Table 4 1-Year Clinical Outcomes in the ITT Population

	CoCr-EES (n = 762)	PtCr-EES (n = 768)	p Value
All-cause death, MI, TVR	36/732 (4.9)	37/745 (5.0)	0.97
All-cause death or MI	22/732 (3.0)	18/745 (2.4)	0.49
All death	9/732 (1.2)	10/745 (1.3)	0.85
Cardiac death	5/732 (0.7)	7/745 (0.9)	0.58
Related to the TV	3/732 (0.4)	6/745 (0.8)	0.51
Not related to the TV	2/732 (0.3)	1/745 (0.1)	0.62
Noncardiac death	4/732 (0.5)	3/745 (0.4)	0.72
MI	13/732 (1.8)	8/745 (1.1)	0.25
Related to the TV	12/732 (1.6)	6/745 (0.8)	0.14
Not related to the TV	1/732 (0.1)	2/745 (0.3)	1.00
Q-wave MI	5/732 (0.7)	1/745 (0.1)	0.12
Non–Q-wave MI	9/732 (1.2)	7/745 (0.9)	0.59
TVR, overall	21/732 (2.9)	20/745 (2.7)	0.83
TLR, overall	14/732 (1.9)	14/745 (1.9)	0.96
TLR, PCI	12/732 (1.6)	10/745 (1.3)	0.64
TLR, CABG	2/732 (0.3)	4/745 (0.5)	0.67
Non-TLR TVR, overall	8/732 (1.1)	7/745 (0.9)	0.77
Cardiac death or MI	18/732 (2.5)	15/745 (2.0)	0.56
Target lesion failure	23/727 (3.2)	26/742 (3.5)	0.72
Target vessel failure	29/727 (4.0)	31/742 (4.2)	0.85
Stent thrombosis (ARC definite or probable)	3/725 (0.4)	3/735 (0.4)	1.00
Definite	3/725 (0.4)	3/735 (0.4)	1.00
Probable	0/725 (0.0)	0/735 (0.0)	—

Values are n/N (%).

ARC = Academic Research Consortium; CABG = coronary artery bypass graft; ITT = intention-to-treat; MI = myocardial infarction; PCI = percutaneous coronary intervention; TLR = target lesion revascularization; TV = target vessel; TVR = target vessel revascularization; other abbreviations as in Table 1.

coronary anatomy is required to reach a consensus regarding stent deliverability and other ease-of-use characteristics.

Study limitations. The 1-year TLF rate with the control CoCr-EES (2.9% in the per-protocol population, and 3.2% in the ITT population) was less than the 5.5% rate assumed during sample size estimation, which was based on prior data from the SPIRIT II and III trials. In the larger SPIRIT IV trial, in which slightly more complex lesions were enrolled than in either of the earlier SPIRIT trials (or the present study), the 1-year TLF rate was only 4.2%, lower than had previously been reported. As such, small absolute differences in event rates between the PtCr-EES and CoCr-EES cannot be excluded by the present study. Nonetheless, the observed 2-sided 95% CI of the difference in the rate of 12-month TLF (−1.3% to 2.3%) ensures that a large absolute difference in TLF between the 2 stent types is unlikely in the lesions tested. Longer-term follow-up and in more complex lesions is required for a comprehensive evaluation between these 2 devices. In this regard, to meet regulatory requirements, the SPIRIT and PLATINUM trials excluded many high-risk patients, such as those with acute or recent MI or visible thrombus, chronic total occlusions, true bifurcations, and lesions in the left main coronary artery or a saphenous vein graft. In contrast, in a large-scale randomized trial in which these patients were actively enrolled, the 1-year rate of TLF with the CoCr-EES was greater (8.2%) than observed in the present study (13). In the future, adoption of the so-called "all-comers" design for regulatory approval stent trials would permit low-frequency but clinically relevant differences between devices to become statistically apparent (or more reliably be excluded), while maintaining reasonable sample size.

Conclusions

In summary, a novel PtCr-EES has been developed and shown to have noninferior 1-year clinical outcomes compared with the predicate CoCr-EES in patients undergoing PCI of up to 2 noncomplex de novo native coronary artery lesions.

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Key Words: angioplasty ▪ coronary artery disease ▪ restenosis.

APPENDIX

For complete list of the study organization and participating sites and investigators, please see the online version of this article.

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ORIGINAL ARTICLE

Long-term prognostic stratification by a combination of ^{123}I-metaiodobenzylguanidine scintigraphy and ejection fraction in dilated cardiomyopathy

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Abstract
Objective ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy is a useful tool for predicting the prognosis in patients with congestive heart failure; however, little is known regarding long-term prognostic evaluations. The aim of this study was to evaluate long-term prognosis in a roughly 10-year period, in dilated cardiomyopathy (DCM) by MIBG imaging, compared to other conventional functional parameters.
Methods Eighty-six DCM patients (50 ± 14 years of age, 57 males) underwent MIBG imaging, at 15 min and 4 h after tracer injection, from which the delayed heart to mediastinum ratio (H/M) and washout rate (WR) were obtained. The left ventricular ejection fraction (EF) and end-diastolic diameter (LVDd) were also measured by echocardiogram. All patients were followed up for 8–14 years, and the death event was investigated.
Results Kaplan–Meier curves revealed a poor prognosis only in the group above the third quartile of WR (=50%) (10-year prognosis, 35%); however, there were no statistically significant differences in prognosis among the other 3 groups (10-year prognosis, 75–84%). A Cox hazard univariate analysis selected WR ($p = 0.0004$), H/M ($p < 0.0001$), EF ($p = 0.0024$), and LVDd ($p = 0.0189$) as significant prognostic indicators. Multivariate analysis revealed the H/M ($p = 0.0023$) and EF ($p = 0.024$) to be an independent prognostic predictor. The 10-year prognosis of patients with both WR < 50% and EF > 30%; WR < 50% and EF < 30%; and both WR > 50% and EF < 30% were 89, 71, and 33%, respectively. These three groups were well stratified, significantly (log-rank test: $\chi^2 = 30.0$, $p < 0.0001$). However, even patients with WR ≥ 50% had few death events after 3 years following MIBG imaging.
Conclusions The MIBG parameter, delayed H/M or WR combined with the EF is a useful tool for the prediction of a long-term prognosis in DCM, which is superior to MIBG parameters alone. However, patients with WR > 50% but no event in a 3-year follow-up period should undergo an additional MIBG imaging for prognostic prediction.

Keywords ^{123}I-metaiodobenzylguanidine (MIBG) · Dilated cardiomyopathy · Washout rate

Introduction

^{123}I-metaiodobenzylguanidine (MIBG) is an analog of guanethidine, and is taken up by uptake-1 as norepinephrine, followed by storage in adrenalin-related sympathetic nerve endings. Cardiac MIBG accumulation and washout reflect kinetics similar to norepinephrine [1]. Many previous reports have suggested that MIBG washout was increased, and myocardial delayed uptake was reduced in patients with heart failure, the severity of which was closely related to cardiac events or prognosis [2–4]. A multicenter study co-registered from Europe and the US has also confirmed the usefulness of MIBG for the prediction of prognosis in heart failure [5]. However, most prognostic studies using MIBG have been undertaken based on data with a follow up of less than 5 years, and these studies

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defined only cardiac events as end points during the follow-up periods. We analyzed the data of all causes of mortality including cardiac events for a 10-year follow up after MIBG scintigraphy in patients with dilated cardiomyopathy (DCM), and assessed how MIBG contributed to the long-term prognostic evaluation in DCM patients, compared with other conventional parameters such as left ventricular ejection fraction (LVEF) and end-diastolic diameter.

Methods

Study population

Among 111 consecutive DCM patients who had undergone MIBG scintigraphy from 1993 to 1997, those patients who had death events or could be followed up for more than 8 years (n = 91) without death events were registered in this study. DCM was diagnosed by clinical history, ECG, chest roentgenogram, echocardiogram, or heart catheterization based on the Handbook for Diagnosis of the Japanese Ministry of Health and Welfare. Patients with severe chronic renal failure (≥CKD stage IV, $n = 4$) and autonomic nervous system disorders ($n = 1$) were excluded. Finally, a total of 86 patients (67 ± 12 years old, 34 males) were enrolled in this study.

All patients were under hemodynamically compensated conditions at the time of MIBG imaging. Informed consent was obtained from each patient. The study protocol was approved by the institutional committee on human clinical investigations.

^{123}I-MIBG scintigraphy

After oral administration of 50 mg potassium iodide for thyroid block, planar scintigraphic imaging in the anterior view was obtained at 15 min (early) and 4 h (delayed) after the intravenous injection of 111 MBq of MIBG. Images were acquired using a single head gamma camera (DS7, Sophy Medical) equipped with a low-energy, high-resolution collimator. A preset time of 5 min was used for image acquisition, with a 159 ± 10 keV energy window.

For the quantitative analysis of MIBG, the delayed heart to mediastinum ratio (H/M) and washout rate (WR) for 4 h were calculated as described in a previous study [2].

Echocardiography

Echocardiography was performed at about the same time as MIBG scintigraphy. From the left ventricular short-axis image, the left ventricular end-diastolic diameter (LVDd) and end-systolic diameter were measured and the LVEF was calculated using the standard method.

Patient follow up

The patients were followed up after the MIBG studies. No patient underwent heart transplantation. The end point was defined as death, from all causes. Sudden cardiac death was defined as death without definite premonitory symptoms or signs. Mortality data were gathered from the patient records in our hospital, telephone interviews, or correspondence by letter.

Statistics

The following variables were analyzed: age, gender, delayed H/M, WR, LVDd, and LVEF. Statistical values are shown as mean ± SD. Prognostic values were determined using a statistical software package (StatView, ver. 4.0). Univariate and multivariate Cox proportional hazards regression models were used to analyze the relations between all causes of death and the MIBG indices. Survival curves for patient subgroups were created by the Kaplan–Meier method to determine the time-dependent cumulative survival rate. These curves were compared using a 2-sample log-rank test. The mean values for the two groups were compared using an unpaired Student t test. A p value of less than 0.05 was considered statistically significant.

Results

The follow-up periods ranged from 0.12 to 14.4 years (average: 9.16 ± 4.21 years). Death events occurred in 26 patients (30%) due to 7 cardiac deaths (six heart failures and one fatal arrhythmia), 2 sudden deaths, 3 cerebrovascular diseases, 2 infectious diseases, one malignancy, one suicide, and one fatal complication of cardiac catheterization. The other 9 patients died of unknown causes.

All patients were divided into a Death group ($n = 26$) and an Alive group ($n = 60$), and the two groups were compared (Table 1). Older patients were included in the Death group ($p = 0.0019$). NYHA class was significantly higher in the Death group ($p = 0.011$), and the frequency of higher functional class was assigned in the Death group ($p = 0.0033$). The WR in the Death group was significantly higher than that in the Alive group ($p = 0.0012$). In addition, the H/M in the Death group was significantly lower than that in the Alive group ($p = 0.0001$). The LVEF and LVDd were lower and higher in the Death group, respectively ($p = 0.0017$, 0.0241, respectively).

Table 1 Clinical characteristics of the Death and Alive groups

	Death	Alive	*p* value
No. of patients	26	60	
Age	56 ± 12	47 ± 15	0.0091
Male/female	22/4	44/16	NS
NYHA	2.15 ± 0.83	1.77 ± 0.53	0.011
I/II/III/IV	6/11/8/1	17/40/3/0	0.0033
Hypertension	2 (8%)	14 (23%)	NS
Diabetes	4 (15%)	8 (13%)	NS
Ventricular tachycardia	16 (62%)	24 (40%)	NS
Atrial fibrillation/flatter	4 (15%)	16 (27%)	NS
Medical treatments			
At the time of MIBG			
Digoxin	17 (65%)	32 (53%)	NS
Diuretics	24 (92%)	36 (60%)	0.0012
ACE	11 (42%)	27 (45%)	NS
β-blocker	6 (23%)	18 (30%)	NS
After MIBG			
ACE or ARB	21 (84%)	50 (86%)	NS
β-blocker	13 (50%)	41 (68%)	NS
WR (%)	43 ± 17	29 ± 18	0.0012
H/M	1.54 ± 0.21	1.79 ± 0.29	0.0001
LVEF (%)	24 ± 11	32 ± 11	0.0017
LVDd (mm)	68 ± 9	63 ± 9	0.0241

The frequency of diuretics in the Death group was higher than that in the Alive group ($p = 0.0012$). There were no significant differences in the frequency of either β-blocker or ACE inhibitor/angiotensin II receptor blocker (ARB) for medical therapy, either before or after the MIBG studies between the Death and Alive groups (Table 1).

With the end point defined as all causes of death, the survival curves are shown in Fig. 1. The threshold value of each parameter was determined as an optimal cutoff point statistically obtained by log-rank test between the two classified groups. The cutoff point was 30%, 50% and 1.45 for ejection fraction (EF), MIBG WR, and H/M, respectively. These curves show that those patients with either an EF less than 30%, a WR of more than 50% and a H/M less than 1.45 had a poor prognosis($p = 0.0014$, $p < 0.0001$, $p = 0.0001$, respectively). All patients were quartered via LVEF and WR values to obtain the mortality in each classified group. The 10-year mortalities in each classified group for LVEF and WR are shown in Fig. 2. The second quartile (30% in LVEF) and the third quartile (50% in WR) are appropriate cutoff values for death events.

The prognostic value of MIBG and echocardiographic parameters with the Cox hazard univariate regression model for all causes of death is shown in Table 2. The four variables shown were all significant prognostic parameters. Among those variables, Cox hazard multivariate regression

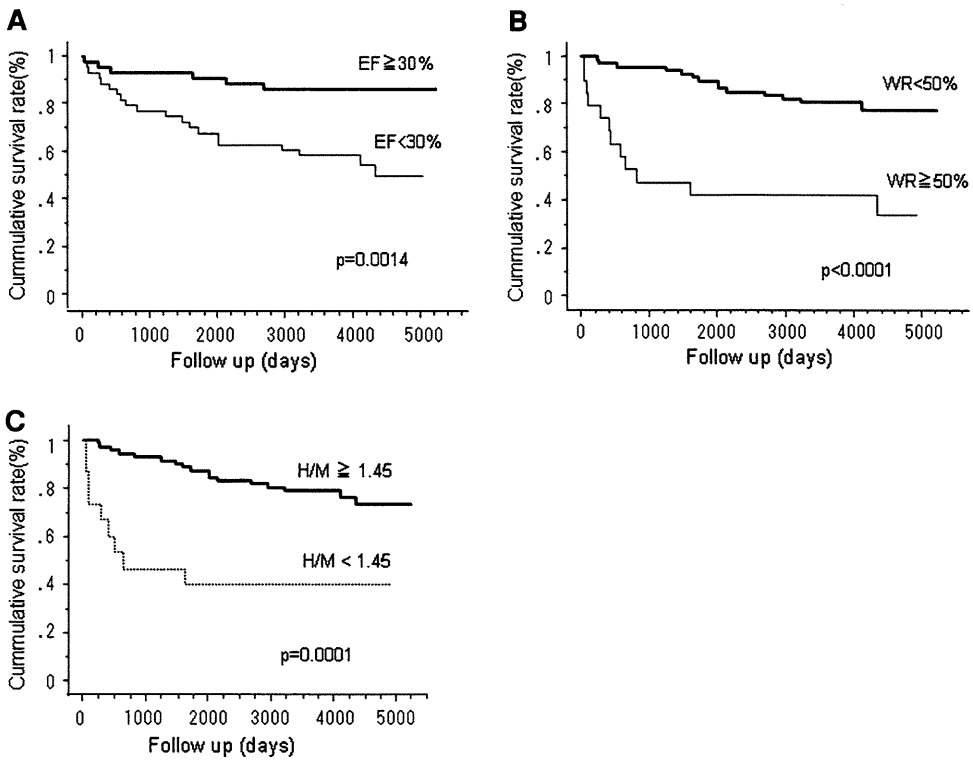

Fig. 1 Kaplan–Meier curves stratified by EF (**a**), WR (**b**) and H/M (**c**) with the end point defined as all causes of death. These curves show that patients with an EF less than 30%, a WR of more than 50% or an H/M less than 1.45 had poor prognoses

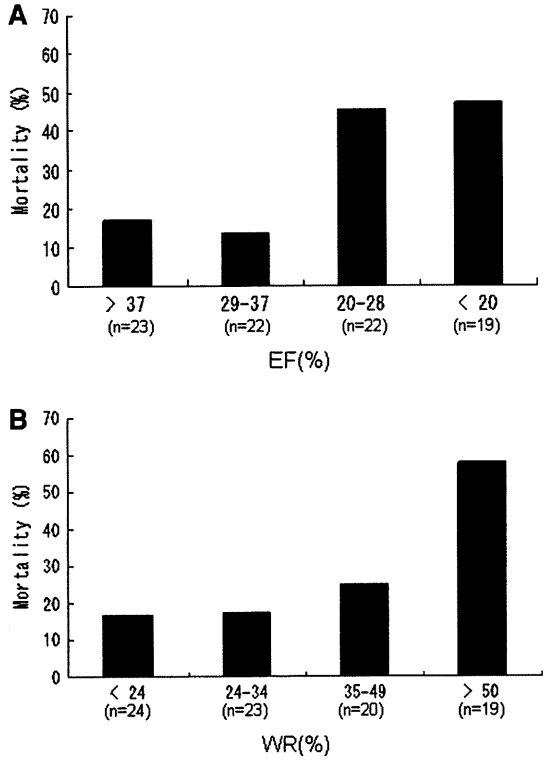

Fig. 2 The 10-year mortality in four groups, classified via LVEF (**a**) and WR (**b**). The second quartile (30%) in LVEF and the third quartile (50%) in WR were appropriate cutoff values for cardiac death

Table 2 Cox hazard univariate (a) and multivariate (b) regression analysis

Variables	χ^2	Hazard ratio (95% CI)	p value
(a)			
NYHA	7.98	2.529 (1.3283–4.814)	0.0047
LVDd	8.24	1.063 (1.020–1.109)	0.0041
LVEF	9.23	0.940 (0.904–0.978)	0.0024
WR	12.94	1.041 (1.018–1.064)	0.0003
H/M	16.43	0.044 (0.010–0.199)	<0.0001
(b)			
LVEF	4.28	0.960 (0.923–0.998)	0.039
H/M	11.18	0.059 (0.011–0.309)	0.0008

analysis revealed that the H/M and EF were independent prognostic parameters for all causes of death ($\chi^2 = 18.43$, $p < 0.0001$).

Kaplan–Meier curves stratified by a combination of WR and EF values are shown in Fig. 3: WR < 50% and EF ≥ 30% (group A, $n = 36$); WR < 50% and EF < 30% (group B, $n = 35$); and WR ≥ 50% and EF < 30% (group C, $n = 15$). No patient was categorized into a group with WR ≥ 50% and EF ≥ 30%. The 10-year survival rates for patients in groups A, B, and C were 89, 71, and 33%, respectively. Patients in group A had a good prognosis. The

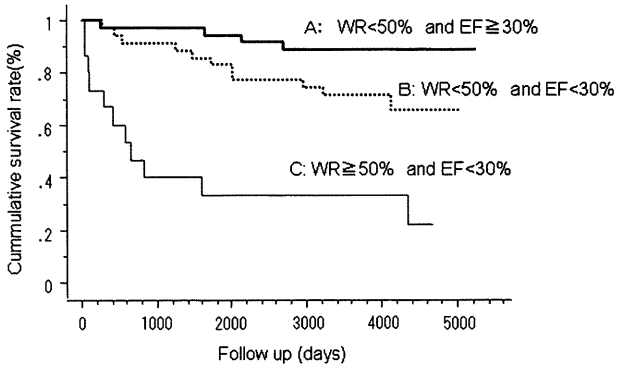

Fig. 3 Kaplan–Meier curves stratified by a combination of WR and EF values: WR < 50% and EF ≥ 30% (group A, $n = 36$); WR < 50% and EF < 30% (group B, $n = 35$); WR ≥ 50% and EF < 30% (group C, $n = 15$). These three curves are well differentiated ($\chi^2 = 30.0$, $p < 0.0001$)

three curves are well stratified significantly ($\chi^2 = 30.0$, $p < 0.0001$). However, even patients in group C had few death events after 3 years following MIBG imaging.

After excluding patients with unknown causes of death ($n = 9$), we evaluated the prognostic significance for cardiac death including sudden death ($n = 9$). Multivariate analysis revealed that only WR was an independent prognostic indicator ($\chi^2 = 9.00$, $p = 0.0027$). Kaplan–Meier curves divided by a threshold value of 50% in WR and 1.45 in H/M were significantly different ($\chi^2 = 17.30$, $p < 0.0001$ and $\chi^2 = 11.3$, $p = 0.0008$, respectively). EF was not a prognostic indicator for cardiac death in either the Cox hazard regression analysis or Kaplan–Meier analysis (Fig. 4).

Discussion

The present study indicated that MIBG scintigraphy is a useful prognostic tool over a 10-year follow-up period in patients with DCM. Another conventional functional parameter, the EF, was also an independent prognostic indicator, which had a less significant prognostic value than the H/M in MIBG. However, the combination of two prognostic parameters was found to be an independent prognostic indicator, as determined by multivariate analysis. In particular, one of the parameters of MIBG, the WR, when combined with the EF was a strong prognostic marker (Fig. 3), and is very useful in the stratification of disease severity. Patients with WR ≥ 50% and EF < 30% had particularly poor prognoses.

Kaplan–Meier curves show that frequent death events occurred during the first 3 years in patients with higher WR values (≥50%). In contrast, these events occurred gradually over the 10-year study period in patients with low EF values. This result indicated that MIBG data are especially

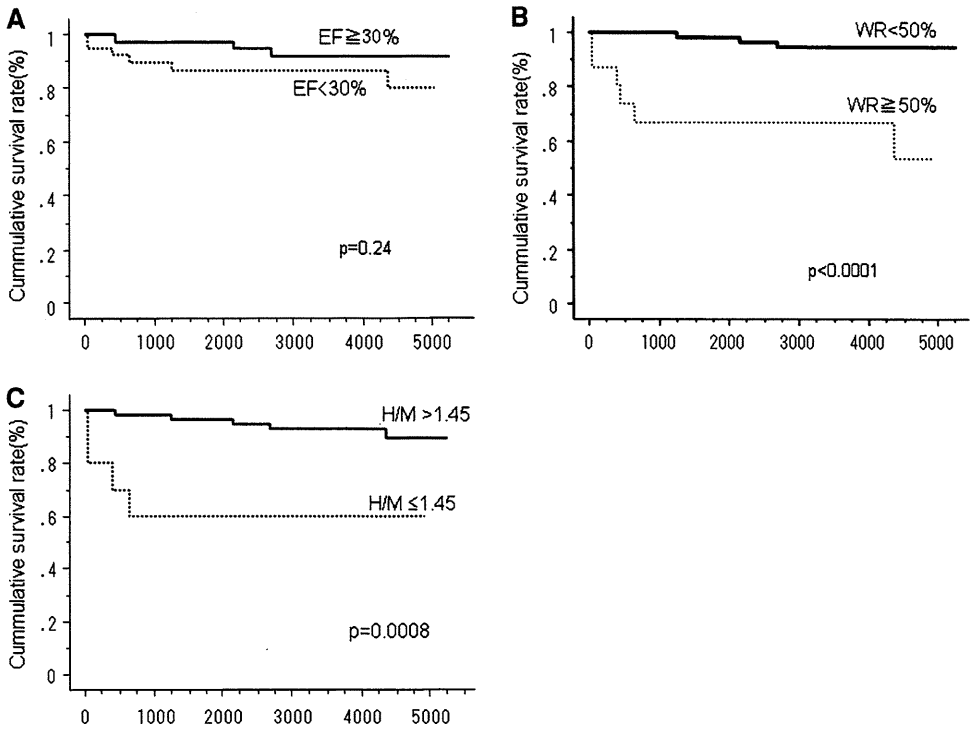

Fig. 4 Kaplan–Meier curves stratified by the EF (**a**), WR (**b**) and H/M (**c**) with the end point defined as cardiac death. The curves show that the patients with a WR of more than 50% and H/M less than 1.45 had poor prognoses. However, the EF does not exhibit prognostic significance, according to the Kaplan–Meier analysis

useful in predicting short-term death events within several years, while the EF reflects the long-term prognosis, but is less useful in predicting short-term prognosis. Even patients with higher WR values (≥50%) had few death events after 3 years following MIBG imaging, and the reason for this is not clear. However, patients with effective initial medical therapy may survive even longer than patients with higher WR values. Akutsu et al. [6] reported a 10-year long-term prognostic evaluation in patients with ventricular tachycardia, where the first 3-year survival curve with lower H/M also showed frequent cardiac events, but very few events during the rest of the follow-up periods. The survival curve pattern was very similar to our study. A recent study revealed that patients with no cardiac event even after 2 years of follow up should undergo MIBG repetitively for prognostic prediction [7], which is a partially supportive result. In this respect, patients with WR > 50% who survive for 3 years should undergo MIBG, and should be re-evaluated regarding prognosis.

We also investigated the threshold value of the parameters for predicting prognosis. When WR was stratified into 4 classes, only the fourth most severe group (>50%) had a higher death event rate, whereas the other 3 groups were comparable. These data suggested that MIBG may primarily detect poor prognostic cases, the patients of which die within several years. Another important point is the good prognosis observed for patients with higher EF values (>30%), regardless of WR value. Many enrolled patients underwent MIBG imaging before complete medical therapy in this clinical setting, because 28 and 44% of the patients took β-blockers and ACE, respectively, before the imaging, while another 35 and 39% of the patients, respectively, took these medical treatments (total: 63 and 83%) after the imaging; therefore, proper medical treatment was certainly performed for these patients during the follow-up period after MIBG imaging. Under this condition, patients with relatively higher EF values (>30%) are less likely to have death events over the 10-year follow-up period.

In the current study, a composite end point including all causes of mortality was used, which is not a direct cardiac end point. An important advantage of all-cause mortality, however, is the fact that it is not affected by verification bias [8]. Furthermore, most deaths in adults are linked to cardiovascular disease. All causes of mortality is, therefore, a commonly used end point, which allows a comparison of the current results to previous investigations [9–12].

We also assessed the prognostic significance for only cardiac as the cause of death. As for all causes of death, WR was also a good prognostic indicator for cardiac death. However, EF was not a prognostic indicator for only cardiac death. The EF may not exhibit a direct influence on cardiac death. Actually, the Kaplan–Meier curve for the lower EF group descends gradually in a stepwise manner over the 10-year follow-up period (Fig. 1a). The lower EF group might tend to involve, rather the non-cardiac cause of death events.

Plasma BNP concentration is also a good prognostic parameter among heart failure patients [13, 14]. A combination of the H/M in MIBG and plasma BNP is reported

to yield greater prognostic information than that from MIBG alone within 16 months [15]. However, it is not clear whether BNP is useful for long-term prognosis in these patients. We did not obtain BNP data in our present study, because no BNP sampling was routinely performed at the beginning of the study in the period from 1993 to 1997. Further investigation is needed to clarify the usefulness of plasma BNP level, in combination with MIBG parameters for long-term prognosis in DCM patients.

A decreased MIBG WR is often observed in patients who have received an effect on cardiac function due to complete medical therapy, indicating a prolonged survival rate [16, 17]. However, we enrolled patients who underwent MIBG imaging under a compensate state of heart failure, but with both complete and non-complete medical therapies. Therefore, the events would depend on additional medical therapy received after MIBG imaging, although medical treatment was altered by patient cardiac status in a long-term follow-up period. We did not include heart failure or fatal arrhythmic events; thus, the final event may strongly depend on the MIBG parameters, regardless of medical therapy, according to the present study results.

Conclusion

The MIBG parameter, delayed H/M or WR combined with the EF is a useful tool for the prediction of a long-term prognosis in DCM, which is superior to MIBG parameters alone. However patients with WR > 50% but no event in a 3-year follow-up period should undergo an additional MIBG imaging to predict the prognosis.

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ORIGINAL ARTICLE

Efficacy of paclitaxel-eluting stent implantation in hemodialysis patients

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Abstract Hemodialysis patients were recognized as a high-risk group for restenosis after percutaneous coronary intervention in the era of the bare-metal stent. Recently, sirolimus-eluting stents (SES) have reduced restenosis and target lesion revascularization (TLR); however, it has been reported that their efficacy in hemodialysis patients is limited. The purpose of this study was to investigate whether paclitaxel-eluting stents (PES) improved angiographic outcomes of hemodialysis patients compared with SES. This study is a retrospective cohort study. We analyzed 54 hemodialysis patients with 87 lesions implanted with PES from February 2007 to September 2008, and 49 hemodialysis patients with 68 lesions implanted with SES from August 2004 to January 2007. Angiographic follow-up after 8–10 months was obtained for 59 lesions (67.8%) in the PES group and 43 lesions (63.2%) in the SES group. At baseline, the PES patients had more peripheral artery disease compared with the SES group (66.7 vs. 34.7%; $p = 0.0012$). There were no significant differences in the angiographic characteristics or procedural index. The binary restenosis rate was lower in lesions implanted with PES than in those with SES (13.6 vs. 39.5%; $p = 0.034$). Accordingly, the TLR rate was lower in lesions implanted with PES than with SES (9.3 vs. 26.5%; $p = 0.041$). Our results suggest that PES is more effective than SES in reducing restenosis and TLR in hemodialysis patients.

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Introduction

Drug-eluting stents (DES) have been hailed as an effective means to prevent restenosis after percutaneous coronary intervention (PCI). A significant reduction in major adverse cardiac events (MACE) and restenosis with DES were found in a meta-analysis [1, 2]. Renal insufficiency is an independent predictor of mortality after PCI [3, 4]. DES implantation for de novo coronary lesions in patients with mildly impaired renal function reduced clinical events compared with bare metal stent (BMS) implantation [5], whereas DES showed no benefit over BMS in patients with moderate to severe renal insufficiency [5, 6]. In Japan, cardiac disease is the leading cause of death among hemodialysis patients, accounting for about 30% of all-cause deaths [7]. Among hemodialysis patients with coronary artery disease (CAD), the cardiac survival rate was significantly higher in a PCI group than in a medication group [8]. These results suggested that dialysis patients who have a higher risk of CAD should undergo more aggressive treatment with PCI than was previously considered [8]. However, the effectiveness of DES implantation for a subset of patients on hemodialysis therapy for end-stage renal disease has not been fully investigated because many clinical randomized trials excluded this subset of patients. Recently, sirolimus-eluting stents (SES) were found to markedly reduce restenosis and target lesion revascularization (TLR) compared with BMS[1, 2]; however, it has been reported that their efficacy in patients with hemodialysis was limited [9–12]. The purpose of this study

was to evaluate the clinical and angiographic efficacy of the polymer-based paclitaxel-eluting stents (PES) compared with SES in hemodialysis patients.

Methods

Patient population

Since May 2007, it has been the policy in our institution to use PES in hemodialysis patients for PCI, except for patients with intolerance to antiplatelet therapy, planned surgery within 3 months requiring withdrawal of antiplatelet therapy, or inappropriate vessel size for the available PES in Japan. The study population consisted of 103 consecutive hemodialysis patients with 155 lesions. A total of 54 hemodialysis patients with 87 lesions were treated with PES between May 2007 and December 2008 (PES group). As a control group, we selected 49 consecutive hemodialysis patients with 67 lesions who were treated with SES from August 2004 to April 2007 before the approval of PES (SES group).

PCI

Lesions were treated using standard interventional techniques. In Japan, PESs were available in diameters of 2.5–3.5 mm and lengths of 8–32 mm. Predilatation and postdilatation were allowed at the discretion of the operators. Intravenous boluses of heparin were administered to maintain an activated clotting time that exceeded 250 s during the procedure. Treatment with aspirin, 81–100 mg/day, was started before the procedure and continued permanently. Ticlopidine 200 mg/day or clopidogrel 75 mg/day was prescribed for a minimum of 6 months after the procedure. In cases of intolerance or allergy to ticlopidine or clopidogrel, cilostazol 200 mg/day was used as an alternative antiplatelet therapy.

Coronary lesion analysis

Lesions were classified according to the modified American Heart Association/American College of Cardiology (AHA/ACC) classification [13]. Assessments using quantitative coronary angiography (QCA) before the procedure, after the procedure, and during follow-up were performed using a computerized, automated, edge-detection algorithm (QAngioXA V7.1.40.0, Medis, Leiden, The Netherlands) [14], by experienced cardiologists blinded to the devices used and the clinical outcomes. A 5-mm vessel segment proximal and distal to the stenosis was used to calculate the average reference vessel diameter (RVD). Minimal lumen diameter (MLD) was measured within the stent (in-stent analysis) and within the segment, including 5 mm proximal and distal to the stent edge (in-segment analysis). Late lumen loss was defined as the difference between MLD after the procedure and MLD at 8–10 months' follow-up. The percentage of diameter stenosis was defined as [(MLD/reference vessel diameter) $\times$ 100]. Coronary artery calcification was assessed according to the presence of thick calcification on fluoroscopy or widespread superficial calcification on intravascular ultrasound.

Endpoint definitions and follow-up

The diagnosis of acute myocardial infarction (MI) included all patients with ST-elevation MI, which was defined as new ST-segment elevation on the electrocardiogram, and a creatinine kinase level more than twice normal. Stent thrombosis was analyzed retrospectively using the new definition of stent thrombosis generated by the Academic Research Consortium [15]. Consequently, stent thrombosis was classified as definite, probable, or possible. Deaths were classified as either cardiac or non-cardiac. Binary restenosis was considered as the occurrence of stenosis >50% of the diameter in the stented lesions. Late lumen loss was defined as MLD at follow-up minus post-procedural MLD measured by QCA. The angiographic patterns of restenosis were classified as focal, diffuse intra-stent, diffuse proliferative, and totally occluding [16]. Target lesion revascularization (TLR) was defined as any repeat PCI or surgical bypass of the original target lesion. The target lesion was considered to be the area covered by the stent plus 5-mm margins proximal and distal to the edges of the implanted stent. Composite MACE included all-cause death, MI, and TLR. All patients were asked to return for QCA between 8 and 10 months after the procedure or earlier if angina symptoms occurred. Clinical follow-up was obtained 12 months after the procedure. All patients provided written informed consent.

Statistical analysis

Continuous variables were presented as mean $\pm$ standard deviation and compared using Student's t test. Categorical variables were expressed as numbers and percentages and compared using the chi-square test or Fisher's exact test, as appropriate. Time-to-events estimates were evaluated using Kaplan-Meier methods and compared using the log-rank test. Univariate Cox regression analysis was performed to assess predictors of TLR and MACE after PCI. Included variables were patient-related factors listed in Table 1, and lesion and procedural characteristics listed in Table 2. The multivariate model included variables with $p \leq 0.20$ in univariate analysis. Multivariate analysis was performed using a Cox proportional hazards model with a stepwise

Table 1 Clinical characteristics

	PES ($n = 54$)	SES ($n = 49$)	p value
Age (years)	64 ± 10	65 ± 9	0.75
Male	41 (76%)	41 (84%)	0.47
Diabetes mellitus	41 (76%)	32 (65%)	0.24
Diabetes mellitus with insulin treatment	23 (43%)	14 (29%)	0.14
Hypertension	48 (89%)	43 (88%)	0.90
Hyperlipidemia	31 (57%)	22 (45%)	0.28
Current smoker	11 (20%)	19 (39%)	0.040
Family history of ischemic heart disease	6 (11%)	15 (31%)	0.027
Peripheral artery disease	36 (67%)	17 (35%)	<0.01
Duration of dialysis (years)	7.1 ± 5.2	8.7 ± 7.1	0.18
Unstable angina pectoris	21 (39%)	9 (18%)	0.38
MI	1 (2%)	5 (10%)	0.17
Previous cerebrovascular disease	9 (17%)	6 (12%)	0.72
Previous myocardial infarction	21 (39%)	19 (39%)	0.99
Previous PCI	24 (44%)	17 (35%)	0.31
Previous CABG	14 (26%)	12 (25%)	0.87
No. of diseased vessels	2.3 ± 0.8	2.2 ± 0.8	0.47
Left main coronary artery disease	20 (37%)	3 (6%)	<0.01
LVEF (%)	40 ± 12	46 ± 12	0.014
Low EF (<40%)	26 (48%)	16 (33%)	0.11

Values are number of patients (%) or mean ± SD

FH family history of ischemic heart disease, *PES* paclitaxel-eluting stents, *SES* sirolimus-eluting stents, *MI* myocardial infarction, *PCI* percutaneous coronary intervention, *CABG* coronary artery bypass grafting, *LVEF* left ventricular ejection fraction

Table 2 Angiographic and procedural characteristics

	PES ($n = 87$)	SES ($n = 68$)	p value
AHA/ACC types			
A	0	0	0.31
B1	7 (8%)	4 (6%)	
B2	57 (66%)	37 (54%)	
C	23 (26%)	27 (40%)	
Target vessel			
LAD	32 (37%)	26 (38%)	0.24
LCX	20 (23%)	12 (18%)	
RCA	25 (29%)	29 (43%)	
LMCA	6 (7%)	0	
SVG	0	0	
ITA	4 (5%)	1 (1%)	
Lesion characteristics			
Severe calcification	64 (74%)	46 (72%)	0.42
Ostial	19 (22%)	6 (9%)	0.049
Bifurcation	27 (31%)	19 (28%)	0.68
Chronic total occlusion	8 (9%)	1 (1%)	0.090
In-stent restenosis	7 (8%)	11 (16%)	0.19
Reference diameter (mm)	2.64 ± 0.77	2.86 ± 0.77	0.085
Minimum lumen diameter (mm)	0.88 ± 0.50	1.02 ± 0.47	0.066
Diameter stenosis (%)	68.0 ± 15.3	64.3 ± 13.3	0.11
Lesion length (mm)	23.9 ± 17.4	23.3 ± 14.5	0.80
Procedural characteristics			
No. of stents/lesion	1.4 ± 0.7	1.5 ± 0.8	0.56
Stent length (mm)	30.8 ± 20.0	34.5 ± 21.3	0.26
Stent diameter (mm)	3.1 ± 0.4	3.0 ± 0.4	0.15
Maximal dilatation pressure (atm)	15.4 ± 2.9	16.4 ± 3.5	0.047
Kissing balloon technique	21 (24%)	4 (6%)	<0.01
IVUS use	36 (41%)	22 (32%)	0.25
Rotational atherectomy	33 (38%)	20 (29%)	0.27

Values are number of lesions (%) or mean ± SD

ACC/AHA lesion class American College of Cardiology/American Heart Association lesion class, *PES* paclitaxel-eluting stents, *SES* sirolimus-eluting stents, *LAD* left anterior descending artery, *LCX* left circumflex artery, *RCA* right coronary artery, *LMCA* left main coronary artery, *SVG* saphenous vein graft, *ITA* internal thoracic artery

variable selection method. Hazard ratios were reported with 95% confidence intervals.

A p value <0.05 was considered significant. All statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL) software for Windows (Microsoft Corp, Redmond, WA).

Results

Baseline patient, angiographic, and procedural characteristics

Baseline clinical characteristics are listed in Table 1. Current smoker and family history of ischemic heart disease were more frequent in the SES group ($p = 0.04$ and $p = 0.027$, respectively). Peripheral artery disease and left main CAD were more complicated in the PES group ($p < 0.001$ and $p < 0.001$, respectively). In addition, the left ventricular ejection fraction (LVEF) was lower in the PES group than in the SES group ($p = 0.014$). The prevalence of diabetes was as high as 70% in both groups. Diabetes showed an increased mortality following PCI [17]. Peripheral artery disease is common in hemodialysis patients and is associated with increased risk of cardiovascular mortality, morbidity, and hospitalization [18]. It was reported that LVEF reduction was an independent predictor of mortality in chronic kidney disease patients with CAD [19], and LVEF in the PES group of this study was lower compared with a previous report [9]. Duration of hemodialysis therapy in this study was about 7–9 years

longer than in a previous report of about 5 years [10]. We believe that this study population, especially the PES group, was clinically in a more critical state than previous study populations.

The prevalence of complex lesions (AHA/ACC B2/C) and severe calcification on angiography were similar in both the PES and SES groups. Compared with the SES group, the TLR rate and binary restenosis in the PES group were significantly lower. It was reported that the TLR rate was 12.0% and in-segment restenosis identified on follow-up angiography was 16.5% in diabetes patients implanted with PES [20]. Although this study population, undergoing hemodialysis, was more critical, the TLR rate was 9% and binary restenosis rate was 14%.

Their angiographic and procedural characteristics are listed in Table 2. No significant differences were observed between the two groups, but the PES group had more ostial lesions than the SES group ($p = 0.049$). In addition, the maximal dilatation pressure was higher in the SES group than in the PES group ($p = 0.047$). It is thought that this resulted from a difference in the stent balloons' nominal dilatation pressure, being higher for the SES balloon. The kissing balloon technique was performed more frequently in the PES group than in the SES group. Procedural success was achieved in all treated patients. There was no difference in the rate of rotational atherectomy use before stent placement between the two groups.

QCA analysis

Angiographic follow-up was obtained in 37 patients (68.5%) with 59 lesions in the PES group and in 33 patients (67.3%) with 43 lesions in the SES group. Serial QCA data are shown in Table 3. No significant difference was observed between the two groups before and after the procedure. However, the diameter of stenosis at follow-up and the angiographic binary restenosis rate were significantly lower in the PES group than in the SES group ($p = 0.028$ and $p = 0.034$, respectively). A pattern of focal restenosis was found in 82% of restenotic lesions treated with SES.

Type IC focal in-stent restenosis [16] was observed in half of the SES group and in none of the PES group with a pattern of focal restenosis in this study. In contrast, focal and diffuse patterns of restenosis were equal in patients treated with PES. However, a significant difference was not found between the two groups regarding focal or diffuse restenosis ($p = 0.16$).

Clinical follow-up outcomes

Clinical follow-up was obtained in all patients (Table 4). The TLR rate was significantly lower in the PES group (9%) than in the SES group (27%) ($p = 0.041$). No significant differences were observed between the two groups in all-cause death, cardiac death, MI, and MACE. In

Table 3 Serial quantitative coronary analysis data

	PES ($n = 59$)	SES ($n = 43$)	p value
Lesion follow-up rate	68%	63%	0.55
Lesion length (mm)	24.9 ± 18.7	24.0 ± 11.7	0.77
Preprocedure reference diameter (mm)	2.68 ± 0.72	2.83 ± 0.64	0.28
Minimal lumen diameter (mm) pre	0.89 ± 0.52	0.98 ± 0.47	0.36
Minimal lumen diameter (mm) post	2.89 ± 0.57	2.74 ± 0.58	0.21
Minimal lumen diameter (mm) follow-up	2.15 ± 0.75	1.95 ± 0.93	0.22
Late lumen loss	0.73 ± 0.83	0.79 ± 0.99	0.72
Diameter stenosis (%) pre	68.4 ± 15.5	66.1 ± 14.5	0.45
Diameter stenosis (%) post	10.6 ± 7.7	12.7 ± 9.9	0.23
Diameter stenosis (%) follow-up	27.1 ± 19.5	37.3 ± 26.7	0.028
Angiographic binary restenosis	8 (14%)	17 (40%)	0.034
Patterns of restenosis focal	4 (50%)	14 (82%)	0.16
I A	0	0	
I B	3	3	
I C	0	7	
I D	1	4	
Diffuse	4 (50%)	3 (18%)	0.16
II intra-stent	1	3	
III proliferative	3	0	
IV total occlusion	0	0	

Values are number of lesions (%) or mean ± SD

PES paclitaxel-eluting stents, *SES* sirolimus-eluting stents

Table 4 Major adverse cardiac events at 12 months

	PES ($n = 54$)	SES ($n = 49$)	p value
All-cause death	9 (17%)	8 (16%)	0.83
Cardiac death	4 (7%)	4 (8%)	0.82
Non cardiac death	5 (9%)	4 (8%)	0.88
Myocardial infarction	1 (2%)	4 (8%)	0.30
Target lesion revascularization	5 (9%)	13 (27%)	0.041
Composite major adverse cardiac events	15 (28%)	18 (37%)	0.33
Stent thrombosis	3 (6%)	3 (6%)	0.77
Definite	0	1	
Probable	0	0	
Possible	3	2	

Values are number of Patients (%)

PES paclitaxel-eluting stents, *SES* sirolimus-eluting stents

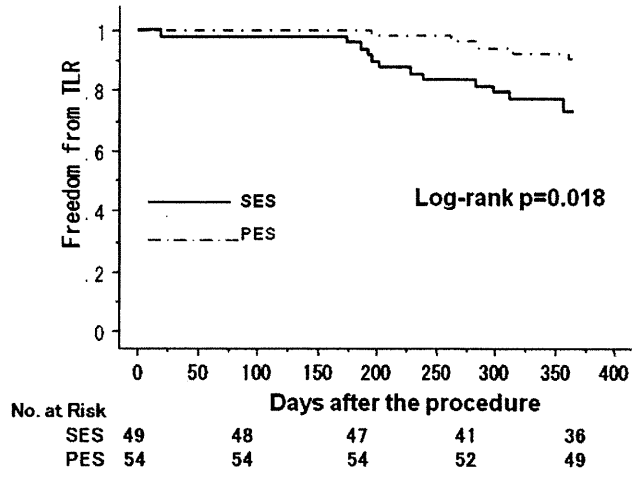

Fig. 1 Survival free from TLR in patients treated with SES versus PES from Kaplan-Meier estimates. PES, paclitaxel-eluting stents; SES, sirolimus-eluting stents

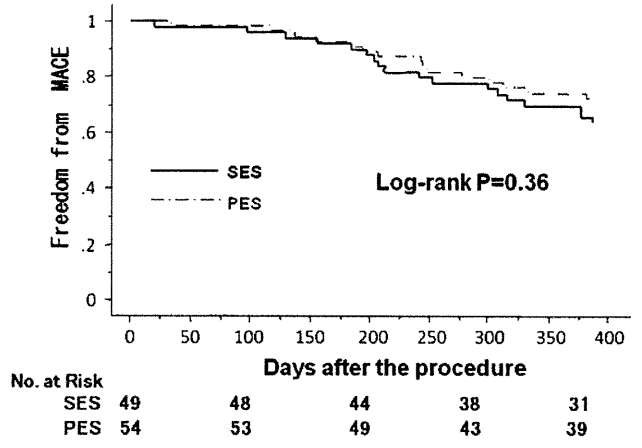

Fig. 2 Survival free from MACE in patients treated with SES versus PES from Kaplan-Meier estimates. PES, paclitaxel-eluting stents; SES, sirolimus-eluting stents

patients with cardiac death, two patients in the PES group and one in the SES group died of congestive heart failure, two patients in the PES group and one in the SES group suffered sudden death, one patient in the SES group died of acute MI, and one patient in the SES group died after stent thrombosis. The incidence of stent thrombosis at 1 year was 6% (3 cases) in the PES group and 6% (3 cases) in the SES group ($p = 0.77$). Of these cases, possible late stent thrombosis occurred in three patients in the PES group and in two patients in the SES group, and definite early stent thrombosis occurred in only one patient (2.0%) in the SES group and in none of the PES group. Kaplan-Meier curves of cumulative incidences of TLR are shown in Fig. 1. There was a significant difference in TLR (log-rank $p = 0.018$). Kaplan-Meier curves of cumulative incidences of MACE are shown in Fig. 2, and there was no statistical difference between the two groups (log-rank $p = 0.36$). Univariate Cox regression analysis to assess predictors of TLR showed two variables with $p \leq 0.2$. The variables were prior to MI and PES use, and were included in the multivariate Cox analysis. After adjusting for the variables, patients treated with PES had a significantly lower rate of TLR than those with SES (hazard ratio 0.29, 95% confidence interval 0.10–0.82, $p = 0.02$, Table 5). Univariate Cox regression analysis to assess predictors of MACE showed five variables with $p \leq 0.2$. The variables were prior MI, prior PCI, prior coronary artery bypass graft, LVEF, and duration of hemodialysis. These variables were included in the multivariate Cox analysis. After adjusting for the variables, duration of hemodialysis was the only significant predictor (hazard ratio 1.08, 95% confidence interval 1.02–1.15, $p < 0.01$, Table 6).

Discussion

The primary finding of this study was that treatment with PES was associated with a moderate reduction in TLR compared with SES in hemodialysis patients. To our knowledge these data are the first to compare PES versus SES outcomes in hemodialysis patients.

In this previous report, it was reported that the rate of MACE at 9 months in patients implanted with SES was significantly lower than in those implanted with PES (6.2% in the SES group and 10.8% in the PES group, $p = 0.009$) [21]. The difference was driven by a lower rate of TLR in the SES group than in the PES group (4.8 vs. 8.3%, $p = 0.03$). However, the risk of death was not significantly different between the PES group and the SES group [21, 22].

Recent studies in Japan in hemodialysis patients as a higher risk subset have reported that the TLR rate was similar between SES and BMS [9, 11, 12]. However, some studies have shown the efficacy of PCI using DES in dialysis patients with a similar reduction in repeat revascularization as in non-dialysis patients [23–25]. As discussed in those reports, a higher rate of angiographic follow-up may explain the increasing rate of TLR in Japan. In addition, different percentages of patients with complex and highly calcified lesions may contribute to the relatively high rate of TLR. Tamekiyo et al. [26] indicated that the studies in Japan included only SES [9, 11, 12], whereas other studies included SES and PES [23–25]. The different results in these studies may reflect a class side effect between the types of DES used in dialysis patients.

Two of the following are nominated for the reason why PES was effective than SES in this study. One reason is that the effectiveness of SES may attenuate in hemodialysis patients as a higher risk subset. The other reason is that

Table 5 Predictive factors for TLR at 12 months

Variables	Univariate analysis		*p*	Multivariate analysis		*p*
	Hazard ratio	95% Confidence interval		Hazard ratio	95% Confidence interval	
PES SES	0.31	0.11–0.87	0.03	0.29	0.10–0.82	0.02
Prior MI	0.36	0.14–0.94	0.04	0.34	0.13–0.88	0.03
Age	0.97	0.93–1.01	0.14			
Male	1.14	0.38–3.46	0.82			
Hypertension	0.98	0.38–1.15	0.38			
Hyperlipidemia	1.03	0.41–2.60	0.95			
Smoking	0.57	0.28–2.01	0.57			
FH	0.61	0.22–1.71	0.35			
Diabetes	1.27	0.48–3.39	0.63			
Peripheral artery disease	1.09	0.43–2.75	0.85			
LVEF	1.01	0.97–1.05	0.56			
Duration of dialysis	1.034	0.97–1.11	0.33			
Prior PCI	0.79	0.31–2.01	0.62			
Prior CABG	0.86	0.31–2.34	0.77			

Multivariate model includes variables with $p \leq 0.2$ in the univariate analysis

FH family history of ischemic heart disease, *PES* paclitaxel-eluting stents, *SES* sirolimus-eluting stents, *MI* myocardial infarction, *PCI* percutaneous coronary intervention, *CABG* coronary artery bypass grafting, *LVEF* left ventricular ejection fraction

Table 6 Predictive factors for MACE at 12 months

Variables	Univariate analysis		*p*	Multivariate analysis		*p*
	Hazard ratio	95% Confidence interval		Hazard ratio	95% confidence interval	
Duration of dialysis	1.09	1.03–1.15	<0.01	1.08	1.02–1.15	<0.01
Prior MI	0.53	0.27–1.04	0.06	0.73	0.34–1.54	0.4
Prior PCI	0.61	0.31–1.21	0.2	0.68	0.34–1.36	0.3
Prior CABG	0.59	0.29–1.22	0.2	0.79	0.36–1.73	0.6
LVEF	0.98	0.95–1.01	0.2	0.98	0.95–1.01	0.2
Age	1.00	0.97–1.04	0.86			
Male	0.91	0.40–2.04	0.81			
Hypertension	1.22	0.43–3.47	0.71			
Hyperlipidemia	1.26	0.63–2.49	0.52			
Smoking	1.36	0.61–3.02	0.45			
FH	0.99	0.40–2.12	0.85			
Diabetes	1.15	0.55–2.41	0.72			
Peripheral artery disease	0.88	0.44–1.74	0.71			
PES SES	0.36	0.37–1.44	0.73			

Multivariate model includes variables with $p \leq 0.2$ in the univariate analysis

FH family history of ischemic heart disease, *PES* paclitaxel-eluting stents, *SES* sirolimus-eluting stents, *MI* myocardial infarction, *PCI* percutaneous coronary intervention, *CABG* coronary artery bypass grafting, *LVEF* left ventricular ejection fraction

PESs show a similar effect in high risk subsets such as in this study. It is possible that the effectiveness of SES is limited in more complex and more highly calcified lesions for three possible reasons. First, the delivery of coronary stents through the rough surface of a calcified coronary artery lumen possibly strips off the polymer and drug of the SES [27]. Second, the recent imaging modality of optical coherence tomography has detected stent strut malapposition, which is a potential risk factor for restenosis or thrombosis after treatment of heavily calcified lesions,

despite the use of high-pressure dilatation or ROTA [28]. In fact, it was reported that the 2-year TLR rate was similarly high in a SES group and a BMS group treated with ROTA in dialysis patients (36 vs. 40%, $p = 0.55$) [26]. Third, the activities of sirolimus as preventing restenosis decrease in diabetic patients [29]. In this study, approximately 70% of patients had diabetes. Sirolimus and paclitaxel drugs activate mitogen-activated protein kinase pathways and inhibit mitogen-induced smooth muscle cell proliferation. Sirolimus potently activates AKT-dependent signaling, overriding the downregulation of this pathway by insulin resistance. This effect is associated with attenuation of the antimigratory effects of sirolimus in the presence of hyperglycemia, which might account for the decreased efficacy in diabetic patients compared with nondiabetic patients. On the other hand, paclitaxel inhibits restenosis independently of the pathways impacted in hyperglycemia [30]. In this theory PES may be more beneficial than SES for diabetic patients. In the SCAAR study [31], the sirolimus-eluting stent resulted in higher rates of restenosis in patients with diabetes compared with those in patients without diabetes. With the paclitaxel-eluting stent the incidence of restenosis was similar in patients with diabetes compared with that in patients without diabetes.

The study by Iakovou et al. [32] demonstrated that renal insufficiency was a key predictor of stent thrombosis in patients with DES implantation and that the incidence of stent thrombosis at 9 months after successful DES implantation in consecutive patients was 1.3%. In this study, the stent thrombosis rate was similar in the two groups and a little higher compared with a previous study in hemodialysis patients [9, 10]. The reason for this could be that the study population was more critical in this study than in previous studies.

In summary, the beneficial effect of SES may be reduced in patients with more complex lesions, whereas PES may be more effective than SES in patients with advanced disease such as that requiring hemodialysis. In this study, MACE occurred in about 30% of the PES group and is a high figure; thus, we have to examine optimal PCI and medical therapy, for example, aimed at further improvement in prognosis.

Study limitations

The study had four limitations. First, the study was limited by its small sample size, non-randomized nature, and performance at a single center. Second, this study comprised a retrospective analysis of a patient registry with historical controls. Third, quantitative analysis was not performed in an independent core laboratory; however, analysis was performed by experienced cardiologists blinded to angiographic and clinical outcomes. Fourth, not all the patients underwent follow-up angiography (65.5% overall).

Conclusion

Clinical and angiographic data in this study suggest that PES are more effective compared with SES in reducing restenosis and TLR in hemodialysis patients.

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Eicosapentaenoic acid reduces warfarin-induced arterial calcification in rats

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ABSTRACT

Background: Eicosapentaenoic acid (EPA), a major n-3 polyunsaturated fatty acid, is reported to have various protective effects for cardiovascular disease. However, few studies have focused on the influence of EPA on vascular calcification.

Methods and results: Arterial medial calcification (AMC) was induced by administering warfarin (3 mg/g food) and vitamin K1 (1.5 mg/g food) for 2 weeks in Sprague–Dawley rats (control group), and EPA (1 g/kg/day) was administered for 2 weeks simultaneously with warfarin and vitamin K1 (EPA group) or after initiation of AMC (late EPA group). EPA showed a marked reduction of medial calcification in the EPA group, and showed a similar effect in the late EPA group. Immunohistochemical and RT-PCR analyses showed that EPA lowered the expression of osteogenetic markers, such as osteopontin, alkaline phosphatase and core binding factor-α1 in the aorta. Significant migration of macrophages with expression of matrix-metalloproteinase (MMP)-2 or MMP-9 was observed in the aortic adventitia around calcification. EPA also reduced macrophage infiltration, MMP-9 expression as well as gene expression of monocyte chemotactic protein (MCP)-1.

Conclusions: These observations indicate that EPA attenuates arterial medial calcification through an effect associated with the suppression of MMP-9 activity and inhibition of macrophage infiltration as well as osteogenic protein expression in warfarin-induced rat models.

Vascular calcification plays an important role in the deterioration of cardiovascular disorders. Vascular calcification consists mainly of atherosclerotic intimal calcification and arterial medial calcification (AMC), also known as Mönckeberg's sclerosis. AMC usually occurs independently, unrelated to intimal calcification, and is found frequently in the muscular arteries of the extremities and characterized by diffuse mineral deposition in the media along the elastic fibers. Several epidemiological and clinical observations have shown that medial calcification is correlated with cardiovascular disease, diabetes, and end-stage renal disease [1,2]. Thus, further elucidation of mechanism responsible for medial calcification and new preventive therapy are required.

Although vascular calcification was thought to be a result from passive degeneration, it has been shown to be an active remodeling process resembling osteogenesis or chondrogenesis [3]. In atherosclerotic intimal calcification, there have been various animal models suggesting that intimal calcification is associated with inflammatory factors, such as oxidized lipids, cytokines in monocytes, oxidant stress, and apoptosis of vascular smooth muscle cells (VSMC) [4–6]. However, only a few murine models of AMC have been reported to show that changes in calcium and phosphate levels and elastin degradation by matrix metalloproteinases (MMPs) may affect pathogenesis of AMC [7]. In addition, no preventive therapy for AMC has yet been established despite its clinical significance.

Eicosapentaenoic acid (EPA), a major omega-3 polyunsaturated fatty acid, is contained in fish such as sardines, tuna, and mackerel. There is increasing epidemiological evidence that EPA has various beneficial effects on cardiovascular disease and cardiovascular mortality [8]. Although the mechanisms are not fully defined, several possible protective effects are considered as follows; attenuation of lipid metabolism, lowering of blood pressure, improvement of vascular endothelial function, reduction of neutrophil and monocyte cytokine production, inhibition of thrombogenesis and the inflammatory response, and an antiarrhythmic effect [9,10]. The significant association between cardiovascular mortality and vascular calcification [1,2] might suggest the possibility that one of the reasons for the inhibitory effect of EPA on cardiovascular disease is attenuation of vascular calcification.

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In order to assess the influence of EPA on vascular calcification, especially AMC, we used a rat model of vascular calcification induced by warfarin to block vitamin K-epoxide reductase [11]. This treatment leads to the exhaustion of the vitamin K stores and to inhibit carboxylation of the matrix Gla protein (MGP), an inhibitor of vascular calcification. Vitamin K1, which cannot counteract the effect of warfarin in extra hepatic tissues [12], was also administered to prevent bleeding.

In this study, we demonstrated a significant inhibition of EPA on AMC and also showed that EPA decreased osteogenesis-related gene expression and adventitial macrophage infiltration with MMP-9 in the calcified aorta.

1. Methods

1.1. Materials

Male Sprague–Dawley rats were purchased from Clea Japan, Inc. (Tokyo, Japan). Vitamin K-deficient food without fish flour (3.8 kcal/g) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Warfarin and vitamin K1 (phylloquinone) were provided by Eisai Co., Ltd. (Tokyo, Japan). Ultra-pure eicosapentaenoic acid ethylester (EPA; >99% purity) was received from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan).

1.2. Induction of vascular calcification in rats and EPA treatments

To induce vascular calcification, 7-week-old, male Sprague–Dawley rats were given a diet containing warfarin (3 mg/g food) and vitamin K1 (1.5 mg/g food) for 2 weeks (control group, $n=27$). In additional rats, EPA (1 g/kg/day) was given orally using the gavage method in addition to warfarin and vitamin K1 for 2 weeks (EPA group, $n=27$). To examine whether EPA can reduce AMC in secondary prevention, another group of 7-week-old rats ($n=10$) were treated with warfarin and vitamin K1 for 2 weeks, then half of the group was given EPA orally for another 2 weeks especially for histological evaluation (late EPA group, $n=5$).

Rats were killed by exsanguination while under ether anesthesia, and the aortas were removed from the aortic arch, most proximal to the heart, to the femoral artery for later studies. Body weight was measured prior to the experiment and immediately before euthanasia. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University.

1.3. Measurement of biochemical parameters

Blood samples were collected from the right atrium and centrifuged at 3000 rpm for 30 min to separate the plasma. Each of the parameters was measured with an auto-analyzer (SRL, Tokyo, Japan).

1.4. Histology and immunohistochemistry

The aorta was fixed with 20% neutral buffered formalin for 24 h, then embedded in paraffin, cut into serial sections of 5 μm longitudinally, deparaffinized and stained with hematoxylin–eosin (HE) and elastica van Gieson (EvG). In order to assess the calcified area, longitudinal sections of the aorta were stained for minerals using the von Kossa method. The calcified area was measured at the abdominal aorta (4 mm in length, directly above the bifurcation) and at the common iliac artery (8 mm in length, directly below the bifurcation) and expressed as the percentage of total surface area using the public domain Scion Image program (Scion Corporation, http://www.scioncorp.com/). The numbers of macrophages were counted at the same area as the calcification measurement site.

For immunohistochemical analysis of osteopontin (OPN), MMP-2, MMP-9, and CD68, formalin-fixed paraffin embedded materials were used and antigen retrieval was achieved by boiling in citrate buffer (pH 6.0). For immunohistochemical analysis of alkaline phosphatase (ALP) and monocyte chemotactic protein (MCP)-1, fresh specimens of the aorta were embedded in OCT compound and immediately frozen in liquid nitrogen, cut 3 μm in thickness, fixed in acetone. These sections were then incubated in blocking serum, and incubated overnight at 4 °C with primary antibodies. The sections incubated with primary antibody against MMP-9 were followed by staining with peroxidase-conjugated anti-goat IgG (1:1000) for 1 h and detected using the EnVision plus kit (Dako, Tokyo, Japan). For immunofluorescence, anti-mouse (for OPN, ALP, and CD68), anti-goat (for MCP-1), and anti-rabbit (for MMP-2 and MMP-9) secondary antibodies conjugated to FITC (1:1000, Jackson Laboratories) were applied for 1 h at room temperature. The primary antibodies used in this study were as follows: OPN (1:300, Santa Cruz), ALP (1:100, R and D systems), CD68 (1:300, CHEMICON), MCP-1 (1:300, Santa Cruz), MMP-2 (1:100, Abcam), MMP-9 (1:100, Santa Cruz).

1.5. Reverse transcriptase polymerase chain reaction

The aortas were removed and immediately placed in RNA*later* (Qiagen, Tokyo, Japan) at 4 °C. Total RNA was obtained using a RNeasy Mini kit (Qiagen) and reverse transcribed in 20 μl volumes using an Omnisctipt RT kit (Qiagen) with 1 μl of random primers. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with the following primers: *OPN*, 5′-CTGCCAGCACACAAGCAGAC-3′ (forward), 3′-TCTGTGGC ATCGGGATACTG-5′ (reverse); *Core binding factor-α1 (Cbfa1)*, 5′-GAGCTACGAAATGC CTCTGC-3′ (forward), 3′-GGACCGTCCACTGTCACTTT-5′ (reverse); *ALP*, 5′-GGACTG GTACTCGGACAATGA-3′ (forward), 3′-CTGGCCTTCTCATCCAGTTC-5′ (reverse); *GAPDH*, 5′-GAGCTACGAAATGCCTCTGC-3′ (forward), 3′-GGACCGTCCACTGTCACTT T-5′ (reverse). Commercial primer pairs (mouse/rat JE/MCP-1/CCL2 primer pair, 246 bp; R and D systems, MN) were used for MCP-1. mRNA was standardized against the level of the respective GAPDH. Band intensities were evaluated using Image Gauge software (Fujifilm, Tokyo, Japan).

1.6. Western blotting

Frozen aorta specimens were homogenized in lysis buffer (50 mM Tris–HCl, pH 7.5, containing 105 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and 2 mM EDTA) using Tissue Lyser (Qiagen) and centrifuged at 14,000 rpm for 30 min. 300 μg of protein from each sample was suspended in a loading buffer, separated on a 10% polyacrylamide gel (Readygels J, BIO-RAD, Tokyo, Japan) , and electrophoretically transferred to a nitrocellulose membrane. After blocking the membranes with 2.5% non-fat milk in PBS for 2 h at room temperature, the primary antibody was applied overnight at room temperature. Membranes were then washed with TBS–Tween and incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins secondary antibody (Dako, Japan) for 1 h at room temperature. After washing, membranes were developed by enhanced chemiluminescence with SuperSignal kit according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.) The primary antibodies were MMP-2 and MMP-9 (1:300, Daiichi Fine Chemical). Quantification was performed with relative density employing Image Gauge software.

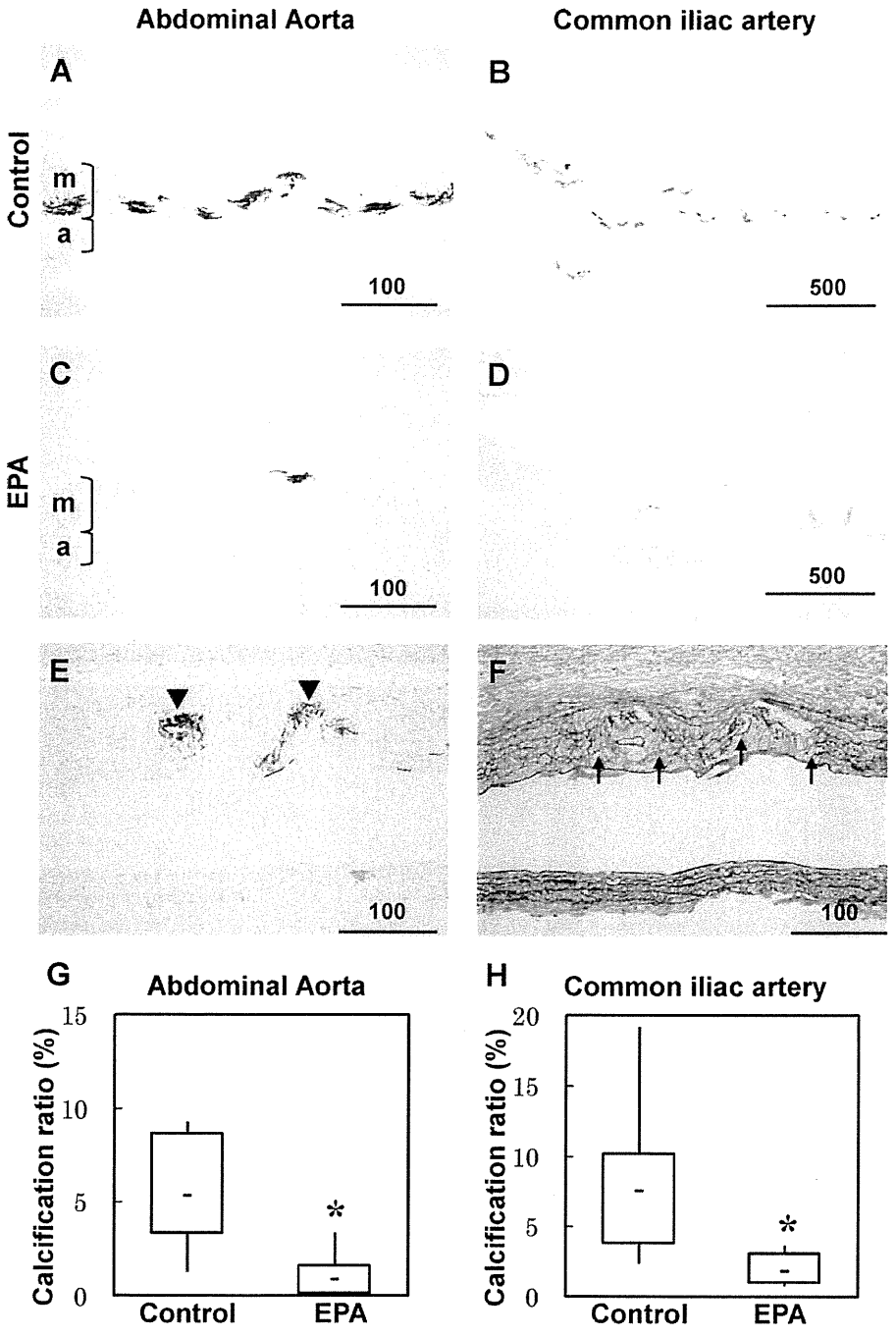

Fig. 1. Inhibitory effects of EPA on warfarin-induced arterial medial calcification. (A–D) von Kossa stained sections of abdominal aorta (A and C) and common iliac artery (B and D) from control (A and B) and EPA (C and D) groups. (E and F) Representative pictures of von Kossa stained (E) and elastica van Gieson stained (F) sections of the calcified aorta. E and F are serial sections. Calcification is shown by a triangle (▲) and elastin degradation was shown by an arrow (↑). (G and H) Quantitative analysis of calcified area in the abdominal aorta (G) and in the common iliac artery (H) ($n = 6$ per group). m, media; a, adventitia. Scale bar, 100 μm (A, C, E and F); 500 μm (B and D). *$p < 0.05$.

1.7. MMP activity

MMP-2 and MMP-9 activities were measured by zymography electrophoresis using a Gelatin Zymo Electrophoresis kit (Life Laboratory Company, Japan) according to the manufacturer's instructions. SDS-polyacrylamide gels were incubated for 24 h at 37 °C. Active MMP-2 and MMP-9 were localized at 45 kDa and 68 kDa, respectively, and visualized as areas of white lytic bands on an otherwise blue background. Gel was digitally photographed and bands were quantified using Image Gauge software.

1.8. Statistics

All data are reported as means ± SD. Means were compared using the Mann–Whitney's *U*-test using the SPSS program, version 13.0J for windows (SPSS Inc., Tokyo, Japan). $p < 0.05$ was considered to indicate a statistically significant difference.

Table 1
Biochemical parameters and body weight gain. Data are shown as means ± SD. Significant differences were analyzed by Mann–Whitney's *U*-test. $^{*}p < 0.01$; $^{**}p < 0.001$ vs. control.

	Control ($n = 14$)	EPA ($n = 14$)
Total cholesterol (mg/dl)	89.1 ± 13.0	71.9 ± 9.9*
Triglyceride (mg/dl)	100.4 ± 44.3	79.1 ± 35.4
Alkaline phosphatase (IU/l)	959.4 ± 244.9	940.9 ± 189.5
Creatinine (mg/dl)	0.25 ± 0.03	0.24 ± 0.03
Calcium (mg/dl)	12.4 ± 0.8	12.2 ± 0.7
P (mg/dl)	16.3 ± 4.6	15.7 ± 3.6
EPA/AA ratio	0.04 ± 0.16	0.58 ± 0.28**
Body weight gain (g)	79.1 ± 10.9	74.6 ± 13.3

* $p < 0.01$.
** $p < 0.001$.

2. Results

2.1. EPA inhibits warfarin-induced arterial medial calcification

After treatment with warfarin for 2 weeks, von Kossa staining of the aortas revealed the presence of extensive arterial medial calcification throughout the abdominal aorta (Fig. 1A) and common iliac arteries (Fig. 1B) in control rats. EvG staining analysis revealed that calcification is associated with the medial elastic fibers, which showed disorganization and fragmentation (Fig. 1E and F). In contrast, EPA reduced arterial medial calcification significantly in the abdominal aorta (histological calcification ratio: control vs. EPA = 5.6 ± 3.4% vs. 1.1 ± 1.3%, $p < 0.05$, $n = 6$; Fig. 1C and G) and in the iliac arteries (control vs. EPA = 8.3% ± 6.2 vs. 1.9 ± 1.5%, $p < 0.05$, $n = 6$; Fig. 1D and H). EPA also blunted degeneration of the elastic network. The EPA/arachidonic acid ratio significantly increased in EPA rats (control vs. EPA = 0.04 ± 0.16 vs. 0.58 ± 0.28, $p < 0.01$, $n = 14$; Table 1). EPA reduced the total cholesterol level (control vs. EPA = 89.1 ± 13.0 vs. 71.9 ± 9.9 mg/dl, $p < 0.01$, $n = 14$; Table 1) and tended to decrease triglyceride level (control vs. EPA = 100.4 ± 44.3 vs. 79.1 ± 35.4 mg/dl, $p = 0.19$; Table 1). To examine the effects of EPA on AMC in secondary prevention, we also assessed whether EPA reduces preformed AMC by administering EPA for 2 weeks after treatment with warfarin and vitamin K1 for 2 weeks. Histological analysis showed that EPA significantly decreased AMC in abdominal aorta (histological calcification ratio: control vs. EPA = 5.4 ± 3.2% vs. 1.4 ± 1.6%, $p < 0.05$, $n = 5$; supplemental Fig. A). Although it was not statistically significant, EPA showed a reduction tendency in common iliac artery (control vs. EPA = 7.6 ± 2.0% vs. 5.5 ± 2.7%, $p = 0.22$, $n = 6$; supplemental Fig. B).

2.2. EPA attenuates osteogenetic markers in the calcified aorta

It has been reported that osteogenetic processes are involved in AMC, and bone-associated proteins are expressed around calcified lesions [3,7]. Therefore, we examined the osteogenic phenotype of calcified arteries in this model. Immunohistochemical analysis revealed that OPN, a glycosylated phosphoprotein associated with mineral deposit [13], was expressed in the calcified lesions in the aorta (Fig. 2A). Similarly, ALP, a functional phenotypic marker of osteoblast [14], was observed in the calcified vascular smooth muscle cells (Fig. 2B). RT-PCR analysis revealed that the mRNA expressions of these osteoblastic markers and Cbfa1, a key transcription factor of osteoblastic differentiation, were significantly downregulated in the EPA rats (OPN, $p < 0.05$, n = 6; ALP, $p < 0.01$, $n = 6$; Cbfa1, $p < 0.05$, $n = 6$; Fig. 2C–F). These results suggest that the process similar to osteogenesis, is implicated in the development of medial calcification in this model, and EPA attenuates this process.

2.3. EPA reduces adventitial macrophage infiltration in the calcified aorta

Prior studies showed that MMP-2 and MMP-9 bind to insoluble elastin playing an important role in elastinolysis leading to the initiation of vascular calcification [15,16]. In fact, disorganization and fragmentation of elastic fibers were observed in the calcified artery of rats treated with warfarin. Although MMPs are secreted from various cells, such as immune cells, fibroblasts, endothelial cells, and smooth muscle cells, macrophages, in particular, have been reported for their involvement in vascular calcification [17,18]. Therefore, we assessed whether macrophages correlate with medial calcification. Immunohistochemistry for CD68 revealed that numerous macrophages migrated into the aortic adventitia around the calcified lesion in control rats (Fig. 3A and C). Macrophages were found not only around progressive calcified areas (supplemental Fig. C and D) but also around extremely small calcifications in the early stage (supplemental Fig. E and F). On the other hand, such significant macrophage infiltration was not observed in the non-calcified aorta (Fig. 3B and D). EPA decreased adventitial macrophages infiltration significantly (abdominal aorta, control vs. EPA = 14.5 ± 6.3 vs. 4.7 ± 2.6/mm, $p < 0.01$, $n = 6$; iliac artery, control vs. EPA = 15.5 ± 7.3 vs. 5.2 ± 1.7/mm, $p < 0.01$, $n = 5$ or 6; Fig. 3G), showing a positive correlation between the number of macrophages and the calcification ratio ($p < 0.01$, $r = 0.79$, $n = 12$; Fig. 3H). Furthermore, some of these adventitial macrophages around the calcified lesions showed positive immunohistochemical staining for MMP-2 (Fig. 3E) or MMP-9 (Fig. 3F). VSMC around the calcification also showed immunoreactivity of MMP-2 (Fig. 3E). These findings suggest that macrophages are an important source for MMP-2 and MMP-9, associated with the development of medial calcification.

2.4. Effects of EPA on MMP expressions in the aorta

To investigate protease expressions in the aorta, a Western blot analysis was performed for MMP-2 and MMP-9. As shown in Fig. 3A, EPA markedly decreased MMP-9 expression in the aorta compared with that of control rats ($p < 0.01$, $n = 5$; Fig. 4C), whereas MMP-2 expression showed no significant difference between the groups (Fig. 4A and B). Similarly, gelatin zymography on aortic extracts revealed a decrease in MMP-9 activity in EPA rats compared with that of control rats ($p < 0.05$, $n = 6$; Fig. 4D and F). On the contrary, direct effect of EPA on active MMP-2 expression was not clear (Fig. 4D and E). These findings indicate that activation of MMP-9 is associated with pathogenesis of medial calcification in this study.

2.5. EPA attenuates MCP-1 expressions in the calcified aorta

We then accessed MCP-1, a chemokine which recruits and activates monocyte/macrophage from the circulation to inflammatory sites. MCP-1 is secreted from various cells, such as fibroblasts, endothelial cells, VSMC, and macrophage. Immunohistochemistry for MCP-1 revealed colocalization with macrophages in adventitia (Fig. 5A) and also expressed in VSMC around sites of calcification in the control group, although the expression of MCP-1 decreased in the EPA group (Fig. 5B). The MCP-1 mRNA expression in the aorta was significantly inhibited in EPA rats compared to control rats ($p < 0.01$, $n = 6$; Fig. 5C and D). These observations indicate the possibility that EPA decreases macrophage infiltration through the suppression of MCP-1 expressions in the aorta.