Short communication

Are platelet aggregation tests the best way to assess new drugs for arterial disease?

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Abstract

Over many years, laboratory testing of platelet aggregability have been carried out in attempts to develop drugs that would prevent thrombosis in arteries. The problems encountered included the question of methodology. Blood samples have to be anticoagulated in order to study the platelets. Anti-coagulation with citrate and tests on derived platelet rich plasma did not correlate at all well with thrombus growth in the stenosed coronary arteries of experimental animals and citrate removes the calcium ions which are vital for platelet function. Anticoagulation with heparin also interfered with platelet function, so that now, hirudins are the preferred anticoagulant. However it was observed that if, in stead of stimulating platelet aggregation with adrenaline or ADP, serotonin was applied to the the preparation, very little aggregation took place in spite of serotonin 5HT2A antagonists being the most potent inhibitors of thrombus growrh in experimental animals. Another indicator that primary platelet agggregation is not a predictor of *in vivo* efficacy was the finding that 5HT2A antagonism inhibited aggregate growth. In a stenosed artery the platelets are activated by increased shear stress and blood turbulance with release of platelet serotonin causing

Introduction

In the obituary of Gustav Born [1], one finds the following statement about his work on blood platelets, "When stimulated by blood vessel damage or blood clotting, these tiny cells "aggregate" together, plugging the damaged vessel and thereby preventing any further escape of blood. To measure this quantitatively, Gus devised and developed a simple, but extremely effective, device (known as the Born aggregometer) and, in doing so, launched an entirely new sub-discipline of haematology often referred to as "platelet aggregometry" (a term he hated). The aggregometer, used in laboratories and hospitals around the world, revolutionised the diagnosis of platelet-related diseases as well as providing a tool eminently suited to the study of the basic biology of these cells and their response to drugs". Very true and Born deserves all the credit he achieved, but is the statement still true today?

The Limitations of Born Aggregometry

The method involved anticoagulating blood samples with citrate and using a centrifuge to separate out a layer of platelet-rich plasma (PRP). When aggregating agents such as collagen are added to PRP in a cuvette, the platelets aggregate and settle at the bottom of the cuvette causing an increase in light transmission through the PRP which can be recorded on a chart or computer [2].

The first problem with this is that citrate anticoagulates the blood sample by removing the calcium ions (Ca2+). However, (Ca2+) is essential for normal platelet function! An equally obvious problem is that such *in vitro* bench tests take place in very different circumstances than real life where one has whole blood flowing through vessels. John Folts had the idea that platelet aggregation would be best studied in partially obstructed arteries in an anaesthetised animal [3,4,5], and showed the various

potentcies of platelet aggregation inhibitors [5].

How well does PRP Aggregometry Correlate with *In Vivo* Platelet Thrombosis?

Torr et al. [2] compared the results of ex vivo classical aggregometry of PRP prepared from experimental animals with the number of flow reductions in a stenosed coronary artery of anaesthetised dogs. Measurements were made before and after administraion of a putative platelet aggregation inhibitor ritanserin. They used collagen and ADP as aggregation stimulants, collagen at 10μM and 50μM, also 5μM plus adrenaline, ADP at 2μg plus adrenaline, 4μg and 6μg. The inhibition by ritanserin in number and slopes of the flow reductions in the animals was very much greater than the inhibition of **ex vivo** platelet aggregation.

Clearly the platelet aggregation results were a very poor way of assessing the inhibition of thrombus growth in the artery by ritanserin. Why use ritanserin? This drug was an inhibitor of the platelet serotonin 5HT2A receptor and was a way of testing the theory that platelet activation in arteries results from the haemodynamic consequences of partial obstruction of the artery.

Arterial Stenosis

At an arterial narrowing (stenosis), $\partial P = a_1Q + a_2Q^2$ where ∂P is the pressure drop across the stenosis, Q is the flow through the artery, a1 and a2 are proportionality parameters. This means that the relationship between the variables ∂P and Q is quadratic and stenotic resistance increases with flow a1Q expresses viscous resistance, while a2Q2 expresses turbulance. Another quantitative expression of a stenosis is the area ratio As/Ao, where As is the cross-sectional area of the stenosis and Ao is the cross-sectional are of the open, normal artery; this can be expressed as a percentage as (1 - As/Ao)x100. As the same *flow* has to go through both the normal section, Ao and the much smaller As of the stenosis, the blood has to go faster, i.e., *velocity* of blood flow increases; this is called convective *acceleration*. One can easily observe this phenomenon by watching a placid full river running into a gorge. Acceleration is determined by force according to Newton's second law of motion, F = ma ,
where F is force, m is mass and a is acceleration. So we envisage a mass of fluid accelerating into a narrowing of the artery exerting greater force, and just as objects in a river gorge feel force, so do blood cells in a stenosis. This effect is called shear stress because it is equal to the force divided by the area of the surface of the object.

Activation of platelets by exposure to increased shear stress and turbulance causes release of serotonin which is present in high quantities in the dense granules [6]. The serotonin is released upon activation and activates more platelets via the 5HT2A receptors, thus maintaining thrombus growth. Thrombus growth is completely abolished by 5HT2A platelet receptor antagonism [7]. The drug used by McAuliffe et al [7] was trialed in stable coronary disease patients and found to cause no change in bleeding time and to have no cerebral or other signicant side effects [8].

The Effect of Serotonin on Platelet Aggregation

This subject has been neglected because serotonin is said to be weak agonist for platelet aggregation [9]. At least these authors [9] did not use PRP, but whole blood using flow cytometry, impedance aggregometry and filtragometry. They concluded that their data suggest that 5-HT per se does not activate platelets, but dose-dependently enhances platelet activation induced by ADP and, in particular, thrombin in whole blood. This effect is mediated by 5-HT2A receptors. While it is true that the coronary thrombosis stimulated by adrenaline is also abolished by 5HT2A antagonism [7], there is a very great difference between the high potency of inhibition of thrombus growth not so stimulated, with the weakness of serotonin in *in vitro* testing [9].

Another problem with whole blood aggregometry is the activation of platelets by heparin [10] making heparins unsuitable for *in vitro* testing. The mst suitable anticoagulants for in vitro work are the hirudins, because the effects of hirudins on platelet function are different from those of unfractionated heparin. Spontaneous aggregation was rarely observed in hirudinized PRP compared to citrate-PRP. In comparison with citrate-PRP, two hirudins reduced the maximal response to epinephrine, but had no influence on the maximal response to ADP. Spontaneous aggregation is rarely observed in hirudinized PRP compared to citrate-PRP. In comparison with citrate-PRP, both hirudins reduced the maximal response to adrenaline, but had no influence on the maximal response to ADP [11]. Other authors agree that of the anticoagulants investigated, the selective thrombin inhibitor hirudin is the most suitable anticoagulant for studies of platelet aggregation *in vitro* in whole blood, e.g., [12]. Menys [13], studying platelet aggregation to collagen, used hirudin to maintain normocalcaemia, thereby avoiding erroneous findings often obtained with citrated blood. Platelet aggregation was quantified by optical aggregometry, and aggregate growth by single platelet counting, i.e., as the aggregates grew, fewer single platlets remained. He concluded that 5-HT contributes to collagen induced aggregate growth and 5-HT2 receptor antagonism with ICI 170809 retards the rate of growth. This could explain the efficacy of 5-HT2 antagonists in limiting coronary thrombosis despite the limited role of 5-HT in primary aggregation. ICI 170809 is the same drug as that used by McAuliffe [7] in the Folts model and by Noble et al in patients [8]. So the classical platelet aggregation tests measure the wrong thing. It is the aggrgation

of platelets that leads to thrombus growth, not the primary aggregation.

It is the reliance on platelet aggregation tests that has led to the development of the present dual anti-platelet therapy of aspirin (antagonising thromboxane) and clopidogrel (antagonising ADP), both of which are ubiquitously present in tissues and antagonism of them inevitably leads to escessive bleeding. Serotonin, on the other hand is only present to any extent in brain and platelets and not in tissue. Excess bleeding is therefore absent [8].

What Happens at the Vessel Wall?

The endothelial layer is covered by a layer of gel called the glycocalyx, dysfunction of which has been postulated to be the first step in the development of atheromatous lesions [14,15]. Such dysfunction occurs by oxidised LDL[16] and with hyperglycaemia [17] leading to cell adhesion to the endothlium [18] and thrombosis [17]. However, in arteries with normal luminal cross sectional area, this does not result in thrombotic occlusion. That only occurs in stenosis where very high concentration of serotoin occur in a confined space y the mechanism described above. A literature search for a bench model of hirudinised whole blood being forced through a stenosis failed to find any such attempt at a more realistic test for predicting clinical efficacy.

What Test Must One Use?

Unfortunately, none of the laboratory tests on platelets realistically simulates the *in vivo* situation of obstructive arterial thrombosis. The only way at present to test potential treatments is by using the Folts model, i.e., animal experimentation. This has been a reliable predictor of clinical efficacy, and has brought to light the importance of serotonin antagonism in spite of the fact that seerotonin is a weak agonist in bench tests [19 -30].

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