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Cellular and Molecular Life Sciences

Memories of a Senior Scientist

Platelets – From haemostasis to inflammation

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Studies at the University of Oslo

I graduated from the Faculty of Mathematics and Natural Sciences, University of Oslo in 1962, where I had studied chemistry, zoology and physics ending with a major in biochemistry. This was a time when academic culture and scientific philosophy was emphasized. Thus, I am proud of having had the later Nobel Price Laureate Odd Hassel (1969) as lecturer in physical chemistry.

My first encounter with platelets took place around 1960 when I worked on my student thesis on phosphodeoxyribomutase with Professor Søren Laland at the Department of Biochemistry. A student working in the same room had got quite a different project, namely to identify the component of erythrocyte lysates that could aggregate platelets, based on an observation made at the Institute for Thrombosis Research at Rikshospitalet (The National Hospital of Norway). She soon realised that the aggregating component emerged from Dowex columns at the same position as ADP, and that ADP taken from the laboratory shelf could aggregate platelets. So, this all started with observations made at the Institute for Thrombosis Research at Rikshospitalet and the Department of Biochemistry at Blindern, Oslo.

After my graduation from the University in 1962, I was offered a job at the above-mentioned institute at Rikshospitalet headed by Professor Paul Arnor Owren, who was followed by Professor Helge Stormorken, both highly respected internationally. Thanks to them I had the opportunity to pursue my ideas in an excellent milieu for many decades.

At this stage, I would like to present some terms that are used in the review. 'Platelet aggregation' is cell-to-cell adhesion of metabolically intact platelets (different from agglutination). Binding of fibrinogen to the

activated GP IIb-IIIa complex (integrin $\alpha_{IIb}\beta_3$) leads to platelet aggregation at low shear. At high shear von Willebrand factor is important. 'Platelet adhesion' describes the adherence of platelets to surfaces, often to collagen, or collagen fibres coated with von Willebrand factor, in the subendothelial layer of a damaged vessel wall. The important adhesion receptor at high shear is the GPIb-V-IX complex with von Willebrand factor as a major, but not only, ligand. 'Secretion' is due to intracellular signalling leading to release of soluble material from the intracellular granules called α- and dense granules, and even from lysosomes. During this process, the α-granule membrane fuses with the surface membrane with the result that integral granule membrane proteins are exposed on the platelet surface. Shedding of soluble forms of these proteins occur by proteolysis mediated by metalloproteinases. Formation of a 'procoagulant surface' occurs as a result of an increased level of cytosolic Ca^{2+}, which leads to the activation of the scramblase, catalysing the breakdown of the phospholipid asymmetry of the surface membrane. This results in a catalytic surface for reactions between coagulation factors coming from plasma and from within the platelet. 'Microparticles' are membrane-derived particles with cytoplasmic content shed from the surface membrane, also as a result of an increased cytosolic calcium concentration with activation of calpain and dearrangement of the platelet cytoskeleton. Very simplified, one may say that haemostasis starts with platelet adhesion to adhesive structures in the subendothelial layer of the damaged vessel wall. This leads to secretion and platelet aggregation at this site. Further, the coagulation cascade is activated, and fibrin is formed on the procoagulant platelet surface, sealing the wound. Control mechanisms halt the process at this stage. In thrombosis the same reactions may take place, but the damage in the vessel wall is typically due to arteriosclerosis, and the limiting mechanisms may not act properly. Inflammation, with inflammatory components also from platelets, is believed to take part in the formation and rupture of aortic plaques. Practically all of these concepts have developed during the last 45 years.

Fibrinogen as a cofactor in platelet aggregation

Thrombin, like ADP, was soon linked to platelet aggregation. In the early sixties reports also started to emerge indicating that fibrinogen was not only the main substrate for coagulation of plasma, but could also be involved in platelet aggregation. It had also been shown that the platelets themselves contained some intracellularly located fibrinogen that could be released from platelets after treatment with thrombin. We also studied this, and found that added fibrinogen could support aggregation of washed platelets suspended in a buffer when the aggregation was induced by ADP and adrenaline, but that externally added fibrinogen was not required when the aggregation was induced by thrombin or collagen particles. Combining this with the observed release of intracellular fibrinogen, we thought that thrombin and collagen also required fibrinogen for aggregation, but that, in this case, this came from within the platelets [1]. In an historical context, it is interesting that our studies were performed using a primitive device called "Chandler's loop", which consisted of plastic tubing forming circular loops containing the washed platelet suspension with fibrinogen and aggregating agent added. These were placed on a rotating device, and the time from mixing to the formation of visible aggregates served as a measure of aggregation. During this period I also learned a lot from Holm Holmsen, who was the one who developed the idea of platelet secretion from storage granules, and Erik Mürer, who made important observations on platelet energy metabolism, all of us working on our Ph.D. theses.

Intracellular platelet fibrinogen

These studies inspired me to a closer study of the intracellular platelet fibrinogen, and a comparison to its counterpart in plasma. Because of the low amount available from human blood, we had to use bovine platelets. This meant long days starting at the slaughter house in the early morning, collecting blood directly from the wound, and ending at midnight with washed and stored platelets. Platelet fibrinogen was then purified and analysed, and compared to purified plasma fibrinogen [2]. Only small structural differences were observed, mainly concerning the carbohydrate content. Later, it became a matter of debate whether the intracellular platelet proteins that have a plasma counterpart are "real" platelet proteins, or if they are taken up from plasma and located in the α-granules after a receptor-mediated internalisation. It has been claimed that platelet fibrinogen, contrary to von Willebrand factor, represents internalised plasma fibrinogen. It should be noted, however, that even if this were the case, subtle differences are to be expected as there is a small fraction of plasma fibrinogen that cannot be internalised. I had the pleasure of sharing the work on platelet fibrinogen with Stanislaw Lopaciuk as a visitor from the Institute of Haematology in Warsaw.

The coagulogen of *Limulus polyphemus* haemocytes

With our interest in the intracellular fibrinogen in platelets, my colleague Erik Mürer and myself became very exited when we became aware of the fact that in the "living fossil" *L. polyphemus* (horseshoe "crab"), all of the clottable protein is found as an intracellular protein in the haemocyte residing in big electron-dense granules. However, in an environment of Gram-negative bacteria it can be released from "exploding" haemocytes followed by polymerisation due to a reaction with the bacterial endotoxin. The idea is that this is part of a defence mechanism whereby invading microorganisms are immobilized in a sort of coagulum, and an early example of inflammation. We soon realised that the coagulogen was a much smaller molecule than the vertebrate fibrinogen [3]. Still, a series of analogies existed. Thus, the coagulogen is also split by a proteolytic enzyme formed as the result of an enzyme cascade activated by endotoxin. The polymerisation as such was a fast process, and could be compared to that of vertebrate fibrin. Part of our *Limulus* studies were done in USA when I worked for a period as Assistant Professor at the Specialized Center for Thrombosis Research at Temple University in Philadelphia, but had to be ended when I returned to Norway in 1972. It should be added, though, that the so-called Limulus test for endotoxin might be said to represent an extension of such studies. At Temple, I also worked with effects of small fibrinogen degradation products on platelet aggregation with Andrzej Budzynski and Victor Marder.

Platelet membrane receptors

A well-known term is GP IIb-IIIa antagonist. GP stands for glycoproteins, i.e. for platelet membrane glycoproteins. Interestingly, whereas membrane receptors often started their lives as entities that were defined by mathematical treatment of data such as the Scatchard plot (numbers and affinities) without knowledge of their true chemical nature, this was different for the platelet membrane receptors. These were first studied as glycoproteins, often by biochemists and carbohydrate chemists. It was then realised that some of them could not be found on platelets of patients with defined congenital bleeding disorders. Being able to observe proteins that bound specifically to glycoprotein complexes, these were defined as receptors with specific ligands. The first receptors studied according to these principles were GP Ib (later recognized as a GP Ib-V-IX complex), which is lacking on the giant platelets of Bernard-Soulier patients, and the GP IIb-IIIa complex (later defined as integrin $\alpha_{IIb}\beta_3$), which is lacking on platelets of Glanzmann's thrombasthenia patients. Much of this knowledge emerged as a result of a small number of people who met regularly at small seminars around the world. This stands out in my memory as a fantastic time, also because of friendships leading to a lot of scientific cooperation worldwide. Our group consisted of Inger Hagen, Geir Gogstad, Frank Brosstad and myself. For me personally, the story started some years earlier, however, demonstrating with my colleague Kjell Grøttum that the platelets of Bernard-Soulier patients showed a slow electrophoretic mobility in an electrophoresis chamber combined with a reduced amount of the negatively charged sialic acid on their surface. At that time, we did not understand why, but when GP Ib was discovered and defined as a heavily sialylated glycoprotein, and shown to be absent from the Bernard-Soulier platelets, we realised that this might be the reason for our findings. Much of the progress during that period was due to the use of labelling and electrophoretic techniques that suited the purpose very well. Our group contributed with the crossed immunoelectrophoresis technique (CIE) performed under conditions where also hydrophobic and amphiphilic membrane proteins could be electrophoresed. A major advantage was that the membranes were solubilized in a non-ionic detergent (Triton X-100) that did not denature the proteins, nor dissociate non-covalent protein complexes, and that the use of gels containing a hydrophobic matrix could be used to discriminate between hydrophilic and hydrophobic proteins. This way we could demonstrate that glycocalicin, thought to be a protein of its own, was a hydrophilic split product from the hydrophobic GP Ib containing the binding sites of GP Ib for both von Willebrand factor and thrombin. Using SDS-PAGE we also observed that glycocalicin was not produced from Bernard-Soulier platelets, indicating that the whole GP Ib molecule might be absent. Inger Hagen observed that both GP IIb and IIIa immunoprecipitated together indicating a complex. Using an overlay technique with radioactive fibrinogen we could demonstrate that fibrinogen was bound to the GP IIb-IIIa complex in the gel. Other protein–protein interactions were also studied such as the interaction between GP Ib and filamin, and GP Ib and thrombin (reviewed in [4, 5]).

Von Willebrand factor and platelets

Already when working with the bovine platelet fibrinogen, we observed that addition of this to human platelets would induce a platelet agglutination, a platelet clumping that was observed even with

formaldehyde-fixed platelets and platelet membrane fractions (platelet ghosts). Later, this was explained by the presence of the protein von Willebrand factor in the fibrinogen preparation, and that this was an agglutination depending on ligand size due to binding of several subunits of the multimeric von Willebrand factor to GP Ib. Subsequently, we observed that after incubation of platelets with dibucaine, which increased the membrane fluidity, even small multimers could support the agglutination [6]. This we explained by the idea that an increased lateral movement of the GP Ib molecules in the membrane would increase the number of receptor-ligand interactions.

Microparticles from platelets

When Pål André Holme, as a medical student, started to study platelet-derived microparticles, only a few papers had been published on this. Using a difference in the structure of the so-called fibrin-stabilising factor (transglutaminase) from platelets and plasma, he demonstrated that the microparticles were shed as outside-out membrane particles. We could also demonstrate microparticles in a clinical condition, namely in the blood from patients with disseminated intravascular coagulation (DIC) [7]. We also observed microparticles in blood in a flow chamber, but only at shear forces characteristic of heavily stenosed arteries [8]. (As to phospholipid scrambling and shedding of microparticles see [9].)

Electrophoretic separation technology

As mentioned above, various electrophoretic techniques had to be applied for the studies of the platelet membrane receptors. This put us in contact with international electrophoresis societies, and I was asked to function as president of the first meeting of the International Council of Electrophoresis Societies in 1993. It was very inspiring to meet experts in a more technical area, particularly pioneers like Stellan Hjertén of the University of Uppsala, who was central in the development of capillary electrophoresis that now is essential in most DNA sequencing technologies.

Platelets and inflammation

As our institute opened for a broader spectrum of studies in internal medicine, the name was changed in 1978 to Research Institute for Internal Medicine. One impact was the arrival of a group from the Section of Clinical Immunology and Infectious Diseases of the National Hospital lead by professors Stig Frøland and Pål Aukrust. Their interest in the immunological components of arteriosclerosis also lead to an interest in inflammatory reactions of platelets, and cooperation between the groups. For us this especially meant studies on two ligands in the TNF superfamily, namely CD40L (CD154) and LIGHT [10, 11]. In accordance with others, we have interpreted an observed shedding of soluble (s) CD40L as a result of a proteolytic action on the surface-exposed protein. Interestingly, we found that intracellular phenomena that could be affected by agents like cytochalasin D or PGE_1, are important for the proteolytic step. Interaction with its receptor CD40, which is constitutively expressed on the platelet surface, also appears to be important for the proteolytic process as previously shown by others for the total process. LIGHT appears to behave very similarly to CD40L with regard to its release after platelet activation, and GP IIb-IIIa antagonists are believed to inhibit the shedding of sCD40L and LIGHT. However, as we have now approached to problems of today, I like to raise some questions exemplified by CD40L biology, but probably of more general importance. Membrane-bound CD40L is known to induce inflammatory reactions in endothelial cells *in vitro*. In such a system we have also observed that recombinant sCD40L induced inflammatory responses, but at much higher concentrations than those that can be expected for the soluble forms from platelets in plasma. However, recombinant sCD40L has been shown to prime the polymorphonuclear leukocyte oxidase at lower concentrations than those used by us [12]. Thus, are both the membrane-bound and the soluble forms of the inflammatory proteins (e.g. CD40L) of biological importance? If so, what is their relative importance? What role is played by costimulatory components? Is the shedding of the proteins a means to limit its cell-associated effects preventing an over-shoot of stimulation of other cells? Or what is really the purpose of the shedding? Is it "good or bad" to prevent the shedding? I leave these questions as a challenge to the younger generation who are about to start their career today some 45 years after we started to look into the fascinating field of platelet biochemistry and biology.

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