Clinical Chemistry Trainee Council
Pearls of Laboratory Medicine
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TITLE: Detection of Activated Platelets

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Hi, my name is János Kappelmayer. I am the director of the Department of Laboratory Medicine at the Medical and Health Science Center, University of Debrecen, Hungary. Welcome to this Pearl of Laboratory Medicine on "Detection of Activated Platelets."

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Anti-platelet agents are one of the most widely prescribed drugs in developed countries. This comes as no surprise as vascular disorders are the leading causes of morbidity and mortality, and to avoid recurrence of these diseases, it is essential to inhibit platelet function. In arterial disorders like acute coronary syndrome, ischemic stroke, and peripheral arterial disease, platelets are the major contributors to disease development.

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Upon activation by platelet agonists like thrombin, ADP, or collagen, the cell shape will change. The usual discoid form of the platelet will become stellate-shaped because of the formed pseudopods. Along with this morphological change, a lot of biochemical pathways are activated. Antigens that are normally absent or are very scanty on the resting platelet surface will be expressed. One of these is an intracellular platelet marker (P-selectin) that in resting cells is present in the alpha-granules, but is exposed to the surface and secreted to the plasma very soon during platelet activation. The number of platelet integrin receptors IIb/IIIa and its activated form detectable by the PAC1 antibody also increases upon activation, while staining for some other glycoproteins like the number of GPIb/IX/V complex are down-regulated in activated platelets.

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Routine laboratory tests provide basically no information on the degree of platelet activation although some platelet-derived parameters like the mean platelet volume (MPV), the platelet-large cell ratio (P-LCR), and the platelet distribution width (PDW) may be altered. Young platelets are usually more active and this fraction can now be automatically studied in some hematology analyzers as the immature platelet fraction (IPF). By ELISA, soluble platelet activation markers like soluble P-selectin and soluble CD40 ligand (CD40L) can be measured. Flow cytometry is probably the most widely used method to

detect activated platelets, since here the overexpressed surface antigens can be quantitated along with the measurement of microparticles. Flow cytometry is also useful for the identification of platelet-leukocyte aggregates. Platelet aggregometry was originally designed to detect dysfunctional platelets, but hyper-aggregation as well as primed platelets can also be detected. A global platelet function analyzer designated as PFA-100 is increasingly used in the detection of platelet function defects. It measures closure times of agonist covered cartridges using whole-blood. Recently, it was found that the shortening of these closure times was observed in some thrombotic disorders reflecting the presence of activated platelets.

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In the investigation of activated platelets, three types of samples can be analyzed. For the soluble markers, citrated plasma is required and – as previously mentioned – these measurements are carried out by immunoassays. Second, we can investigate intact cells usually by flow cytometry or alternatively, by fluorescent/confocal or electron microscopy. Last but not least, cell fragments can be analyzed. These can be of several types and those that were shown to contribute to vascular problems are called microparticles. These 0.1-1 micron sized cell fragments can be enumerated and characterized by flow cytometry; however, smaller microvescicles (sometimes referred to as exosomes) are usually studied by electron-microscopy.

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Didactically, we can split platelet activation tests to direct and indirect subtypes. Most of these can be investigated by flow cytometry. Many of the direct tests are detecting elevated antigen expression on the platelet surface like CD62, CD63, and CD154. Although these direct tests are very useful in identifying activated platelets, in several pathological states, indirect tests may be even more useful. It has been detected that under in vivo conditions in a primate thrombosis model, circulating platelets rapidly lose their surface P-selectin but continue to circulate and function. Under these conditions and also in many human disorders, circulating monocyte-platelet complexes were found to be even more sensitive than direct platelet markers.

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Circulating leukocytes, particularly myeloid cells, tend to bind platelets and thus, may express antigens that are not of myeloid origin. These leukocytes – as well as many other cells like platelets and endothelial cells – release microparticles into the circulation. These microparticles may bind to platelets and thus, platelets may expose antigens that are usually not of platelet origin, like tissue factor or P-selectin glycoprotein ligand-1 (better known as PSGL-1). When platelets are stimulated in vitro with increasing thrombin concentration, microparticle numbers are elevated at higher agonist concentrations. This is always accompanied by lactate dehydrogenase (LDH) release that reflects some degree of cell lysis.

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As mentioned previously, flow cytometry is the most widely used method to detect platelet activation. It is also a suitable technique to verify platelet glycoprotein deficiencies or clopidogrel resistance. There are many advantages when platelets are studied by flow cytometry. Contrary to several other techniques, it can be executed even in case of very low platelet count with only a few microliter of blood

sample. Furthermore, platelets can be studied in their natural environment, heterotypic aggregates can be investigated, and platelet microparticles can be also enumerated. However, there is a major disadvantage compared to ELISA assays since plasma samples can be pooled by freezing, while platelets need to be studied immediately after blood drawing or should be fixed, but also this way can only be stored for a limited time. Thus, these flow activation assays may be prone to pre-analytical errors. On the other hand, if many activated platelets are eliminated from the circulation under pathological states, we can underestimate the platelet activation status, as in the flow assays we can only evaluate circulating platelets.

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Platelet aggregometry is the gold-standard of method for investigating platelet dysfunction. In the classical lumi-aggregometric assay, platelet aggregation is monitored by measuring the optical density of the PRP solution and the release reaction is followed by measuring ATP in a luminescent assay. Platelet aggregation is the primary test in identifying platelet function abnormalities by the lack of response to one or more platelet agonists; however, this test can also be used in identifying activated platelets since these cells possess hyperaggregability to low concentration of agonists. Such platelets were identified in myocardial infarction and ischemic stroke.

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Numerous clinical studies were carried out where platelet activation was verified. Many of these are related to cardiovascular disorders, where the activated platelet can be a pathogenic factor of the disease. Since the description of the Trousseau phenomenon, it is also known that in malignancies, the coagulation system can often be activated due to several factors like expression of tissue factor on tumor cells, or the presence of a cysteine protease called cancer procoagulant. In septic conditions, the elevation of C-reactive protein directly contributes to monocyte tissue factor expression and hence to thrombosis. It is also noteworthy, that seemingly healthy obese people are also having hyper-activated platelets and this was shown to correlate to the increase of media thickness in their cerebral arteries. Several polymorphisms in P-selectin have been described that may modulate the platelet activation status in healthy persons.

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A special subpopulation of platelets is generated under dual activation. This subpopulation was designated as COAT or COATED platelets. The COAT acronym derives from names of the required agonists collagen and thrombin. These platelets are capable of binding the α-granule proteins at higher extent. Today, mostly the snake venom convulxin is used instead of collagen. It has been shown that COAT platelet number can be altered in several thrombotic and bleeding disorders.

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This test requires gel-filtered platelets to get rid of plasma proteins, since COAT platelets are identified by the binding of exogenously added labeled fibrinogen. During gel-filtration, a slight activation of platelets may occur, but considerably less than during platelet washing procedure. In the first step, a biotinylated fibrinogen antibody is added to the gel filtered platelets and COAT platelets are visualized by the addition of strepatividin conjugated phycoerythrin. As can be seen on the slide, non-activated

platelets and platelets stimulated by single agonists only display small fibrinogen signal while dual activation results in two separate populations and nearly half of the platelets will display substantial fibrinogen binding.

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A rare but very important case of platelet activation is when platelets are activated by auto-antibodies generated during heparin treatment. These antibodies are directed towards the heparin-PF4 complex. They activate platelets via their Fc receptors and as a result, platelet number gradually decreases resulting in heparin-induced thrombocytopenia (HIT). There are multiple methods for the detection of these autoantibodies; some methods provide results within minutes and are now applied 24/7 in selected laboratories. These are usually particle enhanced immunoassays and are prone to provide false-positive results. A standard method for the detection of these antibodies is ELISA; however, a functional test can also be constructed using flow cytometry, that proves that these antibodies do activate control platelets. In this assay, healthy donor platelets are incubated by the patient's plasma and platelet activation is monitored by annexin V binding.

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A typical plot in this assay will provide two values; one being the percentage of annexin V positive donor platelets. As can be seen on panel A in the dual labeled platelet rich plasma, this percentage is very low in control platelets, but is significantly up-regulated if incubated with a HIT positive plasma and heparin (panel B). In addition, more microparticles are formed that also reflects platelet activation. This can be appreciated when we compare the number of dots in the R2 region on panels C and D.

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- Flow cytometry is a versatile technique that is suitable for detecting in vitro and ex vivo platelet activation
- An advantage of this technique is that it can be used in case of low platelet counts using a miniscule amount of blood
- Pre-analytical issues are of major importance in eliminating false positive results
- Cellular and soluble platelet markers may provide different results depending on the actual pathological state
- Simultaneous testing of different platelet activation markers is not only capable of detecting current activation status of platelets, but can also predict subsequent adverse clinical events

Slide 16: References

Slide 17: Disclosures

Slide 18: Thank You from www.TraineeCouncil.org

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