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DUAL-AGONIST ACTIVATED PLATELETS: AN EMERGING COMPONENT OF THE PRIMARY HEMOSTATIC RESPONSE

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INTRODUCTION

Platelets activated at sites of vascular injury play two key roles in normal hemostasis. First, by adhering to exposed subendothelium, binding adhesive proteins and aggregating, they create a physical barrier that limits blood loss. Second, platelets accelerate coagulation by providing a surface which promotes two procoagulant reactions, generation of Xa and thrombin. A specific subset of dual-agonist activated platelets described in this thesis may affect both of these roles.

Platelets activated simultaneously with collagen and thrombin reveal two distinc populations of activated cells. One population expresses very high levels of several α-granule born procoagulant proteins including factor V (FV), fibrinogen (Fbg), von Willebrand factor (vWF), fibronectin (Fn), α_2-antiplasmin (α_2-AP), and thrombospondin (Tsp) while other does not. Those cells retaining high levels of procoagulant proteins are referred to as COAT-platelets, an acronym for **co**llagen **a**nd **t**hrombin activated platelets. This thesis discusses the characteristics and possible physiological implications of COAT-platelets.

Alberio *et al.* initially observed that human gel-filtered platelets (GFP) stimulated simultaneously with two agonists, thrombin and collagen, produced two polulations of activated cells regard to factor V expression. The population of cells with high levels of surface FV represented 20 to 40% of the total activated platelets. These two populations could also be observed upon platelet activation with thrombin plus convulxin. Convulxin is a snake venom protein activating the glycoprotein VI (GPVI) receptor on platelets. Subsequently we have examined additional α-granule proteins on the surface of COAT platelets and demonstrated that not solely FV but fibrinogen, vWF, fibronectin, α_2-antiplasmin and thrombospondin are also present on thrombin plus collagen (or convulxin) activated platelets. Antisera against IgG and albumin, which are also α-granule born proteins, did not exhibit this „COAT-like" population upon dual stimulation. All of these adhesive and procoagulant proteins as well as FV are substrates for transglutaminases (TG), and inhibitors of transglutaminase prevented the production of COAT-platelets.

To identify the possible binding site for these proteins on the platelet mebrane we have synthetised a transglutaminase substrate (CP_{15}). This 15-residue peptide derived from β-casein included a transglutaminase-active glutamine and was prepared either biotinylated (biotin-CP_{15}) or fluorescein labeled (Fl-CP_{15}). When biotin-CP_{15} was included in a COAT-platelet assay, the biotin label was incorporated into COAT platelets, and this incorporation could be blocked with transglutaminase inhibitors. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of biotin-CP_{15} incorporated COAT-platelets revealed no high molecular weight platelet membrane proteins. Therefore Fl-CP_{15} labeled-COAT platelets were extracted with ethanol and analyzed on reverse phase HPLC. The protuct than was analyzed by mass spectrometry and found to be the starting material Fl-CP_{15} plus 176 Da. The most common constituent in platelets with a molecular weight of 176 Da is serotonin. Direct evidence of serotonin's role was provided by identifying conjugated serotonin on fibrinogen isolated from COAT platelets.

Alberio *et al.* also demonstrated that young platelets, identified by thiazole orange (TO) binding, produced a higher fraction of COAT-platelets than did aged, TO-negative cells. COAT-platelets were also found to express maximum levels of phosphatidyl-serine (PS) as demonstrated by annexin V binding. With the exception of ionophore A23187, the gold standard of PS exposure, no single agonist produced platelets with significant PS expression. However, dual-agonist stimulation with convulxin plus thrombin or collagen plus thrombin elicited strong binding of annexin V in the same population of cells retaining FV. Additionally we have confirmed that all of other α-granule proteins but IgG and albumin were present in a higher fraction on the surface of thiazole orange binding young platelets than on aged ones.

Similar subpopulation of platelets can also be generated by the combined stimulation of FcγRIIA and thrombin receptors. Platelets activated in this manner are referred to as Fc receptor and thrombin-activated (FcRT) platelets, and they share many of the characteristics of the formerly observed COAT-platelets, including aminophospholipid exposure, adhesive and procoagulant protein enrichment, increased frequency among young platelets, and sensitivity to transglutaminase inhibitors. Although FcγRIIA receptor activation can be achieved either with anti-CD9 monoclonal antibodies (ALB-6 and ML-13) or with direct Fc receptor cross-linking, FcRT-platelet generation occurs

only with concurrent or slightly delayed thrombin stimulation. FcRT-platelet formation in platelet poor plasma and whole blood was also investigated, and results were similar to those observed with gel-filtered platelets. Previous experiments with COAT-platelet formation used physiologic agonists (collagen and thrombin) that might be encountered under either physiologic or pathologic conditions; however, these experiments with Fc receptor stimulation have offered the first example in which these highly prohemostatic platelets are likely to be strictly pathogenic.

We do not yet know the physiologic significance of COAT-platelets, but there are several observations that suggest these cells are important components of the primary hemostatic system. The most obvious finding is exposure of surface PS and the generation of significant prothrombinase activity by COAT-platelets. Clearly, PS exposure is a critical component of the hemostatic system, and the selective exposure of PS by COAT-platelets after collagen plus thrombin stimulation suggests an important role for these cells.

This generation of two populations of activated cells is emphasized by the disparate nature of the final products. COAT-platelets with their armor strongly adherent, procoagulant proteins differ dramatically form the non-COAT population where classical bleeding and retention of adhesive and prohemostatic proteins is observed. The full impact of COAT-platelets in physiologic and pathologic aspects of hemostasis remains to be determined.

MAIN OBJECTIVES AND SPECIFIC AIMS

Platelets co-stimulated with collagen and thrombin reveal a sub-population of cells which express high levels of surface-bound, functional factor V. This sub-population represents ~30% of all platelets and is referred to as COAT-platelets. While the exact role of COAT-platelets in hemostasis is yet to be determined, this thesis suggests that COAT-platelets offer a new mechanism for providing concentrated, pro-hemostatic factors at a site of maximal platelet activation. To produce COAT-platelets, it is hypothesized that coincident α-granule secretion, aminophospholipid exposure and transglutaminase availability are required. The following will address the exact mechanism of COAT-platelet formation.

1. Additional proteins on the surface of COAT platelets:

COAT-platelets will also express von Willebrand factor, fibrinogen, α_2-antiplasmin, thrombospondin, and fibronectin on their surface. COAT-platelets are observed upon dual activation with thrombin plus collagen type I, type V, type VI or convulxin, an agonist specific for the collagen receptor glycoprotein VI; however, no single agonist examined will able to produce COAT-platelets.

2. Transglutaminase activity and COAT platelet formation:

COAT-platelet formation, as monitored by FV, vWF, α_2-AP, Fbg or Fn binding, will inhibited by dansyl cadaverine, putrescine and acetyl-casein, all inhibitors of transglutaminases

3. Fibrinogen on COAT-platelets:

The binding of Fbg to dual-agonist stimulated platelets will be investigated to determine if Fbg is bound to the GP IIb/IIIa receptor on COAT-platelets

4. Formation of COAT-platelets in platelet rich plasma:

Studies detailed above have utilized gel-filtered platelets, an experimental system which has many advantages but does not reflect the natural environment for platelets. Therefore we will investigate whether COAT-platelets can be formed in PRP upon activation with two agonists.

5. FcRT- platelets:

The collagen receptor GP VI shares several signaling pathways with the Fc receptor (FcγRIIa; CD32) present on human platelets. As a result, the ability of FcγRIIa engagement to facilitate generation of COAT-platelets will be tested.

6. Identification of casein peptide cross-links in COAT platelets:

Preliminary data indicate that a fluorescently labeled casein peptide (Fl-CP_{15}) is coupled to a small, hydrophobic molecule in the platelet membrane during COAT-platelet formation. This hydrophobic anchor will be identified with a combination of enzymatic digestion, reverse phase HPLC and mass spectrometry.

7. Analysis of the factor V transglutaminase product in COAT platelets:

Preliminary experiments have failed to identify a macromolecular cross-link partner for FV in COAT-platelets. It is assumed, therefore, that the same low molecular weight, transglutaminase partner observed with Fl-CP_{15} is reacting with FV. Technical limitations will prevent a direct evaluation of this product, but a combination of enzymatic and chemical modifications in conjunction with reverse phase HPLC will allow an indirect evaluation of the chemical nature of the modification.

8. Identification of membrane attachment site for transglutaminase reaction:

The reactivity of biotinylated-CP_{15} with COAT-platelets offers an opportunity to determine if a membrane protein was serving as the lysine acceptor for transglutaminase immobilization of the α-granule born proteins. The advantage of a low molecular weight marker like biotinylated-CP_{15} is that it would not significantly affect the SDS-PAGE migration of the membrane protein to which it was coupled. However, SDSPAGE/Western blot analysis of biotin-CP_{15} derivatized COAT-platelets did not reveal any protein labeled with biotin. As a result of this negative Western blot experiment, the possibility that a small molecule is serving as the anchor for CP_{15} will be investigated.

RESULTS AND DISCUSSION

We have demonstrated that the combined action of two physiologic agonists, thrombin and collagen, is able to promote high levels of α-granule born prohemostatic protein expression on the surface of a discrete fraction of platelets; we have referred to this population as COAT-platelets. Convulxin, a specific agonist for the collagen receptor GPVI (4) can substitute for collagen in this reaction. We have also shown that generation of COAT-platelets parallels the exposure of negatively charged membrane phospholipids, although aminophospholipid exposure is not sufficient to generate these subpopulation of activated cells. Similarly, α-granule release is required but not sufficient for COAT-platelet generation, since we observe a dose-dependent increase in formation of COAT-platelets with agonist concentrations well above that necessary to induce P-selectin expression in more than 95% of all platelets (95). In addition, platelets expressing FV, in particular COAT-pletelets, are functionally relevant and quantitatively more important under these conditions than platelet-derived MP in

promoting procoagulant activity. These results also demonstrate that COAT-platelet formation is enriched in reticulated platelets, suggesting that young platelets are more likely than aged ones to undergo this transformation. Previous studies from our laboratory have demonstrated that aging platelets lose reactivity toward thrombin (95) and as well as collagen/convulxin (112). It is therefore conceivable that these age-related changes in reactivity toward single agonists are especially critical for an activation endpoint (COAT-platelet formation), which relies on both of these agonists.

1. Additional proteins on the surface of COAT platelets:

COAT-platelet has been initially observed and characterised regarding of FV bindig to dual-agonist activated platelets (3). We have here subsequently demostrated that COAT-platelets also express von Willebrand factor, fibrinogen, α_2-antiplasmin, thrombospondin, and fibronectin on their surface (**5**). COAT-platelets were observed upon dual activation with thrombin plus collagen type I, type V, type VI or convulxin, an agonist specific for the collagen receptor glycoprotein VI; however, no single agonist examined were able to produce COAT-platelets (3, **5**).

2. The role of microparticles:

In order to define the relative contributions of platelets and platelet-derived microparticles to the observed procoagulant activity, the level of residual procoagulant activity after separation of platelets and microparticles by centrifugation was assessed (51). We have also shown that dual stimulation with thrombin plus collagen, platelet-derived MP appear to contribute less than 20% of the prothrombinase activity in the absence of exogenous Va (3). The difference between ours and previous studies (59) in the relative contribution of platelets and platelet microparticles to prothrombinase activity may reflect that the latter study was performed in the presence of exogenous factor Va. Despite the fact that MP generated in vivo can stimulate coagulation (115) and that MP-related procoagulant activity has been implicated in pathologic prothrombotic states (116), our results agree with previous observations (53, 117-118) and are consistent with the concept that under physiologic conditions an adequate hemostatic response must be rapid and localized to the site of vascular injury. This concept is supported by the observation that platelet FV appears to be uniquely important to hemostasis even in patients with near normal levels of plasma FV. Additionally our clinical experience supports the importance of another coagulation

factor, serum FVII activity as well, in severely bleeding patients with low platelet count (**22**).

3. Transglutaminase activity and COAT platelet formation:

One characteristic shared by all the α-granule proteins present on COAT-platelets is that each is a known transglutaminase substrate, and this seemed to offer a plausible explanation for the strong affinity of these proteins for the platelet surface. We therefore examined the impact of transglutaminase inhibitors on COAT-platelet formation and found an attenuation (**5**). More importantly, it was also demonstrated that a synthetic transglutaminase substrate CP_{15}, a 15-residue peptide serving as a glutamine donor, was incorporated into COAT-platelets (**5**), thereby providing positive evidence that a transglutaminase was active during COAT-platelet formation.

The identity of the transglutaminase responsible for coupling serotonin to α-granule proteins is still uncertain. Platelets have significant levels of the factor XIIIa subunit in their cytoplasm (119) as well as a tissue transglutaminase (120). Immunochemical studies of COAT-platelets identified both transglutaminases on the surface of these cells (**5**), and an anti-FXIII antibody was able to inhibit COAT-platelet formation (**5**). However, Jobe et al. (121) report in an abstract that FXIIIa knockout mice are unaffected in their ability to produce COAT-platelets. While this point remains unresolved, it is noteworthy that Walther et al. (122) have recently demonstrated that small, cytoplasmic GTP-ases are derivatized with serotonin via a transglutaminase activity during platelet activation; this derivatization has been termed serotonylation.

4. Fibrinogen on COAT-platelets:

The binding of Fbg to dual-agonist stimulated platelets has been investigated to determine if Fbg is bound to the GP IIb/IIIa receptor on COAT-platelets. The unusual retention of these α-granule proteins on COAT-platelets was demostrated by the inability of PAC-1 to displace or prevent the binding of fibrinogen (**5**). PAC-1 is a monoclonal antibody which recognizes the activated conformation of GP IIb/IIIa with an affinity 50 times greater than does fibrinogen, and PAC-1 can actually be used as a GP IIb/IIIa antagonist to prevent platelet aggregation (103). Even though experiments with LIBS-6 indicated that GP IIb/IIIa molecules on COAT-platelets were occupied (**5**)

these observations led to the conclusion that fibrinogen, and perhaps other α-granule proteins, were being retained on the platelet surface with an exceptional affinity.

5. Formation of COAT-platelets in platelet rich plasma:

We have performed additional experiments utilizing PRP and demostrated that COAT-platelets can also be formed in an experimental system which reflect more natural environment for platelets. The current gold-standard test for COAT-platelets, transglutaminas inhibitors, were shown to inhibit formation of this specific population of activated platelets, allowing us to demonstrate that these cells are similar to those observed utilizing GFPs. These PRP-derived COAT-platelets have less surface FV than normal COAT-platelets, and they represent only 60-70% of the normal COAT percentage observed with gel filtered platelets.

6. FcRT- platelets:

We have also demonstrated that COAT-platelet production is not restricted to activation with thrombin plus collagen (or convulxin); these unusual platelets can also be generated by engagement of the Fc receptor (FcγRIIA) in combination with thrombin (**7**). The distinct subpopulation of activated platelets observed with this latter method is referred to as FcRT-platelets. We have observed that FcRT-platelets share a number of properties with COAT-platelets, including high levels of several α-granule proteins on their surface, exposure of negatively charged platelet phospholipids, susceptibility to transglutaminase inhibitors, and increased prevalence among young cells (**5**). FcγRIIA, a low-affinity receptor for IgG, is the only Fc receptor present on platelets, and it can be clustered physiologically by immune complexes (83) resulting in platelet activation. In addition, numerous anti-platelet antibodies are known to activate platelets through engagement of the Fc receptor, a mechanism mimicked here by ALB-6 and ML-13, two anti-CD9 mAbs (84). Considering that the platelet collagen receptor glycoprotein VI shares many signaling pathways with FcγRIIA (87), it is not unexpected that Fc receptor stimulation together with thrombin can generate a "COAT-platelet"-like population. However, none of these agonists alone was sufficient to induce COAT-platelet or FcRT-platelet production. One surprising discrepancy between COAT-platelets and FcRT-platelets is the absolute percentage of cells produced; there were approximately half as many FcRT-platelets as COAT-platelets generated for a given donor. This difference may be a function of the absolute number of glycoprotein VI

versus FcγRIIA molecules present on the membrane. Although the number of FcγRIIA molecules is approximately 1500 copies per cell (75), the number of glycoprotein VI molecules has not been reported, although it is likely to be significantly higher because it is observable with simple protein staining of sodium dodecylsulfate–polyacrylamide gel electrophoresis gels (37), and FcγRIIA is not. The absolute number of receptors available for stimulation may affect the temporal or quantitative distribution of intracellular second messengers generated on engagement and thereby differentially affect complex activation events required for COAT-platelet or FcRT-platelet production.

7. Identification of casein peptide cross-links in COAT platelets:

For the purpose of identification of transglutaminase cross-links in COAT platelets we have synthetised a 15-residue peptide derived from β-casein (CP_{15}) which included a transglutaminase-active glutamine (**5**). Preliminary data has indicated that a fluorescently labeled casein peptide (Fl-CP_{15}) is coupled to a small, hydrophobic molecule in the platelet membrane during COAT-platelet formation. This transglutaminase substrate was also used to address the question of which platelet component(s) served as the amino-donor in this transglutaminase reaction. While the anticipated result was that a platelet membrane protein would be the anchoring site for these transglutaminase substrates, the actual molecule conjugated to CP_{15} has turned out to be serotonin (**5**). This unexpected finding was corroborated by the demonstration that fibrinogen recovered from COAT-platelets had covalently bound serotonin (**5**).

8. Identification of membrane attachment site for transglutaminase reaction:

To identify the possible binding site for these proteins on the platelet mebrane CP_{15} was prepared either biotinylated (biotin-CP_{15}) or fluorescein labeled (Fl-CP_{15}). When biotin-CP_{15} was included in a COAT-platelet assay, the biotin label was incorporated into COAT platelets, and this incorporation could be blocked with transglutaminase inhibitors. SDS-PAGEanalysis of biotin-CP_{15} incorporated COAT-platelets revealed no high molecular weight platelet membrane proteins. Therefore Fl-CP_{15}-labeled COAT-platelets were extracted with ethanol and analyzed on reverse phase HPLC. The protuct than was analyzed by mass spectrometry and found to be the starting material Fl-CP_{15} plus 176 Da. The most common constituent in platelets with a molecular weight of 176

Da is serotonin. Direct evidence of serotonin's role was provided by identifying conjugated serotonin on fibrinogen isolated from COAT platelets.

Two different mechanisms appear to control surface binding of procoagulant protein released from α-granules. Low-level surface expression of these proteins can be induced by all agonists examined and is independent from the exposure of negatively charged membrane phospholipids, confirming the existence of a binding site other than aminophospholipids (113). COAT-platelet generation is only induced by the combined stimulus of two agonists, requires the presence of extracellular calcium, and parallels the exposure of aminophospholipids, although the latter is not sufficient for its generation. Moreover, only platelets expressing high leveles of surface bound FV are able to maximally bind exogenous FXa. This is reminiscent of the model recently proposed by Bouchard et al. For effector cell protease receptor 1 (EPR-1) mediated binding of FXa (114).

COAT-platelets, coinciding with aminophospholipid exposure and the highest ability to bind FXa, theoretically represents the most efficient substrate for prothrombinase complex assembly. When two stimuli inducing similar amounts of negatively charged phospholipids are compared, the stimulus able to induce high levels os surface bound FV (COAT-platelets) is more efficient in promoting thrombin generation. Our observations demonstrate that COAT-platelets expressing high levels of surface bound FV, even though it is present in just a minority of activated cells, is functionally more relevant than low-level FV expressing non-COAT-platelets.

These data allowed a preliminary model of COAT-platelet formation and structure to be proposed. This model is based upon four primary findings: several α-granule proteins are coordinately retained on the COAT-platelet surface; fibrinogen is bound to the COAT-platelet with an exceptional affinity; serotonin is conjugated to at least some of these α-granule proteins; and fibrinogen and thrombospondin have binding sites for serotonin-derivatized proteins. The proposed model addresses all four findings. With this model, α-granule proteins bind to their traditional receptors, e.g. fibrinogen to GP IIb/IIIa and FVa to PS. In addition, serotonin-derivatized α-granule proteins are also able to interact with serotonin binding sites on neighboring fibrinogen and/or

thrombospondin molecules. For example, serotonin-derivatized fibrinogen on COAT-platelets is not only bound to GP IIb/IIIa, but also to neighboring fibrinogen or thrombospondin molecules as a result of serotonin-dependent interactions. The consequence of these multivalent interactions is stabilization of the surface bound complex.

As a result, COAT-platelets represent a unique component of hemostasis. The requirement for dual stimulation indicates that COAT-platelets will only be formed under circumstances of extreme haemostatic need, such as immobilization of platelets on the collagen surface of a ruptured vessel in the presence of continuous thrombin generation. Moreover, the physiological significance of COAT-platelets is yet unknown, although inspection of the prothrombotic proteins present on the surface of these cells leads to speculation that they could be significant contributors to thrombotic processes. Most remarkable are the presence of an active prothrombinase complex and the availability of surface PS. While there are observations which suggest an important role for COAT-platelets, hard data on this subject are still lacking.

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