparin-binding erythropoietin Heparin-binding motif of human PLGF-2 was added to the C-terminus of entire polypeptides of human EPO. The R/K (arginine/lysine)-repeat of the heparin-binding motif of hPLGF-2 contributes to the affinity to glycosaminoglycans in the cell surface and/or extracellular matrix. The italic C (cysteine) is involved in the disulfide bond of EPO.

polarity to the receptor-expressing cells via the paracrine system. Endothelial cells and most other cells express membrane type proteoglycans (PG) such as syndecans and glypicans. PG consist of glycosaminoglycans (GAG) and their core proteins. Extracellular matrix (ECM) is composed of fiber proteins (collagen, etc) and ground substances (mucopolysaccharides) such as GAG (heparan sulfate, chondroitin sulfate, etc) and PG. HBM of growth factors and chemokines are bound to GAG via interaction between arginine/lysine-repeat ($-NH_3^+$) of HBM and saccharide sulfate ($-SO_3^-$) of GAG.[6]

Intervention of tumor angiogenesis is one of the hopeful strategies to treat malignant tumors,[7] and several angiogenesis inhibitors have been developed such as anti-VEGF antibodies,[8] VEGF receptor-tyrosine kinase inhibitors,[9] angiostatin,[10] and endostatin.[11] Among them, endostatin, a C-terminal fragment derived from type XVIII collagen, is an endogenous inhibitor of angiogenesis, similar to angiostatin and thrombospondin. Endostatin has high affinity for heparin through an 11 arginine basic patch,[12] and also binds all heparan sulfate PG with low affinity.[13] Administration of recombinant endostatin in combination with a standard chemotherapeutic regimen significantly improved the clinical course in patients with non-small cell lung cancer over that of the chemotherapeutic regimen alone.[14]

Among several angiogenic factors, PLGF, another VEGFR1 ligand, has been found to play an important role in angiogenesis, vasculogenesis, and especially in cancer angiogenesis,[15] and an antibody against PLGF (TB403) is hopeful to treat patients with cancer.[16] Unlike VEGF, HBM of PLGF-2 quite abundantly consists of arginine-repeats, and plays an important role in adult arteriogenesis.[17,18] Therefore, HBM of PLGF-2 may have stronger heparin-binding affinity, and may occupy GAG to purge the concentration gradient of several angiogenic factors from tissues. We then tried to synthesize chimeric EPO introduced with HBM of PLGF-2.

Chimeric EPO introduced with HBM may express affinity to ECM in organs and endothelial cells, and may work as a long-acting EPO to treat anemia and tissue injury. At first we synthesized recombinant mouse heparin-binding EPO (heparinophilic EPO: HEPO) employing cDNA of mEPO and HBM of mPLGF using a COS-expression system. rmHEPO simultaneously expressed heparin-binding features and erythropoietic activity *in vitro*. We then attempted to synthesize rhHEPO to further obtain basic data of HEPO *in vivo* and *in vitro*. We utilized a baculovirus vector and serum free culture system to simplify the purification of recombinant protein while preserving the glycoprotein structures, because unglycosylated EPO does not show bioactivity.

Materials and methods

Vector, expression, and HEPO purification

Human EPO cDNA, rhEPO, rhAEPO (asialoerythropoietin), rhCEPO (carbamyl erythropoietin), and the EPO-dependent cell line AS-E2[19] were kindly provided by Chugai Pharmaceuticals (Tokyo, Japan). HEPO cDNA was synthesized by the PCR method using the human EPO cDNA and primers as shown in Table 1. The structure of synthesized cDNA was: 5′- (SphI site)- (entire EPO cDNA without a signal sequence)- (cDNA of PLGF-2 heparin-binding motif)- (stop codon)- (EcoRI site)-3′ (Figure 1). The sequence of the cDNA was then confirmed, the cDNA was installed into a pMIB Baculovirus Vector (Invitrogen, CA, USA) using restriction enzymes, SphI and EcoRI, and the vector was introduced into *Escherichia coli*, JM109 (Takara Bio, Otsu, Japan). Several clones were determined for the cDNA sequence, and the HEPO vector was obtained. The vector was increased using an *E. coli* clone, purified using a Quantum Prep, Plasmid Maxiprep Kit (BIO-RAD, CA, USA), treated with an endotoxin removal kit (Mira CLEAN, Mirus Bio, WI, USA), and the DNA concentration was measured at OD260, and then stored at −80°C.

Table 1. Sequences of primers and the resulted polypeptide.

5′primer (to add Sph-I site on the 5′-terminus of EPO cDNA):
ttaatggcatgctagccccaccacgcctcatctgtgac
3′primer (to elongate cDNA of human EPO on the 3′-terminus with cDNA of PLGF heparin-binding motif and EchRI site)
3′primer-1:
ttctcttccccctgcccttgggtctcctccttctgtcccctgtcctgcaggcctc
3′primer-2:
ggcagtctgtgggtctctgcttctctctcctcctcttcccccctgcccttgggtc
3′primer-3:
gagaattcctacctccggggaacagcatcgccgcacaggtggcagtctgtgggtctctgcttct

Insect ovary cells (HighFive, Invitrogen) were maintained in a serum free medium (ExpressFive, Invitrogen), seeded in collagen type I-coated culture bottles (BioCoat, Becton Dickinson, CA, USA), and incubated at 28°C in a humidified room air.[20] The vector was expressed in the cells using a transfection reagent (FuGENE HD, Invitrogen), and the supernatant was harvested. Small particles were removed from the supernatant using 0.45 mm filters (MILLEX-HA, Millipore, MA, USA), and the supernatant was concentrated to ×30 using Amicon Ultra-15 filters (cut off 10 kD, Ultracel-10k, Millipore).

HEPO was purified using a Heparin-Sepharose gel pre-packed column (HiTrap Heparin, GE Amersham, NJ, USA). Briefly, the column was filled with 1M Tris buffer (pH 7.5), and the concentrated supernatant was then infused. The column was washed with a sufficient volume of 1M Tris buffer (pH 7.5), and HEPO solution was collected using 1M Tris buffer (pH 7.5) supplemented with 1M NaCl and 0.3% BSA. The HEPO solution was concentrated using Ultracel-10k, the EPO concentration was measured using an hEPO ELISA kit (R&D Systems, MN, USA), and 10 mg/mL HEPO was prepared. Vector not installed with HEPO cDNA was also expressed in insect cells, the supernatant was concentrated, collected through a heparin column, and further concentrated in the same manner as the HEPO solution, and an equivalent amount of Mock solution was utilized as the control for the HEPO solution.

Confirmation of heparin-affinity and *in vitro* bioactivity

rhEPO or rhHEPO (both 5 ng) was infused into a heparin–agarose gel pre-packed column (Sigma, MO, USA), and serially eluted with 5 mL of 0, 0.5, 5, and 50 U/mL of heparin (Sigma) in PBS, and the EPO concentration of each fraction was measured by ELISA.

To simultaneously determine the heparin-affinity and bioactivity, 0.01 mL of heparin–agarose gel beads were washed with PBS, incubated overnight in 0.5 mL of PBS with 40 ng EPO derivatives (rhEPO, rhAEPO, rhCEPO, rhHEPO, and an equivalent amount of Mock), washed again with PBS, added to 1×10^5/ml of AS-E2 cells in IMDM (Invitrogen) supplemented with 20% FBS (Invitrogen), and incubated for 5 days.

To compare the erythropoietic activity of EPO and HEPO, 2×10^4/mL of AS-E2 cells were cultured in IMDM with 20% FBS in the presence of 0.003 to 10 ng/mL of rhEPO or rhHEPO for 4 days, and the cell growth was analyzed.

In vivo hematopoietic activity

All procedures using animal models in the present study were performed under sterile conditions with the approval of the Institutional Animal Care and Use Committee in compliance with procedures and methods outlined by the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23; National Institutes of Health, Bethesda, MD). Male eight-week old male ICR mice (30–35 g, Charles River, Yokohama, Japan) were intramuscularly injected with 2 mg/kg rhEPO or rhHEPO on days 0, 2, and 4, and the blood count were analyzed on days 7 and 14.

In vivo effects on spontaneous angiogenesis

White mice such as ICR and black mice such as C57 are generally weak and strong in response to ischemic stress, respectively. We chose C57/BL mice as the lower limb ischemia model to observe spontaneous angiogenesis. Eight-week old male C57/BL mice (20–25 g, Charles River) were anesthetized, and the proximal regions of the left femoral and saphenous arteries were ligated as reported previously.[21] Two mg/kg rhEPO or rhHEPO were intramuscularly injected into the ischemic left limbs two times (days 0 and 3) or 3 (days 0, 2, and 4). Limb blood flow was measured on day 7 using a moorLDI laser Doppler system (Moor Instruments, DE, USA), expressed as a flux ratio (the value in the ischemic left legs/value in the intact right legs) as reported previously.[21] Limb blood flow was also measured in leg-ischemic mice (no EPO injection) 24 h after ligation as a landmark of acute leg ischemia.

Statistics

The mean and standard deviation (S.D.) levels were calculated and used to express the results. The values were compared among groups by one-way ANOVA followed by the Fisher multiple comparison test.

Results

Heparin-affinity and *in vitro* activity

rhHEPO, but not rhEPO, expressed affinity to heparin–agarose gel, and at least 50 U/mL heparin was required to release rhHEPO from the gel (Figure 2). As shown in

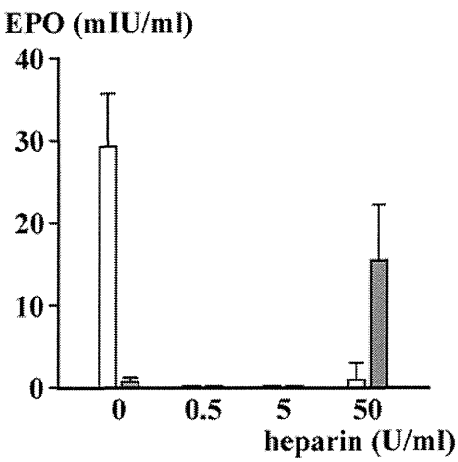

Figure 2. Heparin affinity of EPO's rhEPO and rhHEPO were put into a heparin–agarose gel column, serially eluted with 0 to 50 U/mL of heparin, and then the EPO concentration was measured in each fraction by ELISA. White bars, rhEPO; gray bars, rhHEPO ($n = 4$).

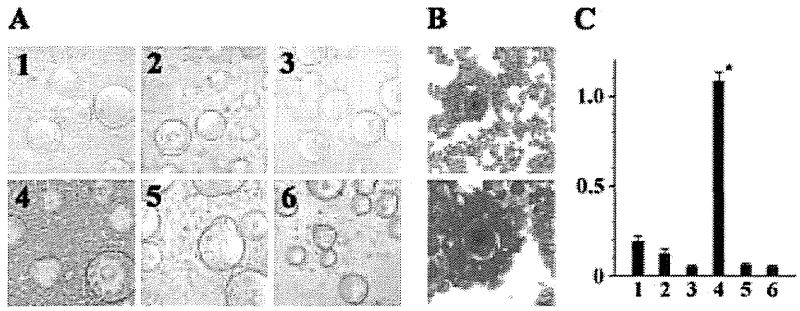

Figure 3. Affinity of rhHEPO to heparin–agarose gel. Heparin–agarose gel was incubated overnight with several EPO derivatives (1, rhEPO; 2, rhAEPO; 3, rhCEPO; 4, rhHEPO; 5, Mock; and 6, PBS), washed, added to the EPO-dependent cell line, AS-E2 (1 × 105/mL), and cultured for 5 days. Panel A: Phase-contrast microscopic pictures of gel (large particles) absorbed with the different types of EPO and the cells (small particles). Panel B: May-Giemsa stain of AS-E2 cells incubated with HEPO-adsorbed gel (upper: ×100, lower: ×400). Note that the gel-contacted cells grew and expanded. Panel C: Cell number (× 106/ml) of panel A ($n = 3$).

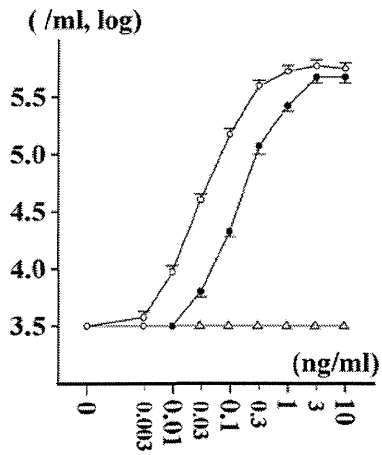

Figure 4. Growth curve of AS-E2 cells AS-E2 cells (2 × 104/ml) were incubated for 4 days in the presence of rhEPO (open circles), rhHEPO (closed circles), or Mock (open triangles). The submaximal concentration of both EPO and HEPO was 3 ng/mL. Note that HEPO in the culture was lost presumably via adsorption on plastic surfaces especially in the lower concentrations ($n = 3$).

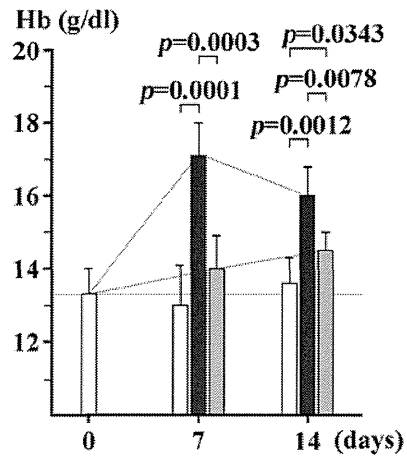

Figure 5. *In vivo* erythropoietic activity in mice. ICR mice were intramuscularly injected with 2 mg/kg of rhEPO (black bars), rhHEPO (grey bars), or the equivalent dose of Mock (white bars) on days 0, 2, 4, and the blood hemoglobin concentration was measured on days 7 and 14 ($n = 6$ for each group). Hemoglobin peaked by the administration of rhEPO on day 7, while it increased even on day 14 by the administration of rhHEPO ($n = 6$ for each group).

Figure 3, only rhHEPO was absorbed into the gel, and AS-E2 cells attached to the gel and multiplied. The activity of rhHEPO was almost the same as rhEPO and the submaximal concentration of the EPOs was 3 ng/mL (Figure 4). While undertaking the production and purification procedures, we found that the concentration of rhHEPO in the solution decreased to about 50% over the period of 1 month presumably via absorption to plastic surfaces. The lower bioactivity of HEPO at a low concentration might be caused by this plastic-absorption.

In vivo activity

The peak Hb value was observed on day 7 by rhEPO administration, while the peak was not observed even on day 14 by administration of rhHEPO (Figure 5). The spontaneous restoration of blood flow in the lower ischemia model of mice was inhibited by the administration of rhHEPO but not by rhEPO or Mock (Figure 6).

Discussion

In the present study, we designed chimeric recombinant EPO that binds specifically to tissue ECM and endothelium surfaces via strong HBM of PLGF-2. It expressed strong heparin-binding affinity and preserved EPO bioactivity *in vitro*. Chimeric EPO also revealed an inhibitory effect on spontaneous angiogenesis *in vivo* presumably through the competitive function of PLGF-2 HBM. Although *in vivo* effects of the present characteristic derivative of EPO on tumor growth were not determined, antiangiogenic activity observed in the present study encourages us in the utility of this derivative in patients with cancer-related anemia. Moreover, tissue ECM and endothelium may store HEPO to help the long-acting nature of the drug.

So far, several EPO derivatives have been synthesized including mimetic peptides.[22] One of the nonhematopoietic EPO's, AEPO, expresses erythropoietic activity *in vitro*, but not *in vivo* due to the rapid clearance via the galactose

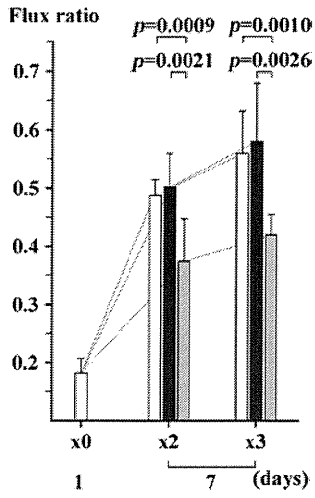

Figure 6. Angiogenic effects of EPO in the mouse limb ischemia model. Lower limb ischemia C57/BL mice were intramuscularly injected with 2 mg/kg of rhEPO (black bars), rhHEPO (grey bars), or the equivalent dose of Mock (white bars) two times (days 0 and 3) or three (days 0, 2, and 4) into the ischemic limbs, and blood flow was measured using the laser Doppler system (flux ratio: blood flow measured in the ischemic limb/and that in the normal limbs). Untreated mice showed cyanosis in the ischemic legs on day 1. The blood flow in Mock and EPO injected mice recovered spontaneously on day 7, while HEPO injection inhibited the recovery of blood flow. (n = 6 to 9 for each group).

binding protein of hepatic cells.[23] CEPO, another type of nonhematopoietic EPO, binds to the EPOR/βc heteroreceptors expressed in the brain, kidney, and liver, but not to the EPOR homoreceptor expressed in erythroid cells, thus CEPO is another nonhematopoietic derivative of EPO.[5] Darbepoietin (NESP) is one of the long-acting EPO's having a long half-life in blood caused by an excess of *N*-Glycans.[24] Methoxy-polyethylene glycol polymer-conjugated EPO (CERA) is another long-acting derivative.[25] We have developed a long-acting derivative, HEPO, by utilizing the affinity of arginine/lysine-repeat to GAG.

The HBM of hVEGF-A165 consists of 43 amino acid residues that include two RR-sequences. Endostatin, an arginine/lysine-rich polypeptide fragment of collagen type-18, competitively inhibits the angiogenic effect of VEGF.[26] As shown in Figure 1, the HBM of hPLGF-2 is intensively abundant in arginine/lysine-repeat that might result in a much stronger affinity to GAG, and might act, so to speak, as a superendostatin that inhibits not only VEGF but also PLGF-2, FGF-2, and other angiogenic growth factors.

EPO shows tissue-protective effects as well as angiogenic effects by itself,[21] and may enhance tumor angiogenesis.[27] The American Society of Hematology and the American Society of Clinical Oncology have proposed a clinical practice guideline on the use of EPO derivatives in patients with cancer.[28] HEPO may have an advantage in inhibiting tumor growth while preserving hematopoietic and tissue-protective effects.

EPO administration improves cardiac damage in animal models of myocardial infarction/reperfusion.[29,30] EPO also protects the myocardium in a rat myocarditis model.[31] We then undertook clinical trials of EPO administration for patients with acute myocardial infarction (AMI) to observe its safety and efficacy: the EPO/AMI-1 study,[32] and under the results of this trial, we began a three-arm, double-blind clinical trial of EPO administration for 600 patients with AMI: the EPO/AMI-2 study.[33] The safety and efficacy of EPO administration for patients with AMI is still controversial.[34,35] The limitation of EPO is possible tumor-angiogenic activity in cancer patients and its thrombopoietin (TPO)-like activity.[36] Bolus administration of EPO may cause cerebrovascular and cardiovascular events.[35] Moreover, there might be an optimal dose of EPO administration to treat coronary ischemia/reperfusion injury.[37] AEPO is one of the candidates to avoid platelet activation because the serum level of AEPO does not elevate while preserving their angiogenic (PCT Patent 2006/129755) and cardioprotective effects but not erythropoietic effects.[38] HEPO is another candidate. HEPO reveals tissue affinity, and thus probably does not to show a rapid rise in blood.

In conclusion, the novel concept to give tissue affinity to a substance by a protein engineering method in the present study might supply a useful drug delivery system.

Limitations

Dose-response and phase of action of HEPO were insufficiently studied in animal models. The peak of the hemoglobin level after HEPO administration was not observed within 2 weeks unexpectedly. The antiangiogenic effect of HEPO was not confirmed by histology. Although this study was insufficient, we went ahead to the next step to develop other derivatives of cytokines.

Declaration of interest

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