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Perspectives and Commentaries

Exhausted Platelets in Cancer and Other Conditions

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THE CONCEPT OF EXHAUSTED PLATELETS

THE PARTICIPATION of platelets in the haemostatic process is characterized by a sequence of events that has been called the 'basic platelet reaction' [1]. This reaction is induced by the adhesion of platelets to subendothelial components, or by the action on membrane receptors of several agonists. It includes a.o. the activation of phospholipases and subsequent synthesis of thromboxane A_2 and other products of prostaglandin metabolism, shape change, release of substances stored in the platelet granules and aggregation of the platelets. The platelets can be activated directly by some agonists or indirectly by some substances synthetized (thromboxane A_2) or released (ADP) upon platelet activation.

Except for serotonin (5-HT), the granule-bound substances which are released during activation are not exchanged between the granules of resting platelets and their cytoplasm nor the plasma.

The dense granule substances are 5-HT, Ca^{2+}, ATP, ADP and pyrophosphate. Almost 65% of the adenine nucleotides of human platelets are stored in these granules, where the ATP:ADP ratio approximates 2:3. This non-metabolic pool does not exchange rapidly with the cytoplasmic metabolic pool, where ATP is much more abundant than ADP (ratio = 8-10:1). Only the second pool can take up adenosine, adenine or orthophosphate from the plasma.

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The α granules contain several proteins (platelet factor 4, β thromboglobulin, thrombospondin, cationic proteins, platelet-derived growth factor or PDGF, permeability factor, mitogenic factors, chemotactic factor, bactericidal factor) in addition to some coagulation factors (fibrinogen, factor V or Va, factor VIII-Von Willebrand). The platelet vesicles contain hydrolases.

The mechanisms of secretion of the granules' contents are not completely understood, but they are known to require energy in the form of cytoplasmic ATP, which is then converted into inosine monophosphate (IMP) and hypoxanthine, the latter appearing extracellularly.

Although it was formerly thought that the activation of the platelets was an irreversible phenomenon, leading *in vivo* to their disintegration into a haemostatic plug, it has been realized during the last years that this is not always true. Platelets which have undergone the release reaction can sometimes (re)circulate; these exhausted, empty platelets present similarities with those observed when insufficient granule constituents have been synthetized and packaged during thrombopoïesis.

SIGNIFICANCE OF CONGENITAL STORAGE POOL DEFECTS AS MODELS OF EXHAUSTED PLATELETS

The congenital storage pool deficiency syndromes are a heterogeneous group of diseases in which the development of dense granules, α

granules or both is decreased; no deficiency of vesicles has been described hitherto [1-3].

The congenital deficiency of α granules or 'grey platelet syndrome' is exceedingly rare. It can be diagnosed by electron microscopy or by the observation of decreased platelet levels of α granule substances; the platelet aggregation is nearly normal.

The congenital deficiencies of dense granules occur in combination with α granule deficiency or most frequently alone. Their diagnosis relies on the following observations:

(i) Electron microscopy discloses decreased numbers of dense bodies.
(ii) The fluorescent dye mepacrine normally accumulates in the dense granules; in these syndromes the number of mepacrine-labelled granules is reduced.
(iii) The aggregation which normally depends on the release-reaction (second-phase aggregation induced by ADP and adrenaline, aggregation induced by collagen or thrombin) is reduced; in contrast, the aggregation induced by arachidonate and by ristocetin is generally normal.
(iv) The amounts of ATP, and more strikingly of ADP, that are present in resting platelets are decreased. As only the non-metabolic pool of adenonine nucleotides is involved, the platelet ATP:ADP ratio is higher than normal, and is close to the ratio in the metabolic pool.
(v) When platelets are incubated with radioactive adenine the radioactivity is incorporated into the metabolic pool but not into the storage pool; as the latter is reduced in the dense granule deficiencies, the specific radioactivity of ADP is abnormally high.
(vi) The platelet levels of 5-HT, Ca^{2+} and pyrophosphate are decreased.
(vii) Dense granule-deficient platelets incorporate lower amounts of radioactive 5-HT than normal platelets; furthermore, the incorporated 5-HT is maintained in the cytoplasm, where it is exposed to the action of the mitochondrial enzymes; consequently, an abnormally high radioactivity appears in the metabolites of 5-HT, such as 5-hydroxyindole acetic acid.
(viii) The density of the platelets is decreased.

These abnormalities might also be present in platelets which have lost their granules as a result of intravascular activation. It must, however, be stressed that the platelets 'born' with a storage pool deficiency are not completely identical to platelets having undergone the release reaction since they most often present no deficiency of α granules nor of vesicles, and the mechanisms inducing the release reaction might be more depressed in the latter than in the former.

FUNCTIONS OF (RE)CIRCULATING ACTIVATED PLATELETS

The demonstration that platelets which have undergone the release reaction can survive in the circulation derives mainly from experiments conducted in the rabbit. Reimers *et al.* [4] stimulated rabbit platelets repeatedly with low concentrations of thrombin, causing the release of more than 60% of serotonin and adenine nucleotides from their dense granules; the main divalent cation of these granules—which in the rabbit is Mg^{2+} and not Ca^{2+}—was also lost, and electron microscopic examination showed that few granules remained. When these degranulated platelets were labelled by [^{3}H]-DFP and reinjected, they were shown to survive normally. Their metabolism was enhanced, as shown by an increased uptake of glucose and an increased lactate production. These platelets were less sensitive to aggregation by low concentrations of thrombin than control platelets, but they were more sensitive to ADP-induced aggregation.

A more complete degranulation can be induced using higher thrombin concentrations [5]. These platelets respond to other stimuli (ADP, collagen, arachidonate) but not to a second exposure to thrombin, indicating that the thrombin receptor is no longer available. They are not able to retract a thrombin-induced fibrin clot, and their adherence to subendothelial tissue is reduced. Furthermore, they are less effective than normal platelets in the control of the bleeding time of thrombocytopenic rabbits.

When thrombin-degranulated rabbit platelets are infused into thrombocytopenic animals they do not resynthetize ATP or ADP, and they maintain their aggregation defects after 20 hr [6]. Finally, Cieslar *et al.*, using arabinogalactan density-gradient centrifugation, showed that the density of thrombin-treated rabbit platelets is lowered [7].

Investigations on human platelets are less numerous. Harbury and Shrier studied granule-deficient human platelets obtained *in vitro* by pretreatment with thrombin, trypsin or Latex particles [8]. Their study led to the conclusion that thrombin is able to interact with its receptor only once. Stored human platelets also lose their granules, and this might contribute to their decreased haemostatic effect [9].

In conclusion, the aforementioned studies demonstrate that platelets having undergone the release reaction can recirculate but are haemo-

statically hypofunctional, disclosing a.o. abnormalities of their membrane receptors. However, it must be emphasized that the triggers used to activate the platelets *in vitro* may be different from that occurring *in vivo*.

METHODS OF DETECTION OF AN ACQUIRED STORAGE POOL DEFECT

Exhausted platelets are cirulating platelets which present a storage pool defect resulting from an intravascular platelet activation.

The methods used to demonstrate the existence of a storage pool defect in acquired diseases must be sensitive, specific and relatively simple.

The requirement for sensitive methods results from the fact that the defect may only be partial. So the measurement of platelet hydrolases [10] or of platelet α granule substances such as β thromboglobulin or platelet factor 4 [11, 12] may be less sensitive than the measurement of platelet-dense granule substances. With regard to the latter, the measurement of platelet adenine nucleotides may be more sensitive than that of platelet serotonin since 5-HT released from the activated platelets could re-accumulate in the co-existing normal platelets, thus masking the defect [13]. The study of α granule substances can, however, not be discarded since a selective α granule release without depletion of the dense granule storage pool has been described in patients undergoing cardiopulmonary bypass [14].

The need for specific methods derives from several considerations. First, the observation of aggregation abnormalities may have many other causes, such as perturbations of prostaglandin synthesis due a.o. to the effect of certain drugs [2], or to abnormalities of platelet membrane receptors due, for instance, to an immune damage [10]. Second, the platelet serotonin level may be decreased in conditions such as migraine, but whether or not this is due to an activation of platelets or to an overall change in 5-HT metabolism is unclear [15]. Third, the heterogeneity of platelets may at least partly be due to their turnover rate, and large, dense, hyperfunctional platelets are produced in conditions of accelerated platelet turnover [16]; the detection of a partial storage pool defect may thus be complicated when there is a disturbance of platelet kinetics.

In view of the aforementioned remarks, it seems that the measurement of platelet α granule substances, such as platelet factor 4 and β thromboglobulin, and platelet ADP and ATP is recommended in order to detect an acquired storage pool defect. The usefulness of electron microscopy [17], serotonin measurement and metabolism [10-13], the mepacrine test and the observation of labelled adenine uptake, undertaken in addition to measurements of platelet levels of factor 4, β thromboglobulin and adenine nucleotides, is less obvious.

The interest of the analysis of platelet density using standardized arabinogalactan density-gradient centrifugation has been stressed in recent publications [11, 12, 18]. Though the mechanisms leading to an abnormal density distribution pattern remain conjectural, it has been shown that an increased ATP:ADP ratio is almost always accompanied by an increased proportion of light platelets [11, 12].
not been performed in all the situations of acquired platelet dysfunction [2], the accurate incidence of storage pool defects in acquired diseases remains unknown; it might be higher than hitherto appreciated.

DEMONSTRATION OF ACQUIRED STORAGE POOL DEFECT CAUSED BY PLATELET ACTIVATION

A decreased content of granule substances may be caused by several mechanisms: abnormal platelets may be produced by the marrow; the platelets produced by the marrow may be normal, but they may have undergone an intravascular release; or the most haemostatically reactive, granule-rich platelets may have been selectively removed from the circulation, leaving only the less active, granule-poor platelets [19].

The first two mechanisms could conceivably have opposite significances: platelets 'born' with a structural defect might predispose to haemorrhage, while platelets activated in the circulation may result from thrombosis. Furthermore, an inability of the megakaryocyte to package the platelet factor 4 and platelet-derived growth factor could lead to the release of these substances into the marrow environment, thus stimulating the local proliferation of fibroblasts and collagen formation [20].

In spite of these different significances, the distinction between abnormal thrombopoïesis and intravascular release is often difficult to establish and has not been assessed in all published studies.

An abnormal thrombopoïesis is considered when there is other evidence of disturbed hematopoïesis but no other evidence of peripheral platelet activation. This situation occurs in myeloproliferative disorders and in acute leukaemias [21, 22]. In contrast, a platelet exhaustion is considered in the absence of evident disturbances of haematopoïesis and in the presence of coexisting abnormalities, suggesting that the platelets are activated *in vivo*. The fact

that these criteria are insufficient will be illustrated later.

The following abnormalities have been considered as evidence that a storage pool defect is due to an intravascular release: compensated or uncompensated intravascular coagulation, in the course of which thrombin may activate the platelets [11, 13, 14, 23]; antibodies and/or immune complexes observed in idiopathic thrombocytopenic purpura and allied conditions [10, 13, 17]; endothelial damage, as in haemolytic-uraemia syndrome and thrombotic thrombocytopenic purpura [13]; cardiopulmonary bypass [14, 19]; cavernous haemangioma [24]; thrombosis [18]; and biological signs of platelet activation, such as spontaneous platelet aggregation, increased platelet aggregate ratio [18], and mainly the plasma levels of β thromboglobulin and platelet factor 4 [13, 18, 25-27]. Though the plasma levels of the latter substances have been the most frequently studied, it must be emphasized that they can also be released by platelets irreversibly incorporated into the haemostatic plug, that they do not explore the release of dense granules and that their fate differs from that of exhausted platelets: the plasma level of β thromboglobulin depends on a balance between the magnitude of the trigger and the renal function; the percentage of exhausted platelets depends on a balance between the magnitude of the trigger, their survival (probably much longer than that of β thromboglobulin) and the production of intact platelets by the bone marrow [13].

The aforementioned contention that an acquired storage pool defect can be attributed to a peripheral release when there is other evidence of platelet activation *in vivo* but no evidence of abnormal haematopoïesis has limitations. First, the assumption that the myeloproliferative disorders and acute leukaemias are the only acquired conditions in which the granule synthesis is disturbed at the megakaryocyte level is entirely speculative: the existence of an abnormal thrombopoïesis has been postulated in conditions such as alcoholism [28] or diseases in which anti-platelet antibodies are observed [17]. Second, the existence of a myeloproliferative disorder or of acute leukaemia does not exclude the possibility of a coexisting peripheral platelet activation. Thus elevated plasma β thromboglobulin levels have been described in myeloproliferative disorders [29] and splenectomy has been shown to improve the storage pool defect observed in hairy cell leukaemia [30].

In the present state of knowledge the major problems raised by the concept of exhausted platelets thus derive from the difficulty to demonstrate that a storage pool defect has really been acquired after the output of the platelets into the circulation. Other determinations, such as studies of membrane receptors [26], may be useful in future studies.

INFORMATION OBTAINED FROM THE PRESENCE OF EXHAUSTED PLATELETS, ESPECIALLY IN CANCER

The concept of exhausted platelets implies that the platelets have been activated somewhere in the circulation, which may a.o. result from thrombosis, and that the resulting exhausted platelets are hypofunctional, which can lead to haemorrhage. The paradoxical occurrence of thrombosis and haemorrhage in conditions such as myeloproliferative disorders has been explained on this basis [29].

As previously stressed, the quantity of exhausted platelets detectable in the circulation depends on a balance between the magnitude of the trigger, the life-span of the circulating activated platelets and the platelet production by the marrow. Thus the amount of exhausted platelets is variable from one patient to another suffering from the same disorder, and the relation between their detection and either a haemorrhage risk or a thrombotic risk remains at present obscure [13].

Nevertheless, the concept of exhausted platelets always implies that they have been activated by some triggering mechanism and that they have released the content of their granules into the circulation. Though the triggering mechanism often remains unclear, one possible explanation of the recent observation of exhausted platelets in patients with malignant solid tumors [12] would be that the tumor cells can lead to a platelet degranulation *in vivo*, as they can do *ex vivo*.

In the past the interactions between platelets and cancer cells have been studied *in vitro* or in experimental models. In these studies it has been suggested that tumor cells can activate the platelets, and that the activation of platelets may play a role in tumour cell metastasis. The activation of platelets leads indeed to the liberation of prostaglandins, mitogenic factors and platelet-derived growth factor, which might control the tumour growth, and to the release of vasoactive substances (serotonin, permeability factor), which might potentiate the extravascular transit of tumour cells [31].

There is now good evidence that platelets may be activated *in vivo* in patients with malignant solid tumors, even without evidence of active consumption coagulopathy. Future studies are

needed in order to determine whether this activation is involved in metastasis and the precise place, if any, of antiplatelet therapy in human cancer.

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