PLATELETS ACTIVATION IN PATIENTS UNDERGOING PTCA AND THEIR RESPONSIVENESS AFTER IN VITRO STIMULATION

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Summary: We evaluated expression of platelet activation markers in blood samples of 15 patients who underwent percutaneous transluminal coronary angioplasty (PTCA) by flow cytometry. Analysis was performed before the beginning of PTCA, during initial coronary angiography and after the end of PTCA or after a stent placement, respectively. We evaluated platelet-derived microparticles, platelet-leukocyte aggregates, platelet aggregates and a membrane expression of CD62P and CD63 molecules. Responsiveness of platelets to the activation in vitro with thrombin-receptor activating protein – 6 (TRAP-6) was tested simultaneously. Statistically significant differences between patient samples were found only in the expression of the activation markers CD62P (before PTCA 0.22 %, during 0.39 %, after 0.67 %), CD63 (0.26 %/ 0.45 %/ 0.85 %) and platelet-leukocyte aggregates (13.57 %/ 18.39 %/ 23.63 %). In the same group the expression of all constitutive membrane markers was statistically significantly decreased: in patients undergoing PTCA was the expression of CD9:87.98 % (in comparison with control group 94.98 %), CD31: 87.10 % (92.78 %), CD36: 87.37 % (90.98 %), CD41: 88.09 % (95.62 %), CD42a: 88.54 % (94.98 %), CD42a: 88.31 % (94.13 %).

Key words: Platelets; Activation markers; Angioplasty; Flow cytometry; Platelet activation in vitro

**Introduction**

PTCA is an invasive procedure during that dilated balloon is causing a “controlled lesion” in the arterial wall narrowed by stenosis. Endothelial cell-lining of artery is damaged by inflated balloon and is changed from thromboresistant to prothrombogenic. Thrombi could be formed which are, in the most aggravated situation, responsible for the occlusion of dilated artery (6,18,29). The activation of platelets can be followed by flow cytometry. The formation of heteroaggregates between platelets and leukocytes, platelets homoaggregates together with release of procoagulant platelet microparticles could be determined. The ample evidence of previous platelet activation is simultaneous membrane expression of both P-selectin (CD62P) adhesion molecule and CD63 receptor on the surface of platelets (1,9,24,26,27). In patients who underwent PTCA we followed before procedure, during and after finishing of PTCA, the expression of the constitutively expressed platelet membrane molecules CD9, CD31, CD36, CD41, CD42a and CD42b, and activation-induced platelet membrane molecules CD62P and CD63. Other markers of platelet activation such as platelet microparticles (MPs), platelet homoaggregates and aggregates between platelets and leukocytes (PLAs) were also determined by flow cytometry. Finally, the ability of platelets to respond to TRAP-6 stimulation in vitro was also determined (30).

**Patients and methods**

**Patients**

The group of 15 patients (14 men, 1 woman, mean age 59.3 yrs.) who underwent PTCA for stenosis of myocardial arteries at the Department of Cardiosurgery, University Hospital, Charles University, Faculty of Medicine, Hradec Králové, was examined. The study was approved by Institutional Ethical Committee and written informed consent was obtain from any participant. Patients were medicated 24 hrs. before PTCA by 100mg of acetylosalicylic acid in all but one exception who was medicated by low-molecular heparin (nadroparin) 11.4kI.U. subcutaneously. Anxiolytic drugs concomitantly with antihistaminic drugs were administered to all patients immediately before PTCA. Average dose of heparin during PTCA administered to each patients was 11 060 I.U. After the procedure 10 patients were treated by 500mg of ticlopidine, heparin 3000 I.U. was intravenously administered to two patients and one patients was treated by 10.9ml nadroparine subcutaneously. Long-lasting medication of patients included acetylosalicylic acid (13 patients), nitrates (13 patients), β-blocators (13 patients), hypolipidemics (9 patients), ACE-inhibitors (6 patients), antiarrythmiacs (3 patients) and many others. Minimal (maximal) number of long-lasting medication administered to one patients in patients group was 6 (13), mean 9.
For important characteristics of the patient’s group see the Tab. 1.

As a control, peripheral blood samples obtained from a group of 25 adults (17 women, 8 men) recruited from the staff members were examined. All volunteers were otherwise healthy, without any medication 2 weeks before blood sampling. The values of their blood leukocytes, blood cell count and platelet parameters were falling into the physiological ranges. Parameters of the patients and control group are shown in the Tab. 2.

Tab. 1: Patients underwent PTCA – essential parameters.

| gender (M:F) | 14:1  |
| age (yrs) (mean) | 59.5  |
| weight (kg) (mean) | 83.5  |
| height (cm) (mean) | 173.6  |
| number of occluded coronary arteries in one patient: |  |
| 1 coronary artery | 9  |
| 2 coronary arteries | 5 |
| 3 coronary arteries | 1 |
| total number of balloon dilatations | 36 |
| localization of dilated stenosis: ACD | 6  |
| RC | 3 |
| RD | 3 |
| RIA | 5 |
| RIM | 2 |
| RMS | 2 |
| graft on RMS | 1 |
| administered dose of heparin in 1 PTCA (mean) | 11 060 I.U. |

Tab. 2: Comparison of selected parameters between patients underwent PTCA and healthy controls.

<table>
<thead>
<tr>
<th>parameter</th>
<th>healthy controls mean / SD</th>
<th>patients mean / SD</th>
<th>comparison L / S / H</th>
<th>statistical significance (p)</th>
<th>statistical test</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>32.04 / 9.69</td>
<td>59.3 / 8.32</td>
<td>/ H</td>
<td>&lt;0.001</td>
<td>t-test</td>
</tr>
<tr>
<td>PTC</td>
<td>199.46 / 44.65</td>
<td>221.21 / 50.66</td>
<td>S /</td>
<td>0.18</td>
<td>t-test</td>
</tr>
<tr>
<td>MPV</td>
<td>8.09 / 0.24</td>
<td>8.49 / 0.76</td>
<td>/ H</td>
<td>&lt;0.001</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>PCT</td>
<td>0.14 / 0.04</td>
<td>0.19 / 0.04</td>
<td>/ H</td>
<td>&lt;0.05</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>PDW</td>
<td>17.36 / 0.68</td>
<td>15.8 / 1.18</td>
<td>L /</td>
<td>&lt;0.001</td>
<td>Mann-Whitney</td>
</tr>
</tbody>
</table>

Method

Three blood samples were drawn from each patient to VACUETTE® (Greiner Labortechnik, Austria) tubes treated with citrate sodium. The first sample (A) was collected from vena brachialis before PTCA. The second sample (B) was an arterial blood obtained from balloon catheter located into proximity of arterial stenosis immediately after visualisation of coronary arteries by roentgen-contrasting compound. The third sample (C) was obtained from the same arterial catheter 10min after PTCA or 10min after stent placement, respectively (28). The samples were processed within 2hrs. after collection. Carefully selected whole blood method to avoid an artificial platelets activation was used (2,12).

Blood samples from fasting healthy control were drawn from cubital vein at 8 to 9 o’clock a.m. Needles LUER 22G (Greiner Labortechnik, Austria) were used. The first 2ml of blood were used to determine basal hematological parameters (platelets count (PTC), mean platelet volume (MPV), platelet hematocrite (PCT), width of platelet volume distribution (PDW). Next 2ml of blood were used both for flow cytometry analysis and TRAP-6 induced platelet activation in vitro.

To identify membrane molecules of platelets the panel of monoclonal antibodies purchased from Immunotech® (Immunotech – a Beckman Coulter Company, Marseille, France) was established. This panel includes either FITC or PE conjugated monoclonal antibodies against CD9, CD31, CD36, CD41, CD42a, CD42b, CD45, CD62P, CD63 molecules. Negative isotypic controls of the same subclasses were included in every analysis.

Blood samples were diluted by Sörensen phosphate buffer solution (PBS, pH 7.4) to obtain sample concentration 10x10^9 platelets/l (2) and incubated with optimal concentration of monoclonal antibody for 20min. The sample was subsequently diluted by PBS and immediately analysed by flow cytometry. The same sample staining procedure was used to analyse platelets surface molecules after TRAP-6 induced activation in vitro. Flow cytometer Coulter® Epics® XL (Coulter, Fullerton, USA) equipped with software version Epics XL Flow Cytometry Work Station – System II™ ver. 3.0. was used through the study.
The platelet population was delineated by forward scatter (FS) and side scatter (SS) parameters in whole unlysed blood. Platelet gate is always contaminated by the small fraction of debris formed from erythrocytes or leukocytes membranes and by large immune complexes (3). Intensity of fluorescence was measured in log scale by single parameter histogram (30). The activation of platelets was assessed by double immunofluorescence analysis using two parameters (dot-plot) histograms. Double positive CD41⁺CD62P⁺ or CD41⁺CD63⁺ platelets were considered as activated (4,20). Expression of CD45 molecule plotted to SS parameter was used to delineate erythrocytes, leukocytes, aggregates of leukocytes and platelets. PLAs were identified as CD45⁺CD41⁺ elements (21). The threshold of 5000 platelets was set up for a single analysis. It means that between 60 000 and 140 000 blood elements were analysed (15,17,21) in a single determination. The relative proportion of PLAs was determined according to Matzdorf et al. (24) together with the evaluation of free platelets, platelet homoaggregates and platelet microparticles.

**Statistical analysis**

Statistical evaluation was performed by statistical software NCSS 6.0.21 (vers. 1996). Paired t-test was used in the case of normal distribution of experimental data. The non-parametric Wilcoxon or Mann-Whitney tests were used if the normal distribution of the data was lacked. Kruskal-Wallis test with subsequent Bonferroni test were used to compare samples A, B, C.

**Results**

The methods using whole blood, platelet rich plasma (PRP) obtained either by simple sedimentation or by careful centrifugation, respectively, were compared in the preliminary study (results are not shown). As the best approach, technique using sodium citrate treated unseparated, unfixed whole blood processed during 2 hrs. after sampling was selected.

Statistically significantly decreased expression of all constitutively expressed membrane molecules was found on platelets of patients with PTCA in comparison with healthy controls (see Tab. 3). In an agreement with others (5,18,19, 24) significant increase in the activation between patient samples obtained before, during and after PTCA, respectively, was proven only for both CD62P and CD63 molecules (Tab. 4, Fig. 1,2) and PLAs (CD45⁺CD41⁺ elements) formation. Statistically significant differences in the expression of other activation markers were not reached (Fig. 3).

The expression of both CD62P and CD63 molecules was significantly lower on platelets of patients before PTCA in comparison with healthy controls (statistical significance p<0.05 and p<0.01 respectively). There is statistically significant increase in the expression of CD62P molecule on platelets after PTCA in comparison with samples obtained before PTCA procedure (p<0.05) (Fig. 1). The gradual statistically significant increase in the expression of CD63 molecule before, during and after PTCA (p<0.001) was found (Fig. 2).

### Tab. 3: Comparison of platelet membrane expression of selected molecules between patients with PTCA and healthy controls (relative values).

<table>
<thead>
<tr>
<th>parameter</th>
<th>healthy controls mean / SD</th>
<th>patients mean / SD</th>
<th>comparison L / S / H</th>
<th>statistical significance (p)</th>
<th>statistical test</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD9</td>
<td>94.98% / 2.95</td>
<td>87.98% / 3.99</td>
<td>L / /</td>
<td>&lt;0.001</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>CD31</td>
<td>92.78% / 2.97</td>
<td>87.10% / 4.24</td>
<td>L / /</td>
<td>&lt;0.001</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>CD36</td>
<td>90.98% / 5.25</td>
<td>87.37% / 4.44</td>
<td>L / /</td>
<td>&lt;0.05</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>CD41</td>
<td>95.62% / 2.23</td>
<td>88.09% / 5.27</td>
<td>L / /</td>
<td>&lt;0.001</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>CD42a</td>
<td>94.98% / 2.62</td>
<td>88.54% / 5.06</td>
<td>L / /</td>
<td>&lt;0.001</td>
<td>t-test</td>
</tr>
<tr>
<td>CD42b</td>
<td>94.13% / 2.58</td>
<td>88.31% / 4.65</td>
<td>L / /</td>
<td>&lt;0.001</td>
<td>Kolmogorov-Smirnov</td>
</tr>
</tbody>
</table>

Abbreviations:  
L / S / H – low / same / high  
SD – standard deviation

### Tab. 4: Comparison of parameters of activations between A,B,C samples of patients and healthy controls (relative values and standard deviation).

<table>
<thead>
<tr>
<th>activation marker</th>
<th>sample A (before PTCA)</th>
<th>sample B (during PTCA)</th>
<th>sample C (after PTCA)</th>
<th>healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD62P</td>
<td>0.22% (0.21)</td>
<td>0.39% (0.31)</td>
<td>0.67% (0.67)</td>
<td>0.34% (0.18)</td>
</tr>
<tr>
<td>CD63</td>
<td>0.26% (0.23)</td>
<td>0.45% (0.35)</td>
<td>0.85% (0.49)</td>
<td>0.45% (0.20)</td>
</tr>
<tr>
<td>PLAs (CD45⁺CD41⁺)</td>
<td>13.57% (6.76)</td>
<td>18.39% (10.96)</td>
<td>23.63% (13.18)</td>
<td>8.16% (3.43)</td>
</tr>
</tbody>
</table>

Abbreviations:  
PTCA: percutaneous transluminal coronary angioplasty  
Sample C: after PTCA or after stent placement  
Sample A: before PTCA  
Sample B: during PTCA
There were no statistically significant differences in the ability of patient's platelets to respond to TRAP-6 stimulation in vitro in comparison with controls. The only exceptions were significantly lower increase in the presence of PLAs (p<0.001), and platelet microparticles (p<0.01–0.05) both in whole blood and in platelets gate in blood samples collected before, during and after PTCA, respectively, in comparison with healthy controls after stimulation in vitro (Fig. 3).

**Discussion and conclusions**

The plasma or urine concentration of various humoral substances such as PF4, β-thromboglobulin, or thromboxane A2 metabolites were previously used as markers of platelets activation in vivo (2). The commercial availability of monoclonal antibodies directed to platelet molecules together with a great progress in flow cytometry was the substantial breakthrough in immediate monitoring of platelets activation both in vivo and in vitro (3,5,8,25). As the best approach, whole blood technique avoiding platelet separation and blood lysis, is commonly recognised. This method reflects the real situation in human body (2). As is stressed by Holada et al. (12), platelets rapidly response to changes in temperature, mechanical stress, exposure to artificial surfaces, and even to the traces of activation agents by rapid activation with consequent changes in the shape of platelets, aggregates formation, rearrangement of cytoskeleton and release of platelet granules. Careful choice of anticoagulant agents is prerequisited (3). For example EDTA does not interfere with phenotypisation of platelets but due to binding of Ca²⁺ ions EDTA interferes with platelets aggregation. Sodium citrate and heparin enable both the determination of platelets phenotypisation and platelets aggregation.

We found statistically significant lower expression of all constitutively expressed surface molecules of patients undergoing PTCA in comparison with healthy controls. This findings could be a least partially explained by the medication of patients with PTCA. The average nine different drugs were administered to PTCA patients in contrast to no medication in controls. Among these drugs administration of antithrombotics (acetylsalicylic acid), nitrates, often in...
combination with β-blockers or calcium-channels blockers together with hypolipidemias was the apparent rule. Some of patients were also treated with low molecular heparin (nadroparin). In addition antithrombotics and anticoagu-
lants were given to all patients before, during and after the
course of PTCA. The influence of these drugs on platelets is
discussed by Knight et al. (16), Koza et al. (19), Wabba et
al. (24), Zhao et al. (33).

Surface membrane expression of activation molecules CD62P and CD63 were determined by both single (17) and
double immunofluorescence staining techniques (4,20). The values obtained by those different methods did not
reveal the significant differences in the case of CD62P expression (p=0.15), but were significantly different in the
case of CD63 molecule. The expression of CD63 was sign-
ificantly higher (p<0.05) using double immunofluores-
cence staining technique. In agreement with others we
proved low expression of CD62P on resting and unstimula-
ted platelets. Itoh and coworkers (14) and Furman and co-
workers (7) refer that the expression of CD62P on platelets
is 0.11 ± 0.20 % and 1.5 ± 0.2 % respectively in healthy do-
nors. Expression of CD62P and CD63 in healthy controls
2.4 ± 0.9 % and 1.3 ± 0.7 %, respectively was reported by
Kolarov et al. (18). The expression of these markers in pa-
ients undergoing PTCA without complications was 2.5 ±
1.0 % and 1.1 ± 0.9 %, respectively. The expression of
CD62P and CD63 was further increased in patients with
PTCA suffered from complication (reocclusion, restenosis)
to 11.3 ± 7.3 % and 2.6 ± 2.4 % respectively.

Matzdorff et al. (23) examined activation of platelets in
vitro. The labeled MPs were used to assess the absolute
number of platelets. The relative number of CD62P+ platelets
wanted to be in a range between 1–5 %, this number is equivalent
to 2 500–14 000 MPs/μl of unstimulated blood (24). The
number of CD45+/CD41+ elements in CD45+ population
in patients with PTCA in comparison with
controls (p<0.05), see Tab. 4, Fig. 3. There were no signifi-
cant differences in the number of these elements before, du-
dering and after PTCA (p=0.86).

There are evidences that platelets are activated during
PTCA (5,11). We found significant increase in the expres-
sion of CD62P and CD63 molecules and concomitant increase in the number of PLAs. Inoue et al. (13) referred
the significant increase in the activation of platelets and
neutrophils in the patients with PTCA which was further
enhanced after stent placement. It is claimed that interaction
between activated platelets and neutrophils could be re-
sponsible for the proliferation of the neointima leading ulti-
mately to the restenosis after the placement of the stent. The
activation of platelets associated with PTCA was also proven
by DPPS (Duesseldorf PTCA Platelet Study) study (18).

The number of PLAs which is 14.7 ± 11.4 % in unfixed
and unstimulated blood is increased to 19.9 ± 13.3 % after
3 hrs. of standing on bench (21). The same authors (21)
conducted more detailed study to reveal the composition of
PLAs. They found with the help of antiCD14 and antiCD16
monoclonal antibodies that 2.4 ± 0.9 % were heteroaggre-
gates between platelets and lymphocytes, 7.2 ± 1.6 % het-
eroaggregates between platelets and monocytes and 3.7 ± 0.3 % heteroaggregates between platelets and neutrophils.
They investigated circumstances which influence the for-
imation and stability of PLAs. PLAs are more efficiently
formed at 37 °C in comparison to laboratory temperature
22 °C. Their formation is also increased in the presence of
lysing solution and fixation solution. The same effect has
also centrifugation and repeated washing of platelets. All
above listed variables increase the formation of platelets
3–5 times.

Platelets are blood elements playing the pivotal role in
the process of hemostasis. Platelets also contribute to the
patophysiological mechanisms. It is necessary to have stan-
dardised method to evaluate the activation of the platelets
in vivo. This approach enables us to follow e.g. effects of the
treatment strategy or to identify patients who are at the risk
of restenosis after PTCA. In this sense it could be of a great
clinical value to continue the long-lasting follow-up of plate-
lets activation markers in patients after PTCA to delineate
possible association with adverse effects such as a stent
occlusion or restenosis.

The evaluation of the activation markers on platelets is
highly specialized laboratory method. The appropriate in-
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terpretation of results relies on the close cooperation between clinician and cytometrist, because it is very likely that a lot of individual variables including patient medication significantly impair platelets. It is possible to prevent uncorrect interpretation by this collaboration (2,3,25).

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References