Platelet-specific markers are associated with monocyte-platelet aggregate formation and thrombin generation potential in advanced atherosclerosis

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Summary
Platelet activation and thrombin generation are crucial steps in primary and secondary haemostasis. However, both also play major roles in intravascular thrombus formation and therefore in the development of adverse cardiovascular events. In the current study, we first sought to investigate the associations of the platelet biomarkers platelet factor (PF)-4, thrombospondin (TSP)-1, soluble CD40 ligand (sCD40L), and soluble P-selectin (sP-selectin) with each other and with monocyte-platelet aggregate (MPA) formation in 316 patients undergoing angioplasty and stenting. To better understand the interplay between platelet activation and thrombin generation, we subsequently investigated the associations of the platelet biomarkers with thrombin generation potential. The mostly platelet-specific markers PF-4, TSP-1 and sCD40L correlated strongly with each other (all p < 0.001), and the best correlation was observed between PF-4 and TSP-1 (r=0.91). In contrast, sP-selectin, which derives from platelets and endothelial cells, correlated rather poorly with TSP-1 (r=0.12, p=0.04), and did not correlate with PF-4 and sCD40L. While PF-4, TSP-1 and sP-selectin correlated significantly with in vivo MPA formation (all p<0.001), no such association was found between sCD40L and MPA formation. PF-4, TSP-1 and sCD40L correlated strongly with peak thrombin generation (all p<0.001) with the best correlation between PF-4 and peak thrombin generation (r=0.55), whereas sP-selectin did not correlate with peak thrombin generation. Likewise, PF-4, TSP-1 and sCD40L correlated significantly with the area under the thrombin generation curve (AUC; all p<0.01), whereas sP-selectin did not correlate with the AUC. In conclusion, platelet-specific markers are associated with MPA formation and thrombin generation potential in patients with advanced atherosclerosis.

Keywords
Platelet factor-4, thrombospondin-1, soluble CD40 ligand, soluble P-selectin, thrombin generation potential

Introduction
Undesired platelet activation plays a major role in the development of acute ischaemic events in patients with cardiovascular disease (1). In response to atherosclerotic plaque rupture, platelets adhere to exposed collagen structures of the injured vessel wall and initiate clot formation, thereby leading to further platelet recruitment and activation with subsequent vessel stenosis or occlusion (2).

Upon platelet activation, platelets secrete their granules’ contents and components of their α-granules’ releasate can subsequently be determined in plasma (2). Platelet factor-4 (PF-4) and thrombospondin (TSP)-1 are platelet α-granule proteins being released from activated platelets in large amounts (3). PF-4 was found to have procoagulatory functions in vitro, but its function in vivo is not fully understood yet (4). TSP-1 has anti-angiogenic and also procoagulatory properties (5). CD40 ligand (CD40L), a transmembrane protein located in platelet α-granules, is translocated to the platelet surface and can therefore also be considered mostly platelet-specific. Once on the surface, it is cleaved and a soluble form of CD40L (sCD40L) is released into the circulation. Elevated levels of sCD40L have been linked to the occurrence of future ischaemic events in patients with acute coronary syndromes (ACS) (6-8), and increased sCD40L was therefore proposed as predictor of worse clinical outcomes in these patients (9).

P-selectin is a cell adhesion molecule, which is also stored in platelet α-granules (2). Following platelet activation, it is translocated to the platelet surface from where it is cleaved and released into the circulation in its soluble form (sP-selectin) (10). However, in contrast to the aforementioned mostly platelet-specific biomarkers, substantial amounts of P-selectin derive not only from platelets, but also from the endothelial Weibel-Palade bodies (11).

Platelet adhesion to monocytes is a common phenomenon and increased in atherosclerosis (12). The P-selectin – P-selectin glycoprotein ligand (PSGL)-1 interplay has been revealed as major axis for leukocyte-platelet aggregate formation (13). Circulating...
monocyte–platelet aggregates (MPA) were shown to be a more sensitive marker of in vivo platelet activation than platelet surface P-selectin expression in several pathophysiological circumstances including myocardial infarction (14). Besides its procoagulant activity, MPA formation has pro-inflammatory effects, thereby linking coagulation and the development of atherosclerosis. Among others, a biological meaning of MPA formation could be the uptake of tissue factor into platelets’ storage after its generation by monocytes (15). Indeed, activated platelets release tissue factor, thereby inducing thrombin generation (16, 17).

Thrombin generation in vivo is the result of a variety of procoagulatory processes. Intravascular thrombin generation is considered a powerful driver of recurrent atherothrombotic events in patients with cardiovascular disease (18). Thrombin activates platelets via protease-activated receptors (PAR)-1 and –4, and glycoprotein Ib–IX-V (2). Thereby, subnanomolar concentrations of α-thrombin may suffice for PAR-1 and –4 coordinated platelet activation.

In the current study, we first sought to investigate the associations of PF-4, TSP-1, sCD40L, and sP-selectin with each other and with MPA formation in patients undergoing angioplasty and stenting for cardiovascular disease. To better understand the interplay between platelet activation and thrombin generation, we subsequently investigated the association of PF-4, TSP-1, sCD40L and sP-selectin with endogenous thrombin generation potential.

Methods

Patients

The study population consisted of 316 patients on dual antiplatelet therapy after percutaneous intervention with endovascular stent implantation.

All patients received daily aspirin (100 mg/day) and clopidogrel (75 mg/day) therapy. Exclusion criteria were a known aspirin or clopidogrel intolerance (allergic reactions, gastrointestinal bleeding), a therapy with vitamin K antagonists (warfarin, phenprocoumon, acenocoumarol), a treatment with ticlopidine, dipyridamol (75 mg/day) therapy. Exclusion criteria were a known aspirin or nonsteroidal antiinflammatory drugs, a family or personal history of bleeding disorders, malignant paraproteinaemias, myeloproliferative disorders or heparin-induced thrombocytopenia, severe hepatic failure, known qualitative defects in thrombocyte function, a major surgical procedure within one week before enrollment, a platelet count < 100,000 or > 450,000 µl and a haematocrit < 30%.

The study protocol was approved by the Ethics Committee of the Medical University of Vienna in accordance with the Declaration of Helsinki and written informed consent was obtained from all study participants.

Blood sampling

Blood was drawn by clean venipuncture from an antecubital vein using a 21-gauge butterfly needle (0.8 x 19 mm; Greiner Bio-One, Kremsmünster, Austria) one day after the percutaneous interven-

tion. To avoid procedural deviations all blood samples were taken by the same physician applying a light tourniquet, which was immediately released and the samples were mixed adequately by gently inverting the tubes. After the initial 3 ml of blood had been discarded to reduce procedurally induced platelet activation, blood was drawn into 3.8% sodium citrate Vacuette tubes (Greiner Bio-One; 9 parts of whole blood, 1 part of sodium citrate 0.129 M/l) for flow cytometric analysis of MPA formation. Plasma was obtained from these samples by centrifugation at 3,000g for 10 minutes (min), and aliquots were stored at –80°C until analyses.

Measurement of PF-4, TSP-1, sCD40L and sP-selectin

PF-4, TSP-1, sCD40L and sP-selectin were measured in duplicates by using commercially available ELISA kits (Quantikine®, R&D Systems, Minneapolis, MN, USA), following the manufacturer’s instructions. All assay kits were from the same batch and all samples were tested within the same run by the same operator. The lower limits of detection are 0.01 ng/ml, 0.095 ng/ml, 2.1 pg/ml, and 0.01 μg/l for PF4, TSP-1, sCD40L, and sP-selectin, respectively. The intra-assay coefficients of variation were < 10% for all assays (n=21). PF-4, TSP-1, and sP-selectin were available for all patients, sCD40L was available for 204 patients (65%).

Determination of MPA formation

MPA were identified in citrate anticoagulated blood as previously described (19). In brief, HEPES buffer was added to 5 μl whole blood, diluted in 55 μl HEPES-buffered saline. After 10 min, monoclonal antibodies (anti-CD45-peridinin chlorophyll protein (clone 2D1, Becton Dickinson [BD], Franklin Lakes, NJ, USA), anti-CD41-phycocerythrin, (clone P2, Immunotech, Vaudreuil-Dorion, QC, Canada), and anti-CD14-allophycocyanin (clone MqP9, BD), or isotype-matched controls were added. After 15 min, samples were diluted with FACSlysing solution and at least 10,000 CD45+ events were acquired immediately on a FACSCalibur flow cytometer (BD) with excitation by an argon laser at 488 nm and a red diode laser at 635 nm at a rate of 200–600 events per second. Within these events, lymphocytes, granulocytes, and monocytes were identified, based on their side scatter versus CD14 characteristics. Monocytes were identified as CD14+ and the CD45+CD14+ events were subjected to further analyses for CD14+ events. The percentage of CD14+CD41+ events of all CD14+ events was recorded. Standard BD Calibrite beads were used for daily calibration of the cytometer. The intra-assay coefficient of variation was 5.4% for MPA formation (n=21). MPA formation was available for 259 patients.

Measurement of thrombin generation potential

Thrombin generation was measured with a commercially available assay (Technothrombin TGA kit, Technoclone, Vienna, Austria) on a fully automated, computer-controlled microplate reader (BioTek, FL X800) and a specially adapted software (Technothrombin TGA, Vienna, Austria) using the fluorogenic substrate Z-Gly-Gly-
Arg-AMC (Bachem, Bubendorf, Switzerland) according to the manufacturer’s instructions as previously described (20). The reaction was triggered with the TGA RC low reagent, which contained 71.6 pmol recombinant human tissue factor lipidated in 3.2 μmol/l phospholipid micelles (phosphatidylcholine [2.56 μmol/l] and phosphatidylserine [0.64 μmol/l]). In this assay, thrombin activity is registered in a thrombin generation curve. From this curve, various parameters can be read out that describe thrombin activity. For analysing the association of thrombin generation potential with PF-4, TSP-1, sCD40L and sP-selectin, the parameters peak thrombin generation (the maximum concentration of thrombin generation) and area under the thrombin generation curve (AUC) were used. All assay kits were from the same batch and all samples were tested within the same run by the same operator. The lower limit of detection with this assay is 0 for peak thrombin generation and the AUC. The intra-assay coefficients of variation were 12% and 5.5% for peak thrombin generation and the AUC, respectively (n=21). Thrombin generation potential was available for all patients.

Statistical analysis

Using a 0.05 two-sided Fisher’s z test of the null hypothesis that the correlation coefficient r=0.2, we calculated that we would have a power of 99% to detect an r of 0.5 with a sample size of 200 patients.

Statistical analysis was performed using the Statistical Package for Social Sciences (IBM SPSS version 23, Armonk, NY, USA). Median and interquartile range of continuous variables are shown. Categorical variables are given as number (%). Spearman rank correlation was used to test for correlations. Two-sided p-values <0.05 were considered statistically significant.

Results

Clinical, laboratory and procedural characteristics of the patient population are given in Table 1.

PF-4, TSP-1 and sCD40L correlated strongly with each other (Table 2; all p <0.001), and the best correlation was observed between PF-4 and TSP-1 (Table 2; r=0.91, p<0.001). In contrast, sP-selectin correlated rather poorly with TSP-1 (r=0.12, p=0.04), and did not correlate with PF-4 and sCD40L (Table 2).

Moreover, we investigated the associations of PF-4, TSP-1, sCD40L and sP-selectin with MPA formation in vivo. While PF-4, TSP-1 and sP-selectin correlated significantly with in vivo MPA formation (Figure 1A, B, D; all p<0.001), no such association was found between sCD40L and MPA formation (Figure 1C).

Peak thrombin generation and the AUC correlated significantly (r=0.53, p<0.001). In a further step, we investigated the associations of PF-4, TSP-1, sCD40L and sP-selectin with peak thrombin generation and the AUC, respectively. PF-4, TSP-1 and sCD40L correlated strongly with peak thrombin generation (Figure 2A-C; all p<0.001) with the best correlation between PF-4 and peak thrombin generation (r=0.55, p<0.001), whereas sP-selectin correlated rather poorly with TSP-1 (r=0.12, p=0.04).

Table 1: Clinical, laboratory and procedural characteristics of the overall study population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall (n=316)</th>
</tr>
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<tbody>
<tr>
<td>Demographics</td>
<td></td>
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<tr>
<td>Age, years</td>
<td>66 (58 – 75)</td>
</tr>
<tr>
<td>Male sex</td>
<td>206 (65.2)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.8 (24.2 – 29.7)</td>
</tr>
<tr>
<td>Medical history</td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>284 (89.9)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>294 (93)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>102 (32.3)</td>
</tr>
<tr>
<td>Active smoking</td>
<td>133 (42.1)</td>
</tr>
<tr>
<td>Laboratory data</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin, g/dl</td>
<td>12.8 (11.8 – 13.9)</td>
</tr>
<tr>
<td>White blood cell count, G/l</td>
<td>8.1 (6.7 – 9.9)</td>
</tr>
<tr>
<td>Platelet count, G/l</td>
<td>208 (176 – 251)</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>1 (0.9 – 1.2)</td>
</tr>
<tr>
<td>C-reactive protein, mg/dl</td>
<td>1 (0.4 – 1.9)</td>
</tr>
<tr>
<td>Procedure</td>
<td></td>
</tr>
<tr>
<td>Stent implantation</td>
<td>316 (100)</td>
</tr>
<tr>
<td>Number of stents/patient</td>
<td>1 (1 – 2)</td>
</tr>
<tr>
<td>Medication pre-intervention</td>
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<tr>
<td>Clopidogrel</td>
<td>316 (100)</td>
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<td>Aspirin</td>
<td>316 (100)</td>
</tr>
<tr>
<td>Statins</td>
<td>301 (95.3)</td>
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<tr>
<td>ACE inhibitors/ARB</td>
<td>273 (86.4)</td>
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<tr>
<td>Beta blockers</td>
<td>217 (68.7)</td>
</tr>
<tr>
<td>Proton pump inhibitors</td>
<td>166 (52.5)</td>
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</tbody>
</table>

Table 2: Correlations between platelet factor (PF)-4, thrombospondin (TSP)-1, soluble CD40 ligand (sCD40L), and soluble P-selectin (sP-selectin).

<table>
<thead>
<tr>
<th></th>
<th>PF-4</th>
<th>TSP-1</th>
<th>sCD40L</th>
<th>sP-selectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-4</td>
<td>1</td>
<td>0.91*</td>
<td>0.7*</td>
<td>0.08</td>
</tr>
<tr>
<td>TSP-1</td>
<td>1</td>
<td>0.65*</td>
<td>0.12*</td>
<td></td>
</tr>
<tr>
<td>sCD40L</td>
<td>1</td>
<td></td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>sP-selectin</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p<0.001; *p=0.04.
did not correlate with peak thrombin generation (Figure 1D). Likewise, PF-4 (r=0.2), TSP-1 (r=0.21) and sCD40L (r=0.2) correlated significantly with the AUC (all p<0.01), whereas sP-selectin did not correlate with the AUC (r=0.09, p=0.1).

**Discussion**

To the best of our knowledge, our study is the first to investigate the associations between four parameters of continuously ongoing platelet activation as well as the associations of these biomarkers with MPA formation and thrombin generation potential in a large population of patients undergoing angioplasty and stenting for advanced atherosclerotic cardiovascular disease. We observed a close correlation between the plasma levels of PF-4, TSP-1 and sCD40L, pointing towards their common origin and “platelet specificity”. In contrast, sP-selectin, which may not exclusively come from platelets (11, 21), did not correlate with PF-4 and sCD40L, and correlated only rather poorly with TSP-1. Only the platelet-specific biomarkers were closely correlated with thrombin generation potential. However, both, the platelet-specific biomarkers PF-4 and TSP-1 as well as sP-selectin correlated with in vivo MPA formation, although only moderately.

The advantage of assessing platelet biomarkers in plasma, rather than their determination on the platelet surface, is their stability in plasma over time, while platelet surface expression represents only a snapshot at the time of blood sampling. Thus, plasma levels of platelet biomarkers provide a good estimate of continuously ongoing platelet activation.

PF-4, TSP-1 and sCD40L, which are almost or completely platelet-derived, correlated with each other, but showed no or only a very weak correlation with sP-selectin. These findings constitute further evidence that plasma levels of P-selectin come from platelets, but at various levels also from the Weibel-Palade bodies of endothelial cells (11, 21). The extent of this contribution to plasma levels of P-selectin may vary in different diseases. (11) High levels of sP-selectin have been associated with the risk of future ischaemic events in apparently healthy women (22), with recurrent
venous thromboembolism (VTE) in patients with a history of VTE (23–25), and with VTE in cancer patients (26).

Upon platelet activation, P-selectin is translocated from alpha granules to the platelet surface, where it subsequently facilitates platelet adhesion to leukocytes. The interplay of platelet P-selectin with PSGL-1 on leukocytes is the most important axis for leukocyte-platelet interactions (13). Thus, levels of P-selectin are expected to correlate with those of platelet adhesion to monocytes. Numerous papers have in fact shown a close correlation between P-selectin exposed on the platelet surface and MPA formation (13). Likewise, sP-selectin would be expected to correlate with MPA formation. However, as suggested by our data, sP-selectin derives only in part from activated platelets (11), and therefore its levels were rather weakly correlated with MPA formation. An at least similar degree of correlation with MPA formation was expected for the mostly platelet-specific biomarkers PF-4 and TSP-1. Indeed, the correlation of PF-4 and TSP-1 with MPA formation in our study population was comparable to the correlation between sP-selectin and MPA formation. Unexpectedly, however, sCD40L was not correlated with MPA formation, pointing towards a different mechanism of its release. This difference is also exemplified by the clinical findings that high sCD40L is only a risk factor for subsequent ischaemic events in patients with ACS (6–9, 27, 28), while high sP-selectin seems to be generally associated with an increased risk of atherothrombotic events and VTE (22–26).

For analysing the association of thrombin generation potential with PF-4, TSP-1, sCD40L and sP-selectin, the parameters peak thrombin generation and AUC were used. Peak thrombin generation by this assay has been repeatedly linked to the occurrence of VTE in large patient populations (29, 30), and is recommended by the manufacturer as best parameter to reflect thrombin activity. The AUC is similar to the endogenous thrombin generation potential, which can be measured by a different thrombin generation assay (31), and was therefore chosen as second parameter of thrombin generation. Consequently, our study is the first investigation on the associations of four widely-available and well-established platelet biomarkers with two strong indicators of thrombin activity in patients with cardiovascular disease.

Figure 2: Correlations of (A) platelet factor-4, (B) thrombospondin-1, (C) soluble CD40 ligand and (D) soluble P-selectin with peak thrombin generation.
What is known about this topic?

- Platelet activation and thrombin generation are crucial steps in primary and secondary haemostasis.
- Both also play major roles in intravascular thrombus formation and therefore in the development of adverse cardiovascular events.
- Platelet factor (PF)-4, thrombospordin (TSP)-1, soluble CD40 ligand (sCD40L) and soluble P-selectin (sP-selectin) are soluble biomarkers that are widely used to estimate the level of platelet activation.

What does this paper add?

- The mainly platelet-specific biomarkers PF-4, TSP-1 and sCD40L correlated strongly with each other, but not with soluble P-selectin.
- These findings indicate that a significant part of sP-selectin is not platelet-derived in patients with advanced atherosclerosis.
- PF-4, TSP-1 and sP-selectin correlated significantly with in vivo monocyte-platelet aggregate formation.
- PF-4, TSP-1 and sCD40L correlated strongly with peak thrombin generation suggesting that smoldering but continuous platelet activation may lead to an increased thrombin generation potential and thus a coagulation system poised for further activation in patients with advanced atherosclerosis.

The meaning of platelet adhesion to monocytes could be manifold. It is known, that activated platelets release tissue factor (16, 17), which then induces thrombin generation. This tissue factor originates mainly from monocytes, which need hours for its synthesis. Thus, the immediate source of tissue factor is most likely platelets, while its continuous production is ensured by monocytes. The uptake of tissue factor is facilitated by platelets’ adhesion to monocytes. These hypotheses are strengthened by our observation that levels of platelet activation as reflected by platelet-specific biomarkers correlated with thrombin generation potential. We made a very similar observation in patients with cancer at risk for VTE. Only the platelet-specific markers PF-4 and TSP-1 were correlated with peak thrombin generation in these patients, while levels of sP-selectin were not (Riedl et al., unpublished data, and 32). We therefore propose that activated platelets lead to an increased thrombin generation potential. Any additional prothrombotic influence, like the rupture of an atherosclerotic plaque of the vessel wall or the continuous impact of cardiovascular risk factors, may shift the balance further towards more pronounced platelet activation and thrombin generation, thereby increasing the risk of vessel occlusion with subsequent ischaemic complications.

It has been shown that antiplatelet therapy with aspirin and P2Y12 receptor blockers significantly reduces platelet granule secretion. In detail, Azar et al. and we demonstrated that clopidogrel diminishes the release of sCD40L by platelets in patients with stable coronary artery disease and in patients undergoing cardiac catheterisation compared to aspirin monotherapy (33, 34). Moreover, Valdes et al. reported a significant reduction of sCD40L and sP-selectin levels after one week of low-dose aspirin (35). Consequently, it would be interesting to assess the plasma concentrations of PF-4, TSP-1, sCD40L and sP-selectin and their association with thrombin generation potential in patients at risk of ischaemic events, who do not receive antiplatelet therapy. However, since patients with known cardiovascular disease, who are referred to our clinic, are usually pre-treated with antiplatelet agents, we were not able to recruit such a patient population.

Limitations of our study are its correlational design and the lack of clinical outcome data. Moreover, all parameters were assessed at a single time point after the revascularisation procedure, and we therefore cannot rule out variations of platelet activation markers, MPA formation and thrombin generation potential over time.

In summary, platelet-specific markers are associated with MPA formation and thrombin generation potential in patients with advanced atherosclerosis. One may speculate that smoldering but continuous platelet activation leads to increased levels of thrombin generation potential and thus a coagulation system poised for further activation. The adhesion of platelets to monocytes seems more complex and may rather result from activated platelets, leukocytes and endothelial cells together.

Conflicts of interest

None declared.

References


