Assessment of Blood Platelet Function

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Platelets are non-nucleated components of blood. Their best recognized physiological function is primary hemostasis, i.e., formation of the hemostatic plug in the site of blood vessel injury. The plug not only limits blood extravasation, but it also creates a procoagulant surface on which the coagulation cascade develops to form fibrin mesh which stabilizes the plug. Effective formation of the hemostatic plug is assured by the ability of platelet to adhere to the site of injury and by firm binding to adjacent platelets. Such a profound phenotype switch from a circulating blood component to an element of a structure which withholds blood outflow demands significant alterations in platelet biochemistry. These alterations are generally termed platelet activation.

Keywords: platelets ; intravital studies ; thrombosis ; atherosclerosis ; inflammation ; metastasis

1. Introduction

Much of our current understanding of platelet biology and platelet's contributions to thrombus formation is derived from in vitro or ex vivo experiments in which platelet aggregation in response to selected stimuli or platelet adhesion to various molecules under static or under flow conditions is measured. These approaches provided information on platelet biochemistry and intracellular signaling pathways which are activated upon platelet stimulation. In vitro approaches, however, have certain drawbacks. Platelets are very susceptible to manipulations which accompany the collection of blood samples and their preparation for experimental procedures. Such manipulations, which include blood withdrawal from the vessel and cell isolation either by centrifugation or by gel filtration, may lead to artifactual platelet activation [1]. Moreover, under in vitro conditions, platelets are devoid of the influence of natural suppressors of platelet activation produced by the vascular endothelium, such as nitric oxide and prostacyclin. This may also lead to a bias of the results obtained under in vitro conditions. For these reasons, the knowledge acquired under in vitro conditions is often verified by in vivo studies in animal models.

Platelet biology owes to intravital studies not only a better understanding of platelets' role in primary hemostasis but also the findings that platelets are important factors in inflammation and atherosclerosis.

There is a heterogeneity among research protocols used in intravital studies of platelets, due to the variety of aspects of platelet function that are studied. The choice of the research protocol is driven first by a question of which aspect of platelet biological activity will be examined. This determines whether platelets should be activated by the researcher prior to measuring their response or whether the researcher is interested in a native state of platelets. If activation is required, a proper stimulus must be chosen to relevantly simulate physiological or pathophysiological conditions to be investigated. Finally, platelet response must be quantified. The method of quantification depends on the manifestation of platelet activity, such as aggregation or adhesion, which is relevant in a given experimental setup.

2. Thrombosis Triggered by Vessel Injury

Arterial thrombosis is an effect of disruption of the endothelial layer as a consequence of rupture of the atherosclerotic plaque. This process in experimental conditions is mimicked by denudation of the endothelial layer. This can be achieved with mechanical, physical or chemical intervention and may involve all layers of the vessel wall or may be limited solely to the endothelial layer [2]. Formation of thrombus can be quantified either by means of the measurement of blood flow decrease in the injured vessel or can be based on the measurement of thrombus size visualized with the use of intravital microscopy. In the first approach, the measurement of blood flow can be performed with the use of laser or ultrasound Doppler technique. The measurement is based on the Doppler effect occurring mainly upon moving red blood cells inside the vessel. The signal is proportional to the linear velocity of blood stream and decreases when blood flow is slowed down due to occlusive thrombus formation. In the second approach, a thrombus size is estimated based on two or three-dimensional images.

3. Platelet Activation by Systemic Application of an Agonist

Injury of the vascular wall leads to the activation of platelets in several inseparable pathways. However, in some studies researchers are interested in assessing the platelet response in vivo to an isolated, defined chemical stimulus rather than to a combination of factors. In such a case, selected agonists are injected into an animal and the platelet response is measured intravitally. This approach to some extent resembles measurements performed in whole blood in vitro, where an isolated stimulus is added to a blood sample and the aggregation of platelets is monitored. An obvious advantage of the in vivo approach is an ability to evaluate whether the platelet aggregates, which are formed as an effect of the used stimulus, are capable of occluding blood vessels in physiological conditions. The use of animals with certain vascular pathology also allows one to test whether pathological conditions affect platelet occlusive properties.

The simplest way of quantification is based on a measurement of a death rate of the animals injected with a tested substance. Since death of animals is presumed to be predominantly an effect of occlusion of pulmonary circulation with platelet aggregates, the death rate was considered proportional to the pro-aggregatory effect of the used agonist. Liang et al. [3] utilized this approach to evaluate antithrombotic properties of pentamethylquercetin (PMQ). Platelets were activated by the tail vein infusion of collagen and epinephrine. Injection of the mixture of collagen and epinephrine resulted in a death rate of 100% the examined mice. As the authors expected, PMQ had an antithrombotic effect and significantly increased the survival rate in mice after collagen-epinephrine-induced pulmonary thrombosis.

More advanced methods are based on the measurement of accumulation of radiolabeled platelet aggregates in the pulmonary circulation. In this approach, platelets are collected from a donor animal, labeled with a radioligand and injected into a recipient animal. Accumulation of platelets in the pulmonary circulation, resulting from injection of an agonist, is measured as an increased scintillation counts over the thoracic region of the animal. Tymvios et al. [4] studied dose-dependent responses to three platelet agonists (ADP, collagen and thrombin) in this model. To verify whether the responses were platelet-dependent, some of the animals were injected with aspirin. All tested agonists induced dose-dependent changes in platelet counts due to the accumulation of thrombi in the pulmonary vasculature, and aspirin was able to inhibit such collagen-induced responses. The location of platelet aggregates in the pulmonary bed was further confirmed histologically. This work confirmed that this model could be used to investigate the pharmacology of exogenous and endogenous modulators of platelet function.

4. Studies on the Platelet Interaction with the Intact Vascular Wall

The involvement of platelets in the development of inflammation and atherosclerosis has been very extensively studied in recent decades [5]. The role of platelets in this process is to a large extent associated with their ability to interact with the vascular wall and to recruit other types of cells to it. When studying these processes, researchers are interested in assessing interactions of native platelets with the vascular wall, where the endothelium is mechanically intact but “activated.” Activation of the endothelium is a phenotype that is associated with decreased production of anti-adhesive mediators, such as nitric oxide or prostacyclin, and with increased expression of pro-adhesive receptors and ligands, such as ICAM, VCAM and vWF. Hence, protocols used in these studies do not include direct activation of platelets or disruption of the endothelial layer. Instead, the endothelium would be challenged with factors aimed at changing its phenotype. The extent of platelet interactions with endothelium is assessed by quantification of still images or movie sequences acquired by intravital microscopy. In order to visualize platelets, they are stained with fluorescent dyes. This can be performed either extracorporeally, when the platelets collected from donor animals are incubated with a fluorescent probe and infused to a recipient animal, or it can be achieved by the injection of platelet-specific antibodies to the experimental animal. Quantification of platelet interactions with the vascular wall is a challenging task. As will be shown in the examples presented below, the calculation of parameters of platelet motility strongly varies between various protocols.

Evaluation of platelet interactions with the vascular wall was used to test the hypothesis of the contribution of endothelial COX activity to platelet function. Buerkle et al. [6] studied the effects of selective COX-2 inhibitors on platelet adhesion to the vascular wall in hamsters pretreated with the selective COX-2 inhibitor NS-398. The animals were infused with calcein-labeled human platelets, and platelet interactions with arterioles in the dorsal skinfold chamber were recorded. Firm adhesion of a platelet to the vessel wall was defined when a platelet did not change its position for a period of at least 30 s. In case of other platelets, their velocities were calculated. It turned out that the selective COX-2 inhibition led to an increase in platelet interactions with the vascular wall. Therefore, the results suggest that it was an effect of a decreased production of endothelial prostacyclin. Interestingly, COX-2 inhibition in this model also increased the thrombotic vessel occlusion after the disruption of the vessel wall. These results are contradictory to the studies of Armstrong et al. [7], described above, which showed a lack of the effect of COX-2 inhibition on platelet aggregation in vivo in the model of pulmonary thromboembolism.

One of the aspects of endothelial dysfunction is a post-ischemic endothelial injury. Platelets were supposed to play an important role in this process. This notion was verified by Massberg et al. [8] in the in vivo model of ischemia/reperfusion. The experiments were performed in a model of ischemia of the small intestine in mice infused with fluorescently labeled platelets. After one-hour, ischemia interactions between platelets and the endothelium were monitored with the use of intravital fluorescence microscopy. Platelets were classified as free flowing, rolling (intermittent platelet adhesion) or adherent cells, according to their time of interaction. Rolling platelets were defined as platelets crossing the venular segment at a velocity significantly lower than the centerline velocity; their numbers were expressed as cells per second per vessel diameter. Adherent platelets were defined in each vessel segment as the number of objects that remained stationary for 30 s. Numbers of both rolling and adherent platelets were higher in the postischemic vessels. Platelet rolling and adhesion were impaired in the P-selectin-deficient mice, and in the mice injected with antibodies functionally blocking P-selectin, which pointed to an important role of this selectin in interactions of platelets with dysfunctional endothelium. When platelets from P-selectin-deficient mice were injected to wt mice, the platelet rolling and adhesion did not differ from that observed in wt mice, which showed that it is endothelial P-selectin which determines the process, while platelet P-selectin is not inevitable for these interactions.

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