Effects of Eicosapentaenoic Acid on Platelet Function in Patients Taking Long-Term Aspirin Following Coronary Stent Implantation

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SUMMARY

Eicosapentaenoic acid (EPA) has been widely accepted to have antiatherosclerotic effects. The aim of this study was to investigate the antiplatelet effect of EPA combined with acetylsalicylic acid (ASA) following stent implantation. Eighteen patients who had undergone coronary stent implantation at least 8 months previously were included. All patients were given EPA ethyl ester (EPA-E) 1.8 g/day in addition to ASA 100 mg/day for 12 weeks. After the treatment, the plasma EPA/arachidonic acid (AA) ratio increased significantly from 0.40 ± 0.2 to 1.08 ± 0.39 ($P < 0.001$). There were no changes in the maximum platelet aggregation (MPA) induced by adenosine diphosphate (5 and 20 $\mu\text{mol/L}$), AA (0.3 and 0.5 mg/mL), or collagen (2 and 4 $\mu\text{g/mL}$). Furthermore, no significant differences were observed in the expression of PAC-1 and CD62P on the platelet surface membranes or in the soluble P-selectin concentration. With further analysis, a significant negative correlation was found between collagen (2 $\mu\text{g/mL}$)-induced MPA and plasma EPA/AA ratio ($r = -0.507$, $P = 0.032$). The patients were then divided into 2 groups according to the median EPA/AA ratio value of 0.92. In the high EPA/AA ratio group ($n = 10$), collagen-induced MPA was significantly suppressed after EPA-E administration (45.3 ± 15.9 versus 39.0 ± 16.3 , $P = 0.033$). In contrast, there were no significant changes in platelet aggregation (56.0 ± 9.8 versus 57.1 ± 11.4 , $P = 0.745$) in the low EPA/AA ratio group ($n = 8$). EPA treatment had a potential to suppress collagen-induced platelet aggregation in patients with a high plasma EPA/AA ratio. (Int Heart J 2014; 55: 228-233)

Key words: Omega-3 polyunsaturated fatty acids, Coronary artery disease

Coronary revascularization with drug-eluting stents (DES) has become a widespread technique for treating coronary artery disease (CAD). The guidelines recommend that patients with DES should receive dual antiplatelet therapy (DAPT) with acetylsalicylic acid (ASA) and thienopyridine for at least 1 year in order to prevent stent thrombosis.^{1,2)} When the risk of stent thrombosis decreases in the chronic phase following DES implantation, secondary prevention becomes a primary target of patient care. In fact, in the chronic phase, the risks of CAD or cerebrovascular disease are much higher than that of stent thrombosis.^{3,4)} Therefore, life-long treatment with ASA is also recommended for the secondary prevention of atherothrombotic events following DAPT termination.^{1,2)}

Eicosapentaenoic acid (EPA) has been widely accepted to have antiatherosclerotic effects.^{5,6)} In relation to secondary prevention, the use of eicosapentaenoic acid ethyl ester (EPA-E) in combination with statins successfully reduced the recurrence of cardiovascular events in the Japan EPA lipid intervention study (JELIS).⁷⁾ In particular, in patients who underwent coronary artery intervention, the JELIS sub-study showed that the incidence of major coronary events was significantly re-

duced by 41% in the EPA treatment group.⁸⁾ EPA is known to improve vascular endothelial function⁹⁾ and to reduce platelet function.¹⁰⁾ These multi-functional characteristics of EPA may have contributed to the positive results of the JELIS study, in which EPA-E was used concomitantly with ASA in the majority of the JELIS patients who had histories of atherothrombotic events. Therefore, it is important to know how platelet function is affected in patients receiving combination therapy of ASA and EPA. However, only a few studies have reported an additional effect of EPA on platelet function in patients with CAD.

In this study, we investigated the effects of EPA-E on platelet aggregation and platelet activation in patients under ASA treatment after coronary stent implantation.

METHODS

Study population: Twenty male patients with stable CAD were recruited for this study. Eligible patients, who were taking statins for dyslipidemia, had undergone coronary stent implantation at least 8 months previously and were placed on continuous ASA (100 mg/day) administration after stent im-

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plantation. Exclusion criteria were as follows: age < 50 or > 75 years, familial hyperlipidemia, liver injury (AST $\geq$ 100 U/L, ALT $\geq$ 100U/L, γ GTP $\geq$ 150U/L, ALP $\geq$ 300, total bilirubin $\geq$ 3), renal insufficiency (serum creatinine $\geq$ 2 mg/dL), recent bleeding episode, uncontrollable hypertension (systolic BP $\geq$ 180 mmHg or diastolic BP $\geq$ 110 mmHg), diabetes mellitus with HbA1c $\geq$ 10%, scheduled for surgery, use of nonsteroidal anti-inflammatory drugs, and previous treatment with antiplatelet therapy other than ASA within the past 30 days. This study was approved by the ethics committee of Teikyo University Hospital (No.11-095), and written informed consent was obtained from all patients included in the study.

Study design: All patients were treated with EPA-E at a dose of 1.8 g/day (900 mg, twice per day after meals) for 12 weeks. All patients also received ASA at a dose of 100 mg daily. The patients who were taking statin were not allowed to change the dose during the study period. Indexes of platelet function were evaluated before and after EPA-E administration by determining the levels of platelet aggregation induced by adenosine diphosphate (ADP), collagen, and arachidonic acid (AA) in platelet-rich plasma (PRP) evaluating platelet surface expression levels of activation markers such as PAC-1 and CD62P (P-selectin) and measuring soluble P-selectin concentration. Additionally, levels of plasma EPA, AA, total cholesterol (T-CHO), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG) were also measured before and after EPA-E administration.

Blood sampling: Blood samples for platelet function assays were obtained from an antecubital vein using a 21-gauge needle. To avoid spontaneous platelet activation, the first 4 to 5 mL of blood was used for clinical chemistry analysis. Additionally, the blood used for measuring soluble P-selectin was centrifuged for 10 minutes at 3000 rpm. The plasma was then frozen and stored at -20°C until analysis.

Platelet aggregation assessment: Maximum platelet aggregation (MPA) was assessed by light transmittance aggregometry (LTA) with an MCM HEMA-TRACER 212 aggregometer (MC Medical, Tokyo) after stimulation with 5 or 20 μ mol/L ADP, 2 or 4 μ g/mL collagen, or 0.3 or 0.5 mg/mL AA. The maximum platelet aggregation rate (MPR) curves were recorded for 5 minutes. PRP was obtained as a supernatant after centrifugation of citrated blood at 1000 rpm for 10 minutes. Isolated PRP was kept at room temperature before use. Platelet-poor plasma (PPP) was obtained by a second centrifugation of the blood fraction at 3000 rpm for 15 minutes. Light transmission was adjusted to 0% with PRP and 100% with PPP for each measurement. Platelet aggregation was determined as the maximum percentage change in light transmittance from baseline, using PPP as a reference.

Platelet activation assessment: Platelet activation was assessed by flow cytometry (BD FACS Calibur flow cytometer; BD Bioscience, San Jose, CA, USA), determined by evaluating the platelet surface expression levels of PAC-1 (monoclonal antibody specific for fibrinogen receptors expressed on GPIIb/IIIa of the surface membranes of activated platelets) and CD62P (glycoprotein on the alpha-granule membranes that is exposed on the surface membranes when platelets are activated). Platelet activation was expressed as the percentage of platelets positive for MoAb binding. The soluble P-selectin concentration was measured using a GMP-140 (P-selectin) EIA kit (Takara,

Kyoto, Japan) on the basis of a sandwich enzyme-linked immunosorbent assay, according to the manufacturer's protocol.

Statistical analysis: Continuous variables are expressed as the mean $\pm$ standard deviation (SD), and the values obtained before and after EPA-E administration were compared using the paired *t*-test. Associations between continuous variables were tested at ad hoc basis with multivariate linear regression analysis using the forced-entry method, producing standardised partial regression coefficient (β). Categorical variables are expressed as frequencies and percentages. Differences were considered significant at *P* < 0.05 (two-tailed). All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL).

RESULTS

Twenty patients were recruited from January 2010 to May 2011. According to a questionnaire survey, 2 of the 20 study patients were found to be noncompliant in taking ASA, and were excluded from the study. Thus, a total of 18 patients were finally selected for analysis. Baseline demographic and clinical characteristics of the study patients are listed in Table I. **Changes in plasma fatty acids and lipid levels:** After 12 weeks of EPA-E treatment, the plasma EPA concentration increased and the plasma AA concentration decreased significantly. Accordingly, the ratio of EPA to AA (EPA/AA) increased significantly. Plasma T-CHO, LDL-C, and TG levels decreased significantly; however, the HDL-C level did not change (Table II). **Effect of EPA-E on platelet functions:** Platelet count and the other blood counts did not change following EPA administration. There were no significant changes in the MPA induced by ADP (5 and 20 μ mol/L), AA (0.3 and 0.5 mg/mL), or collagen (2 and 4 μ g/mL). Furthermore, no significant differences were

Table I. Baseline Characteristics of Study Patients

<i>n</i> = 18	
Age (years)	63.6 $\pm$ 7.9
Male	18/18 (100%)
BMI (kg/m ²)	25.6 $\pm$ 2.5
Dyslipidemia	18/18 (100%)
Hypertension	14/18 (77.8%)
Diabetes	5/18 (27.8%)
PAD	0/18 (0%)
Smoking	8/18 (44.4%)

Table II. Changes in Blood Count, Plasma Fatty Acids and Lipid Levels Before and After EPA-E Administration

	Pre	3Mo	<i>P</i>
PLT ($\times 10^4/\mu$ L)	20.73 $\pm$ 5.14	21.28 $\pm$ 5.25	0.271
WBC ($\times 10^3/\mu$ L)	63.50 $\pm$ 25.43	61.44 $\pm$ 17.64	0.686
Hb (g/dL)	14.01 $\pm$ 1.10	14.17 $\pm$ 1.20	0.234
T-CHO (mg/dL)	161.00 $\pm$ 24.5	150.67 $\pm$ 22.4	0.004
HDL-C (mg/dL)	51.67 $\pm$ 12.8	48.67 $\pm$ 10.9	0.611
LDL-C (mg/dL)	82.06 $\pm$ 21.9	75.61 $\pm$ 20.5	0.034
TG (mg/dL)	160.61 $\pm$ 132.7	149.44 $\pm$ 74.1	0.047
EPA (μ g/mL)	67.24 $\pm$ 32.0	147.81 $\pm$ 45.0	< 0.001
AA (μ g/mL)	181.03 $\pm$ 54.6	144.13 $\pm$ 35.3	< 0.001
EPA/AA ratio	0.40 $\pm$ 0.20	1.08 $\pm$ 0.39	< 0.001

Table III. Comparisons of Platelet Function Before and After EPA-E Administration

	Pre	3Mo	P
MPR (%) (<i>n</i> = 18)			
ADP 5 μ M	66.94 $\pm$ 4.50	66.72 $\pm$ 4.36	0.795
ADP 20 μ M	71.72 $\pm$ 4.30	72.50 $\pm$ 3.17	0.474
Collagen 2 μ g/mL	50.06 $\pm$ 14.29	47.06 $\pm$ 16.74	0.183
Collagen 4 μ g/mL	65.67 $\pm$ 11.57	63.00 $\pm$ 12.28	0.122
Arachidonic acid 0.3 mg/mL	4.11 $\pm$ 1.53	3.72 $\pm$ 1.53	0.233
Arachidonic acid 0.5 mg/mL	4.50 $\pm$ 1.58	4.50 $\pm$ 1.86	1.000
Expression of platelet surface (%) (<i>n</i> = 17)			
PAC1	15.56 $\pm$ 11.80	13.03 $\pm$ 6.68	0.358
CD62P	4.40 $\pm$ 1.20	4.36 $\pm$ 1.17	0.923
Soluble P-selectin (ng/mL) (<i>n</i> = 15)	217.27 $\pm$ 132.1	207.00 $\pm$ 83.7	0.483

Figure 1. Correlations between collagen-induced MPA and plasma EPA concentration (A) and EPA/AA ratio (B). MPA indicates Maximum platelet aggregation; EPA, eicosapentaenoic acid ethylester; and AA, arachidonic acid.

observed in the expression of PAC-1 and CD62P on the platelet surface membranes or in the soluble P-selectin concentration (Table III).

To further evaluate the effects of EPA on platelet function, correlations between platelet aggregation and plasma fatty acid levels or the plasma EPA/AA ratio were studied. The collagen (2 μ g/mL)-induced MPA showed a significant negative correlation with the plasma EPA level ($r = -0.518$, $P = 0.027$) and with the EPA/AA ratio ($r = -0.507$, $P = 0.032$) at 12 months (Figure 1A, 1B), although no significant correlations were found at baseline ($r = -0.366$, $P = 0.135$; $r = -0.462$, $P = 0.054$, respectively). There was no correlation between MPA and plasma AA level ($r = 0.151$, $P = 0.550$).

We then divided the patients into 2 groups according to the median value of the plasma EPA/AA ratio of 0.92 at 12 weeks after being treated with EPA-E. There were no significant differences in baseline characteristics between the 2 groups. The EPA/AA ratio increased significantly in both the higher ($n = 10$) and lower ($n = 8$) EPA/AA ratio groups (baseline 0.25 ± 0.12 to on-treatment 0.74 ± 0.11 , $P < 0.001$ 0.52 ± 0.19 to 1.36 ± 0.29 , $P < 0.001$ respectively) (Figure 2A, 2B). The patient baseline characteristics were similar between the 2 groups (Table IV). However, after treatment, both collagen- and AA-induced MPA were significantly suppressed in the

higher EPA/AA ratio group ($n = 10$), while no changes were found in the lower group (Figure 3A, 3B). Multivariate linear regression analysis showed that a high EPA/AA ratio was independently and negatively associated with collagen (2 μ g/mL)-induced MPA ($\beta = -0.639$, $P = 0.036$) (Table V). No suppressive effect on ADP-induced MPA was observed in either group (Figure 3C).

DISCUSSION

In the current study, EPA-E prescribed concomitantly with ASA for secondary prevention following percutaneous coronary intervention (PCI) failed to show a clear antiplatelet effect overall. However, in patients with a high on-treatment EPA/AA ratio, EPA-E significantly suppressed platelet aggregation.

Historically, those with a high omega-3 fatty acid intake or a high plasma EPA concentration have been reported to exhibit reduced thrombogenicity.¹⁰ The main explanation for this reduction in thrombogenicity is that omega-3 fatty acid (or EPA) replaces AA, which is a strong agonist of platelet aggregation. Thus, an increase in EPA intake is usually followed by a decrease in AA in the body, theoretically reducing platelet

Figure 2. EPA/AA ratio before and after EPA-E administration (A), EPA/AA ratio before and after EPA-E administration which is divided into two groups according to 3M EPA/AA ratio (B).

Table IV. Baseline Characteristics of the Patients Divided into 2 Groups According to the Median Value of the Plasma EPA/AA Ratio of 0.92 After EPA-E Administration

	EPA/AA ≥ 0.92 (n = 10)	EPA/AA < 0.92 (n = 8)	P
Age (years)	64.4 $\pm$ 11.6	62.8 $\pm$ 5.4	0.067
Male	10/10 (100%)	8/8 (100%)	
Dyslipidemia	10/10 (100%)	8/8 (100%)	
Hypertension	7/10 (70%)	7/8 (87.5%)	0.787
Diabetes	1/10 (10%)	4/8 (50%)	0.600
PAD	0/10 (0%)	0/8 (0%)	
Smoking	3/10 (30%)	5/8 (62.5%)	0.168

Table V. Multivariate Linear Regression Analysis on Collagen (2 μ g/mL)-Induced MPA Variation

	Standardized coefficient (β)	P
EPA/AA ≥ 0.92	-0.639	0.036
Smoking	-0.223	0.392
Diabetes	-0.316	0.230
Hypertension	0.090	0.708
Age	0.333	0.189

Dependent variable: collagen (2 μ g/mL)-induced MPA.

function. Several studies have demonstrated antiplatelet effects following EPA treatment. Terano, *et al* studied the antiplatelet effect of EPA on healthy subjects.¹¹⁾ In their study, following supplementation with EPA-E 3.6 g/day for 4 weeks, platelet aggregation and platelet retention were significantly suppressed. The same authors showed that the EPA content in platelet phospholipids increased markedly, whereas the AA content did not change. Suehiro, *et al* reported that the addition of EPA-E 1.8 g/day to ASA therapy significantly inhibited platelet aggregation in patients with chronic cerebral infarction.¹²⁾ Gajos, *et al* also showed that the addition of omega-3 polyunsaturated fatty acid to ASA and clopidogrel significantly suppressed platelet aggregation in patients undergoing PCI.¹³⁾

Figure 3. Comparisons of collagen-induced MPA (A), AA-induced MPA (B) and ADP-induced MPA (C) before and after EPA-E administration. ADP indicates adenosine diphosphate.

Nomura, *et al.* clearly showed that platelet-derived microparticles, indicating platelet activation, significantly decreased with EPA treatment in hyperlipidemic and diabetic patients.¹⁴⁾

Conversely, several studies failed to show any antiplatelet effects of EPA. Davi, *et al.* studied the add-on effect of EPA with ticlopidine on platelet function in patients with CAD.¹⁵⁾ The aggregation responses to collagen, ADP, and AA were not altered significantly by the intake of EPA (as fish oil). Serebrunyan, *et al.* evaluated platelet function following 2 weeks of omega-3 fatty acid supplementation in patients with CAD who

were taking ASA.¹⁶⁾ They used various platelet tests, including aggregometry and flow cytometry, and cartridge analyzer readings. These gave a mixture of positive and negative results, but most importantly, collagen- and AA-induced aggregation were not affected by the treatment. The lack of uniformity in study subjects and the amount of EPA supplementation, or differences between the platelet function evaluation methods may account for this disparity.

In the current study, we used several different methods for testing platelet function, including aggregometry and flow cytometry, but we could not show any positive effect of EPA-E treatment combined with ASA overall. We found a negative correlation between the on-treatment EPA/AA ratio and collagen-induced platelet aggregation, and the effects of EPA-E appeared to be insignificant in those with a low EPA/AA ratio.

The concept of EPA/AA ratio was originally introduced a long time ago, when native Inuit people in Greenland were found to have a strikingly low incidence of ischemic heart disease. The fact that they had high levels of plasma EPA and low AA concentrations suggested that their omega-3 fatty acid-rich food was implicated in this favorable outcome.^{10,17)} Subsequently, a number of studies reported findings indicating that a low EPA/AA ratio was related with a higher incidence of cardiovascular events in the Japanese population,¹⁸⁾ as well as in those with a greater number of diseased coronary vessels,¹⁹⁾ a higher risk of cerebrovascular accident,²⁰⁾ or more visceral fat, which is a major factor in metabolic syndrome.²¹⁾

With a certain amount of EPA supplementation, the EPA/AA ratio increases without fail as shown in the current study (Figure 2A). An increase in the EPA/AA ratio following EPA supplementation was reported to reduce cardiovascular events in the JELIS sub-study.⁸⁾ In this sub-study, the incidence of sudden cardiac death or fatal/nonfatal myocardial infarction was significantly lower in the high on-treatment EPA/AA ratio (≥ 1.06) group than in the low EPA/AA ratio (≤ 0.55) group. Interestingly, the current study showed similar results, revealing that a high on-treatment EPA/AA ratio is essential if EPA is to be effective in reducing platelet aggregation. The cut-off EPA/AA ratio value of 0.92 is close to that of the JELIS study. We may need to raise the EPA/AA ratio and maintain it around 1.0 in order to achieve positive effects with EPA at the clinical level. Further large-scale studies are warranted.

Limitations: The small sample size is one of the limitations of this study, and the lack of power could be related to our overall negative results. However, it is noteworthy that we were able to demonstrate a correlation between platelet function and plasma EPA concentration as well as EPA/AA ratio. Second, all the patients were men in this study, so the results may not apply to female patients. Third, the possibility of a statin-related reduction in AA metabolism has been raised.²²⁾ However, in this study, all patients were taking statins throughout the study period; therefore, any influence on our results should be minimal. **Conclusion:** EPA treatment had a potential to suppress collagen-induced platelet aggregation in patients with a high plasma EPA/AA ratio. A further, large-scale study is warranted.

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