performed on approximately 5,000 candidates and applicants every year, along with the institutional annual general medical checkup.

In addition, we could not measure the inflammatory markers (IL-6, adiponectin and hs-CRP) repeatedly because written informed consent limited the measurements to once only, at the time of entry.

Finally, we can conclude that subclinical atherosclerosis is independently accelerated via continuous smoking or LDL-C, and that the smoking-induced promotion of atherosclerotic change is closely associated with inflammatory reactions. Furthermore, the entire inhibition of activated inflammatory responses by smoking cessation is still insufficient to abrogate accelerated progression of subclinical atherosclerosis after 2 years in men, and reduced adiponectin level can be potentially proposed as an underlying mediator. Further investigation into the role of adiponectin in relation to smoking cessation in a larger cohort for a longer period is therefore warranted, to enable investigation of a direct mechanistic link between them, and to verify strategies to increase adiponectin as a therapeutic intervention against atherosclerosis and subsequent CVD events.

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Disclosures

Conflict of Interest: None.

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Supplementary Files

Supplementary File 1

Table S1. Correlation Between Mean Risk Factors During Study Period of 2 Years and Progression of IMT

Table S2. Correlation Between Smoking Habit and Progression of IMT in 2 Years

Table S3. Correlation Between Smoking Habit and Inflammatory Markers

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ORIGINAL ARTICLE
Ischemic Heart Disease

Elevated Serum Heart-Type Fatty Acid-Binding Protein in the Convalescent Stage Predicts Long-Term Outcome in Patients Surviving Acute Myocardial Infarction

Sen Matsumoto, MD; Daisaku Nakatani, MD, PhD; Yasuhiko Sakata, MD, PhD; Shinichiro Suna, MD, PhD; Masahiko Shimizu, MD, PhD; Masaya Usami, MD; Masahiko Hara, MD; Satoru Sumitsuji, MD; Shinsuke Nanto, MD; Tatsuya Sasaki, MD, PhD; Toshimitsu Hamasaki, PhD; Hiroshi Sato, MD, PhD; Masatsugu Hori, MD, PhD; Issei Komuro, MD, PhD
on Behalf of the Osaka Acute Coronary Insufficiency Study (OACIS) Group

Background: Little is known about the prognostic significance of elevated serum heart-type fatty acid-binding protein (H-FABP) in post-acute myocardial infarction (post-AMI) patients.

Methods and Results: A total of 1,283 post-AMI patients with available serum samples collected in the convalescent stage were studied. During a median follow-up period of 1,785 days, 176 patients (14%) had adverse events (all-cause mortality, n=81; non-fatal MI, n=44; readmission for heart failure [HF], n=51). Patients were divided into 2 groups according to a serum H-FABP level of 6.08 ng/ml, which was determined to be the optimal cut-off for discriminating all-cause mortality based on the maximum value of the area under the receiver operating characteristic curve. Patients with elevated H-FABP (>6.08 ng/ml, n=224) had a significantly higher incidence of death (18.3% vs. 3.8%, P<0.001) and readmission for HF (10.3% vs. 2.6%, P<0.001), but not of non-fatal MI (4.5% vs. 3.2%, P=0.187), compared to those with H-FABP <6.08 ng/ml. Multivariate Cox regression analysis indicated that elevated serum H-FABP was associated with an increased risk of mortality (hazard ratio [HR], 1.91; 95% confidence interval [CI]: 1.03–3.51, P=0.039) and readmission for HF (HR, 2.49; 95% CI: 1.15–5.39, P=0.020).

Conclusions: Elevated serum H-FABP during the convalescent stage of AMI predicted long-term mortality and readmission for HF after survival discharge in the post-AMI patients. (*Circ J* 2013; **77**: 1026–1032)

Key Words: Acute myocardial infarction; Cardiac event; Heart-type fatty acid-binding protein

Heart-type fatty acid-binding protein (H-FABP) is an abundant cytoplasmic protein in myocardial tissue,[1] and is rapidly released into the circulation at an earlier stage of acute myocardial infarction (AMI) than other biomarkers such as troponin and creatinine kinase (CK). Because H-FABP quickly disappears from the blood,[2] this protein biomarker has been used in clinical practice to diagnose AMI at an early phase.[3–8] Several reports have shown that elevated H-FABP in the acute phase of AMI is associated with an increased risk of cardiac events,[9–11] but few studies have investigated the clinical significance of serum H-FABP level in the convalescent stage of AMI.

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In patients with chronic heart failure (HF), it has been reported that increased serum H-FABP could reflect ongoing myocardial damage that causes progressive deterioration of ventricular function, and thus predict worse outcome.[12–14] Therefore, we hypothesized that elevated H-FABP level documented in the convalescent stage could reflect ongoing myocardial damage after AMI and thus predict worse outcome in post-AMI patients.

In the present study we (1) investigated whether elevated serum H-FABP was a predictor of adverse events in patients

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Department of Cardiovascular Medicine (S.M., D.N., Y.S., S. Suna, M.S., M.U., M. Hara, S. Sumitsuji, S.N., I.K.), Department of Biomedical Statistics (T.H.), Osaka University Graduate School of Medicine, Suita; Cardiovascular Division, Osaka Minami Medical Center, National Hospital Organization, Kawachinagano (T.S.); School of Human Welfare Studies Health Care Center and Clinic Kwansei Gakuin University, Nishinomiya (H.S.); and Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka (M. Hori), Japan
Members of the OACIS Group are listed in Appendix 1.
Mailing address: Daisaku Nakatani, MD, PhD, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka (A8), Suita 565-0871, Japan. E-mail: nakatani@cardiology.med.osaka-u.ac.jp
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surviving AMI; (2) determined the optimal cut-off value for H-FABP for predicting high risk for mortality; and (3) compared the predictive accuracy of adverse events (as assessed using the C-statistics, calculated as the area under the receiver operating characteristic curve; AUC), between H-FABP, cardiac troponin-T and left ventricular end-diastolic dimension (LVDd).

Methods

Source of Study Data

The Osaka Acute Coronary Insufficiency Study (OACIS) is a prospective, multicenter observational study in which 25 collaborating hospitals from the Osaka region of Japan record demographic, procedural, and outcome data, as well as collect and store blood samples from patients with AMI. A detailed description of the OACIS registry has been published elsewhere.[15–19] The study protocol was approved by the ethics committee of each participating hospital.

Subjects

Among 8,603 patients with AMI who were registered in the OACIS between 1998 and 2008, we included all patients fulfilling the following criteria: (1) discharge alive between 2000 and 2005; and (2) provision of a blood sample before or within 14 days of discharge. The study protocol complied with the Guidelines for Genome/Genetic Research issued by the Japanese government and was approved by each of the participating institutional ethics committees. Written informed consent was obtained from each patient.

Serum Collection and Measurement of H-FABP and Cardiac Troponin-T

At each participating hospital, fasting blood samples were collected into serum separator tubes. After separation of the clot by centrifugation of the tubes at 1,430 g for 15 min at 4°C, the serum was removed and sent to SRL (Tokyo, Japan), where samples were stored at –80°C until the initial assay. The samples were then shipped to DS Pharma Biomedical for the measurement of H-FABP using a MARKIT-M H-FABP enzyme-linked immunosorbent assay kit (DS Pharma Biomedical, Osaka, Japan). The lower and upper detection limits for the H-FABP concentration were 1.25 ng/ml and 250 ng/ml, respectively. The median timing of serum sample collection after AMI onset was 20 days (range, 14–27 days). Cardiac troponin-T was measured with a third-generation immunoassay (Elecsys Troponin T; Roche Diagnostics, Indianapolis, IN, USA). The detection limit of the assay was 0.01 ng/ml.

Data Collection

Research cardiologists and trained research nurses or coordinators recorded data concerning sociodemographic variables, medical history, therapeutic procedures, and clinical events during the hospital stay. Clinical data after discharge were obtained at 3 and 12 months after the onset of AMI, and annually thereafter up to 5 years. The clinical endpoint of this study was primarily all-cause mortality. To explore the underlying mechanism, readmission for HF and non-fatal MI were also included as study endpoints. Non-fatal MI was defined as the occurrence of symptoms used for the diagnosis of AMI.[18] Readmission for HF was defined using the Framingham Heart Study criteria.[20]

Statistical Analysis

After confirming that serum H-FABP was higher in post-AMI patients who died from any cause, we determined the best cut-off point for balancing the sensitivity and specificity of all-cause mortality with the point on the curve closest to the upper left-hand corner of the AUC.[21] To verify the estimated cut-off value, repeated random sub-sampling validation was conducted.[22] C-statistics, as assessed by the AUC, were then used to assess predictive accuracy.[23] According to the optimal cut-off value for H-FABP concentration, patients were then divided into 2 groups. Categorical variables were compared between the 2 groups using the χ^2 test. All continuous variables are described as mean±SD and were compared using Student's t-test. For continuous variables not normally distributed, the variables are given as median (25^{th}–75^{th} percentile) and compared using Mann-Whitney U-test. Rates of cardiac events, including all-cause mortality, non-fatal MI, and readmission for HF, following discharge for the 2 groups are described using the Kaplan-Meier method and compared using the log-rank test. Multivariate logistic regression analysis was used to identify factors associated with elevated H-FABP. The variables used in model 1 were age, male gender, body mass index, diabetes mellitus (DM), hypertension, dyslipidemia, smoking, previous MI, peak CK ≥3,000 (IU/L), Killip ≥II on admission, reperfusion therapy, and estimated glomerular filtration rate (eGFR). Step-wise regression analysis (model 2) was then performed to validate the determinants detected in model 1. Cox regression models were used to examine whether elevated H-FABP was associated with an increased risk of all-cause mortality, non-fatal MI, and readmission for HF. All analyses were performed using PASW Statistics, version 18 (SPSS, Chicago, IL, USA). Statistical significance was defined as $P<0.05$ or 95% confidence intervals (95% CI) that did not include 1.0.

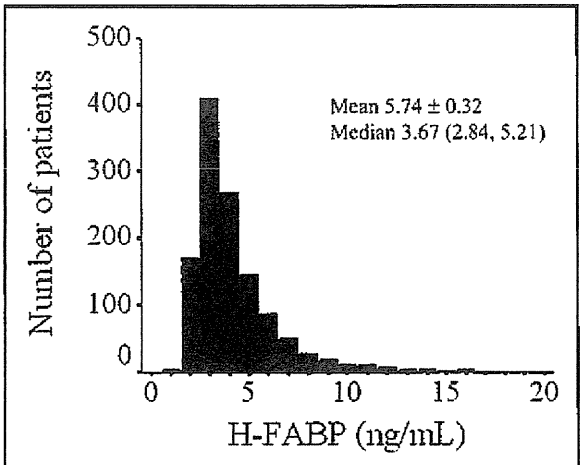

Figure 1. Serum heart-type fatty acid-binding protein (H-FABP) distribution in post-acute myocardial infarction patients.

Results

A total of 1,283 post-AMI patients were included in the present study; 77% were male and the mean age was 64±11 years. The median serum H-FABP concentration collected at a median of 20 days after AMI onset was 3.67 ng/ml (2.84–5.21; Figure 1). Because serum H-FABP was significantly higher in patients who died from any cause than surviving patients (6.09 ng/ml,

Table 1. Baseline Patient Characteristics vs. Serum H-FABP Level

	H-FABP (ng/ml)		P-value
	≤6.08 (n=1,059)	>6.08 (n=224)	
Age (years)	62.0±10.4	71.0±10.4	<0.001
Male	78.6	71.0	0.018
BMI (kg/m^2)	23.9±3.2	23.1±4.0	0.012
Diabetes mellitus	29.4	38.8	0.007
Hypertension	54.4	72.5	<0.001
Dyslipidemia	50.2	38.1	0.001
Smoking	71.5	53.6	<0.001
Previous MI	10.5	13.4	0.233
Onset to admission time <24 h	87.0	86.5	0.825
Peak CK ≥3,000IU/L	35.8	33.3	0.571
Killip class ≥II on admission	8.7	18.8	<0.001
Reperfusion therapy	92.6	86.6	0.005
eGFR (ml·min^{-1}·1.73 m^{-2})	69.9±18.3	46.6±24.1	<0.001
LVDd (mm)	50.3±5.9	51.5±7.4	0.034
cTnT (ng/ml)	0.01 (0.01–0.05)	0.05 (0.01–0.38)	<0.001
ACEI or ARB	75.9	64.5	0.001
β-blocker	44.1	48.6	0.234
Statin	39.9	25.5	<0.001
Antiplatelet therapy	99.1	98.6	0.445

Data given %, mean±SD or median (25th–75th percentile).
ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CK, creatinine kinase; cTnT, cardiac troponin T; eGFR, estimated glomerular filtration rate; H-FABP, heart-type fatty acid-binding protein; LVDd, left ventricular end-diastolic dimension; MI, myocardial infarction.

Table 2. Factors Associated With Elevated H-FABP (>6.08 ng/ml)

	Model 1†		Model 2‡	
	OR (95% CI)	P-value	OR (95% CI)	P-value
eGFR	0.94 (0.93–0.95)	<0.001	0.94 (0.93–0.95)	<0.001
Age	1.07 (1.05–1.10)	<0.001	1.08 (1.06–1.10)	<0.001
Diabetes mellitus	1.69 (1.12–2.54)	0.013	1.71 (1.14–2.56)	0.010
Killip class ≥II	1.60 (0.89–2.89)	0.118	– (–)	–
BMI	0.98 (0.92–1.04)	0.515	– (–)	–
Hypertension	1.40 (0.92–2.16)	0.120	– (–)	–
Dyslipidemia	0.93 (0.62–1.39)	0.726	– (–)	–
Reperfusion therapy	0.92 (0.43–1.96)	0.823	– (–)	–
Male	1.06 (0.65–1.72)	0.816	– (–)	–
Peak CK ≥3,000 (IU/L)	1.09 (0.72–1.66)	0.694	– (–)	–
Previous MI	0.99 (0.56–1.74)	0.973	– (–)	–
Smoking	0.88 (0.57–1.37)	0.583	– (–)	–

†Variables considered to be associated with elevated H-FABP were inserted into multivariate logistic regression model. ‡Stepwise regression analysis was further performed in order to validate the factors identified in model 1.
CI, confidence interval; OR, odds ratio. Other abbreviations as in Table 1.

3.92–13.3 ng/ml vs. 3.57 ng/ml, 2.78–4.95 ng/ml; P<0.001), we determined the optimal cut-off value of serum H-FABP for discriminating between the risk of all-cause mortality and survival. The optimal cut-off was estimated to be 6.08 ng/ml with C-statistics of 0.68.

Patients with elevated serum H-FABP >6.08 ng/ml (n=224) were more likely to be older and female, and to have DM, hypertension, HF defined as Killip class ≥2 on admission, and multivessel disease compared to those with H-FABP ≤6.08 ng/ml (n=1,059; Table 1). In addition, patients with elevated H-FABP were less likely to have dyslipidemia, smoking, obesity, and reperfusion therapy, and were less likely to receive angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or statins (Table 1).

Multivariate logistic regression analysis indicated that advanced age, DM, and decreased eGFR were significantly associated with elevated H-FABP (Table 2). To clarify the effect of infarct size on H-FABP concentration, correlations were examined. No significant correlation between serum H-FABP concentrations and peak CK was detected (Figure 2).

During a median follow-up period of 1,785 days, 81 patients died, 44 had non-fatal reinfarction, and 51 were rehospitalized for HF (Table 3). Kaplan-Meier curves showed that patients with elevated H-FABP had a significantly higher incidence of death (18.3% vs. 3.8%, P<0.001; Figure 3A) and readmission for HF (10.3% vs. 2.6%, P<0.001; Figure 3B) than those

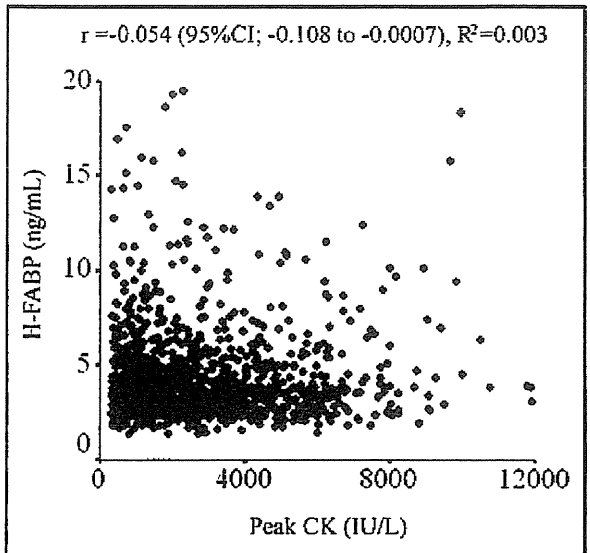

Figure 2. Correlation of serum heart-type fatty acid-binding protein (H-FABP) concentration with peak creatinine kinase (CK).

Table 3. Outcome Following Discharge for AMI

Outcome	H-FABP (ng/ml) ≤6.08 (n=1,059)	H-FABP (ng/ml) >6.08 (n=224)	P-value
All-cause mortality, n (%)	40 (3.8)	41 (18.3)	<0.001
Non-fatal MI, n (%)	34 (3.2)	10 (4.5)	0.187
Readmission for HF, n (%)	28 (2.6)	23 (10.3)	<0.001

AMI, acute MI; HF, heart failure. Other abbreviations as in Table 1.

Table 4. Correlation of Elevated Serum H-FABP and Outcome

Outcome	Adjusted HR† (95% CI)	P-value
All-cause mortality	1.91 (1.03–3.51)	0.039
Non-fatal MI	1.40 (0.55–3.57)	0.475
Readmission for HF	2.49 (1.15–5.39)	0.020

†Adjustment for age, gender, BMI, classical coronary risk factors (hypertension, dyslipidemia, diabetes mellitus, and smoking), history of previous MI, reperfusion therapy, Killip class on admission, chronic kidney disease defined as eGFR <60ml·min^{-1}·1.73m^{-2}, and medication at discharge (ACEI and/or ARB, β-blockers, and statins).
HR, hazard ratio. Other abbreviations as in Tables 1–3.

without, although no significant differences in the rate of non-fatal MI was detected between the 2 groups (4.5% vs. 3.2%, P=0.187; Figure 3C). Multivariate Cox regression analysis also showed that elevated serum H-FABP was associated with an increased risk of all-cause mortality (hazard ratio [HR], 1.91; 95% CI: 1.03–3.51; P=0.039) and readmission for HF (HR, 2.49; 95% CI: 1.15–5.39; P=0.020; Table 4); no association was observed between elevated serum H-FABP and the risk of non-fatal MI (HR, 1.40; 95% CI: 0.55–3.57; P=0.475; Table 4).

The predictive accuracy of all-cause mortality and readmission for HF, assessed with the C-statistics for these 2 factors, was greater for H-FABP (0.730 and 0.724, respectively) compared with that for cardiac troponin-T (0.634 and 0.600, respectively) and LVDd (0.587 and 0.622, respectively) measured before discharge in the present subjects.

Discussion

In the present study, we found that elevated H-FABP is associated with an increased risk of all-cause mortality, and readmission for HF following AMI, but not of non-fatal MI. From the present analysis of 1,283 AMI patients, the optimal cut-off value for serum H-FABP was 6.08ng/ml. The factors associated with elevated H-FABP were advanced age, DM, and impaired renal function.

To our knowledge, this is the first study to investigate the clinical impact of serum H-FABP concentration at the convalescent stage in post-AMI patients. H-FABP level in the present patients was skewed towards lower concentrations, given that the median and mean were 3.67ng/ml and 5.74ng/ml, respectively (Figure 1). Considering that the cut-off of H-FABP to diagnose MI is >6.2ng/ml in the clinical setting, as measured by commercially available kits,[2] 18% of the present patients had elevated H-FABP in the 20 days after AMI onset. Although it is presently unclear why H-FABP remains high in the convalescent stage of AMI, it is possible that myocardial damage is ongoing during this stage.[12,13]

Elevated serum H-FABP in the acute stage of AMI or acute coronary syndrome (ACS) appears to be a useful predictor of cardiac events after the onset of disease. The relationship between serum H-FABP at a mean of 40 hours and cardiac events during a 10-month follow-up period in 2,287 ACS patients was investigated in the Orbofiban in Patients with Unstable Coronary Syndromes (OPUS)-TIMI 16 trial. The authors found that elevated H-FABP (>8.0ng/ml) was associated with an increased risk of death (HR, 2.7) and congestive HF (HR, 2.4). Kilcullen et al demonstrated the prognostic importance of H-FABP level evaluated 12–24 hours after the onset of ACS in the Evaluation of Methods and Management of Acute Coronary Events (EMMACE)-2 study, which consisted of 1,448 ACS patients, in that quartiles of H-FABP level strongly predicted 12-month mortality on multivariate analysis.[9] In addition, Viswanathan et al reported an association between elevated H-FABP and increased risk of 12-month mortality and myocardial reinfarction in 955 patients with suspected ACS.[11] But because these previous studies investigated only the impact of H-FABP level on ≤12-month outcomes in acute ACS patients, it remained unclear whether H-FABP is a useful predictor of cardiac events in patients surviving the acute stage of AMI. In this regard, the present findings are of marked clinical significance in the setting of secondary prevention after AMI, because the prognostic impact of elevated H-FABP on subsequent adverse events in post-AMI patients was examined during a relatively long follow-up period of approximately 5 years. Notably, we found that elevated serum H-FABP is associated with an increased risk of all-cause mortality and readmission for HF. With respect to the predictive accuracy of all-cause mortality and readmission for HF, the C-statistics for these 2 factors were larger for H-FABP compared with those for both troponin-T and LVDd in the current subjects, suggesting that H-FABP is superior to troponin-T and LVDd for predicting subsequent adverse events in post-AMI patients. Therefore, the present findings suggest that the measurement of serum H-FABP in a single blood sample collected during the convalescent stage may predict long-term outcome, such

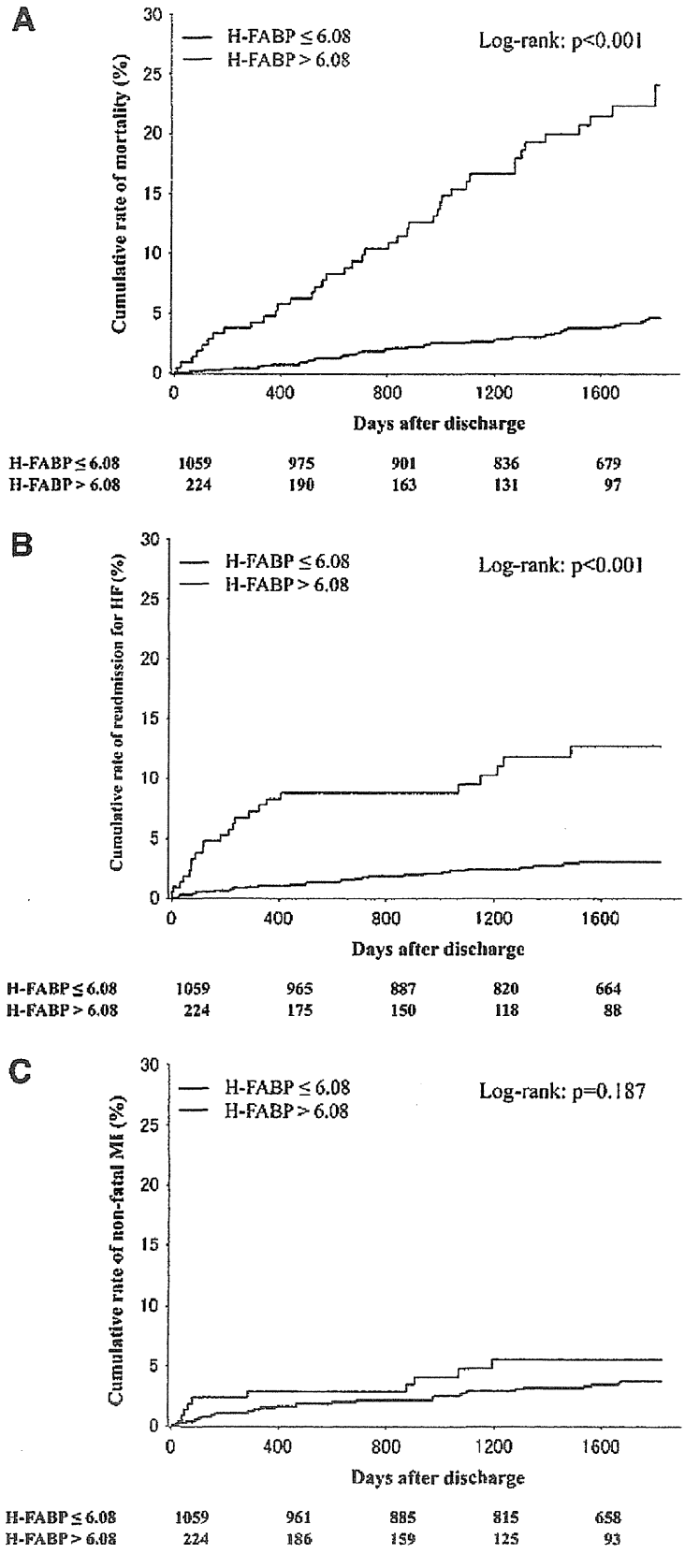

Figure 3. Kaplan-Meier curves showing event rates for patients (red) with and (blue) without elevated heart-type fatty acid-binding protein (H-FABP): (**A**) all-cause mortality; (**B**) readmission for heart failure (HF); and (**C**) non-fatal myocardial infarction (MI).

as all-cause mortality and rehospitalization for HF, with higher accuracy than troponin T and LVDd in post-AMI patients.

The correlation between elevated H-FABP and outcome in AMI patients has been examined using cut-offs determined between the highest and 3 lower quartiles.[9–11] Although this approach has been used to estimate cut-off values in several epidemiological studies, it is not necessarily optimal to predict subsequent outcomes. Therefore, we determined the optimal cut-off value of serum H-FABP, which was found to be 6.08 ng/ml with a C-statistic of 0.68, discriminating all-cause mortality. The C-statistic was 0.67 for a cut-off value of 5.21 ng/ml, which was determined from the value between the 3rd and highest quartiles in the present patients. Although these C-statistics were similar between the 2 cut-offs, the optimal cut-off value calculated in the present study may provide adequate risk stratification to predict subsequent cardiac outcome in the clinical practice setting. Indeed, the sensitivity, specificity, and positive and negative predictive values were 51%, 85%, 18%, and 96%, respectively, for the optimal cut-off value of 6.08 ng/ml, and 57%, 77%, 14%, and 96%, respectively, for the cut-off value determined from the 3rd and highest quartiles, showing that the former cut-off value had higher specificity and positive predictive value. We also attempted to determine whether elevated H-FABP is attributable to necrosis of infarcted areas, but did not detect a significant correlation for H-FABP with peak CK (**Figure 2**), suggesting that serum H-FABP is not associated with infarct size.

We found that advanced age, DM, and impaired renal function, as indicated by decreased eGFR, are associated with increased serum H-FABP concentration (**Table 2**). We speculate that elevated H-FABP in post-AMI patients results from either increased secretion of H-FABP from the myocardium or impaired excretion of H-FABP from the kidney.[24,25] Akbal et al reported that H-FABP is significantly elevated in patients with metabolic syndrome, particularly in diabetic patients.[26] We previously reported that post-AMI diabetic patients have an increased risk of admission for HF and that high-sensitivity C-reactive protein (hs-CRP) in the convalescent stage of AMI is significantly higher in diabetic patients who were readmitted for HF than in those who were not.[17] Scirica et al also reported that hs-CRP in the convalescent stage of ACS is significantly higher in patients rehospitalized for HF.[27] Considering that aging is associated with increased vascular inflammation, it is possible that myocardial injury associated with aging, metabolic disorders, and inflammatory reactions coordinately induced elevation of serum H-FABP, resulting in an increased risk of all-cause mortality and readmission for HF in the present patients.

Kleine et al found that H-FABP concentration remains elevated even 25 hours after AMI onset in patients with renal insufficiency, whereas it normalizes within 10 hours in patients with normal renal function.[25] These findings suggest that the excretion of H-FABP may be prolonged in patients with renal dysfunction, resulting in worse outcome in relation to impaired renal function. Indeed, all-cause mortality ($P<0.001$) and readmission for HF ($P=0.037$) were significantly higher in patients with elevated serum H-FABP than in those without among patients with impaired renal function with eGFR<50 $ml \cdot min^{-1} \cdot 1.73 m^{-2}$. We note that a linear relationship between elevated H-FABP and increased risk of all-cause mortality ($P=0.018$) and readmission for HF ($P=0.039$) was also seen, even in patients with eGFR ≥50 $ml \cdot min^{-1} \cdot 1.73 m^{-2}$, indicating that elevated H-FABP serves as a predictor of these adverse outcomes in patients with normal renal function. Advanced age may be involved in elevated serum H-FABP through both increased secretion from heart tissue and decreased secretion from the kidney. It should be stressed, however, that the factors of age, DM, and impaired renal function might account for a small fraction of the increased incidence of all-cause mortality and readmission for HF observed in the present study, although the coefficient of determination was not significant (data not shown). Therefore, elevation of serum H-FABP might be attributable to yet undetermined underlying factors associated with myocardial injury and inflammation, among others. Interestingly, the present results show that there is no relationship between peak CK in the acute stage of AMI and elevated H-FABP, suggesting that higher serum H-FABP concentration does not necessarily cause subsequent mortality through increased infarct size.

Study Limitations

Several limitations of the present study warrant mention. First, although the OACIS is a multicenter observational prospective study and represents the real-world situation regarding the management and outcome of AMI, the present study was conducted retrospectively and included only patients who provided written informed consent, and may have been influenced by the presence of selection bias. Second, left ventricular ejection fraction (LVEF) was not assessed before discharge, even though LVEF is considered to be associated with an increased risk of adverse events. Instead, Killip class and peak CK activity were used to investigate predictors of elevated H-FABP, and the relationship between elevated H-FABP and mortality. Third, we did not have sufficient information on the treatment of patients, including the use of percutaneous coronary intervention for myocardial ischemia after discharge. Therefore, the present observations may need to be confirmed in various cohorts.

Conclusions

Elevated serum H-FABP served as an accurate predictor of long-term outcome, including all-cause mortality and readmission for HF, in the 1,283 present post-AMI patients. Patients with H-FABP >6.08 ng/ml during the convalescent stage of AMI may be treated as at high risk for subsequent adverse events.

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Disclosures

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Appendix 1

The OACIS Investigators

Chair

Issei Komuro, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan

Secretariats

Yasuhiko Sakata (Chief), Daisaku Nakatani, Shinichiro Suna, Masaya Usami, Sen Matsumoto, Masahiko Hara, Mariko Kishida, Rie Nagai: Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan

Statisticians

Toshimitsu Hamasaki, Kenichi Hayashi, Toshifumi Sugitani: Department of Biomedical Statistics, Osaka University Graduate School of Medicine, Suita, Japan

Investigators (institutions listed in alphabetical order)

Yoshiyuki Kijima, Yusuke Nakagawa, Minoru Ichikawa, Higashi-Osaka City General Hospital, Higashi-Osaka; Young-Jae Lim, Shigeo Kawano, Hideyuki Narmori, Kawachi General Hospital, Higashi-Osaka; Hiroshi Sato, Kwasnsei Gakuin University, Nishinomiya; Takashi Shimazu, Hisakazu Fuji, Kazuhiro Aoki, Kobe Ekisaikai Hospital, Kobe; Masaaki Uematsu, Yoshio Ishida, Tetsuya Watanabe, Masashi Fujita, Masaki Awata, Kansai Rosai Hospital, Amagasaki; Michio Sugii, Meiwa Hospital, Nishinomiya; Masatake Fukunami, Takahisa Yamada, Takashi Morita, Osaka General Medical Center, Osaka; Shinji Hasegawa, Nobuyuki Ogasawara, Osaka Kosei Nenkin Hospital, Osaka; Tatsuya Sasaki, Yoshinori Yasuoka, Kiyoshi Kume, Osaka Minami Medical Center, National Hospital Organization, Kawachinagano; Hideo Kusuoka, Yukihiro Koretsune, Yoshio Yasumura, Keiji Hirooka, Osaka Medical Center, National Hospital Organization, Osaka; Masatsugu Hori (previous Chair), Osaka Prefectural Hospital Organization Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka; Kazuhisa Kodama, Yasunori Ueda, Kazunori Kashiwase, Mayu Nishio, Osaka Police Hospital, Osaka; Yoshio Yamada, Jun Tanouchi, Masami Nishino, Hiroyasu Kato, Ryu Shutta, Osaka Rosai Hospital, Sakai; Shintaro Beppu, Hiroyoshi Yamamoto, Osaka Seamens Insurance Hospital, Osaka; Issei Komuro, Shinsuke Nanto, Yasushi Matsumura, Tetsuo Minamino, Satoru Sumitsuji, Yuji Okuyama, Yasuhiko Sakata, Shungo Hikoso, Daisaku Nakatani, Masahiro Kumada, Michihiro Takeda, Shinichiro Suna, Osaka University Graduate School of Medicine, Suita; Toru Hayashi, Yasuji Doi, Ken-ichiro Okada, Noritoshi Ito, Saiseikai Senri Hospital, Suita; Kenshi Fujii, Katsuomi Iwakura, Atsushi Okamura, Motoo Date, Yoshiharu Higuchi, Koji Inoue, Sakurabashi Watanabe Hospital, Osaka; Noriyuki Akehi, Settsu Iseikai Hospital, Settsu; Eiji Hishida,Teramoto Memorial Hospital, Kawachinagano; and Shiro Hoshida, Kazuhiko Hashimura, Takayoshi Adachi, Yukinori Shinoda, Yao Municipal Hospital, Yao, Japan.

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Decreased mortality associated with statin treatment in patients with acute myocardial infarction and lymphotoxin-alpha C804A polymorphism

Shinichiro Suna [a,1], Yasuhiko Sakata [a,b,*,1], Daisaku Nakatani [a,1], Keiji Okuda [a,1], Masahiko Shimizu [a,1], Masaya Usami [a,1], Sen Matsumoto [a,1], Masahiko Hara [a,1], Kouichi Ozaki [c,1], Hiroya Mizuno [a,1], Tetsuo Minamino [a,1], Seiji Takashima [a,1], Masami Nishino [d,1], Yasushi Matsumura [e,1], Hiroshi Takeda [e,1], Toshihiro Tanaka [c,1], Hiroshi Sato [f,1], Masatsugu Hori [g,1], Issei Komuro [a,1]

[a] Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan
[b] Department of Evidence-Based Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
[c] Laboratory for Cardiovascular Diseases, SNP Research Center, RIKEN Center for Genomic Medicine, Yokohama, Japan
[d] Osaka Rosai Hospital, Sakai, Japan
[e] Department of Medical Information Science, Osaka University Graduate School of Medicine, Suita, Japan
[f] School of Human Welfare Studies, Kwansei Gakuin University, Nishinomiya, Japan
[g] Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka, Japan

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ABSTRACT

Aims: We previously reported the association of single nucleotide polymorphisms in the lymphotoxin alpha (LTα) gene with susceptibility to acute myocardial infarction (AMI) and increased mortality after discharge. In the present study, we investigated whether the adverse effect of LTα C804A polymorphism on mortality could be pharmacologically modified by statin treatment after AMI.

Methods and results: We conducted a multicenter study that included 3486 post-AMI patients between 1998 and 2008. During a median follow-up period of 1775 days, 247 deaths were recorded. The mortality rate was significantly higher in LTα 804A allele carriers compared to non-804A allele carriers (7.9% vs. 5.7%, $p = 0.011$). The LTα 804A allele was significantly associated with increased mortality for post-AMI patients not receiving statins (hazard ratio [HR]: 1.48, 95% confidence interval [CI]: 1.03–2.12, $p = 0.034$), but not for those receiving statins (HR: 1.22, 95% CI: 0.70–2.10, $p = 0.486$). *In-vitro* experimental analyses demonstrated that the LTα 804A polymorphic protein, 26Asn-LTα_3, induced monocyte-endothelial interaction and endoplasmic reticulum (ER) stress in cardiomyocytes more strongly than the LTα_3 804C polymorphic protein 26Thr-LTα_3. However, the effects of both LTα_3 proteins were decreased and became comparable by the pretreatment of cells with pravastatin.

Conclusion: LTα C804A polymorphism was associated with an increased risk of mortality for AMI patients, although this effect was masked in patients treated with statins. This finding is supported by the observed attenuation of 26Asn-LTα_3-mediated monocyte-endothelial interaction and ER stress in cardiomyocytes treated with pravastatin. LTα C804A polymorphism may have potential as a novel therapeutic target for secondary prevention after AMI.

1. Introduction

Myocardial infarction (MI) is one of the major causes of death in developed countries. Although the implementation of evidence-based therapies has greatly reduced mortality [1,2], the long-term mortality rate after discharge for AMI remains high. Because personal risk after AMI survival dramatically varies demographically, the development of improved secondary prevention programs,

Abbreviations: AMI, acute myocardial infarction; Asn, asparagine; CI, confidence interval; ER, endoplasmic reticulum; GRP, glucose-regulated protein; HR, hazard ratio; HUVEC, human umbilical vein endothelial cells; LTα, lymphotoxin alpha; SNPs, single nucleotide polymorphisms; Thr, threonine; TNF, tumor necrosis factor; VCAM1, vascular cell adhesion molecules 1.

* Corresponding author. Department of Evidence-based Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. Tel.: +81 22 717 7152; fax: +81 22 717 7156.
E-mail address: sakatayk@cardio.med.tohoku.ac.jp (Y. Sakata).

[1] On behalf of the Osaka Acute Coronary Insufficiency Study.

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including personalized therapy approaches is necessary to reduce post-AMI mortality. In particular, therapeutic approaches that take into account genetically determined risk may have great potential for personalized treatments that improve adverse outcomes after MI.

Recently, others and we identified that single nucleotide polymorphisms (SNPs) in the genes encoding lymphotoxin alpha (LTα) and its associated proteins are associated with AMI onset [3–8]. LTα is a proinflammatory cytokine with homology to inflammatory cytokine tumor necrosis factor (TNF)-α [9–11] and is related with the development of atherosclerotic lesions in coronary arteries [12,13]. Interestingly, SNPs in the LTα gene are associated with both increased susceptibility to AMI onset post-AMI mortality [14]. Based on the pro-inflammatory characteristics of LTα, we hypothesized that the adverse effects of LTα polymorphism could be mediated by statins, which are one of the most widely prescribed medicines with anti-inflammatory properties [15].

Here, we investigated the pharmacogenetic interactions between LTα C804A polymorphism and statins in post-AMI patients, and attempted to determine the underlying mechanisms through *in-vitro* analyses.

2. Materials and methods

2.1. Epidemiologic data regarding the impact of LTα C804A polymorphism

Among 10,076 consecutive Japanese AMI patients who were registered in the Osaka Acute Coronary Insufficiency Study (OACIS) between 1998 and 2008, we enrolled 3486 patients who were discharged alive and submitted samples for the genetic analysis performed in this study. Details of the OACIS and genotyping have been reported elsewhere [14,16]. All patients provided written informed consent, and the study protocol complied with the Guidelines for Genome/Genetic Research issued by the Japanese government and was approved by the ethics committee of each institution.

The patients were divided into two groups according to the presence (n = 1390; statin(+) group) or absence (n = 2096; statin(−) group) of statin treatment at discharge. In each group, the incidence of all-cause mortality was compared between patients with the AA or CA genotype (A allele carriers) and those with the CC genotype (non-A allele carriers) of LTα C804A polymorphism. LTα C804A polymorphism (rs1041981) was determined using an automated fluorescent allele-specific DNA primer assay (Toyobo Gene Analysis, Tsuruga, Japan) [17].

2.2. Cell culture and materials

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA, USA) and cultured in EGM-2 SingleQuots endothelial cell medium (Clonetics) at 37 °C in a humidified 5% CO_2 incubator. Cells from passages three to eight were used for experiments. THP1 cells, a human acute monocytic leukemia cell line, were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium (Clonetics) containing 10% fetal calf serum, 2 μM glutamine, and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator [12]. Recombinant 26Thr-LTα$_3$ and 26Asn-LTα$_3$ proteins were expressed in *Escherichia coli* using the pET29 system (Novagen, Madison, WI, USA) and purified as previously described [8]. Cytotoxic assays were conducted in the WEHI164s fibrosarcoma cell line (provided by the Institute of Development, Aging and Cancer, Tohoku University). Pravastatin was obtained from Daiichi-Sankyo (Tokyo, Japan).

2.3. Neonatal rat cardiomyocyte preparation

Primary ventricular myocytes were isolated from neonatal rats, purified by Percoll density gradient centrifugation, and pre-plated for 1 h to enrich for cardiac myocytes (95%). The obtained cells were plated at a density of 7.5 × 10^5 cells per well (3.5-cm diameter), and then cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Camarillo, CA, USA) containing 10% fetal calf serum, 2 μM glutamine, and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator [18].

2.4. Monocyte-endothelial cell adhesion assay

We previously reported that LTα$_3$ increases monocyte-endothelial interactions *in vitro* [12]. To investigate the effects of LTα C804A polymorphism and statin treatment on monocyte-endothelial cell interactions, cell adhesion assays involving THP1 cells and HUVEC were conducted. As LTα C804A polymorphism replaces threonine with asparagine at residue 26 (Thr26Asn) in the LTα$_3$ protein, recombinant 26Thr- and 26Asn-LTα$_3$ were compared. For the assay, HUVEC were first cultured in 96-well microplates (Asahi Techno Glass, Tokyo, Japan) for two days to form monolayers, and were then incubated with 1 ng/ml 26Thr-LTα$_3$ or 26Asn-LTα$_3$ for 5 h prior to performing the adhesion assay. The effects of pravastatin treatment on adhesion of THP1 cells to HUVEC were also examined by adding 10 μM pravastatin to HUVEC cultures 12 h before stimulation with LTα$_3$ [12]. Further details of this assay are shown in supplementary method.

2.5. Vascular cell adhesion molecule 1 (VCAM1) assay

To determine the influence of 26Thr-LTα$_3$ and 26Asn-LTα$_3$ on the expression of VCAM1, Western blot analysis was performed as previously described [12]. Briefly, total cellular protein (10–20 μg) was obtained from HUVEC after stimulation with 1 ng/ml 26Thr-LTα$_3$ or 26Asn-LTα$_3$ for 6 h. To evaluate whether the effects of 26Thr-LTα$_3$/26Asn-LTα$_3$ on VCAM1 expression were influenced by pravastatin treatment, 10 μM pravastatin was added 12 h before 26Thr-LTα$_3$/26Asn-LTα$_3$ stimulation of HUVEC cultures. VCAM1 and actin were detected using anti-VCAM1 and anti-actin antibodies, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.6. Monocyte migration assay

The migration of monocytes to HUVEC was analyzed using a modified Boyden chamber method. Briefly, HUVEC were cultured in the lower compartments of 24-well Transwell® microplates (Corning, Acton, MA, USA) for two days to form a monolayer. To investigate the effects of LTα C804A polymorphism on the migration of THP1 cells to HUVEC, HUVEC were incubated with 1 ng/ml 26Thr-LTα$_3$ or 26Asn-LTα$_3$ for 5 h prior to performing the migration assay. We also examined the effects of pravastatin treatment on monocytes migration by adding 10 μM pravastatin to HUVEC cultures 12 h before stimulation with LTα$_3$. Further details of this assay are shown in Supplementary Methods.

2.7. Endoplasmic reticulum (ER) stress in rat neonatal cardiomyocytes

ER stress induced by LTα$_3$ on rat neonatal cardiomyocytes was evaluated by treating rat cardiomyocytes with either 10 ng/ml 26Thr-LTα$_3$ or 26Asn-LTα$_3$ for 24 h. The effects of pravastatin on 26Thr-LTα$_3$/26Asn-LTα$_3$-induced ER stress were evaluated by adding 10 μM pravastatin to cultures of rat cardiomyocytes 24 h before stimulation with LTα$_3$. For Western blot analysis, Glucose-regulated

protein 94 kDa (GRP94) and 78 kDa (GRP78) were detected using anti-GRP94 and anti-GRP78 antibodies, respectively (Assay Designs, Ann Arbor, MI, USA), and GAPDH was detected using anti-GAPDH antibody (Millipore, Billerica, MA, USA).

2.8. Statistical analysis

All statistical analyses were performed with either SPSS 11.0 software (SPSS Inc., Tokyo, Japan) or R software (http://www.R-project.org/). For epidemiologic analysis, discrete variables were expressed as counts or percentages, as indicated, and compared with the χ^2-test. Continuous variables were expressed as the mean ± SD and compared using the unpaired Student's *t*-test. Survival curves were constructed using the Kaplan–Meier method and differences in mortality rates were compared between groups with the Log-rank test. Cox regression analyses were used to determine whether LTα C804A polymorphism was an independent predictor of mortality. For experimental analysis, data are presented as the mean ± SE. Statistical significance was determined by the unpaired Student's *t*-test or one-way ANOVA followed by Bonferroni's correction, except for the analysis of GRP78/94, for which Mann–Whitney's *U* test was used. All reported *p* values are two-sided, and statistical significance was defined as $p < 0.05$.

3. Results

3.1. Impact of LTα C804A polymorphism on mortality and statin treatment in post-AMI patients

Among 10,076 consecutive Japanese AMI patients who were registered in the Osaka Acute Coronary Insufficiency Study (OACIS) between 1998 and 2008, typing for this polymorphism was conducted for 3506 post AMI patients, and successful typing was obtained for 3486 patients, which denotes call rate was 99.4% (Suppl. Fig. 1). The prevalence of the C804A genotypes was as follows: CC 1333(38.2%), CA 1592(45.7%), AA 561(16.1%) ($p = 0.020$ by Hardy–Weinberg equilibrium test) (Suppl. Table 1).

After comparing the incidence of all-cause mortality among patients with the three genotypes of LTα C804A polymorphism, a similar trend of increasing mortality for patients with the AA and CA genotypes was detected (Suppl. Fig. 2). Therefore, we selected LTα C804A polymorphism as a dominant parameter and compared the incidence of all-cause mortality between patients with the AA or CA genotype (A allele carriers) and those with the CC genotype (non-A allele carriers). The baseline characteristics of all subjects and the two patient subgroups, consisting of those with (statin(+)) and without (statin(−)) statin treatment at discharge, are shown in Table 1 and Suppl. Table 2. In both patient subgroups, no significant differences were detected in the baseline characteristics between LTα 804A allele carriers (AA + CA genotypes) and non-804A allele carriers (CC genotypes), except for age and reperfusion therapy. Age was significantly younger and reperfusion rate was significantly higher in the former subgroup.

A total of 247 deaths were recorded during the median follow-up period of 1775 days. Kaplan–Meier curves were constructed for patients based on LTα C804A polymorphism (Fig. 1A), revealing that 804A allele carriers had significantly higher all-cause mortality than that of non-804A allele carriers (7.9% vs. 5.7%, $p = 0.011$). We also plotted Kaplan–Meier curves for all-cause mortality among subgroups that were divided based on treatment with statins at discharge (Fig. 1B). For patients not receiving statin therapy (statin(−)), all-cause mortality was significantly higher in 804A carriers compared to non-804A carriers (9.8% vs. 6.8%, $p = 0.025$), but did not differ among those receiving statin therapy (statin(+)) (5.0% vs. 4.2%, $p = 0.345$), suggesting that the mortality effects of LTα C804A polymorphism are influenced by statin therapy.

Table 1
Patient demographics based on lymphotoxin alpha C804A polymorphism.

	LTA 804 polymorphism		p Value
	Non-A allele carrier (CC)	A allele carrier (AA + AC)	
	N = 1333	N = 2153	
Age (y.o.)	64.7 ± 11.0	63.9 ± 11.4	0.035
Male (%)	77.8	78.1	0.853
BMI (kg/m^2)	23.9 ± 3.4	23.8 ± 3.4	0.686
DM (%)	31.0	34.2	0.053
HT (%)	58.9	59.2	0.869
HL (%)	48.5	47.0	0.404
Smoking (%)	64.6	65.3	0.691
OMI (%)	10.5	11.5	0.343
AP (%)	24.2	24.8	0.695
Admission < 24 h (%)	39.9	38.3	0.335
STEMI (%)	86.4	86.5	0.959
peak CK > 3000 (%)	31.6	34.8	0.061
Killip > 1 at admission (%)	11.8	13.3	0.193
T-Cho (mg/dl) at admission	197.9 ± 42.9	195.6 ± 44.7	0.145
HDL-Cho (mg/dl)	46.8 ± 12.7	46.1 ± 12.3	0.093
Multi-vessel disease (%)	33.9	37.3	0.050
Reperfusion therapy (%)	89.5	91.8	0.023
Stent (%)	78.8	77.9	0.249
Thrombectomy (%)	39.1	39.4	0.883
Final TIMI3 (%)	89.2	88.1	0.338
ACEIs (%)	54.4	54.9	0.768
ARBs (%)	23.9	24.2	0.842
ACEIs or ARBs (%)	76.4	76.6	0.921
Beta blockers (%)	45.0	47.6	0.135
Ca blockers (%)	21.3	21.1	0.904
Statin (%)	41.4	38.9	0.145
Diuretics (%)	24.8	27.7	0.057
Anti-platelets (%)	98.3	98.2	0.800
Nitrates (%)	37.7	39.5	0.304
Anti-coagulants (%)	18.8	18.4	0.816
T-Cho (mg/dl) at discharge	186.9 ± 35.1	185.4 ± 36.4	0.339
HDL-Cho (mg/dl)	38.8 ± 10.7	38.0 ± 10.7	0.110

Patient characteristics between LTα 804A allele carriers (AA + AC) and non-804A allele carriers (CC).
Discrete variables are expressed as counts or percentages, as indicated, and were compared with the χ^2-test. Continuous variables are expressed as the mean ± SD, and were compared with the unpaired Student's *t*-test.
LTα = lymphotoxin alpha; BMI = body mass index; DM = diabetes mellitus; HT = hypertension; HL = hyperlipidemia; OMI = old myocardial infarction; AP = angina pectoris; STEMI = ST elevation myocardial infarction; CK = creatinine kinase; T-Cho = total cholesterol; HDL-Cho = high density lipoprotein cholesterol; PCI = percutaneous coronary intervention; emergent PCI = PCI performed within 24 h after the onset of MI; Multi-vessel disease = two or three vessel disease; Final TIMI3 = final TIMI3 acquisition at reperfusion therapy; ACEI = angiotensin converting enzyme inhibitor; ARB = angiotensin II receptor blocker; Ca blockers = calcium channel antagonists at discharge.

Cox's proportional hazard analysis confirmed that the 804A allele was significantly associated with increased mortality (Table 2, adjusted HR 1.44, 95% CI: 1.06–1.95, $p = 0.018$). Interestingly, this trend was evident for patients without statin treatment at discharge (adjusted HR 1.48, 95% CI: 1.03–2.12, $p = 0.034$), but not for those who were treated with statins at discharge (adjusted HR: 1.22, 95% CI: 0.70–2.10, $p = 0.486$) (Table 2).

3.2. LTα-induced adhesion of THP1 cells to endothelial cells is attenuated by pravastatin

As shown in Fig. 2A, both 26Thr-LTα$_3$ and 26Asn-LTα$_3$, corresponding to LTα 804C- and 804A-polymorphic proteins, respectively, increased the adhesion of THP1 cells to HUVEC monolayers when compared with controls ($p < 0.001$). Notably, exposure of HUVEC to 26Asn-LTα$_3$ led to greater increases in the adhesion rate

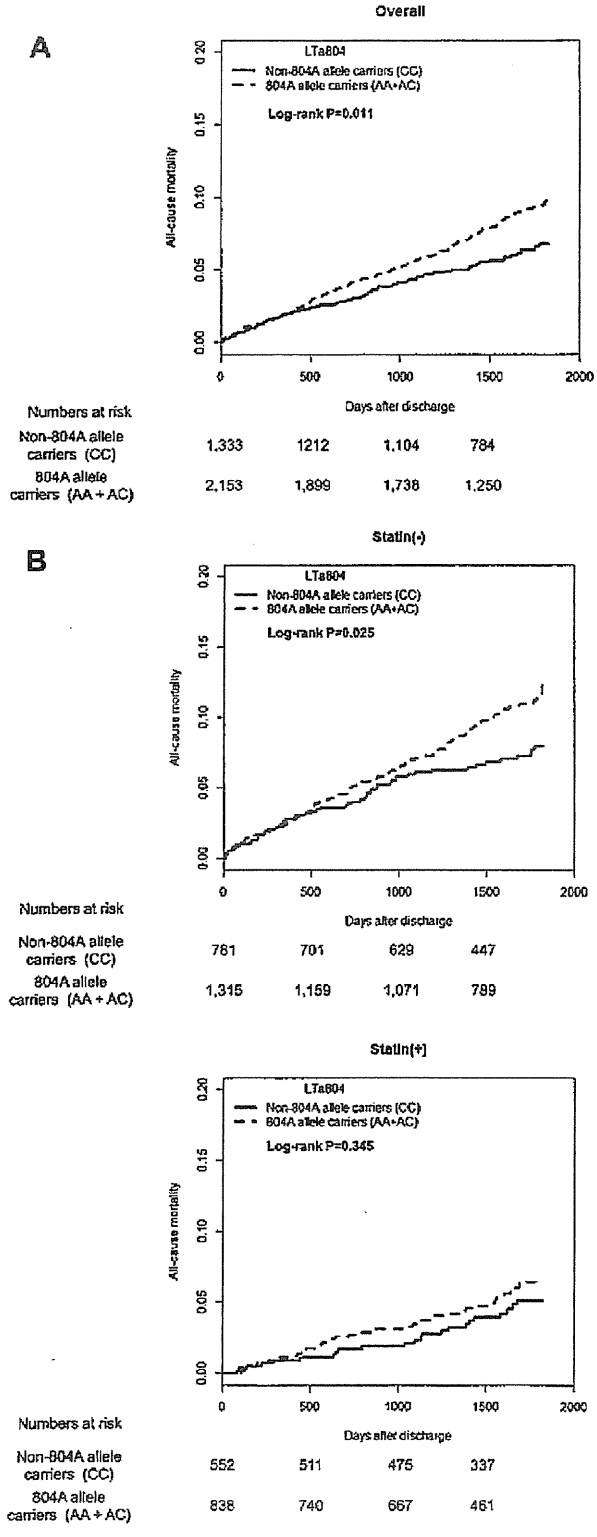

Fig. 1. All-cause mortality for AMI patients in relation to LTα C804A polymorphism and statin treatment. A. All-cause mortality rate was significantly higher in LTα 804A allele carriers (AA + AC, $N = 2153$) (dotted line) than in non-804A allele carriers (CC, $N = 1333$) (solid line) ($p = 0.011$ by the Log-rank test). B. All-cause mortality rate was significantly higher in LTα 804A allele carriers (AA + AC) (dotted line) than in non-804A allele carriers (CC) (solid line) in the subgroup without statin therapy (statin(−)) ($p = 0.025$ by the Log-rank test), whereas no difference was detected between the LTα C804A polymorphism groups for those that received statin therapy (statin(+)) ($p = 0.345$ by the Log-rank test).

Table 2
Hazard ratio for all-cause mortality among LTα 804A allele carriers in the presence or absence of statin prescription at discharge.

	Death	Total *N*	HR	95% CI	*p* Value
Model 1					
Overall	247	3486	1.42	1.08–1.86	0.011
Statin(−)	182	2096	1.44	1.04–1.98	0.026
Statin(+)	65	1390	1.28	0.77–2.12	0.346
Model 2					
Overall	247	3486	1.49	1.14–1.96	0.004
Statin(−)	182	2096	1.54	1.12–2.13	0.008
Statin(+)	65	1390	1.33	0.80–2.20	0.278
Model 3					
Overall	199	3024	1.40	1.04–1.89	0.027
Statin(−)	144	1784	1.48	1.03–2.12	0.034
Statin(+)	55	1240	1.22	0.70–2.10	0.486

Model 1. Hazard ratio of LTα 804A allele carriers (AA + AC) for all-cause mortality (unadjusted).
Model 2. The hazard ratio of LTα 804A-allele carriers on all-cause mortality after being discharged alive was analyzed by Cox regression analysis. Covariates were age and sex.
Model 3. Model 2 + diabetes mellitus, hypertension, hyperlipidemia, smoking history, peak CK ≥ 3,000, multi-vessel disease, reperfusion therapy, and ACEI/ARBs, beta blockers at discharge.
Abbreviations are the same as those used in Table 1.

compared to that of 26Thr-LTα$_3$ (2.36 vs. 2.03 fold, respectively, compared with control; $p < 0.05$), although the rate decreased and was comparable between the groups (1.58 vs. 1.43 fold, respectively, p = n.s.) after the pretreatment of HUVEC with pravastatin. Western blotting revealed that the expression level of VCAM1 on HUVEC was markedly increased by treatment with 26Asn-LTα$_3$ compared to that with 26Thr-LTα$_3$. However, both 26Asn- and 26Thr-LTα$_3$-induced VCAM1 expression was attenuated at similar levels by pravastatin (Fig. 2B).

3.3. LTα-induced monocyte migration is attenuated by pravastatin

In a two-chamber migration assay, the migration rate of THP1 towards the HUVEC-conditioned medium markedly increased by exposure to 26Thr-LTα$_3$ and 26Asn-LTα$_3$ when compared with controls ($p < 0.001$). In the absence of pravastatin pretreatment, the migration ratio displayed a larger increase for 26Asn-LTα$_3$ stimulation compared to 26Thr-LTα$_3$ (2.14 vs. 1.55 fold compared with control, $p < 0.05$), whereas the two LTα proteins displayed similar small effects in the presence of pravastatin (1.48 vs. 1.46 fold compared with control, p = n.s.) (Fig. 2C).

3.4. LTα-induced ER stress in rat cardiomyocytes is attenuated by pravastatin

GRP78 expression levels in rat neonatal cardiomyocytes were increased by LTα$_3$ stimulation after 24 h when compared with controls ($p < 0.001$) (Fig. 3A and B). Exposure to 26Asn-LTα$_3$ led to greater GRP78 expression compared to that induced by 26Thr-LTα$_3$ (1.68 vs. 1.53 fold compared with control, $p < 0.05$ by Mann–Whitney's U test), although no differences in expression were detected after the pretreatment of cardiomyocytes with 10 μM pravastatin (p = n.s.). Similarly, GRP94 expression was also markedly increased following 26Asn-LTα$_3$ stimulation compared to exposure to 26Thr-LTα$_3$ (1.83 vs. 1.59 fold compared with control, $p < 0.05$ by Mann–Whitney's U test) (Fig. 3C and D), and no

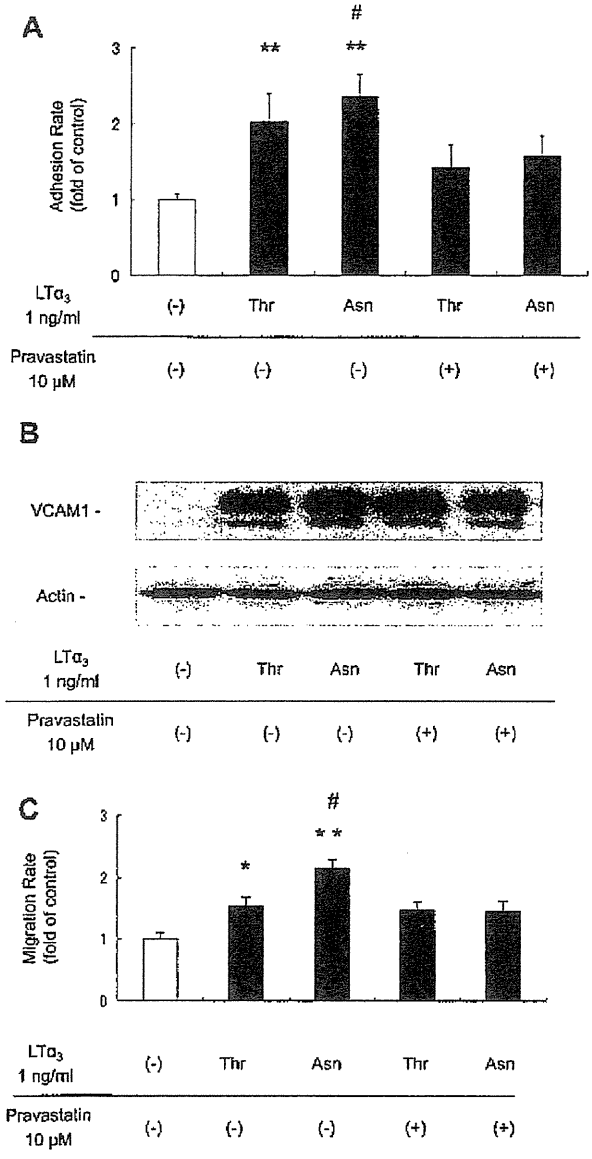

Fig. 2. Effects of LTα C804A polymorphism on monocyte-endothelial adhesion (A) and induction of VCAM1 expression (B) in the absence and presence of pravastatin treatment. Effect of LTα C804A polymorphism on THP1 cell migration rate (C). A. The adhesion rate of THP1 onto HUVEC was increased by both 26Thr-LTα3 and 26Asn-LTα3 stimulation of HUVEC. The adhesion rate was higher when cells were stimulated by 26Asn-LTα3 than by 26Thr-LTα3 (**$p < 0.001$ vs. control, #$p < 0.05$ vs. 26Thr-LTα3 protein by the unpaired t-test), but the difference was reduced by the pretreatment of cells with 10 μM pravastatin. B. The expression levels of VCAM1 were increased by 26Thr-LTα3 or 26Asn-LTα3 stimulation in comparison with control. 26Asn-LTα3 led to greater induction of VCAM1 expression compared to 26Thr-LTα3, but VCAM1 was expressed at comparable levels for both proteins following the pretreatment of HUVEC with 10 μM pravastatin. A representative result among five independent experiments is shown. C. The migration rate of THP1 towards HUVEC was increased by both 26Thr-LTα3 and 26Asn-LTα3, although the rate was higher for cells stimulated with 26Asn-LTα3 (2.14 vs. 1.55 fold when compared with control, **$p < 0.001$ vs. control, *$p < 0.01$ vs. control, and #$p < 0.05$ vs. 26Thr-LTα3 by the unpaired t-test). However, no significant differences were detected between the two proteins following the pretreatment of HUVEC with 10 μM pravastatin (1.48 vs. 1.46 fold when compared with control by the unpaired t-test).

differences in expression were detected after pravastatin pretreatment (p = n.s.).

4. Discussion

We previously reported the clinical impacts of polymorphisms in the genes encoding LTα and its related proteins on susceptibility to AMI and increased post-AMI mortality. The results of the present study further underline the importance of the LTα cascade in the pathogenesis of cardiovascular diseases by showing that a functional SNP in the LTα gene at C804A, which replaces threonine with asparagine at residue 26 (Thr26Asn), is associated with increased all-cause mortality after AMI. Furthermore, our findings suggest that LTα gene polymorphism has clinical significance as a therapeutic target, because a reduction of 26Asn-associated mortality risk by statin treatment was suggested in the cohort of post-AMI patients, as well as in the *in-vitro* experimental studies.

We found that LTα C804A polymorphism is also associated with increased mortality among AMI patients registered in OACIS, a prospective observational study of AMI being conducted in Osaka, Japan. However, this result was not entirely unexpected, as we previously reported that A252G polymorphism in the LTα gene, which has a strong linkage disequilibrium with C804A, is associated with increased post-AMI mortality [14]. Therefore, the most important finding of the present study was the demonstration of the pharmacological modification of the C804A-related mortality risk with statin treatment in the secondary prevention setting of post-AMI. Our findings also suggest that the pharmacological modification mediated by statin might be attributable to decreased monocyte-endothelial interaction and ER stress in cardiomyocytes.

The growth of atherosclerotic lesions involves the inflammatory response and is characterized by the adhesion of monocytes onto endothelial cells, migration of monocytes into intima, scavenging of lipoprotein particles, followed by the formation of foam cells and secretion of various pro-inflammatory cytokines [19]. We recently demonstrated that LTα3 stimulation induces the expression of various genes involved in inflammation and cell adhesion, including VCAM1, which is a key player in the binding of monocytes to endothelial cells [20], in HUVEC and human coronary arterial endothelial cells [12,21]. In the present study, the stimulation of HUVEC with LTα 804A polymorphic protein (26Asn-LTα3) markedly increased both the adhesion and the migration of monocytes to endothelial cells in comparison with LTα 804C polymorphic protein (26Thr-LTα3). Notably, the differences between 26Asn- and 26Thr-LTα3 were attenuated by the pretreatment of cells with pravastatin, a result that is consistent with our epidemiologic data. Additionally, we performed the experiments to elucidate whether or not LTα3 could alter the transformation of monocytes into macrophages by the observation of morphological change with microscopy and by using fluorescent-labeled oxidized low-density lipoprotein (DiI-Ox-LDL, Biomedical Technologies Inc. Stoughton, MA, USA) followed by fluorescence activated cell sorting (FACS). However, we observed no transformation of monocytes into macrophages after LTα3 stimulus, whereas Phorbol 12-myristate 13-acetate (PMA) stimulus induced the transformation.

Several reports have described the role of LTα in inflammation and atherosclerosis. Although the LTα signaling pathway is considered to be similar in part to that of TNFα, LTα appears to play a dominant role in the regulation of atherosclerotic lesion growth. Schreyer et al. [13] showed that TNFα deficiency did not alter lesion development in mice fed an atherogenic diet, whereas the loss of LTα resulted in a three-fold decrease in the amount of lesions. In addition, we previously revealed that LTα is expressed in the atherosclerotic plaques of patients with coronary artery disease [8]. The present data suggest that the increased risk of mortality

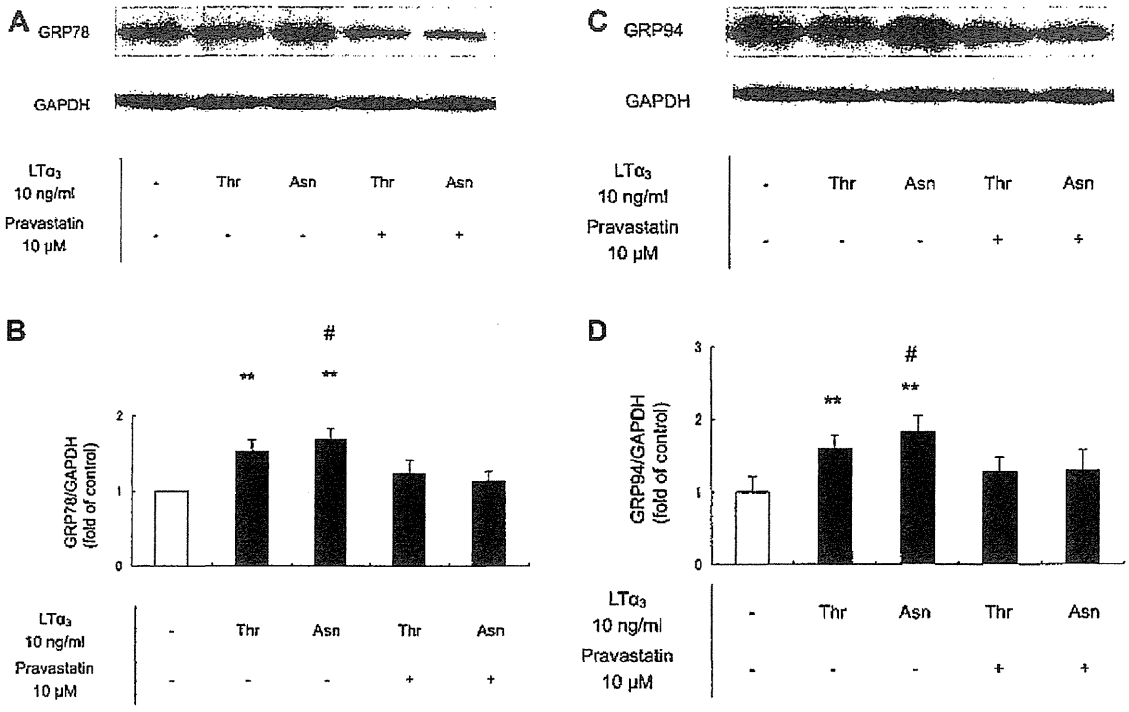

Fig. 3. Effect of LTα C804A polymorphism on the induction of ER stress in rat neonatal cardiomyocytes. The expression levels of glucose-regulated protein (GRP) 78 were increased in rat cardiomyocytes by stimulation with either 26Thr-LTα$_3$ or 26Asn-LTα$_3$ when compared with control cells (**$p < 0.001$) (A and B). 26Asn-LTα$_3$ had a greater effect on GRP78 expression compared to 26Thr-LTα$_3$ (1.68 vs. 1.53 fold when compared with control, #$p < 0.05$), but GRP78 expression induction was attenuated by 10 μM pravastatin pretreatment for both 26Thr-LTα$_3$ and 26Asn-LTα$_3$ (p = n.s.). The expression levels of GRP94 were also increased by 26Thr-LTα$_3$ or 26Asn-LTα$_3$ stimulation when compared with control cells (**$p < 0.001$) (C and D), but were higher for 26Asn-LTα$_3$ compared to 26Thr-LTα$_3$ (1.83 vs. 1.59 fold, #$p < 0.05$). The induction of GRP94 expression was also attenuated by the pretreatment of cardiomyocytes with pravastatin (p = n.s.).

associated with LTα C804A polymorphism can be lowered by statin treatment, and that the beneficial effects of statins for 804A allele carriers might be attributable to the attenuation of monocyte-endothelial interactions and cardiomyocyte degeneration. Previous experimental studies have revealed that statins attenuate the inflammatory response, including the decreased expression of adhesion molecules triggered by TNFα via the NFκB signaling pathway [22–25]. Considering that LTα shares major receptors (TNF receptors type I and II) with TNFα [11], it is reasonable to speculate that statins attenuate the LTα-induced inflammatory response, thereby preventing atherogenesis and cardiovascular events. Our present experimental data support this idea, as 26Asn-LTα$_3$ stimulation led to greater human monocyte-endothelial adhesion and monocyte migration compared to 26Thr-LTα$_3$, and the effects induced by both 26Asn- and 26Thr-LTα$_3$ were blunted and became comparable following pravastatin treatment.

Inflammation induced by pro-inflammatory cytokines is one of the major causes of ER stress, which is characterized by the accumulation of unfolded proteins induced by stimuli such as oxidative stress and ischemia. As ER stress in cardiomyocytes is followed by cardiac remodeling and heart failure [18,26,27], we evaluated whether LTα polymorphism is associated with the occurrence of ER stress. ER stress triggers the unfolded protein response, which involves a group of signal transduction pathways that ameliorate the accumulation of unfolded proteins in the ER by increasing the expression of ER-resident chaperones, such as GRP78/94. Thus, enhanced expression of GRP78/94 can be used as a marker of ER stress. Here, we found that ER stress in rat cardiomyocytes was significantly elevated by 26Asn-LTα$_3$ stimulation compared with that by 26Thr-LTα$_3$, and that these differences were blunted by pravastatin treatment. These findings may partly explain our observation that LTα 804A allele carriers had higher mortality after MI in the absence of statin treatment.

The preventive effects of statin therapy on cardiovascular events have been demonstrated in many clinical studies and are thought to be mediated, at least in part, by the pleiotropic effects, including anti-inflammatory and lipid-lowering activities [28–30]. However, despite considerable research efforts, the pleiotropic effects of statins remain poorly characterized and have yet to be demonstrated in the clinical setting [15]. In the present study, no significant differences were detected in the average total and HDL-cholesterol level between 804A allele and non-804A allele carriers in both statin(+) and statin(−) subgroups, suggesting that LTα polymorphism does not modify cardiovascular risk by altering serum cholesterol levels. Therefore, we speculate that statins may mask the adverse effects of LTα polymorphisms via their pleiotropic properties, independently of their lipid-lowering effects.

Several limitations of our study warrant mention. First, the epidemiologic analyses were based on an observational study, and statin prescriptions were not randomized. However, the bias was likely minimal, because statins were prescribed by attending physicians without knowledge of patients' LTα genotype, and as a consequence, statin prescription was naturally "genetically randomized". Second, data on the daily doses, adherence, and discontinuation of statin treatment after discharge were lacking, despite the possibility that these factors may have modified the actual impact of statin therapy on LTα polymorphism. Third, although we found that LTα polymorphism is associated with mortality after the onset of AMI in our patient cohort comprised of Asian people who were predominantly Japanese, further analysis is

needed to verify the results in other ethnic groups. Fourth, the study included only the patients with informed consent, who were in relatively less severity after the onset of MI, which is also a study limitation.

In conclusion, our results demonstrate that the LTα 804A allele is significantly associated with increased mortality in post-AMI patients, a finding that may be attributable to the increased mortality risk of the 804A allele in the absence of statin treatment. Therefore, post-AMI patients with LTα C804A polymorphism may be good candidates for pharmacological intervention with statins to improve long-term mortality. Although further investigations are needed, genetic polymorphisms of LTα, including C804A, may represent suitable therapeutic targets in the near future.

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Conflict of interest

None of the authors have conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.01.020.

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Impact of Beta Blockade Therapy on Long-Term Mortality After ST-Segment Elevation Acute Myocardial Infarction in the Percutaneous Coronary Intervention Era

Daisaku Nakatani, MD, PhD[a], Yasuhiko Sakata, MD, PhD[a,b,d,e,*], Shinichiro Suna, MD, PhD[a], Masaya Usami, MD[a], Sen Matsumoto, MD[a], Masahiko Shimizu, MD, PhD[a], Masahiko Hara, MD[a], Masaaki Uematsu, MD, PhD[f], Masatake Fukunami, MD, PhD[g], Toshimitsu Hamasaki, PhD[c], Hiroshi Sato, MD, PhD[h], Masatsugu Hori, MD, PhD[i], and Issei Komuro, MD, PhD[a], on behalf of the Osaka Acute Coronary Insufficiency Study (OACIS) Investigators[†]

Although clinical guidelines recommend long-term β-blocker (BB) therapy to decrease mortality after acute myocardial infarction, these recommendations are based predominantly on evidence from before the reperfusion and thrombolytic eras. To investigate the effects of BB therapy for patients with acute myocardial infarctions on mortality in the percutaneous coronary intervention era, a total of 5,628 consecutive patients who were admitted <24 hours after the onset of ST-segment elevation myocardial infarction, treated with emergent percutaneous coronary intervention, and discharged alive were studied. During a median follow-up period of 1,430 days, mortality rates did not differ between patients with and without BB therapy (5.2% vs 6.2%, p = 0.786). Multivariate analysis revealed that BB treatment was not associated with a reduced risk for mortality (hazard ratio 0.935, 95% confidence interval 0.711 to 1.230, p = 0.534). The results of propensity score matching also indicated that the mortality rates did not differ between the 2 groups. However, subgroup analyses among matched populations revealed that BB treatment was associated with a significantly lower mortality risk for high-risk patients, who were defined as those with Global Registry of Acute Coronary Events (GRACE) risk scores ≥121 (hazard ratio 0.596, 95% confidence interval 0.416 to 0.854, p = 0.005) or those administered diuretics (hazard ratio 0.602, 95% confidence interval 0.398 to 0.910, p = 0.016), but not for lower risk patients. In conclusion, BB treatment was associated with reduced long-term mortality in patients after ST-segment elevation myocardial infarction at higher risk, but not in those at lower risk. Although randomized controlled studies are warranted to confirm these results, the implementation of BB therapy for discharged patients with ST-segment elevation myocardial infarction may need to be assessed on the basis of individual mortality risk in the percutaneous coronary intervention era. © 2013 Elsevier Inc. All rights reserved. (Am J Cardiol 2013;111:457–464)

Under current clinical guidelines, oral β-blocker (BB) therapy is widely recommended for indefinite long-term use in all patients who recover from ST-segment elevation myocardial infarction (STEMI) and do not have contraindications.[1–4] However, these recommendations are based predominantly on evidences obtained before the reperfusion and thrombolytic eras,[5–9] and few data have been collected in the percutaneous coronary intervention (PCI) era. Recent advances in the management of STEMI, particularly the use of primary PCI, have significantly reduced long-term mortality.[10,11] Because these treatment advances potentially mask the mortality benefits of BB therapy, reassessing the efficacy of BB exposure for patients who survive STEMI is warranted. In this study, we investigated the relation

[a]Department of Cardiovascular Medicine, [b]Department of Advanced Cardiovascular Therapeutics, and [c]Department of Biomedical Statistics, Osaka University Graduate School of Medicine, Suita, Japan; [d]Department of Evidence-Based Cardiovascular Medicine, and [e]Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan; [f]Cardiovascular Center, Kansai Rosai Hospital, Amagasaki, Japan; [g]Division of Cardiology, Osaka General Medical Center, Osaka, Japan; [h]School of Human Welfare Studies Health Care Center and Clinic, Kwansei Gakuin University, Nishinomiya, Japan; and [i]Osaka Prefectural Hospital Organization, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan. Manuscript received June 27, 2012; revised manuscript received and accepted October 30, 2012.

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See page 463 for disclosure information.

*Corresponding author: Tel: 81-6-6879-6612; fax: 81-6-6879-6613.
E-mail address: sakatayk@cardiology.med.osaka-u.ac.jp (Y. Sakata).

[†]A list of the Osaka Acute Coronary Insufficiency Study (OACIS) Investigators appears in the Appendix.

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between BB treatment at discharge and long-term mortality for consecutive patients with STEMI enrolled in the Osaka Acute Coronary Insufficiency Study (OACIS).

Methods

The OACIS is a prospective, multicenter observational study of consecutive patients with acute myocardial infarctions (AMIs) at 25 collaborating hospitals located in the Osaka region of Japan and is registered with the University Hospital Medical Information Network Clinical Trials Registry in Japan (UMIN000004575). One of the main aims of the OACIS is to examine the effects of cardiovascular prevention drugs on secondary prevention after AMI in the contemporary clinical setting. A detailed description of the OACIS has been published elsewhere.[12] The study protocol was approved by the ethics committee of each participating hospital, and each patient provided written informed consent.

Among the 10,074 patients registered in the OACIS registry from April 1998 to April 2011, we identified 5,628 consecutive patients who were admitted <24 hours of the onset of STEMI, treated with emergent PCI, and discharged alive.

Investigative cardiologists and research coordinators recorded demographic and clinical data for patients during the period of hospitalization. After discharge, further data were obtained at 3 and 12 months after AMI and annually thereafter for up to 5 years. Thrombolysis In Myocardial Infarction (TIMI) and Global Registry of Acute Coronary Events (GRACE) risk scores were calculated with multiple imputation for each patient as described elsewhere.[13–15] The left ventricular ejection fraction was assessed using echocardiography before discharge using the Teichholz method. The primary end point of this study was all-cause death, which was categorized as cardiac, noncardiac, or unknown.

Categorical variables were compared using chi-square tests with continuity correction or Fisher's exact tests. Continuous variables are presented as medians (interquartile range [IQR]) or as mean ± SD and were compared using unpaired Student's *t* tests or 2-tailed Wilcoxon's rank-sum tests between patients with and those without oral BB treatment at discharge. To minimize differences in baseline characteristics between the 2 groups, patients were matched in a 1-to-1 manner on the basis of propensity scores, which were calculated for each patient using a logistic regression model[16] that included a total of 32 variables (baseline demographics, angiographic parameters, and medication at discharge), as listed in Table 1. The variables inserted into the multivariate models to calculate propensity scores were determined after screening for multicollinearity. According to the propensity score, patients were selected using a 5-to-1 digit-matching technique using the nearest neighbor method.[17,18] The area under the receiver-operating characteristic curve and the Hosmer-Lemeshow goodness-of-fit statistic were calculated to assess the performance and calibration of the model, respectively. Mortality rates were determined using Kaplan-Meier curves and were compared using log-rank tests. Cox regression analyses were performed to assess whether BB therapy was associated with a reduced risk for mortality. Variables with p values <0.20 before matching in univariate analyses were included in the multivariate Cox regression models.[19] Propensity score was incorporated as a variable into the models before matching. To identify high-risk populations according to GRACE scores, classification and regression trees for survival data (survival CART) were used.[20] Survival CART analysis revealed that the first split point to partition the mortality risk for patients without BB treatment among the matched populations was a GRACE risk score of 121 and that the second and third split points for each subgroup were risk scores of 100 and 141, respectively. Therefore, the mortality benefits of BB therapy at discharge were initially compared between patients with GRACE risk scores <121 and ≥121 and then among those with scores of <100, 100 to 120, 121 to 140, and ≥141. Subgroup analysis was performed in patients after propensity score matching to identify patients having a mortality benefit of BB treatment. All analyses were performed using PASW Statistics version 18 (SPSS, Inc., Chicago, Illinois) or SAS version 9.1.3 (SAS Institute Inc., Cary, North Carolina). Statistical significance was defined as p <0.05. For the subgroup analyses, p values <0.05 and p values for interactions <0.10 were considered as statistically significant.

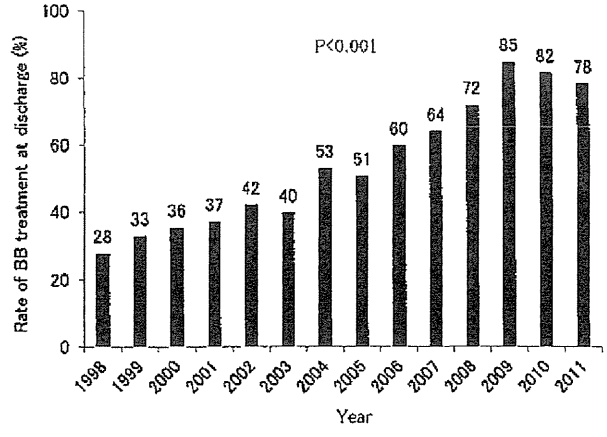

Figure 1. Trend in the annual prescription rate (1999 to 2011) of BBs at discharge in post-AMI patients.

Results

Among the 5,628 study patients, 2,880 (51.2%) were prescribed oral BB therapy at discharge after STEMI. In the BB group, 2,075 (72.0%), 559 (19.4%), 135 (4.7%), 33 (1.1%), and 78 (2.7%) patients received carvedilol, metoprolol, bisoprolol, atenolol, and other BBs, respectively. A trend of increased prescription of BB at discharge by year was clearly evident until 2009, as shown in Figure 1 (p <0.0001). After 2009, approximately 80% of patients received BB treatment. In addition, several significant differences in the baseline characteristics between patients in the BB and non-BB groups were detected (Table 1). Notably, patients in the BB group were more often men, had higher body mass indexes and TIMI and GRACE risk scores, and displayed higher frequencies of hypertension, dyslipidemia, cardiopulmonary arrest on arrival, and Killip class ≥II. With regard to angiographic findings, a greater number of BB group patients had multivessel disease and culprit lesions

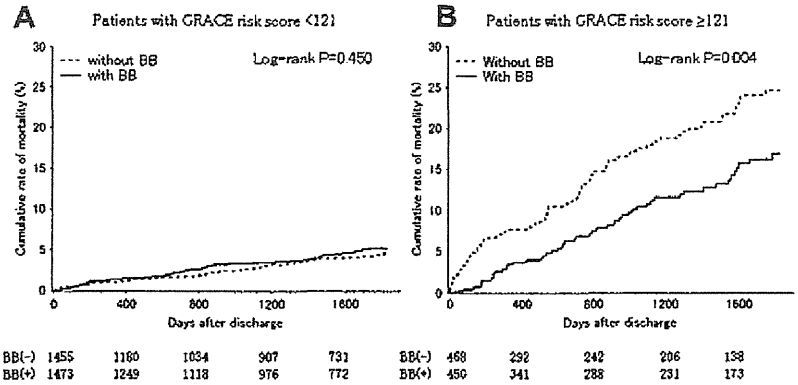

Figure 3. Cumulative incidence of mortality in patients with STEMIs who underwent PCI and were discharged alive with GRACE risk scores <121 *(A)* and ≥121 *(B)*. *Solid and dashed lines* indicate BB and non-BB treatment, respectively. Numbers below the x axis indicate the number of patients in each group at risk at the indicated time.

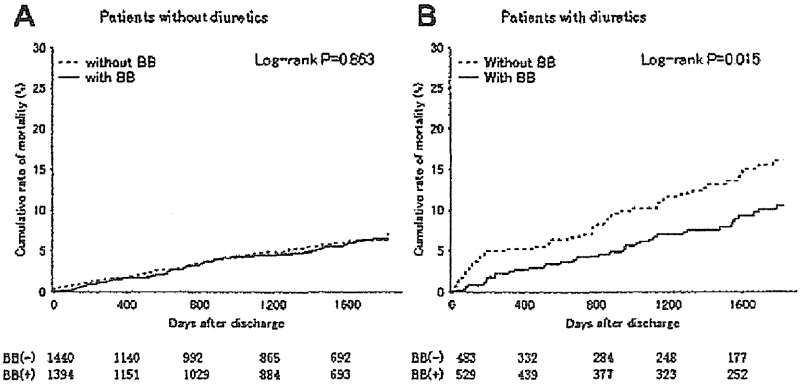

Figure 4. Cumulative incidence of mortality in patients with STEMIs who underwent PCI and were discharged alive treated without *(A)* or with *(B)* diuretics after propensity score matching. *Solid and dashed lines* indicate BB and non-BB treatment, respectively. Numbers below the x axis indicate the number of patients in each group at risk at the indicated time.

interval 0.711 to 0.739), and the p value of the Hosmer-Lemeshow test was 1.000. A total of 3,846 patients with well-matched baseline characteristics, with the exception of year, culprit lesion involving the left anterior descending coronary artery, peak creatine kinase, and prescription of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, were identified between the 2 groups, (Table 1). However, no marked differences in mortality rates were detected between the groups (Table 2, Figure 2). Among patients after propensity score matching, the prescription of BBs at discharge was associated with lower long-term mortality in high-risk patients, who were defined as those with GRACE risk scores ≥121 (Figure 3) or those who were prescribed diuretics (Figure 4), with significant p values for interaction (p = 0.013 and p = 0.077, respectively; Figure 5). Patients with GRACE risk scores ≥121 or those administrated diuretics were more likely to have histories of myocardial infarction (22.2% vs 7.9%, p <0.001, for GRACE score ≥121 vs <121; 17.8% vs 9.2%, p <0.001, for diuretics vs no diuretics), Killip class ≥II on admission (45.6% vs 5.3%, p <0.001, for GRACE score; 31.1% vs 9.1%, p <0.001, for diuretics), and greater peak creatine phosphokinase values (2,709 IU/L [IQR 1,442 to 4,518] vs 2,431 IU/L [IQR 1,192 to 4,261], p <0.001, for GRACE score; 3,549 IU/L [IQR 1,995 to 5,964] vs 2,206 IU/L [IQR 1,080 to 3,809], p <0.001, for diuretics). Kaplan-Meier estimates and Cox regression analysis for the subgroups partitioned by CART analysis suggested that an association existed between BB treatment and reduced mortality for patients with GRACE risk scores ≥121 (Figure 5), particularly for those with scores of 121 to 140 (Table 4).

Discussion

In the present study, we examined the relation between BB therapy and long-term mortality after STEMI in a real-world population of the contemporary PCI era. The results revealed that BB treatment at discharge was associated with decreased mortality in post-STEMI patients at higher risk, but not in those at lower risk. Although further randomized controlled studies are warranted, our findings may suggest reevaluation of the current guidelines, which generally recommend implementing BB therapy for all post-STEMI patients.[1–4,21]

The findings of large clinical trials conducted before the reperfusion and thrombolytic eras confirmed that BB treatment at discharge improved survival in post-AMI patients.[5–9] In the Beta-Blocker Heart Attack Trial (BHAT),

Cardiovascular Project, a retrospective analysis published in 1998 that included >200,000 post-AMI patients, were 14.4% and 23.9% for BB and non-BB patients, respectively, even in low-risk patients.[25] Therefore, it is likely that the recent decrease in long-term mortality may have masked the beneficial effects of BB therapy rather than indicating a change in the efficacy of BB therapy. Indeed, in non-BB group patients at low risk (GRACE score <121) or without diuretics, mortality rates during a median follow-up period of 3.9 years were only 3.2% and 4.8%, respectively (Figures 3 and 4).

Importantly, the present findings also indicate that BB therapy at discharge has beneficial effects for high-risk patients, whose mortality rates remained relatively high throughout the follow-up period. Among patients with GRACE risk scores ≥121, or those taking diuretics, BB group patients had a significantly lower mortality risk than non-BB group patients. Together, these results indicate that in high-risk patients, the beneficial effects of BB therapy may outweigh the risks, even in the contemporary PCI era. For patients taking diuretics, BB therapy resulted in a lower risk for mortality than for patients not taking diuretics. One possible reason may be that patients taking diuretics had more severe conditions. Indeed, patients taking diuretics have had higher rates of history of myocardial infarction, Killip class ≥II on admission, and greater peak creatine phosphokinase compared to those not taking diuretics. Another possibility may be that there could have been an interaction between BBs and diuretics on reduced risk for mortality, although there is no evidence for this. Despite the observed benefits of BB therapy in high-risk patients, it is disputable whether BBs should be prescribed to those at extremely high risk. Through the application of survival CART analysis,[26,27] we identified that patients with GRACE risk scores of 121 to 140 experienced the greatest benefit from BB treatment. In this subgroup of patients, the mortality rate in a matched population was approximately 56% lower for those treated with BBs than those without, whereas no significant mortality benefit was detected in patients with GRACE risk scores ≥141 (Table 4). Accordingly, these results also suggest that the prescription of BBs should be considered with caution, particularly for patients at extremely high risk.

Several limitations of the present study warrant mention. First, our study was not a randomized controlled study, and thus, potential biases in measured and unmeasured variables may have existed. For example, we lacked information on contraindications to BB treatment, such as bronchial asthma, arteriosclerosis obliterans, and severe bradycardia. Second, no data were available for the timing of BB therapy initiation during hospitalization. Third, we lacked data on several factors, including the daily doses, adherence, and discontinuation of BB treatment after discharge in the BB group and on the initiation of BB treatment in the non-BB group after discharge, which may have modified the actual clinical impact of BB therapy on mortality. However, it has been reported that adherence to BB treatment, unlike other cardiovascular secondary prevention medications, is not associated with reduced 30-month outcomes for post-AMI patients in the overall population as well as patients stratified by various concomitant medication use,[28] suggesting that the influence of adherence on treatment outcomes was minimal. Forth, the left ventricular ejection fraction was assessed by echocardiography using the Teichholz method, an M-mode technique widely used in large trials with limited reliance on geometric assumptions. Therefore, caution is needed when interpreting the data compared to those obtained from other methods of assessing of the left ventricular ejection fraction, such as 3- or 2-dimensional echocardiography, radionuclide ventriculography, and magnetic resonance ventriculography.[29]

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Disclosures

The authors have no conflicts of interest to disclose.

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