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ORIGINAL ARTICLE

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The white blood cell count is an independent predictor of no-reflow and mortality following acute myocardial infarction in the coronary interventional era

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BACKGROUND. In the era before the use of coronary reperfusion therapy, an elevated white blood cell (WBC) count was associated with a higher risk of adverse events following acute myocardial infarction (AMI). However, the relationship between WBC count and prognosis after AMI has not been investigated since coronary intervention was introduced.

AIM. To evaluate whether a high WBC count within 48 hours of the onset of AMI predicts future adverse events in patients undergoing percutaneous coronary intervention (PCI).

METHOD. We evaluated 1,016 patients who underwent PCI in the acute phase of MI using the Japanese Acute Coronary Syndrome Study (JACSS) database.

RESULTS. WBC count was significantly associated with smoking, sudden onset AMI, and the no-reflow phenomenon during PCI, as were age, peak creatine kinase level, and Killip class. An elevated WBC count was significantly associated with higher risk of in-hospital mortality. Patients in the highest quartile of WBC count were about three times more likely to have a poor prognosis after AMI compared to those in the lowest quartile.

CONCLUSIONS. The WBC count is of great significance for stratifying patient risk and can be used as a universal marker for predicting future adverse events following any treatment for AMI.

Keywords: acute myocardial infarction (AMI); coronary intervention; mortality; no-reflow phenomenon; white blood cell (WBC) count

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Introduction

Inflammation has been demonstrated to be a powerful risk factor for the development of coronary artery disease (1–3). It is well recognized that various markers of inflammation and acute-phase reactants such as C-reactive protein, serum amyloid A, and von Willebrand factor are closely related to atherosclerosis (1–5). Recently, there has been a renewed interest in the white blood cell (WBC) count as a prognostic indicator of acute coronary syndrome (6). The WBC count is a simple and universally available marker of inflammation and acute-phase reactants. The association between WBC count and the increased risk of developing acute myocardial infarction (AMI) has been noted since the 1970s (7). An elevated WBC

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Abbreviations and acronyms

AMI	Acute myocardial infarction
BMI	Body mass index
CK	Creatine phosphokinase
IRCA	Infarct-related coronary artery
JACSS	Japanese Acute Coronary Syndrome Study
LAD	Left anterior descending artery
LVEF	Left ventricular ejection fraction
MACE	Major adverse cardiac events
PCI	Percutaneous coronary intervention
TIMI	Thrombolysis in myocardial infarction
WBC	White blood cell

Key messages

- The WBC count is a strong and independent predictor of the no-reflow phenomenon during PCI and is closely related to long-term mortality as well as in-hospital adverse events.
- The WBC count measured in the acute phase of MI is of great significance for stratifying patient risk and can be used as a universal marker for predicting future adverse events following any treatment for AMI.

count was also associated with a higher risk of adverse events following AMI in the era before coronary reperfusion therapy (8–10). Various factors such as the severity of the diseased coronary artery, impaired epicardial and myocardial perfusion, and infarct size are postulated to be involved in the higher risk of adverse events. However, the detailed mechanism responsible for poor outcome in patients having elevated WBC count is currently unknown.

Prompt restoration of myocardial blood flow is the therapeutic goal for AMI because early reperfusion by means of aggressive coronary intervention has decreased the mortality rate (11). On the other hand, activated leukocytes can cause reperfusion injury by hypercoagulation, no-reflow, and cardiotoxicity through oxygen-derived free radicals and proinflammatory cytokines (12–15). Therefore, the purpose of the present study was to evaluate whether the WBC count in the acute phase of MI predicts adverse in-hospital clinical outcomes and long-term prognosis in the coronary interventional era.

Patients and methods

Data sources

The Japanese Acute Coronary Syndrome Study (JACSS) is a retrospective and multicenter observational study conducted at 35 medical institutions in Japan. The JACSS database includes 1,640 consecutive patients hospitalized at the participating institutions within 48 hours after the onset of symptoms of AMI from January to December 2001. AMI was defined as raised myocardial enzyme concentrations, with either typical chest pain persisting longer than 30 minutes or electrocardiographic changes (including ischemic ST-segment depression, ST-segment elevation, or pathologic Q waves). Raised enzyme concentrations were defined as peak creatine phosphokinase (CK) levels more than twice the normal upper limit. Medical doctors abstracted demographic, clinical, and treatment variables from the clinical records of each institution and entered them directly into a computer database using interactive software. The study protocol was reviewed and approved by the ethical committee of each participating institution.

Patients

The subjects of the present study were restricted to patients whose blood samples were collected on admission to the hospital for the measurement of a WBC count and who underwent percutaneous coronary intervention (PCI) as soon as possible after admission. The present study included 1,016 out of the 1,640 patients whose records are contained in the JACSS. The remaining 624 patients were excluded because PCI was not performed in the acute phase of MI (464 patients) and/or a WBC count was not measured (192 patients). Patients who were treated with antihypertensive drugs or whose baseline blood pressure was $\geq$140/90 mm Hg were considered to be hypertensive. Diabetes mellitus was diagnosed according to the World Health Organization criteria (16). Hypercholesterolemia was defined as total cholesterol $\geq$ 220 mg/dL and/or triglyceride $\geq$ 150 mg/dL. Cigarette smoking was defined as active smoking, obesity as body mass index $\geq$ 25 kg/m^2, and family history as death of family members due to ischemic heart disease.

WBC count and other laboratory data

Blood samples for measurements of the WBC count and other biochemical assessments were obtained intravenously immediately after admission. The WBC count and other biochemical assessments were measured using the standard technique adopted by the individual institutions and WBC was expressed as absolute count per mm^3. The WBC count was treated both as a continuous variable and as a categorical variable, divided into quartiles according to the leukocyte count.

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Coronary angiography and coronary intervention

Emergent coronary revascularization therapy using PCI was performed in all patients in the acute phase of MI. The allocation of coronary angiography and reperfusion therapy was determined by the physician's decision. The perfusion grade of the infarct-related artery was assessed in accordance with the Thrombolysis in Myocardial Infarction (TIMI) study classification (17). Angiographical no-reflow was considered to be present if the perfusion of the infarct-related artery was TIMI grade 0, 1 or 2 despite the absence of coronary stenosis ≥ 50%, flow-limiting coronary dissection, or hypotension after PCI. Treatment of no-reflow, including intracoronary infusion of vasodilators, was at the physician's discretion. Final TIMI flow grade was assessed on the final shot of the emergent coronary angiography.

In-hospital major adverse cardiac events and follow-up

The primary end point was in-hospital mortality from any cause. Major adverse cardiac events (MACE), including cardiac death, reinfarction, unstable angina, heart failure, and stroke, were also assessed during hospitalization. We also evaluated the mortality following AMI during long-term follow-up. Patients in this analysis were followed with an average duration of 373 ± 204 days (maximum 701 days). The primary end point of this analysis was death (total mortality).

Statistical analysis

Subjects in the analysis were divided into four quartiles according to their leukocyte count. Differences in frequencies were analyzed by the chi-square method. The comparisons of continuous data were performed by one-way analysis of variance (ANOVA) followed by Scheffé's F test.

Clinical characteristics considered to be associated with WBC count were included in the models. These characteristics included age, gender, family history, medical history (hypertension, diabetes mellitus, hypercholesterolemia), smoking, admission characteristics (body mass index (BMI), serum creatinine level, previous myocardial infarction, preinfarction angina), time from symptom onset to PCI, coronary angiographic findings (multi-vessel involvement, culprit location, occlusion of the infarct-related coronary artery (IRCA) in the acute phase, no-reflow phenomenon during PCI), peak CK level and left ventricular ejection fraction (LVEF) in the acute phase of MI and at follow-up. Killip classification on hospital admission, depending on the clinical manifestations of cardiac failure, was also assessed (Killip I, no heart failure; Killip II, S_3 and/or basal lung crepitations; Killip III, acute pulmonary edema; and Killip IV, cardiac shock) (18). Odds ratios (OR) and 95% confidence intervals (CI) of in-hospital adverse events were calculated using logistic regression analysis.

We plotted cumulative event curves using the Kaplan-Meier survival method and tested differences between the curves for statistical significance by log-rank analysis. Cox proportional-hazards analysis was performed to assess the risk of total death by WBC count quartile. Differences with $P < 0.05$ were considered statistically significant in all analyses. All data were expressed as mean ± SD.

Results

Clinical and angiographic demographics

The WBC count ranged from 1,190 to 30,700 per mm^3. The median WBC count was 9,800 per mm^3 and the interquartile interval was 7,800 to 12,200 per mm^3 (Fig 1). Table 1 shows the clinical and angiographic characteristics of the patients by quartiles of the WBC count. Male patients had a higher WBC count than female patients. A higher WBC count was also observed in younger patients with diabetes mellitus, hypercholesterolemia, greater BMI, and current smoking. Approximately 40% of all patients had preinfarction angina but a higher WBC count was observed in patients without preinfarction angina (sudden onset AMI). The WBC count was significantly higher in patients with higher Killip class and occlusion of IRCA on acute phase. No-reflow during PCI and lower final TIMI flow grade was observed in patients with a higher WBC count. A higher peak CK level was also confirmed in patients with a higher WBC count.

Relationship between WBC count and in-hospital adverse events

A higher WBC count was observed in patients with in-hospital adverse events. Patients in Quartile 3 and 4 were more than three times more likely to have in-hospital death compared with those in Quartile 1 (Table 2).

Clinical determinants of WBC count and long-term mortality

To assess the determinants of the high WBC count at the time of admission to the hospital, a multiple regression analysis was performed after a stepwise regression analysis including all clinical variables in Table 1. The results revealed that a high WBC count was significantly associated with age, smoking, peak CK level, Killip class, no-reflow during PCI, and

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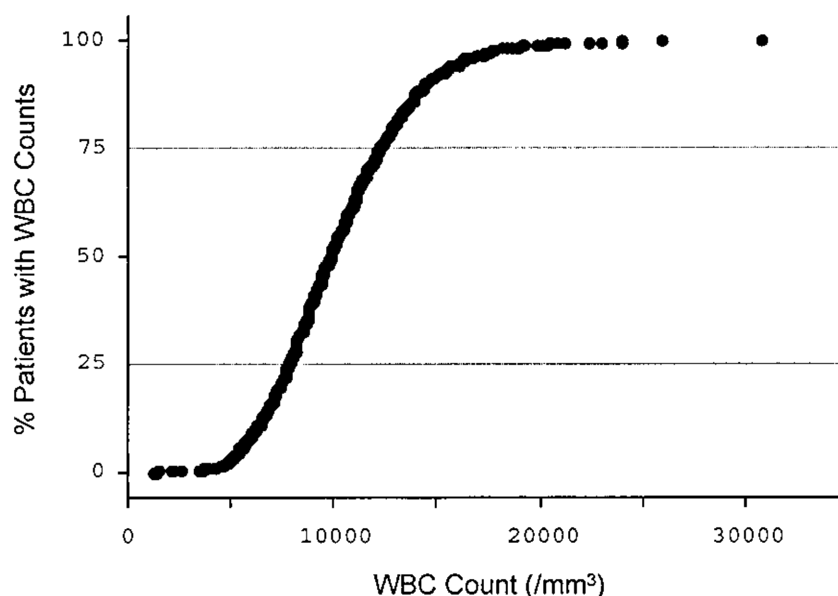

Figure 1. Cumulative distribution of baseline WBC count in 1,016 study patients. Median WBC count was 9,800 and mean WBC count was 10,161 ± 3,433/ mm^3. The 25th and 75th percentiles of WBC count were 7,800 and 12,200/mm^3, respectively.

sudden onset AMI (without preinfarction angina) (r = 0.3967, $P < 0.0001$) (Table 3).

The event-free survival curves of the four groups are shown in Figure 2. Patients in Quartile 4 had a hazard ratio of 2.9 compared with those in Quartile 1 for death following AMI, after adjusting for independent factors which were closely associated with the WBC count (Table 4).

Table 1. Clinical and angiographic characteristics of AMI by quartiles of a WBC count.

	Quartile 1 (≤7800) (*n* = 254)	Quartile 2 (7840–9750) (*n* = 253)	Quartile 3 (9800–12100) (*n* = 255)	Quartile 4 (≥12200) (*n* = 254)
Age, years	71 ± 10	69 ± 12	65 ± 12*†	62 ± 12*†
Male sex, %	67	68	75	82*†§
Hypertension, %	56	56	58	56
Diabetes mellitus, %	28	31	28	36*§
Hypercholesterolemia, %	28	29	36	39*†
Body mass index, kg/m^2	23.2 ± 3.2	23.3 ± 3.3	23.6 ± 3.2	24.1 ± 3.7*
Current smoking, %	29	42*	53*†	60*†
Family history, %	13	12	11	13
Serum creatinine, mg/dL	1.1 ± 1.3	1.0 ± 0.6	1.0 ± 0.8	1.0 ± 0.5
Previous MI, %	11	13	9	13
Pre-infarction angina, %	42	41	39	31*†
Time from symptom onset to PCI, hr	5.4 ± 8.1	5.8 ± 7.4	6.1 ± 7.7	5.9 ± 7.9
Killip class II-IV, %	11	19	21	26*†§
Coronary multi-vessel involvement, %	46	47	44	50
Culprit LAD location, %	45	51	46	52
Occlusion of IRCA on acute phase, %	63	65	69	73*†
No-reflow during PCI, %	12	13	14	19*†
Final TIMI flow grade 0–2,%	8	8	11	17*†
Peak CK (*n* = 990), IU/L	2255 ± 2461	2892 ± 2219	3315 ± 2620*	3879 ± 3415*†
LVEF				
acute phase (*n* = 396), %	56 ± 18	55 ± 20	61 ± 20	56 ± 19
discharge (*n* = 478), %	56 ± 13	53 ± 13	53 ± 12	51 ± 12

* $P < 0.05$ *versus* Quartile 1; † $P < 0.05$ *versus* Quartile 2; § $P < 0.05$ *versus* Quartile 3 as results of chi-square method between quartiles or one-way analysis of variance (ANOVA) followed by Scheffé's F test. AMI = acute myocardial infarction; CK = creatine phosphokinase; IRCA = infarct-related coronary artery; PCI = percutaneous coronary intervention; LAD = left anterior descending artery; LVEF = left ventricular ejection fraction; TIMI = thrombolysis in myocardial infarction; WBC = white blood cell.

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Table 2. In-hospital adverse events by quartiles of WBC count.

	Cardiac death				MACE				Total death			
	n (%)	OR	95% CI	*P*	*n* (%)	OR	95% CI	*P*	*n* (%)	OR	95% CI	*P*
Quartile 1 (≤7800) (*n* = 254)	7 (3)	1.00	-	-	19 (7)	1.00	-	-	8 (3)	1.00	-	-
Quartile 2 (7840–9750) (*n* = 253)	8 (3)	1.152	0.411–3.227	0.7874	17 (7)	0.891	0.452–1.757	0.7388	11 (4)	1.398	0.553–3.535	0.4794
Quartile 3 (9800–12 100) (*n* = 255)	16 (6)	2.362	0.955–5.845	0.0629	30 (12)	1.649	0.902–3.014	0.1040	24 (9)	3.125	1.407–7.255	0.0055
Quartile 4 (≥12200) (*n* = 254)	24 (9)	3.682	1.557–8.709	0.0030	35 (14)	1.977	1.098–3.559	0.0231	26 (10)	3.507	1.556–7.904	0.0025

Odds Ratio (OR) based on a logistic regression analysis regarding in-hospital adverse events (cardiac death, MACE, and total death). MACE = major adverse cardiac events; WBC = white blood cell.

Discussion

In the present study, we found a strong association between elevated WBC count on admission and adverse clinical outcomes in patients who underwent PCI in the acute phase of MI. Patients with a WBC count in the highest quartile had a total mortality rate about three times higher than that of patients with a WBC count in the lowest quartile.

Numerous studies have documented an increased WBC count as a predictor of poor prognosis after AMI (19–22). Recent reports also confirmed the short-term prognostic importance of WBC counts measured during the acute phase of MI (11, 23–25). Here we clarified that a high WBC count is still associated with a poor prognosis after AMI even with coronary intervention as it is following thrombolytic therapy or without the use of coronary reperfusion therapy. Age, smoking, peak CK level, Killip class, no-reflow phenomenon during PCI, and sudden onset AMI (without preinfarction angina) were significantly correlated with the WBC count on admission to the hospital following AMI.

Barron et al. postulated that the mechanism by which a high WBC count leads to increased mortality is through a hypercoagulable or thromboresistant state (24). They found that an increased WBC count was related to lower rates of coronary patency and augmented thrombus burden in patients with AMI treated with thrombolytics (24). We have previously reported that coronary plaque rupture, which we confirmed pathologically through necropsies, leads to the onset of AMI without preinfarction angina and is aggravated by smoking and a high WBC count (26). It may be hypothesized that the greater thrombus formation at the site of the ruptured plaque is mediated by the increased expression of tissue factor on leukocytes in the setting of AMI (27). The procoagulant activity of circulating leukocytes may also play an important role in the thrombus formation (12).

In addition to the reduced patency and greater thrombus burden in patients with an elevated WBC count, these patients had poorer downstream microvascular perfusion as assessed by TIMI perfusion grade (28). The mechanism by which neutrophils cause this phenomenon is unclear. However, Engler et al. have documented that reperfusion following prolonged ischemia leads to neutrophils binding to the ischemic endothelium, which in turn results in progressive leukocyte capillary plugging and the no-reflow phenomenon (13). In animal models of ischemia-reperfusion, neutrophils appear to lead to infarct expansion, and neutrophil depletion is associated with a marked reduction in infarct size (29, 30). In the present study, more than 70% of patients with the no-reflow phenomenon during PCI never achieved a flow TIMI grade 3; however, patients with a no-reflow phenomenon in the lower WBC count groups (Quartile 1 and 2) were significantly more likely to

Table 3. Multiple regression analysis for a WBC count on admission.

Independent variables	Regression coefficients	Standard error	95% CI	*P*
Age	−68.703	8.765	−85.904–51.503	<0.0001
Smoking	907.709	208.547	498.460–1316.958	<0.0001
Peak CK	0.161	0.038	0.087–0.235	<0.0001
Killip class	403.491	122.396	163.302–643.679	0.0010
No-reflow phenomenon during PCI	632.912	280.917	81.646–1184.178	0.0245
Sudden onset AMI (without preinfarction angina)	456.715	205.851	52.756–860.674	0.0267

P values as results of a multiple regression analysis after a stepwise regression including all variables in Table 1. AMI = acute myocardial infarction; CK = creatine phosphokinase kinase; PCI = percutaneous coronary intervention; WBC = white blood cell.

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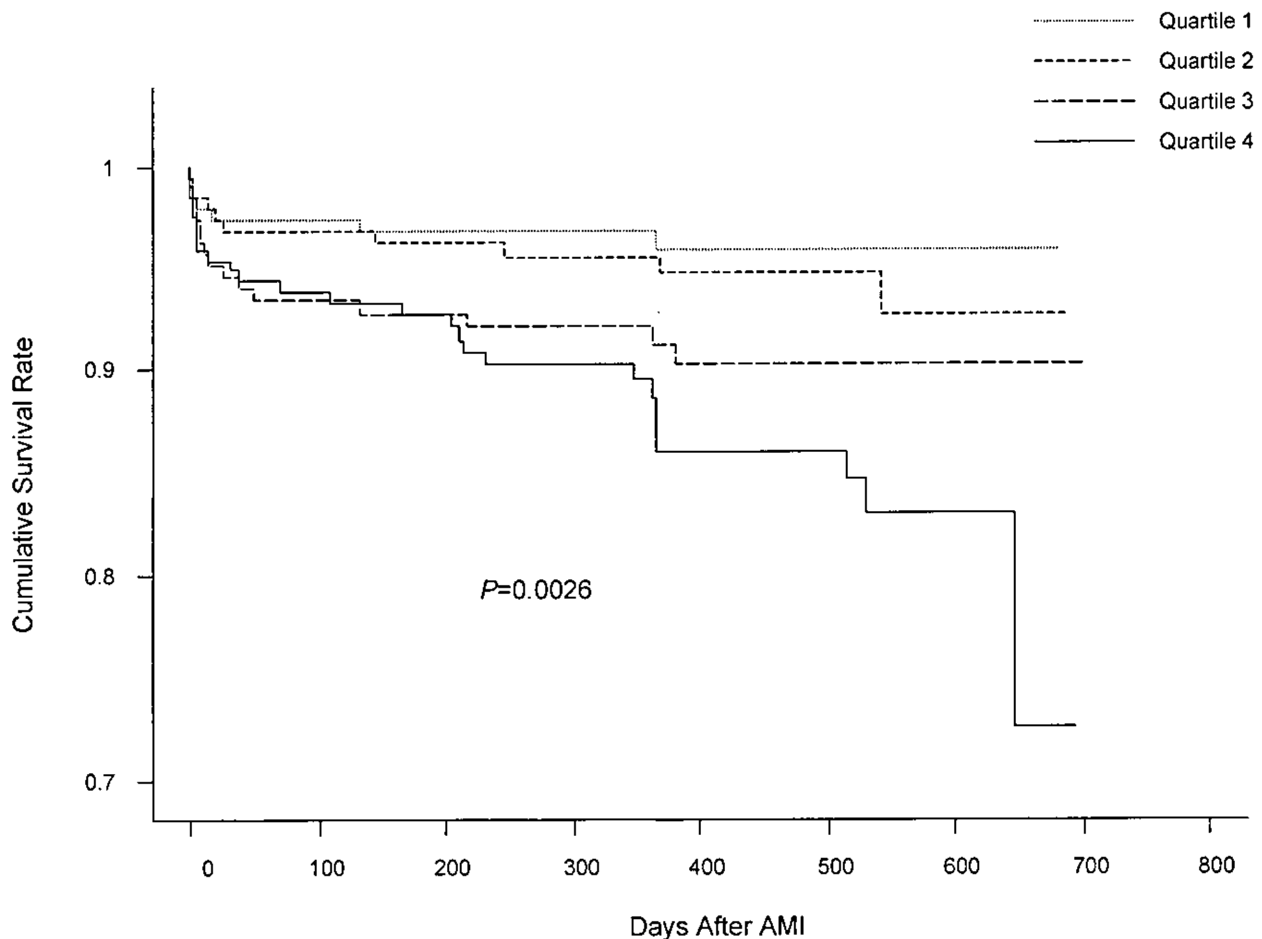

Figure 2. Event-free survival of patients after AMI by quartile of a WBC count.

achieve complete reperfusion compared to those in the higher WBC count (Quartile 3 and 4) (34% *versus* 16%, $P = 0.0144$). Incomplete reperfusion of the infarct-related coronary artery is associated with poor prognosis after AMI and is strongly affected by infarct size. The WBC count at the time of admission to the hospital and the infarct size as measured by the peak CK level showed a significantly positive correlation ($r = 0.2203$, $P < 0.0001$). An increased WBC count showed a weak but significant negative correlation with lower left ventricular ejection fraction at discharge ($r = -0.1079$, $P = 0.0183$). Therefore, the association between WBC count and mortality may be explained by the larger infarct size. Note that we did not collect information regarding the WBC count differential, which may have contributed important additional information. However, it is helpful to stratify patient risk after AMI with a WBC count to predict future adverse events.

This study is limited by being a retrospective, multicenter study. However, this study included all patients with AMI in the database who underwent coronary intervention during 2001. All patients were followed after the onset of AMI, so that the results of

Table 4. Association between WBC group and long-term mortality

	Total death		Unadjusted			Adjusted		
	n	%	OR	95% CI	*P*	OR	95% CI	*P*
Quartile 1 (≤7800) (*n* = 197)	9	5	1.000	-	-	1.000	-	-
Quartile 2 (7840–9750) (*n* = 193)	14	7	1.378	0.524–3.621	0.5154	1.253	0.475–3.308	0.6488
Quartile 3 (9800–12100) (*n* = 195)	24	12	2.307	0.949–5.609	0.0652	2.092	0.810–5.404	0.1274
Quartile 4 (≥12200) (*n* = 207)	36	17	3.647	1.583–8.404	0.0024	2.897	1.177–7.130	0.0206

Hazard ratios compared with Quartile 1 regarding long-term mortality after unadjusting and adjusting for independent factors which are closely associated with a WBC count on admission in a multiple regression analysis. WBC = white blood cell.

the present study should reflect the actual conditions of patients with AMI in the coronary intervention era.

In conclusion, a WBC count measured within 48 hours of the onset of AMI is a strong and independent predictor of the no-reflow phenomenon during PCI, and furthermore is closely related to the risk of long-term mortality and in-hospital adverse events. Although the results of the present study are similar to those of previous studies with conservative treatments or thrombolytic agents, the present data also have important clinical implications for stratifying patient risk according to the WBC count even when they receive coronary intervention. The WBC count measured in the acute phase of MI is of great significance and can be used as a universal marker for predicting future adverse events following coronary interventional treatment, as well as following conservative or thrombolytic treatment for AMI.

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Appendices

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bjh research paper

Molecular mimicry by *Helicobacter pylori* CagA protein may be involved in the pathogenesis of *H. pylori*-associated chronic idiopathic thrombocytopenic purpura

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Summary

The eradication of *Helicobacter pylori* often leads to platelet recovery in patients with chronic idiopathic thrombocytopenic purpura (cITP). Although this clinical observation suggests the involvement of *H. pylori*, little is known about the pathogenesis of cITP. We initially examined the effect of *H. pylori* eradication on platelet counts in 20 adult Japanese cITP patients. Then, using platelet eluates as the probe in immunoblot analyses, we examined the role of molecular mimicry in the pathogenesis of cITP. *Helicobacter pylori* infection was detected in 75% (15 of 20) of cITP patients. Eradication was achieved in 13 (87%) of the *H. pylori*-positive patients, seven (54%) of which showed increased platelet counts within the 4 months following treatment. Completely responsive patients also showed significant declines in platelet-associated immunoglobulin G (PAIgG) levels. Platelet eluates from 12 (nine *H. pylori*-positive and three *H. pylori*-negative) patients recognized *H. pylori* cytotoxin-associated gene A (CagA) protein, and in three completely responsive patients, levels of anti-CagA antibody in platelet eluates declined after eradication therapy. Cross-reactivity between PAIgG and *H. pylori* CagA protein suggests that molecular mimicry by CagA plays a key role in the pathogenesis of a subset of cITP patients.

Keywords: chronic idiopathic thrombocytopenic purpura, *Helicobacter pylori*, molecular mimicry, cytotoxin-associated gene A protein.

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Idiopathic thrombocytopenic purpura (ITP) is an acquired autoimmune disorder characterized by a low platelet count and mucocutaneous bleeding. The cause is destruction of platelets in the reticuloendothelial system mediated by platelet-bound autoantibodies (Cines & Blanchette, 2002). The targets of the anti-platelet autoantibodies include a variety of platelet proteins, among them glycoproteins IIb/IIIa, Ib/IX, Ia/IIa and IV. Several groups have recently reported that eradication of *Helicobacter pylori* leads to platelet recovery in patients with chronic ITP (cITP) (Gasbarrini *et al*, 1998; Grimaz *et al*, 1999; Tohda & Ohkusa, 2000; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002). Although these clinical observations suggest the involvement of *H. pylori*, little is known about the mechanisms responsible for triggering production of the anti-platelet autoantibodies involved in the pathogenesis of cITP.

The gram-negative bacterium *H. pylori* is the human pathogen responsible for chronic gastritis and peptic ulcers; moreover, infection with this organism also increases the risk of gastric cancer and mucosa-associated lymphoid tissue lymphoma (Suerbaum & Michetti, 2002). During the gastritis,

H. pylori infection induces production of anti-gastric epithelial autoantibodies through a process of molecular mimicry involving the gastric epithelium and one or more *H. pylori* antigens (Negrini *et al*, 1996). Notably, *H. pylori* has also been implicated in the pathogenesis of some autoimmune diseases, such as rheumatoid arthritis, autoimmune thyroiditis and Sjögren's syndrome (Gasbarrini & Franceschi, 1999). In the present study, we therefore examined the effect of *H. pylori* eradication in a group of Japanese cITP patients with the aim of understanding better the role an autoimmune response mediated by molecular mimicry in the pathogenesis of cITP.

Patients and methods

Patients

Twenty adult cITP Japanese patients (five men and 15 women; mean age, 53 years) with cITP were enrolled prospectively. ITP was defined by thrombocytopenia (platelet count $<120 \times 10^9$/l) without megakaryocytic hypoplasia in the bone marrow, and by exclusion of other causes. Patients with secondary autoimmune thrombocytopenia were excluded. Platelet-associated immunoglobulin G (PAIgG) levels were determined using an enzyme-linked immunosorbent assay (normal range: 9·0–25·0 ng/10^7 platelets).

^{13}C-urea breath tests (Otsuka, Tokyo, Japan) were used to diagnose *H. pylori* infection. Eradication of *H. pylori* was assessed 8 weeks after treatment using the same test. The clinical responses to *H. pylori* eradication were evaluated 4 months after treatment: a complete response was defined as an increase in the platelet count to more than 120×10^9/l; a partial response was defined as an increase $>20 \times 10^9$/l above pretreatment platelet counts.

The regimen for *H. pylori* eradication, which entailed administration of clarithromycin (400 mg twice daily), amoxicillin (1500 mg twice daily) and lansoprazole (60 mg twice daily) for 7 d, was administered to both *H. pylori*-positive and -negative patients. During the study period, no other new therapies for ITP were added, although patients who were receiving maintenance therapy for ITP continued to do so with no changes.

This study was approved by the Institutional Review Board of Yamaguchi University Hospital; informed consent was obtained from all participants according to the terms of the Declaration of Helsinki.

Platelet eluates

Platelets were harvested from 40 ml of whole blood in ethylenediaminetetraacetic acid (EDTA), washed four times with phosphate-buffered saline (PBS) containing 2% EDTA and 15% acid-citrate-dextrose, and resuspended in PBS containing 0·2% bovine serum albumin (BSA). PAIgG was eluted from 5×10^7 washed platelets using ether according to the method of von dem Borne *et al* (1980), after which the eluates were stored at −20°C until use.

Cell lysates from H. pylori

Helicobacter pylori (NCTC11637) was cultured in Brucella-broth as described previously (Okamoto *et al*, 2002). To obtain cell lysates, the cells were washed twice with 50 mmol/l PBS and suspended in 10 mmol/l PBS, after which they were incubated with lysozyme for 20 min at room temperature, sonicated, and centrifuged at 8000 × g for 15 min at 4°C. The resultant supernatant was collected as the cell lysate.

Immunoblot analysis

Helicobacter pylori cell lysates were subjected to 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0·1% Tween 20 (TBS-T) and then incubated for 12 h at 4°C with platelet eluates from 5×10^7 washed platelets in 4 ml of TBS containing 3% BSA. After washing the labelled membranes with TBS-T, they were incubated for 1 h with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) polyclonal antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA).

Immunoprecipitation

Samples of cell lysate containing 1 mg of *H. pylori* protein in 500 μl of PBS were incubated for 2 h at 4°C with 2 μg of goat anti-cytotoxin-associated gene A (CagA) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), after which immunoprecipitation was facilitated with protein G-Sepharose (Amersham Biosciences, Piscataway, NJ, USA). The immune complexes were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analysed by immunoblotting with platelet eluates.

Statistical analysis

Differences between *H. pylori*-positive and *H. pylori*-negative patients with respect to age, gender, disease duration and platelet count were analysed using non-parametric Mann–Whitney *U*-tests, as were differences in PAIgG levels in responding and non-responding patients before treatment. PAIgG levels before and after treatment were compared using the Wilcoxon signed-rank sum test. *P*-values <0·05 were considered significant.

Results

Patient characteristics and outcome

The characteristics and outcomes of all 20 cITP patients studied are shown in Table I. Before treatment, both infected and uninfected patients were PAIgG-positive and showed no

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Table I. Characteristics and outcomes of patients.

Patient*	Age (years)	Sex	Contraction period (months)	Previous treatment†		Eradication	Platelet count‡			PAIgG (ng/10⁷plts)		Response§
				Therapy	Response§		Before	1 month	4 month	Before	4 month	
P1	48	F	72	PSN, S	NR	Yes	36	183	134	87·9	30·2	CR
P2	64	F	126	–	–	Yes	57	114	127	54·7	60·2	CR
P3	70	F	120	PSN, K	NR	Yes	17	137	211	46·7	23·8	CR
P4	67	M	25	–	–	Yes	76	156	196	29·8	26	CR
P5	46	F	12	PSN	PR	Yes	27	229	276	59·3	22·2	CR
P6	17	F	35	PSN	PR	Yes	66	220	198	53·1	20·3	CR
P7	61	M	37	–	–	Yes	33	78	80	137·7	106·9	PR
P8	54	M	108	–	–	Yes	18	16	13	297·9	411·7	NR
P9	44	F	106	PSN, S	NR	Yes	6	6	2	1006·1	853·1	NR
P10	37	F	24	–	–	Yes	103	95	106	64·4	34·8	NR
P11	62	F	156	PSN	NR	Yes	10	22	26	241	103·6	NR
P12	52	F	108	PSN	NR	Yes	45	45	44	218	125·6	NR
P13	61	F	23	PSN	PR	Yes	49	52	29	71·8	209·7	NR
P14	49	F	180	PSN	NR	No	32	30	28	74·6	54·8	NR
P15	63	M	60	–	–	No	23	30	46	70·8	89·9	PR
N1	28	F	27	PSN, Ig, K	NR		12	15	20	520	142·5	NR
N2	26	F	84	PSN	PR		111	117	83	65·9	114·2	NR
N3	55	M	216	–	–		8	10	8	172·1	212·8	NR
N4	64	F	181	PSN, S	NR		23	22	24	45·6	61·4	NR
N5	57	F	252	PSN	PR		42	43	42	250·5	623·7	NR

*P and N indicate positive and negative for *H. pylori* infection respectively.
†PSN, prednisolone; S, splenectomy; Ig, intravenous immunoglobulin; K, kami-kihito herbal medicine.
‡Platelet counts ($\times 10^9$/l) before, 1 month and 4 months after eradication.
§CR, complete response; PR, partial response; NR, no response.

significant differences with respect to age, gender, disease duration or platelet count. The prevalence of *H. pylori* infection was 75% (15 of 20), and bacterial eradication was achieved in 13 (87%) of the *H. pylori*-positive patients. Four months after treatment, 7 (54%) of the patients in whom *H. pylori* had been eradicated showed increased platelet counts. Of those, six responded completely and one showed a partial response. In addition, one patient (P15) in whom eradication was not achieved also showed a partial response. Despite our failure to eradicate *H. pylori* in that patient, the urea breath test levels decreased after treatment. All patients who responded completely showed recovery of platelet counts within 1 month after eradication. By contrast, none of the five *H. pylori*-negative patients showed improvement in their platelet counts, despite receiving the same treatment regimen.

The PAIgG levels also declined significantly in responding patients following *H. pylori* eradication; no significant decline was observed in non-responding patients (Table II). Notably, the pretreatment levels of PAIgG were significantly lower in responding patients than in non-responding ones (P = 0·016, Table II).

Table II. Comparison of PAIgG levels.

	PAIgG (mean ± SEM, ng/10⁷ platelets)		
	Before treatment	After treatment	*P*-value
Responding patients (n = 6)	67·0 ± 13·5	41·4 ± 12·1	0·04
Non-responding patients (n = 14)	252·3 ± 79·1	245·7 ± 73·8	0·87

H. pylori immunoblot analysis

The presence of antibodies recognizing both platelets and *H. pylori* antigens was evaluated by subjecting platelet eluates from 18 cITP patients to Western blot analysis. Because of severe thrombocytopenia or platelet aggregation, sufficient platelets for analysis could not be obtained from two patients (P9 and P11). Platelet eluates from 12 (nine *H. pylori*-positive and three *H. pylori*-negative) patients recognized one or more *H. pylori* proteins, and all 12 eluates recognized a single 140 kDa *H. pylori* protein (Fig 1A). Contamination of the platelet eluates by serum IgG was excluded, as none of the eluates from three *H. pylori*-positive non-thrombocytopenic volunteers recognized any *H. pylori* proteins (Fig 1B).

The CagA antigen is a highly antigenic *H. pylori* protein. Its molecular weight,140 kDa (Fig 1C, lane 1), suggested that it might be the protein recognized by the platelet eluates from

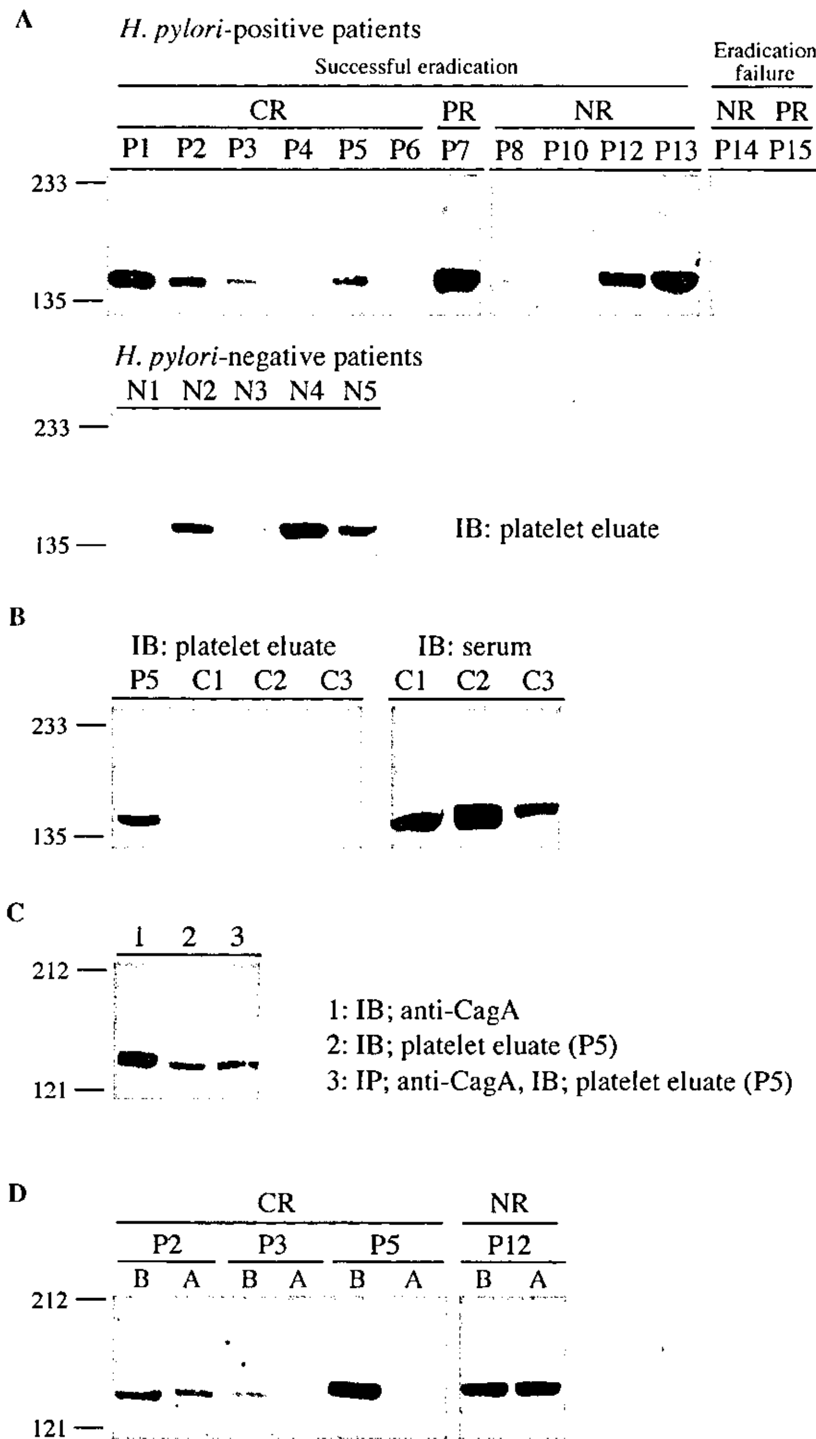

Fig 1. Immunoblot and immunoprecipitation assays. (A) *Helicobacter pylori* proteins were separated by SDS-PAGE and immunoblotted with platelet eluates (PAIgG) from *H. pylori*-positive (P1-P15) and *H. pylori*-negative (N1-N5) cITP patients. (B) *H. pylori* proteins were also immunoblotted with platelet eluates and serum from *H. pylori*-positive non-thrombocytopenic volunteers (C1-C3). P5 is shown as a positive control. (C) *H. pylori* proteins were immunoblotted with anti-CagA antibody (lane 1) or platelet eluate from a *H. pylori*-positive cITP patient (P5) (lane 2). In lane 3, total *H. pylori* lysate was subjected to immunoprecipitation with anti-CagA antibody and then immunoblotted with platelet eluate from P5. (D) Comparison of the levels of anti-CagA antibody present in platelet eluates before and after *H. pylori* eradication. *H. pylori* proteins were immunoblotted with platelet eluates from completely responsive patients (P2, P3, and P5) and one unresponsive patient in whom eradication was successful (P12). B and A, before and after eradication respectively: CR, complete response; PR, partial response; NR, no response; IB, immunoblot; IP, immunoprecipitation. Numbers on the left indicate molecular weights in kDa. For all immunoblot analyses, eluates from 5×10^7 platelets were used as primary antibodies.

cITP patients (e.g., P5) (Fig 1C, lanes 1, 2). This idea was substantiated when the CagA protein that was immunoprecipitated using a specific anti-CagA antibody was recognized by platelet eluate (Fig 1C, lane 3). Furthermore, levels of anti-CagA antibody decreased in platelet eluates from three patients who responded completely to eradication therapy (P2, P3 and P5). By contrast, no reduction in anti-CagA antibody was observed in the eluate from a non-responding patient (P12), although the *H. pylori* was successfully eradicated (Fig 1D).

Discussion

Several clinical studies have demonstrated a beneficial effect of *H. pylori* eradication on platelet recovery in cITP patients

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(Gasbarrini *et al*, 1998; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002) and a similarly good response rate (54%) was achieved in the present study. Moreover, by treating *H. pylori*-negative patients with the same regimen of antibiotics, we showed that the drugs themselves exerted no direct pharmacological effects leading to improved platelet counts, which confirmed that the efficacy of the treatment was eradication-dependent and therefore limited to *H. pylori*-positive cITP patients. Thus, to relieve the autoimmunity seen in patients with *H. pylori*-associated cITP, it is essential to eliminate the persistent infection.

There are a variety of infectious organisms that express molecular mimic antigens involved in the pathogenesis of autoimmune diseases (Wucherpfennig, 2001) – e.g. rheumatic fever, Guillain-Barré syndrome (Yuki *et al*, 1993) and immune thrombocytopenia (Bettaieb *et al*, 1992; Bettaieb *et al*, 1996). Among these, *H. pylori* induces production of anti-gastric epithelium autoantibodies (Claeys *et al*, 1998); moreover, monoclonal antibodies against *H. pylori* reportedly cross-react with several other human tissues, including salivary gland, renal tubular epithelium and duodenal epithelium (Ko *et al.*, 1997). We have shown here that PAIgG from several cITP patients recognized *H. pylori* CagA protein and that cross-reactive antibody levels decreased following *H. pylori* eradication in patients that showed a complete response. This is consistent with the idea that *H. pylori* infection exerts a causative effect on the autoimmunity responsible for cITP via molecular mimicry. In addition, the finding that platelet eluates from three of five *H. pylori*-negative cITP patients also recognized CagA suggests that the anti-CagA antibody present in the eluate is the anti-platelet autoantibody produced in cITP, and is not an anti-*H. pylori* antibody produced during normal immune responses to bacterial infection.

Molecular mimicry is one way to break immunological tolerance and initiate the production of autoantibodies. Normally, autoreactive B cells cannot produce autoantibodies because they receive no help from autoreactive $CD4^+$ T cells, which are functionally deleted. However, if a cross-reacting non-self antigen is encountered, the B cells can present peptides from this molecule to non-self reactive $CD4^+$ T cells, thereby driving them to produce autoantibodies (Roitt *et al*, 1998). We suggest that *H. pylori* CagA may be such a cross-reactive antigen. Anti-platelet autoreactive B cells recognize CagA and can present it to *H. pylori*-reactive $CD4^+$ T cells under conditions of persistent infection. B cells may also produce anti-platelet autoantibodies without the help of autoreactive $CD4^+$ T cells in a subset of cITP patients. Enzyme-linked immunoSPOT assays have recently been used to evaluate these autoreactive anti-platelet B cells (Kuwana *et al*, 2002). Although we could use this approach in the present study, it may be useful in future studies for further analysis of the pathogenesis of *H. pylori*-associated cITP.

The good platelet recovery achieved after eradication of *H. pylori* in the present study is consistent with earlier studies from Japan and Italy (Gasbarrini *et al*, 1998; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002). On the other hand, a Spanish group reported lower response rates (13%), although the prevalence of *H. pylori* infection does not differ among ethnic groups (Jarque *et al*, 2001). This probably reflects the fact that some *H. pylori* strains do not harbour the CagA gene, and that CagA-positivity varies depending upon the geographic location (Perez-Perez *et al*, 1997; Mobley *et al*, 2001). In Japan, most *H. pylori* strains do harbour the CagA gene (Maeda *et al*, 1997), which probably accounts for the efficacy of eradication therapy in the treatment of cITP there. Recently, a French group reported no reactivity between platelet eluates and *H. pylori* proteins in ITP (Michel *et al*, 2002). We think the apparent absence of cross-reactivity might be because of the small number of patients examined in that study or the low incidence of CagA-positivity in France.

Unfortunately, because the amounts of PAIgG available from individual patients were very limited, we were unable to determine the platelet antigens that PAIgG recognizes in common with CagA protein. In addition, cross-reactivity between platelet antigens and *H. pylori* antigens other than CagA may also be involved in the pathogenesis of *H. pylori*-associated cITP, as the platelet eluates from two completely responsive patients (P4 and P6) did not recognize CagA protein.

In summary, we have demonstrated cross-reactivity between PAIgG and *H. pylori* CagA protein and suggest that molecular mimicry by CagA of an unknown platelet antigen is crucially involved in the pathogenesis of a subset of cITP cases. Further investigation should enable identification of the platelet antigen that shares an epitope with CagA, as well as clarification of the host susceptibility factors via which autoimmunity is induced.

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Inhibitory Actions of Several Natural Products on Proliferation of Rat Vascular Smooth Muscle Cells Induced by Hsp60 from *Chlamydia pneumoniae* J138

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Atherosclerosis is a vascular disorder involving inflammation, a narrowed vascular lumen in the entire tunica intima, and reduced elasticity of the arterial wall. It has been found that Hsp60 from *Chlamydia pneumoniae*, an obligate bacterial pathogen associated with atheroma lesions, mimics human Hsp60, thereby causing attacks by immune cells on stressed endothelial cells expressing endogenous Hsp60 on their surface. Furthermore, Hsp60 from *C. pneumoniae* has been shown to promote the growth of vascular smooth muscle cells (VSMCs). To explore probes that can be used for studying signal transduction elicited by the chlamydial Hsp60, we have tested several natural products for their inhibitory actions on the Hsp60-induced proliferation of rat arterial smooth muscle cells. Sesamol, vanillyl alcohol, and *trans*-ferulic acid exhibited moderate inhibitory actions on the Hsp60-induced cell proliferation; zerumbone, humulene, and caryophylene effectively inhibited it at low concentrations with IC_{50} values of 529, 122, and 110 nM, respectively. The results indicated that the 11-membered alicyclic ring is favorable for interactions with receptors involved in the Hsp60-induced VSMC proliferation.

KEYWORDS: ***Chlamydia pneumoniae*; heat shock protein 60; chaperonin; atherosclerosis; vascular smooth muscle cell; humulene; caryophylene; zerumbone; sesamol**

INTRODUCTION

According to the vital statistics of the Ministry of Health and Welfare of Japan, the leading cause of death from disease is malignant tumors (cancer, sarcoma), followed by cardiac diseases and cerebrovascular diseases. The total number of deaths from cardiac and cerebrovascular diseases is 280 000 per year, which is almost the same as the number of deaths from malignant tumors. It is estimated that about 12% of males and about 8% of females aged 45–74 years currently have disorders in coronary arteries such as atherosclerosis, and these percentages are rapidly increasing. Therefore, the prevention of such vascular diseases as well as diabetes and hypertension is very important.

Atherosclerosis is a disorder involving immune inflammation of arterial walls mediated by foam cells that stem from blood-derived monocytes and macrophages (*1*). The inflammation results in a narrowed vascular lumen, thereby causing heart disease and stroke. Although elevated levels of oxidized low-density lipoprotein, modified lipoprotein, and homocyteine have been proposed as risk factors for atherosclerosis, it has been recently demonstrated that the atherogenesis is likely to be associated with microbial infection (*2*). Of the candidate causal pathogens, an obligate bacterium *Chlamydia pneumoniae* is most correlated with atherosclerosis (*3–6*). In support of a tight link with bacterial infection, the application of an antibiotic azithromycin was shown to prevent atherosclerosis (*7*).

Hsp60, referred to as chaperonin 60 or GroEL, is able to refold and prevent the aggregation of denatured polypeptides in the presence of co-chaperonin or Hsp10 (*8*, *9*). Besides such intracellular chaperone functions, chlamydial Hsp60s are able to activate human immune cells to release cytokines (*10*). Interestingly, Hsp60 from *C. pneumoniae* was shown to co-localize with infiltrating macrophages in the atheroma (*11*). Once immune cells interact with the bacterial Hsp60, they attack not only the bacterial pathogen but also stressed endothelial cells expressing endogenous Hsp60s on their cell surface due to the cross-reactivity of the antigens (*12*).

In addition to activating the immune system, Hsp60 from *C. pneumoniae* has been shown to directly promote the growth of vascular smooth muscle cells (VSMCs) (*13*). Although Hsp60-induced cell proliferation was shown to be mediated by Toll-like receptor (TLR) 4 (*13*), there is evidence that the binding

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affinity of chlamydial Hsp60 for macrophages dose not differ from that for macrophages lacking TLR4 (*14*), suggesting that TLR4 plays an important role in the Hsp60-induced signal transduction but does not interact directly with this protein elicitor.

In this study, to develop useful probes for understanding the mechanism of the signal transduction elicited by extracellular Hsp60s, we have evaluated actions of various natural compounds on the proliferation of rat vascular smooth muscle cells induced by Hsp60 from *C. pneumoniae*. The results indicate zerumbone and its related compounds may become lead compounds for developing novel probes.

MATERIALS AND METHODS

Natural Products. Zerumbone (Z) was steam distilled and purified from essential oil of the rhizomes of *Zingiber zerumbet* (*15*). Humulene (HL) and caryophylene (CA) were distilled and purified from essential oil of *Eugenia caryophylla*. Eugenol (Eug) from citrus fruit peels (*16, 17*), sesamol (Ses) from sesames, vanillyl alcohol (VanAlc) from vanilla bean, and *trans*-ferulic acid (FerA) from *Celosia argentea* L. (*18*) were isolated and purified from 70% ethanol–water extract. Anacardic acid monoene (AM) and triene (AT) were isolated and purified from methanol extract of cashew nuts (*19*).

Gene Cloning. The gene encoding Hsp60-1 (GroEL1) of *C. pneumoniae* (CP-Hsp60-1) was amplified by PCR using primers C.P.Gro1-N (5′-GGGAATTCCATATGGCAGCGAAAAATATTAAA-TATAATG-3′) and C.P.Gro1-C (5′-CCGCTCGAGGTAGTCCATTC-CTGCGCTTGGC-3′). PCR was carried out using 1 unit of KOD -Plus- (TOYOBO, Osaka, Japan), 2 ng of the *C. pneumoniae* (strain J138, see ref *20*) genomic DNA as template, 0.2 μM primers (C.P.Gro1-N and C.P.Gro1-C), and 0.2 mM dNTP mixture in a 50 μL solution for 30 cycles of 15 s at 94 °C for denaturing, 30 s at 53 °C for annealing, and 90 s at 68 °C for DNA extension. After gel purification using a low melting point agarose gel, the isolate fragment was digested by *Nde*I and *Xho*I, and cloned into the *Nde*I and *Xho*I sites of a vector pET22b (+) (Novagen, WI).

Expression and Purification of CP-Hsp60. Recombinant CP-Hsp60-1, fused with additional amino acids (leucine, glutamic acid, and six histidine residues) from the vector pET-22b (+) at the carboxyl terminus, was expressed at 28 °C in *Escherichia coli* BL21 (DE3) (Novagen) according to the manufacturer's directions. The crude recombinant protein was purified by a Ni^{2+}-nitrilotriacetic acid (NTA) column (Novagen) followed by gel filtration chromatography using a Superdex 200 HR 10/30 column (Amarsham Biosciences, NJ) with phosphate-buffered saline (PBS). The protein concentration was determined by the Bradford method (*21*) using bovine serum albumin as the standard. The purity of the protein was checked by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*22*). Proteins in the gel were stained with Coomassie Brilliant Blue R-250.

Cell Cultures. Rat VSMCs were obtained by explant from the SD rat artery. Some of the cells were kindly provided by Dr. Ken-ichiro Hayashi (Department of Neurochemistry and Neuropharmacology, Medical School of Osaka University, Japan). VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine, penicillin, streptomycin, and fungizone at 37 °C, and were used after 2–10 passages (*23*).

Cell Proliferation Assay. Rat VSMCs (1 mL, 1 × 10^5 cells) plated in 24-well plates were incubated for 1 day in the presence of 10% FCS and starved in DMEM without FCS for 48 h. The compounds were then applied for 1 h prior to co-application with 10 μg/mL CP-Hsp60-1. After incubation for 48 h, the cells were enumerated using a hemocytemeter. Unless otherwise noted, tests were made in quadruplicate and the IC_{50}, concentration (M) required to reduce the proliferation of VSMCs induced by CP-Hsp60-1 by 50%, was determined for active compounds such as Z, HL, and CA.

RESULTS AND DISCUSSION

Assay Conditions. To establish an assay for the proliferation of rat VSMCs elicited by CP-Hsp60-1, we evaluated the effects of the time of starvation before treatment with the protein and the time of treatment with the chlamydial protein on the growth of VSMCs.

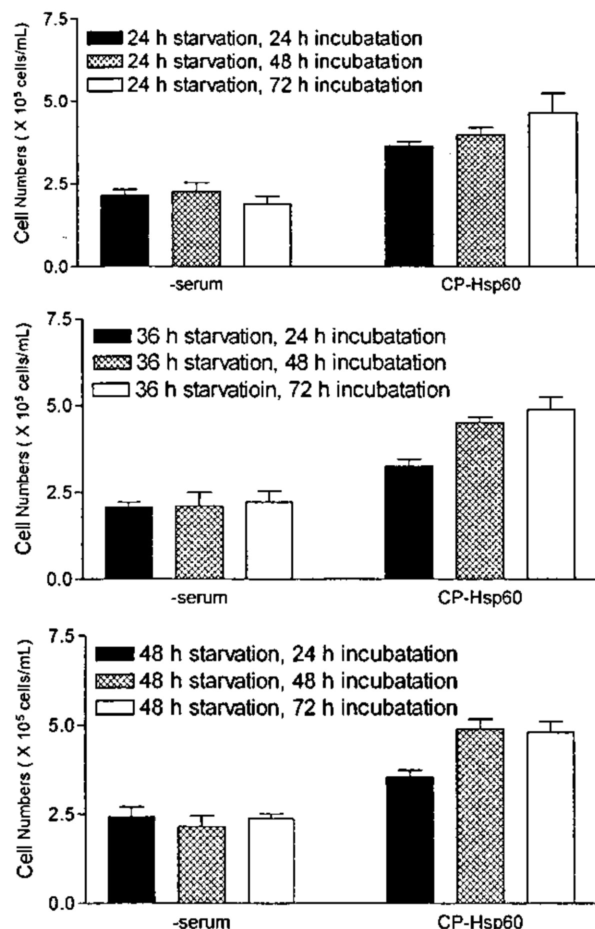

Figure 1. Effects of time of starvation and treatment with Hsp60-1 from *C. pneumoniae* on growth of rat vascular smooth muscle cells. The recombinant Hsp60-1 expressed by *E. coli* was applied at 10 μg/mL. Each data point represents mean ± SEM of four experiments.

Figure 1 shows the cell growth in 24, 36, and 48 h starvation groups, which were then cultured in a serum-deficient but Cp-Hsp60-1-supplemented medium for 24, 36, and 48 h after starvation. The longer the culture period in the presence of Cp-Hsp60-1, the more VSMCs proliferated. The growth of VSMCs measured after starvation for 48 h was higher than that after starvation for 24 h. Taking these results into consideration, the effects of the natural compounds on the Hsp60-induced cell growth were evaluated by co-applying them with the chlamydial protein for 48 h after 48 h-starvation.

Concentration-Dependent Proliferation of Rat VSMCs by CP-Hsp60-1. Rat VSMCs were exposed to CP-Hsp60-1, and the cell proliferation was measured after 48 h of incubation (n = 4). CP-Hsp60-1 was added to the medium at final concentrations of 0.1, 1.0, 5.0, 10, 20, and 50 μg/mL. As the control, PBS (−), which does not include CP-Hsp60-1, was added to the culture medium. There were no significant differences in the proliferation of VSMCs treated with 0.1, 1.0, and 5.0 μg/mL CP-Hsp60-1 and the control. When exposed to 10, 20, and 50 μg/mL CP-Hsp60-1, however, the number of cells was 2.3-, 3.2-, and 3.7-fold larger than the control, respectively (**Figure 2**). The cell proliferation reached almost the maximal level on treatment with CP-Hsp60-1 at 20 μg/mL. By contrast, when the recombinant Hsp60-1 was either heated at 100 °C for 30 min, or treated with proteinase K at 200 μg/mL for 2 h at 37

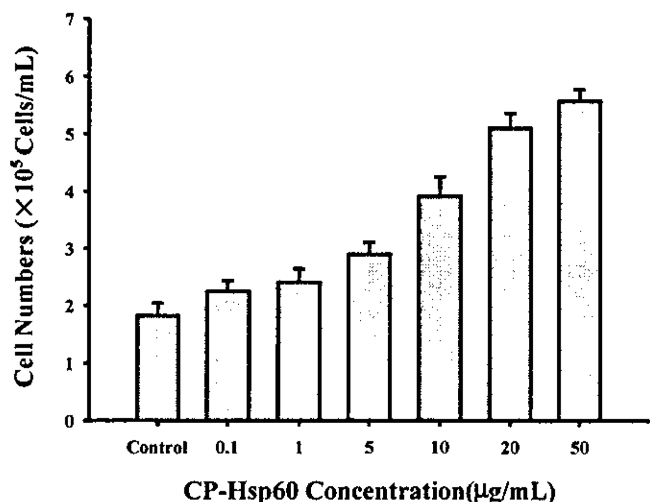

Figure 2. Dose-dependent actions of recombinant Hsp60-1 from *C. pneumoniae* on growth of rat vascular smooth cells. Each data point represents mean ± SEM of four experiments.

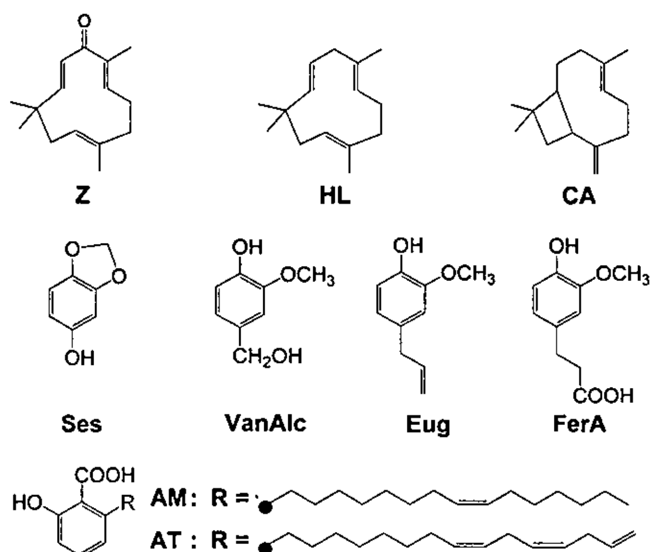

Figure 3. Chemical structures of natural compounds tested for their actions on proliferation of vascular smooth muscle cells induced by Hsp60-1 from *C. pneumoniae*.

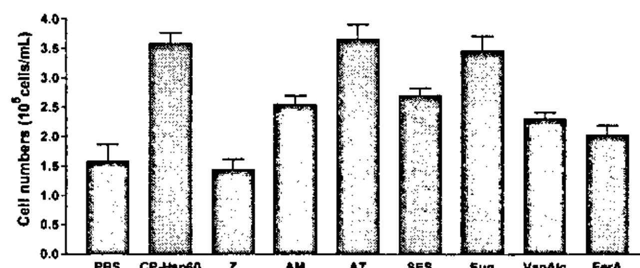

Figure 4. Actions of zerumbone (Z), sesamol (Ses), eugenol (Eug), vanillyl alcohol (VanAlc), *trans*-ferulic acid (FerA), anacardic acid monoene (AM), and triene (AT) on the proliferation of vascular smooth muscle cells induced by Hsp60-1 from *C. pneumoniae*. Each data point represents mean ± SEM of four experiments.

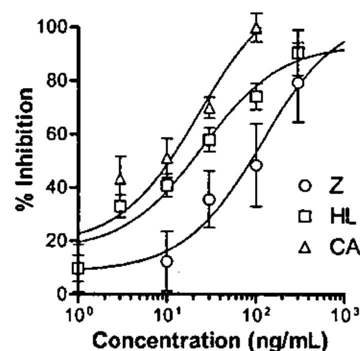

Figure 5. Dose-dependent inhibitory actions of zerumbone (Z, ○), humulene (HL, □), and caryophylene (CA, △) on the proliferation of rat smooth muscle cells induced by Hsp60-1 from *C. pneumoniae*. Each data point represents mean ± SEM of eight experiments. Experiments for Z were conducted separately from the test illustrated in **Figure 4**.

Table 1. Inhibitory Actions of Compounds on VSMC Proliferation Induced by CP-Hsp60-1

compd	IC_{50} (nM)	compd	IC_{50} (nM)	compd	IC_{50} (nM)
Z	530	HL	122	CA	110

°C, no cell proliferation was observed (data not shown), suggesting that the VSMC growth was due to an action of the recombinant CP-Hsp60-1 preparation.

Actions of Compounds on Cell Proliferation. Compounds that have been shown to have physiological activities against cancer cells were used for assaying inhibitory effects on the proliferation of VSMCs. The compounds isolated from natural materials (**Figure 3**) were zerumbone (Z) obtained from *Zingiber zerumbet*, eugenol (Eug) obtained from citrus fruit peels, sesamol (Ses) obtained from sesames, and anacardic acid monoene (AM) and triene (AT) obtained from cashew nuts. Dimethyl sulfoxide (DMSO) was used as the solvent to prepare stock solutions of these compounds. The DMSO concentration in the assay medium was 1% (v/v), irrespective of the presence or absence of the compounds. Prior to co-application of CP-Hsp60-1 with the compounds, the cells were treated for 1 h with the same concentration of the compounds. Among the tested compounds (**Figure 3**), Ses, VanAlc, FerA, and AM applied at 10 μg/mL showed weak or moderate inhibitory action on the cell proliferation induced by CP-Hsp60-1, whereas Z completely suppress it at this concentration (**Figure 4**). By contrast, Eug and AT was inactive on the cell proliferation. Based on the result, the inhibitory activity of Z-related compounds against the CP-Hsp60-1-induced cell proliferation was evaluated. The analogues of Z tested were humulene (HL), which lacks the carbonyl group, and caryophylene (CA), in which the double bonds in HL are cyclized (**Figure 3**). Z and its related compounds H and CA exhibited inhibitory actions even at 1 μg/mL (data not shown). Thus, as illustrated in **Figure 5**, the dose-inhibitory action relationships of Z, HL, and CA were investigated in detail. The inhibitory activity of HL and CA was more potent than Z. The IC_{50}, the concentration (M) required to reduce the growth of VSMCs induced by 10 μg/mL CP-Hsp60-1 by 50%, of the three compounds was calculated by the Hill equation. The IC_{50} values of Z, CA, and HL were 529, 122, and 110 nM, respectively (**Table 1**). At these IC_{50}s, all of these compounds had no significant effect on VSMC cultured in the absence of CP-Hsp60-1 (data not shown), suggesting that the inhibitory action at such low concentrations is selective for the CP-Hsp-1-induced events. The rank order of the inhibitory activity indicated that the carbonyl group at position 1 and olefinic bonds at positions 2 and 6 of Z are not essential and rather unfavorable for the inhibitory activity.

In summary, we have for the first time found by testing nine natural products that compounds having an 11-membered alicyclic ring and a trisubstituted benzene skeleton are effective

in reducing the proliferation of rat VSMCs induced by Hsp60-I (GroEL1) of *Chamydia pneumoniae*. Although the modes of action of these inhibitory compounds are unknown, the discovery of compounds capable of influencing Hsp60-induced cell growth at low concentrations could lead to the development of novel and useful probes for understanding the cell signaling elicited by Hsp60s.

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