The participation of numerous inflammatory mediators in the inflammatory process involving endothelium and smooth muscle is essential in the development of all stages of atherosclerosis, from initiation, progression, and finally to the evolution of complications. Pathologic and immunohistochemical staining (IHCS) studies have clearly shown a preponderance of inflammatory cells in ruptured plaques of patients dying suddenly of severe coronary artery disease. Recently, an autopsy study has demonstrated that an increased serum level of C-reactive protein (CRP), an index of inflammation, directly participates in the atherosclerotic process. Others have shown that P-selectin expression on platelets determines platelet aggregate size and stability.

Recent studies have demonstrated that elevation of the white blood cell (WBC) count during acute myocardial infarction (AMI) is associated with reduced epicardial blood flow and myocardial reperfusion, thromboresistance, and adverse outcomes. Accordingly, an association between an elevated WBC count and the infarct-related coronary artery (ICA) thrombus burden has been suggested but to date no direct evidence exists to further support this observation in survivors of AMI.

Therefore, this study tested the hypotheses that circulating levels of hs-CRP correlate with the immunohistochemical localization of CRP in the ICA, and that the soluble (s) CRP as the only independent predictor of newly ruptured plaque and vascular death in various clinical settings. Recently, an autopsy study has demonstrated that an increased serum level of CRP measured by high-sensitivity assay (hs-CRP) is significantly related to atherothrombi and plaque burden in patients dying suddenly of severe coronary artery disease.

P-selectin is a cell adhesion molecule produced by activated platelets and endothelial cells and is important in modulating the interaction of these cells with neutrophils and monocytes, thereby contributing to thrombus formation. Previous works have indicated that P-selectin mediates the accumulation of leukocytes, which in turn promotes fibril deposition. Others have shown that P-selectin expression on platelets determines platelet aggregate size and stability.

Key Words: Acute myocardial infarction; Immunohistochemical staining; Inflammatory mediators
P-selectin level and WBC count are significantly increased in the ICA than in the circulation in patients with AMI of <6 h duration undergoing primary percutaneous coronary intervention (PCI).

Methods

Patient Population and Inclusion Criteria

All patients with AMI of <12 h duration were considered eligible for primary PCI. The PercuSurge GuardWire™ device (Medtronic AVE) was used as a precaution against distal embolization in patients during primary PCI if the angiographic results complied with the following criteria: (1) the angiographic morphologic features of the ICA showed high-burden thrombus formation as defined in our recent study;\(^2\) (2) reference lumen diameter (RLD) of the ICA ≥3.5 mm; and (3) AMI <12 h. Exclusion criteria were: (1) RLD of ICA <3.5 mm; (2) heavily calcified ICA; and (3) very tortuous ICA.

To avoid other variables that could influence the serum levels of hs-CRP and sP-selectin, and the WBC count, we excluded patients with a history of recent surgery or trauma within the preceding 2 months, renal insufficiency (creatinine >1.5 mg/dl), malignancy or liver cirrhosis, febrile disorders, acute or chronic inflammatory disease on study entry or history of recent infection, and those with AMI onset ≥6 h. Patients were also excluded if fever (body temperature >37.5°C) was observed in the emergency room.

Between October 2002 and April 2004, a PercuSurge GuardWire™ device (Medtronic AVE) was used to prevent distal embolization in 147 consecutive patients presenting with AMI (<12 h). Of these, 63 patients who upon presentation had experienced the onset of AMI >6 h were excluded. Of the remaining 84 patients with AMI <6 h, 35 were further excluded because of either incomplete occlusion of the ICA or atherothrombus was not aspirated from their ICA during the procedure. Thus, 49 patients who had atherothrombi aspirated from the ICA (Fig 1) comprised the study population.

Forty-five subjects who had experienced angina and undergone their first elective PCI were matched for age, gender, and the existence of hypertension, diabetes mellitus, current smoking habit, as well as hypercholesterolemia, and served as at-risk controls. Informed consent was given by each study subject. The Institutional Review Committee on Human Research approved the study protocol.

Procedure and Protocol

A transradial approach using a 6Fr arterial sheath has been the design protocol for AMI at Chang Gung Memorial Hospital since 2001. When diagnostic results demonstrated that the ICA met the criteria for PercuSurge device therapy, the 6Fr arterial sheath was replaced with a 7Fr arterial sheath, and a 7Fr standard interventional guiding catheter was selectively positioned in the culprit artery. Thrombectomy with an export suction catheter was performed after the distal protection balloon was inflated prior to coronary angioplasty. Blood samples for the ICA levels of hs-CRP and sP-selectin and the WBC count were obtained using the Export Suction Catheter beyond the occluded lesion at the beginning of the procedure. To avoid the possibility of dilution of these inflammatory mediators, 2–3 mL of blood inside the guiding catheter was first removed followed by the collection of 6–8 mL of blood from the ICA.

Clopidogrel (300 mg preoperative loading dose, then 75 mg/day) was given for at least 4 weeks to patients who underwent primary stenting. Aspirin (orally 100 mg/day) was given to each patient indefinitely.

Analysis of Blood Samples

Blood samples were obtained from the radial artery in the cardiac catheterization room before the coronary angiographic study. WBC count, biochemical parameters, and electrolytes were determined by standard laboratory methods.

The hs-CRP was measured by immunonephelometry using the BN™ system (Dade Behring Inc Newark, DE, USA). The lower detection limit is <0.15 mg/L. The intra-individual variability of serum hs-CRP levels was assessed.

Figure 1. Atherothrombosis aspirated by export suction catheter from 4 cases (A–D). Yellowish cholesterol and debris in the filter (B, C).
in both study patients and at-risk control subjects whose mean intra-assay coefficients of variance were 2.89% and 2.74%, respectively.

The concentration of sP-selectin was measured using a standard enzyme-linked immunosorbent assay and a commercially available kit (R and D Systems; Minneapolis, MN, USA). The assay was sufficiently sensitive to detect concentrations of less than 0.5 ng/ml, according to the manufacturer’s instructions. Intra-individual variability of the sP-selectin levels was assessed in both study patients and at-risk control subjects whose mean intra-assay coefficients of variance were 4.78% and 4.33%, respectively.

**Specimen Preparation**

The aspirated tissue was placed in 10% buffered formalin for IHCS of CRP and macrophage identification. In each case, the gross dimensions of the atherothrombus were measured in centimeters. The atherothrombi were then paraffinized and the paraffin blocks were serially sectioned at 2μm thickness for IHCS in each case.

**Immunohistochemical Stains**

Two sections were prepared and incubated with the specific anti-CD68 (Dako Corporation) antibody (at a dilution of 1:50) for the identification of macrophages in each case. Three sections were prepared in each case and IHCS...
was carried out using standard avidin–biotin techniques with a commercially available antiserum for CRP (Sigma, St Louis, MO, USA) at a dilution of 1:200. The super sensitive polymer-HRP IHC was used as a detection system (BioGenex). Deparaffinized sections were incubated in 1mmol/L EDTA buffer with steam heat before staining for antigen retrieval.

Qualitative Analysis for Staining

Grading of the staining intensity of CRP was assessed in both macrophages and atherothrombotic tissue (extracellular staining) (Figs 2,3). A qualitative score of 0–3 was given to each case: 0 indicated no staining; 1+ indicated <30% macrophages staining or <30% extracellular staining; 2+ indicated 30–60% macrophages staining or 30–60% extracellular staining; 3+ indicated >60% macrophages staining or >60% extracellular staining. The staining was analyzed by a pathologist who was unaware of the procedure and the patients’ clinical information.

Statistical Analysis

Categorical variables were compared using the chi-square test or Fisher’s exact test. Continuous variables were compared using the t-test. Log-transformation of hs-CRP and sP-selectin was used to improve the normality for statistical analysis. Continuous variables of hs-CRP among the 3 different grades of staining of CRP were compared using repeated measures ANOVA, and Scheffe’s multiple comparison procedure was used for further comparing the significant difference among the 3 groups. SAS statistical software for Windows version 8.2 (SAS Institute, Cary, NC, USA) was used for statistical analysis. A probability value <0.05 was considered statistically significant.

Results

Baseline Characteristics of the Study Patients and At-Risk Control Subjects (Table 1)

The 2 groups did not differ significantly with regard to age, gender, risk factors for coronary artery disease, body mass index, creatinine level, medications, multivessel disease, and incidence of PCI to the left anterior descending artery (LAD) and non-LAD. However, circulating levels of hs-CRP and sP-selectin, and the WBC counts were significantly higher in the study patients than in the at-risk control subjects. The procedural success rate was 98.0%. One patient died in hospital. Thus, the 30-day morality was 2.0%.

Comparison of the Levels of Inflammatory Biomarkers Between the Systemic Circulation and ICA in 49 AMI Patients (Table 2)

Serum levels of hs-CRP did not differ between the ICA and systemic circulation in the study patients, but the sP-selectin level and WBC count were significantly higher in the ICA than in the systemic circulation.

IHCS

Table 3 shows the different CRP staining intensities of the macrophages and atherothrombotic tissue, as well as

<table>
<thead>
<tr>
<th>Table 1 Baseline Characteristics of Study Patients and At-Risk Control Group</th>
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<td></td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Male gender</td>
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<tr>
<td>Hypertension</td>
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<tr>
<td>Current smoker</td>
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<tr>
<td>Body mass index (kg/m²)*</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
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<tr>
<td>HDL cholesterol (mg/dl)</td>
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<tr>
<td>LDL cholesterol (mg/dl)</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
</tr>
<tr>
<td>Diabetic mellitus</td>
</tr>
<tr>
<td>WBC count (x10³ /ml)</td>
</tr>
<tr>
<td>Soluble P-selectin (ng/ml)</td>
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<tr>
<td>hs-CRP (mg/L)</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
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<tr>
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<tr>
<td>Clopidogrel</td>
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<tr>
<td>Statins</td>
</tr>
<tr>
<td>ACE inhibitors</td>
</tr>
<tr>
<td>Multivessel disease²</td>
</tr>
<tr>
<td>PCI to LAD</td>
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<tr>
<td>PCI to non-LAD</td>
</tr>
<tr>
<td>TIMI-3 flow³</td>
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<tr>
<td>Peak level of CK-MB (U/L)</td>
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<tr>
<td>LVEF (%)</td>
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<td>30-day mortality</td>
</tr>
</tbody>
</table>

Data are mean value±SD or % (number) of patients.

ACE, angiotensin-converting enzyme; HDL, high-density lipoprotein; LDL, low-density lipoprotein; WBC, white blood cell; hs-CRP, high-sensitivity C-reactive protein; PCI, percutaneous coronary intervention; LAD, left anterior descending artery; TIMI, Thrombolysis in Myocardial Infarction; CK, creatine kinase; LVEF, left ventricular ejection fraction.

*Body mass index was defined as weight in kilograms divided by square of the height in meters (kg/m²).

†Multivessel disease was defined by stenoses of >50% in ≥2 major epicardial coronary arteries.

‡TIMI-3 flow indicated successful reperfusion.
The circulating level of hs-CRP was significantly higher in grade 3+ than in grade 2+ and grade ≤1+ (p<0.0001), and in grade 2+ than in grade ≤1+ (p<0.001) in macrophage staining of CRP. Additionally, the circulating level of hs-CRP was significantly higher in grade 3+ than in grade 2+ and grade ≤1+ (p<0.0001) and in grade 2+ than in grade ≤1+ in extracellular staining of CRP (p<0.001). Multiple stepwise logistic regression analysis of the variables in Table 1 demonstrated that an increased circulating level of hs-CRP was the only independent predictor of ≥2+ within macrophages (odds ratio (OR) =3.71, 95% confidence interval (CI) =2.57–18.54, p<0.0001) and of ≥2+ in extracellular staining of CRP (p<0.001). Multiple stepwise logistic regression analysis of the variables in Table 1 demonstrated that an increased circulating level of hs-CRP was the only independent predictor of increased macrophage and extracellular staining intensity of CRP. 

Impact of Inflammatory Mediators in the ICA on Thrombus Burden

Pieces of atherothrombi aspirated from the ICA during PCI were measured across all 3 dimensions and then summed into a total volume (width×height×length cm³). The measurement results demonstrated that 69.4% of patients (group 1, n=34) had a total atherothrombus volume ≥0.5 cm³, whereas the other 30.6% (group 2, n=15) had a volume <0.5 cm³. The mean atherothrombus volume was significantly higher in group 1 than in group 2 (0.88±0.24 cm³ vs 0.32±0.18 cm³, p<0.0001). Furthermore, the ICA level of sP-selectin was noticeably higher in group 1 than in group 2 patients (85.67±16.82 vs 67.94±18.54, p<0.0001). Moreover, there was a significant correlation between the thrombus volume and the ICA sP-selectin level (r=0.414, p<0.0001).

**Table 2** Comparison of the Levels of Inflammatory Biomarkers in the Systemic Circulation and ICA in 49 AMI Patients

<table>
<thead>
<tr>
<th>ICA</th>
<th>Systemic circulation</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC count (×10³/ml)</td>
<td>14.4±8.1</td>
<td>10.2±3.6</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>3.42±2.91</td>
<td>3.82±3.07</td>
</tr>
<tr>
<td>Soluble P-selectin (ng/ml)</td>
<td>80.27±18.59</td>
<td>64.7±18.12</td>
</tr>
</tbody>
</table>

Data are mean value ± SD.
ICA, infarct-related coronary artery; AMI, acute myocardial infarction; WBC, white blood cell; hs-CRP, high-sensitivity C-reactive protein.

**Table 3** Association Between Circulating Level of hs-CRP and Staining Intensity of CRP Within Macrophages and Atherothrombotic Tissue

<table>
<thead>
<tr>
<th>% Ciculating hs-CRP level (mg/L)</th>
<th>Macrophage staining</th>
<th>Extracellular staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>(no. of patients)</td>
<td>Grade 3+</td>
<td>Grade 2+</td>
</tr>
<tr>
<td></td>
<td>32.7% (16)</td>
<td>18.4% (9)</td>
</tr>
<tr>
<td></td>
<td>3.61±2.98</td>
<td>2.81±2.56</td>
</tr>
<tr>
<td>Grade ≤1</td>
<td>49.0% (24)</td>
<td>1.92±1.18</td>
</tr>
<tr>
<td></td>
<td>1.92±1.18</td>
<td>1.92±1.18</td>
</tr>
<tr>
<td>Grade 2+</td>
<td>53.1% (26)</td>
<td>16.3% (8)</td>
</tr>
<tr>
<td></td>
<td>3.82±2.71</td>
<td>2.91±1.89</td>
</tr>
<tr>
<td>Grade ≤1</td>
<td>30.6% (15)</td>
<td>30.6% (15)</td>
</tr>
<tr>
<td></td>
<td>3.78±1.09</td>
<td>3.78±1.09</td>
</tr>
</tbody>
</table>

Data are mean± SD of patients.
Symbols (*, †, ‡, §, ||, ¶) indicate significant difference (at 0.05 level) by Scheffe’s multiple comparison procedure.
* vs † and ‡, p<0.0001; † vs ‡, p<0.001; § vs || and ¶, p<0.0001; || vs ¶, p<0.001.

Correlation Between ICA Levels of hs-CRP and WBC Count

Correlation analysis demonstrated that the increase in the ICA level of sP-selectin was significantly related to the increase in the ICA WBC count (r=0.548, p<0.0001) (Fig 4).

**Discussion**

This study is original and has several notable findings. First, both the sP-selectin level and WBC count were significantly higher in the ICA than in the systemic circulation in patients with AMI <6 h duration undergoing primary PCI. Second, the ICA level of sP-selectin was substantially higher in patients with a large atherothrombotic burden (≥0.5 cm³) than in patients with a small atherothrombotic burden (<0.5 cm³). Third, an increased circulating level of hs-CRP was the only independent predictor of increased macrophage and extracellular staining intensity of CRP. Finally, an increased ICA level of sP-selectin was significantly and directly related to an increased WBC count in the ICA.

Accumulating evidence from clinical observations indicates that the CRP level is one of the most powerful predictors of atherosclerosis and vascular death. Other clinical observational studies have shown that CRP is related to adverse outcomes in patients.

![Fig 4. Correlation between infarct intra-coronary artery levels of soluble P-selectin (sP-selectin) and white blood cell counts in patients with acute myocardial infarction onset <6 h. WBC, white blood cell.](image)
Distribution of Inflammatory Mediators in Infarct Artery

The reasons for the elevated sP-selectin level in the ICA compared with that in systemic circulation in the present study remains uncertain. However, we hypothesize that P-selectin is rapidly expressed on the surface of activated platelets and endothelial cells after plaque rupture and thrombus formation in the coronary artery. Additionally, compared with the systemic circulation, the ICA should display a more rigorous response in both the expression and enzymatic cleavage of sP-selectin, resulting primarily from the twin stimulations of endothelial-platelet-leukocyte interaction and thrombus formation. Furthermore, most P-selectin is retrieved and accumulates in the occluded ICA after being cleaved into the soluble form (sP-selectin) rather than then being released into the circulation. Previous studies have demonstrated that glycoprotein P-selectin, a membrane component of cell storage granules, is rapidly translocated from the a-granules of platelets and the Weibel-Palade bodies of endothelial cells to the cell surface following an inflammatory process or other stimuli. Consequently, both enzymatic cleavage of expressed P-selectin and alternative splicing of P-selectin messenger ribonucleic acid occur quickly, giving rise to sP-selectin detectable in the peripheral blood. Thus, our suggestions, based on laboratory findings, are further supported by these previous studies.

Recent studies have demonstrated that an increased WBC count in patients with AMI is strongly associated with reduced epicardial blood flow and myocardial reperfusion, thromboreistance, and adverse clinical outcomes. The present study showed a substantially increased WBC count in study patients compared with at-risk control subjects. Importantly, the WBC count was significantly higher in the ICA than in the systemic circulation. Furthermore, a linear correlation was found between WBC count and sP-selectin level in the ICA. We suggest that sP-selectin plays a key role in thrombus formation in the study patients. The suggestions presented here based on clinical observation and laboratory findings are supported by previous studies that demonstrated that leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. Accordingly, the findings of this study reinforce the recent reports of elevated WBC count being associated with thromboreistance and reduced myocardial reperfusion.

**Study Limitations**

First, the PercuSurge device was not used in the at-risk control subjects. Therefore, this investigation could not provide information regarding the ICA levels of hs-CRP, sP-selectin and WBC count of these subjects. Second, the staining intensity of CRP within macrophages in systemic circulation was not performed. Hence, this investigation could not provide information regarding the difference in staining intensity of CRP within macrophages in the ICA and systemic circulation in the study patients. Finally, without an intravascular ultrasound study, we could not provide a correlation between atherothrombus volume and plaque burden. Autopsy studies previously demonstrated that moderate plaque burden was usually found in most infract-related arteries. Thus, atherothrombus volume may not have an association with plaque burden.

**Conclusion**

The present study demonstrated that the circulating level of hs-CRP was strongly and independently correlated with increased intensity of both macrophage and extracellular tissue staining of CRP. Previous studies have shown that the circulating concentration of human CRP, the classical acute phase protein, is always increased following AMI, starting approximately 6 h after onset of symptoms and peaking about 50 h. Therefore, according to those studies, the serum level of hs-CRP can be classified into 2 different intervals in the clinical setting of AMI (ie, 6 h before and after the onset of symptoms). Patients enrolled in the present study, who experienced AMI with duration <6 h undergoing primary PCI, showed no difference in the serum hs-CRP level in the ICA and in the systemic circulation. This finding suggests that circulating level of hs-CRP within 6 h of onset of AMI, before the participation of myocardial damage in the inflammatory process within the atherosclerotic plaque, reflects the baseline level of serum hs-CRP in the present patients. Moreover, the serum level of hs-CRP was significantly higher among the study patients than the at-risk control subjects. Therefore, we suggest that an elevated serum hs-CRP level prior to AMI portends rupture of vulnerable plaque. This suggestion, based on clinical observation and laboratory findings, is further supported by a recent autopsy study that demonstrated that an increased serum level of hs-CRP is strongly related to atherothrombi and plaque burden in patients with severe coronary artery disease who died suddenly.

Although animal and in vitro studies have stressed the important role of P-selectin in facilitating atherosclerosis lesion development, mediating endothelial-leukocyte-platelet interactions and stabilizing platelet aggregation data on the role and circulating level of sP-selectin following AMI remains limited. Additionally, no data are available on the level of sP-selectin in the ICA of patients following AMI. The third important finding of this study was that among the study patients the serum level of sP-selectin was markedly higher in the ICA than in the systemic circulation. Furthermore, an increased ICA sP-selectin level was significantly associated with increased atherothrombotic burden in the ICA. Thus, we suggest that sP-selectin may play a crucial role in thrombus formation and organized-thrombus maintenance in the ICA. This suggestion is supported by a previous experimental study demonstrating that sP-selectin facilitates fibrin deposition within the thrombus.

The reasons for the elevated sP-selectin level in the ICA compared with that in systemic circulation in the present study remains uncertain. However, we hypothesize that...
of hs-CRP was independently associated with the staining intensity of CRP in the ICA. This finding highlights the possible role of CRP in contributing to the rupture of atherosclerotic plaque. Moreover, this study revealed the sequestration of sP-selectin and WBC in the ICA, indicating that these inflammatory mediators could, at least in part, participate in thrombus formation and stabilization following AMI.

Acknowledgements

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