Background: There are reports on the correlation of the variability of the anti-platelet effect of aspirin and the clinical outcomes. However, the mechanism of this correlation is still unknown. There is still no report on the relationship between the variability of anti-platelet effect of aspirin and the changes of sVCAM-1, hs-CRP and platelet monocyte aggregates.

Methods: A total of 42 cases, 22 men and 20 women, aged between 41 and 81 years at high cardiovascular risk (because of the presence of cardiovascular diseases, diabetes or at least two classical cardiovascular risk factors) were enrolled randomly from outpatient clinics. The patients were in stable condition and under therapy of 100 mg enteric-coated aspirin preparation per day for at least 1 month. Serum levels of markers of inflammation (hs-CRP), endothelial activation (sVCAM-1), platelet activation and platelet monocyte aggregates were measured. In addition, an in vitro cell culture model was used to study the effect of aspirin on platelet-induced VCAM-1 mRNA expression and nuclear factor (NF)-κB activation in endothelial cells (ECs).

Results: High-risk patients taking aspirin were divided into a high 50-percentile group and a low 50-percentile group according to their different degrees of platelet activation. No significant difference was found in hs-CRP and platelet monocyte aggregates between these two groups. However, the high-platelet activation group had significantly higher sVCAM-1 than did the low platelet-activation group. Aspirin not only inhibited platelet activation by adenosine diphosphate (ADP), but also decreased the activated platelet-induced VCAM-1 expression and NF-κB activation in ECs.

Conclusion: Inadequate inhibition of platelet activation by aspirin was associated with higher serum concentration of sVCAM-1 in subjects with high cardiovascular risk.

Key Words: Aspirin • Highly sensitive C-reactive protein • Platelets • Platelet monocyte aggregates • Vascular cell adhesion molecule-1

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease involving endothelial cells (ECs), smooth muscle cells (SMCs), leukocytes, platelets, growth factors, and cytokines. Activated platelets are present in the circulating blood of atherosclerotic individuals and have been documented in patients with peripheral arterial disease. An expanding body of evidence continues to focus on the role of activated platelets in the development of athero-
sclerotic lesions. However, the contribution of platelets to the process of atherosclerosis has been unclear. In addition to thrombosis, recent studies have provided insights into platelet function in inflammation and atherosclerosis.5

When ECs are activated in response to cytokines, the expression of cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) on the EC surface is markedly increased.6 The appearance of soluble cell adhesion molecules in the circulation is thought to result from their release from the surface of activated ECs as a result of their increased expression. Therefore, circulating cell adhesion molecules, such as soluble VCAM-1 (sVCAM-1), are regarded as inflammatory markers related to EC activation and atherosclerosis.7 Activated platelets may participate in the early progression of atherogenesis by modulating adhesive properties of ECs for blood cells.8 In addition, highly sensitive C-reactive protein (hs-CRP) is an independent predictor of risk for atherosclerosis, cardiovascular events, atherothrombosis, hypertension, and myocardial infarction.9,10 The level of hs-CRP is elevated in patients with acute coronary syndromes, as well as in healthy patients who are at increased risk for cardiovascular disease.11 Platelet monocyte aggregates constitute one of the mediators recently proposed to bridge thrombosis and inflammation. Activated platelets rapidly mobilize intracytosol P-selectin to the cell membrane. Then these platelet monocyte aggregates interact with atherosclerotic lesion, indicating that cell-cell interactions between platelet and leukocytes can modulate and sustain inflammatory events at the vascular wall.5

Aspirin therapy is widely used in patients at risk of developing atherosclerosis and thrombosis. The therapeutic efficacy of aspirin in cardiovascular diseases is attributed to its platelet-inhibitory function, which results from irreversible inhibition of cyclooxygenase (COX) activity and thromboxanes (TX) generation.12 Although aspirin is the most commonly used inhibitor of platelet function in clinical practice, the anti-platelet effects of aspirin may not be uniform in all patients. There are reports on the correlation of the variability of the anti-platelet effect of aspirin and on the clinical outcomes.13,14 However, the mechanism of this correlation is still unknown. There is still no report on the relationship between the variability of anti-platelet effect of aspirin and the changes of sVCAM-1, hs-CRP and platelet monocyte aggregates.

In the present study, high-risk patients taking aspirin were divided into two groups: high 50-percentile group with platelet activation over 4.2% (21 cases, 10 men and 11 women, age: 65 ± 10 yr) and low 50-percentile group with platelet activation below 4.2% (21 cases, 12 men and 9 women, age: 67 ± 12 yr). The anti-platelet effect of aspirin and the circulating levels of sVCAM-1, hs-CRP, and platelet-monocyte aggregates were assessed and compared in these two groups. In addition, an in vitro cell culture model was used to study the VCAM-1 mRNA expression and nuclear factor (NF)-κB activation in ECs, and evaluate the inhibitory effects of aspirin on this endothelial VCAM-1 expression and NF-κB activation.

METHODS

Materials
All cell culture materials were purchased from Gibco (Grand Island, NY, USA). sVCAM-1 enzyme-linked immunosorbent assay (ELISA) kit was obtained from R & D Systems (Minneapolis, MN). Hs-CRP assay kit was purchased from Beckman Coulter (Fullerton, CA). NF-κB activation ELISA kit was purchased from Panomics (Redwood City, CA). Acetylsalicylic acid (ASA, aspirin used for in vitro study), pyrrolidine dithiocarbamate (PDTC), and other chemicals of reagent grade were purchased from Sigma (St. Louis, MO).

Patients
The ethics committees of St. Martin De Porres Hospital and National Chiayi University approved the study protocol, and written informed consents were obtained from all patients before enrollment. Patients at high risk for cardiovascular events because of the presence of atherosclerotic disease, diabetes or at least two classical cardiovascular risk factors, including ages over 45 for men and over 55 for women, hypertension (blood pressure over 130/85 mm Hg), hypercholesterolemia (plasma cholesterol levels over 200 mg/dL), current smoker, body mass index (BMI) over 25 kg/m², and waist over 90 cm in men or over 80 cm in women were enrolled randomly from outpatient clinics. The patients were in stable condition and under therapy of 100 mg enteric
coated aspirin preparation per day for at least one month before the blood sample correction. In addition, no other anti-platelet medication was taken besides the aspirin. Outpatients at the same high risk for cardiovascular events without taking aspirin were enrolled as controls. Five mL of peripheral venous blood samples were drawn from the antecubital vein after overnight fasting, collected in a tube containing 3% citrate, and transported to the laboratory room within 30 min.

**Platelet preparation**

Platelets were prepared as previously described. Briefly, platelet-rich plasma was prepared by low-speed centrifugation (200 × g for 10 min at room temperature) of 10 mL blood samples. Platelets were obtained from the platelet-rich plasma by further centrifugation (700 × g for 15 min). The centrifuged platelets were then suspended in 3 mL HEPES buffer (145 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 0.5 mmol/L Na₂HPO₄, 6 mmol/L glucose, pH 7.4, at 37 °C) and counted by using an automatic analyser (model T-890, Coulter Co., Hialeah, FL, USA). A final concentration of 2~3 × 10⁸ platelets/mL was obtained by dilution. During the preparation procedure, the platelets were stored in polyethylene tubes and kept at room temperature. The cells were used for the tests within 2 h.

**Platelet activation analysis and platelet-monocyte aggregates analysis by flow cytometry**

In platelet activation analysis, the blood was left unstirred to avoid platelet activation. Four μmoles of adenodiphosphate (ADP) as a platelet agonist were added into 100 μL whole blood for 5 minutes. For studying the effect of exogenous aspirin, exogenous ASA was added into 100 μL whole blood, followed by the addition of another 4 μmoles of ADP. Then, 1X BD FACS Lysing Solution (BD Biosciences, San Diego, CA) was added for 10 minutes. The supernatant was removed after centrifugation; antibodies for CD42 and CD62 were added for 30 min. Individual platelets were identified on the basis of their differential light scattering characteristics and positive anti-CD42 fluorescence. Platelet activation was measured by the percentage of expression of CD62 on platelet surface (Figure 1A).

The measurement of platelet-monocyte aggregates was performed by flow cytometric analysis. BD FACS Lysing Solution was added into 100 μL whole blood for 10 min. The supernatant was removed after centrifugation. Then, antibodies for CD14 and CD42 were added for 30 minutes. Individual monocytes were identified on the basis of their differential light scattering characteristics and positive anti-CD14 fluorescence. Platelet-monocyte aggregates were expressed as the percentage of monocytes with CD42 fluorescence (Figure 1B).

**Hs-CRP analysis**

The hs-CRP in the samples was measured with a validated, high-sensitivity immunoassay method using an autoanalyzer (IMMAGE Immunochemistry Systems, Beckman Coulter). The hs-CRP concentrations were determined with a typical detection limit of 0.0165 mg/dL.

**sVCAM-1 analyses**

The concentrations of sVCAM-1 in serum and cell culture medium were measured by ELISA as previous described.

**Culture of ECs**

ECs were isolated from human umbilical cords as previous described. Briefly, human umbilical cords were washed, filled with 0.1% collagenase, and incubated at 37 °C for 10 min. The suspension of ECs were collected and centrifuged for 10 min. ECs were then resuspended in M199 medium supplemented with penicillin/streptomycin and 20% FBS. Cultures were main-

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*Figure 1.* (A) Platelets were distinguished from one another on the basis of their differential light scattering characteristics and positive anti-CD42 fluorescence. The horizontal axis reflects the size of the cells; the vertical axis reflects fluorescence intensity of bound FITC-labeled anti-CD42. (B) Monocytes were distinguished from one another on the basis of their differential light scattering characteristics and positive anti-CD14 fluorescence.
tained at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were kept static, and the culture medium was changed every 3 days.\(^\text{18}\)

**Real-time reverse transcription polymerase chain reaction**

Total RNA preparation and the RT reaction were carried out as described previously.\(^\text{19}\) PCRs were performed using an ABI Prism 7900HT according to the manufacturer’s instructions. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). The designed primers in this study were: VCAM-1 forward primer, 5’-ATG CCT GGG AAG ATG GTC G-3’; VCAM-1 reverse primer, 5’-TCT GGG GTG TGT TCG ATT TTA-3’; 18S rRNA forward primer 5’-GGG GTC ATT GAT GGC AAC AAT A-3; 18S rRNA reverse primer, 5’-ATG GGG AAG GTG AAG GTC G-3’. Quantification was performed using the 2\(^{-\Delta\Delta Ct}\) method.\(^\text{19}\)

**Transcription factor assay (TF ELISA assay)**

Nuclear extracts of cells were prepared as previously described.\(^\text{20}\) Equal amounts of nuclear extracts were used for quantitative measurements of NF-κB activation using commercially available ELISA kits that measure p65 NF-κB-DNA binding activities.

**Statistics**

There were two different types of baseline characteristics and risk factors of patients for the high 50-percentile group and low 50-percentile group, as shown in Table 1. In these continuous types (such as Age et al.), the equal variances conditions between high and low group were evaluated, and then the significance of any difference between two groups was tested by independent t test. About these discrete risk factors (such as, hypertension et al.), since there was not a large sample size, Fisher’s exact test was used for comparing two proportions. The distributions of hs-CRP, VCAM, and PMA between the two groups were assessed by a normal Q-Q plot (Table 2). If the distribution coincides with normality, independent t procedures were used. If not, the Mann-Whitney U test was adopted to assess differences in medians.

For in vitro cell culture test, the Statistical significance of VCAM-1 mRNA expression and NF-κB activa-

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**Table 1. Baseline characteristics of patients and concomitant medication in aspirin-treated patients with high and low 50-percentile platelet activation**

<table>
<thead>
<tr>
<th>Continuous variables</th>
<th>High 50-percentile (n = 21)</th>
<th>Low 50-percentile (n = 21)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>64.9 (9.91)</td>
<td>67.0 (11.59)</td>
<td>0.532</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.0 (7.99)</td>
<td>64.9 (10.44)</td>
<td>0.480</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>158.9 (7.57)</td>
<td>158.6 (7.37)</td>
<td>0.918</td>
</tr>
<tr>
<td>BMI</td>
<td>26.5 (2.62)</td>
<td>25.7 (3.10)</td>
<td>0.367</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>86.6 (7.70)</td>
<td>88.2 (9.98)</td>
<td>0.571</td>
</tr>
<tr>
<td>Discrete Variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (Male)</td>
<td>10 (47.6%)</td>
<td>12 (57.1%)</td>
<td>0.758</td>
</tr>
<tr>
<td>Hypertension</td>
<td>13 (61.9%)</td>
<td>15 (71.4%)</td>
<td>0.744</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>9 (42.9%)</td>
<td>5 (23.8%)</td>
<td>0.326</td>
</tr>
<tr>
<td>Active smoker</td>
<td>3 (14.3%)</td>
<td>2 (9.5%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>5 (23.8%)</td>
<td>6 (28.6%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Atherosclerotic disease</td>
<td>11 (52.4%)</td>
<td>5 (23.8%)</td>
<td>0.111</td>
</tr>
<tr>
<td>Concomitant Medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-blocker</td>
<td>12 (57.1%)</td>
<td>13 (61.9%)</td>
<td>0.744</td>
</tr>
<tr>
<td>ACEI or ARB</td>
<td>15 (71.4%)</td>
<td>13 (61.9%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Statin</td>
<td>11 (52.4%)</td>
<td>12 (57.1%)</td>
<td>1.000</td>
</tr>
<tr>
<td>DM medication</td>
<td>5 (23.8%)</td>
<td>6 (28.6%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>11 (52.4%)</td>
<td>13 (61.9%)</td>
<td>0.756</td>
</tr>
</tbody>
</table>

Data presented are mean (standard deviation) or number of patients (percentage).

ACEI, angiotensin-converting enzyme inhibitor; ARB, angitensin II receptor blocker; DM, diabetes mellitus.
tion was determined by Student’s t test. Statistic significance was assessed as \( p < 0.05 \).

**RESULTS**

**Patients’ characteristics and platelet activation by ADP**

A total of 42 cases, 22 men and 20 women aged between 41 and 81 years with validated data were enrolled in the study. The platelet activation of these 42 patients with aspirin usage ranged from 0.93%~6.61%, with a mean and standard deviation of 4.2\( \pm \)1.0%, which was significantly lower than the 9.0\( \pm \)0.5% (range 8.46%~9.96%) of 7 control patients without aspirin usage (Figure 2). Patients taking aspirin were divided into two groups of 21 patients each according to the median value of platelet activation at 4.2%, i.e., a high 50-percentile group and a low 50-percentile group. There were no significant statistical differences in age, gender, BMI, or waist size between these two groups. The history of hypertension, hypercholesterolemia, active smoking, diabetes and concomitant medication usage also did not show any statistical differences. Though there were 11 cases with symptomatic atherosclerotic diseases in the high percentile group, higher than 5 cases in the low percentile group, this difference did not reach statistical significance (Table 1).

**Platelet activation, platelet monocyte aggregates, hs-CRP and sVCAM-1**

There was no significant difference in both platelet monocyte aggregates and hs-CRP concentration between two groups. However, the serum concentrations of sVCAM-1 in the high-percentile group were significantly higher than the low-percentile group (Table 2).

**Aspirin inhibited platelet-induced VCAM-1 mRNA expression in ECs**

Vascular ECs are the major cellular source of VCAM-1 upregulation following inflammatory injury. As shown in Figure 3A, co-culture of ECs with ADP-activated platelets for 4 h induced a 9-fold increase in VCAM-1 mRNA expression in comparison to ECs co-cultured with control platelets.

To determine the role of aspirin in the induction of VCAM-1 expression in ECs by activated platelets, platelets were first incubated with ADP for 30 min, then with different doses of aspirin for another 30 min in the presence of ADP, and finally co-cultured with ECs for 4 h. The expression of VCAM-1 mRNA in ECs was inhibited by aspirin in a dose-dependent manner, suggesting that different levels of platelet activation may modulate VCAM-1 mRNA expression in ECs (Figure 3B). Similarly, when ECs were first incubated with different doses of aspirin for 2 h, and then co-cultured with ADP-activated platelets for 4 h in the presence of the aspirin, the expression of VCAM-1 mRNA in ECs was also inhibited by the aspirin pretreatment in a dose-dependent manner (Figure 3C). Furthermore, after 2 h of pretreatment with aspirin, the subsequent transfer of ECs to a fresh medium without aspirin for co-culture with ADP-activated platelets, led to an inhibition of the expression of VCAM-1 mRNA in ECs by about 30% (Figure 3D).

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**Table 2. Platelet-monocyte aggregates and inflammatory markers in aspirin-treated patients with high and low 50-percentile platelet activation**

<table>
<thead>
<tr>
<th></th>
<th>High 50-percentile</th>
<th>Low 50-percentile</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-CRP (mg/L)</td>
<td>21.12</td>
<td>21.88</td>
<td>0.840</td>
</tr>
<tr>
<td>VCAM-1 (pg/mL)*</td>
<td>25.67</td>
<td>17.33</td>
<td>0.028*</td>
</tr>
<tr>
<td>PMA (%)</td>
<td>19.10</td>
<td>23.90</td>
<td>0.204</td>
</tr>
</tbody>
</table>

PMA: platelet-monocyte aggregates.

* \( p < 0.05 \) under Mann-Whitney U test.

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**Figure 2.** The percentages of platelet activation in patients with or without aspirin usage. The 42 patients with aspirin usage were in wide variation with range from 0.93%~6.61%. The percentages of platelet activation in 7 patients without aspirin usage were 8.46%~9.96%. \( *p < 0.05 \).
These results suggested that aspirin not only inhibited platelet activation by ADP, but also decreased the activated platelet-induced VCAM-1 expression in ECs.

**Aspirin inhibits VCAM-1 mRNA expression via suppressing NF-κB activation in ECs**

Activation of NF-κB is known to be an important participant in cytokine-induced VCAM-1 expression in ECs.\(^6,21\) We tested whether NF-κB activation is involved in the activated platelet-induced VCAM-1 gene expression. ECs were incubated with the specific inhibitor for NF-κB (PDTC, 50 and 150 μM) for 1 h and followed by stimulation of activated platelets for 4 h. The activated platelet-induced VCAM-1 mRNA expression was significantly reduced by NF-κB inhibition with PDTC, indicating that NF-κB is involved in the regulation of VCAM-1 gene expression (Figure 4A). We further evaluated the activated platelet-induced NF-κB activation by TF ELISA assay kits from Panomics. ECs co-cultured with activated platelets showed that stimulation of ECs with activated platelets increased p65 NF-κB-DNA binding activity from 30 min and remained elevated activity for at least 2 h (Figure 4B). To determine the role of aspirin in the activation of NF-κB in ECs by activated platelets, platelets were incubated with ADP for 30 min, then with 100 μM aspirin for another 30 min in the presence of ADP, and co-cultured with ECs for 4 h. The activation of p65 NF-κB in ECs was inhibited by aspirin, suggesting that inhibition of platelet activation may reduce NF-κB activation in ECs (Figure 4C). In addition, ECs were first incubated with 100 μM aspirin for 2 h, and then co-cultured with ADP-activated platelets for 4 h in the presence of the aspirin, the activation of NF-κB in ECs was also inhibited by the aspirin pretreatment (Figure 4C). These results provide additional evidence that the NF-κB activation plays an important role in the regulation of activated platelet-induced VCAM-1 expression in ECs.

**DISCUSSION**

The results of this study have several significant clinical implications. First, the patients at high risk for cardiovascular events had a higher platelet activation percentage without aspirin usage than did those patients
with aspirin usage. Second, high-risk patients with aspirin usage showed wide percentage variations of platelet activation. Third, patients in the high-percentile group of platelet activation showed higher serum sVCAM-1 concentration.

Aspirin is the most commonly-used inhibitor of platelet function in clinical practice. Many clinical studies have shown that aspirin is effective for both primary and secondary prevention for myocardial infarction, stroke and cardiovascular death. It is also effective in the acute management of myocardial infarction, unstable angina and embolic stroke. Although the benefits of aspirin are widely accepted, there are still a proportion of patients who experience poor results despite daily aspirin therapy. Atherothrombosis is a complex physiological process, and the anti-platelet effects of aspirin may not be uniform in all patients. In the present study, platelet activation was measured by the percentage of CD62 expression on platelet surface after ADP stimulation by flow cytometry. The anti-platelet effect of aspirin in high-risk patients is variable, ranging from 0.93% to 6.61% (4.2 ± 1.0%) (Figure 2). In the study by Benedek et al., 10 healthy males received a single oral 80-mg aspirin dose on three separate days and the successive doses were separated by a two-week washout interval. Their data showed that aspirin pharmacokinetics and platelet aggregation response exhibited considerable intra- and inter-subject variability. In addition, inadequate aspirin absorption in patients could also be detected by measurement of serum TXB2 levels. Maree et al. 25 found that 44% of 131 stable cardiovascular patients who took enteric coated aspirin had elevated serum TXB2 levels. Platelet aggregation occurred more frequently in these patients with elevated serum TXB2 levels. Cox et al. 26 demonstrated that the lower bioavailability of enteric coated aspirin and poor absorption from the higher pH environment of the small intestine may result in an inadequate amount of aspirin being delivered into the circulatory system. However, the pH environment of the stomach/intestine in patients of this study needs further investigation. Although the mechanism of the variability of anti-platelet effect of aspirin in this study was unclear, pharmacokinetic issues including absorption problem should definitely be considered.

The high-risk patients with aspirin usage were divided into two groups according to the degree of platelet activation. A previous study showed that patient weight and age were significant independent predictors of an incomplete response to aspirin. 25 However, in this study, no significant differences in terms of hs-CRP and platelet-monocyte aggregates, the serum level of sVCAM-1 was higher in the high-percentile group. This result supports the emerging concept that platelets play a role in the thrombosis and inflammation in atherosclerosis.
The change of inflammatory biomarkers such as sVCAM-1 is an important factor and plays a pivotal role during atherogenesis.27 Activated platelets may also participate in such steps of atherogenesis by modulating gene expressions of ECs.28 Although many investigations have focused on quantifying the percentage of patients who display aspirin resistance, the relationship between the variability of the anti-platelet effect of aspirin and the change of VCAM-1 gene expression level is unclear. In order to evaluate the effect of aspirin on platelet-induced endothelial VCAM-1 gene activation, an in vitro model was used to study the VCAM-1 mRNA expression in HUVECs. The results demonstrated that the expression of VCAM-1 mRNA in ECs was significantly up-regulated by ADP-activated platelets, as compared with the control (untreated) platelets (Figure 3A). Several previous studies have shown that platelet activation was reduced by aspirin in vivo and in vitro.29-31 This study found that the treatment of aspirin in platelets may decrease the effect of ADP on platelet activation because of the reduced VCAM-1 expression in ECs (Figure 3B). According to these data, aspirin may exert anti-inflammatory effects on both platelets and ECs.

CONCLUSION

The anti-platelet effect of aspirin is not uniform among patients. This variability is important. This study demonstrated that inadequate inhibition of platelet activation by aspirin was associated with higher serum concentration of sVCAM-1 in subjects with high cardiovascular risk.

ACKNOWLEDGEMENTS

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