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Molecular aspects in clinical hemostasis research at Karolinska Institutet

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ABSTRACT

The development of hemostasis research at Karolinska Institutet is described, focusing first on the initial findings of the fibrinogen structure and the hereditary bleeding disorders, hemophilia A and von Willebrand's disease. Basic research has focused on new biomarkers for cardiovascular/thromboembolic disorders, such as myocardial infarction and stroke, including preeclampsia and diabetes, with studies on the importance of decreased fibrinolysis in these disorders. Since long, the structure of the fibrin network has been evaluated, and recently the influence of aspirin and new thrombin and factor Xa inhibitors has been investigated. Research on the contact pathway of coagulation has also started at the Unit.

1. Heparin, fibrinogen, coagulation factor VIII, von Willebrand factor, new anticoagulants, chromogenic synthetic peptides

It all started with the purification of heparin in the 1930s by Erik Jorpes, professor of Medical Chemistry at Karolinska Institutet [1,2]. This led to early introduction of heparin treatment in Sweden. Heparin, and later fractionated heparin, are now used since many decades all over the world for prophylaxis and treatment of thromboembolic disorders. Other aspects of heparin have also been studied, including the interaction between heparin and antithrombin [2], leading to non-thrombogenic heparin-coated surfaces [3] now valuable in treatments using extracorporeal membrane oxygenation (ECMO).

In the laboratory of Jorpes, Margareta and Birger Blombäck purified fibrinogen and found, together with Inga-Marie Nilsson, coagulation factor VIII lacking in hemophilia A, as well as a new hemostatic factor, von Willebrand factor (VWF) lacking in the disease now called von Willebrand disease (VWD) [4–6]. This led to early treatment of Swedish bleeders with these disorders. Especially the prophylactic treatment for avoidance of joint destruction became well-known world-wide. Further work on fibrinogen led to the conclusion that fibrinogen was a dimer composed of three different polypeptide chains. The two fibrinopeptides A and B (FPA, FPB), split off by thrombin from the N-terminal part when fibrinogen is transformed to fibrin, were analyzed from different species and used to trace evolutionary patterns [6]. The amino acid sequence of the FPA nonapeptide was well preserved, and led to the idea that thrombin should be possible to inhibit by peptides imitating the area around the cleavage site. The tripeptide segment Gly-Pro-Arg in fibrinogen, positioned after the cleavage site, was found to be substituted by Gly-Pro-Ser in a Detroit girl with severe menstrual bleedings [6]. This established the nature of the then second mutation explaining a dysfunction. In industrial collaboration, the FPA nonapeptide and shorter variants were synthesized and tested. Maximum antithrombin (anticoagulant) activity was found for the nonapeptide but also for the variant tripeptide Phe-Val-Arg. It was hoped that such a peptide, instead of warfarin, could be clinically useful to prevent thrombosis [7]. Several patents were granted. However it was not until much later that this became a reality. Nowadays, there is an overflowing market of new thrombin and factor Xa inhibitors partly based on these early ideas.

It was also found that the tripeptide could be used for measurement of thrombin activity, and by coupling the carboxylic part of the arginine residue of the tripeptide to a chromophor, paranitroanilide, this aim was achieved [8]. The activity of thrombin liberated paranitroaniline, and the color change from the chromophor paralleled the thrombin activity. In the same manner, chromogenic synthetic peptides were constructed for measurement of other proteases, such as factor Xa, plasmin, trypsin and kallikrein. In a few years, about a thousand reports were published world-wide regarding proenzymes, enzymes and their inhibitors using such peptides. In this manner enzymatic assays were rapidly developed for the determination of many coagulation factors and their inhibitors in human plasma. Enzymatic colorimetric assays were easier to perform and control than corresponding clotting tests. An assay for determination of prokallikrein was developed at the Unit and an assay for factor X was of special interest since it correlated well with the Thrombotest assay (PT), indicating that measurement of factor X could be used instead of PT assays for monitoring coumarol therapy [9,10].

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2. Fibrin network structure in myocardial infarction and stroke. Influence of aspirin, thrombin and factor Xa inhibitors on the network structure

The fibrinogen work has subsequently been focused on the fibrin network – the last step of coagulation. Birger Blombäck developed a permeability method, measuring the flow through a fibrin gel formed by adding thrombin and calcium to plasma in a cuvette [6]. The method has later been modified [11], and we showed that young males with earlier myocardial infarction (MI) had a slow flow through the fibrin gel formed from their plasma. Obviously, they had thin fibers in a fine-meshed, tight and rigid fibrin network. Its tightness was found to be correlated with the coronary stenosis score as measured with angiography [12]. Young males had elevated fibrinogen levels and we found that 51% of the variance in the plasma fibrinogen level was accounted for by genetic heritability. The studies supported the view that an increased plasma fibrinogen level constitutes a primary risk factor for CHD. Another research group later demonstrated that increased levels of fibrinogen and VWF were associated with long-term risk of CHD and mortality in middle-aged women [13].

During the last decade a unit at Danderyd Hospital, with focus on hemostatic problems in CHD and stroke, in collaboration with our group showed that the fibrin network in plasma from patients with type I diabetes is tighter than that from healthy control subjects. It becomes more porous, when diabetic glycemic patients are submitted to insulin-pump therapy, treatment with low molecular weight heparin, or with lipid-lowering statins [14,15]. A porous fibrin gel network is easily attacked by degrading enzymes such as plasmin. Also, a decreased porosity of the fibrin network was found in patients with acute ischemic stroke in spite of the fact that they were on treatment with acetylsalicylic acid (ASA) (Rooth et al., submitted for published). Similarly, an interesting method for measurement of microparticles useful to assay in thromboembolic states, has recently been published [16].

ASA is increasingly used in treatment of cardiovascular disorders to prevent recurrence of MI and stroke. It has been found to be clinically more effective at lower dose, but there are different opinions about the dose. Our group early found that withdrawal of ASA gave rise to a tighter fibrin network and that a low dose of ASA gave rise to a more porous network. We also showed that ASA treatment at very low doses led to an increased porosity of the network [17] Intrigued by our findings, we designed an in vitro study using different doses of both ASA and its main metabolic product, salicylic acid, into which ASA is rapidly transformed in vivo and which can inhibit ASA [18]. Also in this in vitro study we could show that low doses of ASA led to a more porous network (Fig. 1). Furthermore we have shown that the fibrin network becomes increasingly porous when thrombin and factor Xa inhibitors are added to plasma in an in vitro system [19].

3. Genetics of von Willebrand's disease

We investigated the heredity of several Swedish families with "pseudohemophilia" in the end of the 1950s. Erik Jorpes initiated investigations on patients in the Åland islands, his birthplace. We demonstrated that the patients of the original bleeder family, described by Dr. Erik von Willebrand, had the same VWD as those we had investigated in Sweden with "pseudohemophilia". There were, however, several other bleeder families on the Åland islands and in further investigations we showed that not all had the genuine VWD [5]. In the early 1990s, we investigated 25 Swedish families who had one or two members with severe VWD (called type 3) and the surviving members of the original Åland family. We then finally confirmed that the patients with VWD type 3 were either homozygous (same genetic VWF defect from each parent) or double heterozygotes (different genetic VWF defects; one from each parent) [20,21]. In the beginning of the 1980s, our group purified VWF while studying the factor VIII/VWF complex, and determined the N-terminal amino acid sequence of VWF [4]. Two female patients with the severe form (type 3) of VWD responded increasingly poor, both clinically and by laboratory values, to transfusions with "Fraction I-0", our name of the VWF preparation. We found that plasma from these patients inhibited ristocetin-induced platelet aggregation in normal platelet-rich plasma and that the inhibitor was a type G immunoglobulin [22].

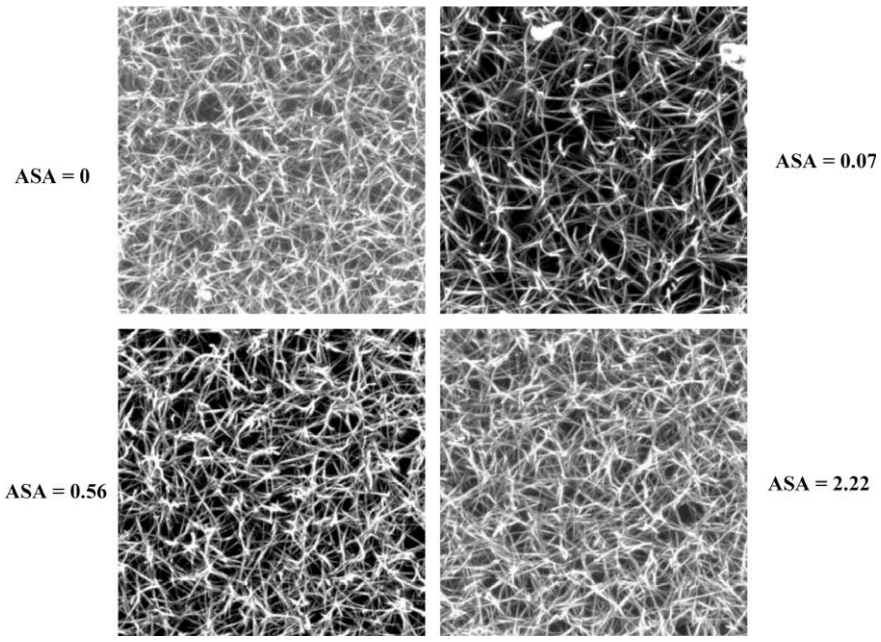

Fig. 1. Confocal 3D microscope images showing porosity of fibrin networks derived from purified fibrinogen incubated with acetyl salicylic acid (ASA) at 0, 0.07, 0.56 and 2.22 mmol/L. Reproduced from [18] with the permission of Wolters Kluwer.

4. Research on the fibrinolytic system

The fibrinolytic proenzyme plasminogen was purified and characterized in the Unit of Erik Jorpes. Wallén and Wiman in Umeå, showed that fibrin potentiates the effect of tissue plasminogen activator (t-PA). This finding, in collaboration with Desiree Collen, led to a breakthrough for thrombolytic therapy with t-PA, which is now coming into use also in stroke therapy, reducing mortality, disability and costs for society. Björn Wiman, who became the successor of Birger Blombäck as professor of Coagulation Research, identified the plasminogen activator inhibitor-1 (PAI-1), an important inhibitor of the fibrinolysis activator t-PA [23,24]. He also characterized PAI-1 and studied its rapid complex binding to t-PA. Together, we further demonstrated that increased plasma levels of PAI-1 was a risk factor for MI, especially for recurrent events. High PAI-1 in MI patients inhibited t-PA and correlated with triglyceride levels, indicating that high plasma levels of PAI-1 could be of importance for the pathogenesis of MI [25,26].

Interest in why PAI-1 is so important for coronary heart disease, made it necessary to turn to genetic analyses, showing that the frequency of a polymorphism earlier described, with 4G instead of 5G in the PAI-1 gene, occurred more frequently among MI patients [27]. It was also observed that very low density lipoprotein (VLDL) induced PAI-1 expression in endothelial cells explaining the link between the two systems. Molecular mechanisms of regulation of the PAI gene expression by VLDL triglycerides were described, as well as a mechanism for influence of environmental interactions on PAI-1 expression.

Wiman identified the PAI-1 binding protein in plasma, vitronectin, and defined its binding site. The results suggested a stoichiometric 1:1 complex between the two molecules. He investigated the relationship between structure and function of PAI-1 using site-directed mutagenesis to produce more than forty PAI-1 mutants, most from different regions of the molecule. The group also studied the transformation of active PAI-1 to its latent form, finding that active PAI-1 was more stable at acidic pH and that one or more histidine residues might be of importance for the stability [24]. They further demonstrated that the transformation from active to latent PAI-1 involves insertion of a reactive center loop in PAI-1, making it inaccessible to plasmin. They found a histidine residue (His364) important for binding of PAI-1 to heparin. Using single site mutants they were able to show that Lys436 of antiplasmin was important for the interaction between plasmin and antiplasmin. Patients with low PAI-1 levels were found to have more bleeding complications than those with high levels.

Using samples from the Stockholm Heart Epidemiology Program (SHEEP), de Faire and Wiman found in a population-based case-control study of MI, that increased plasma concentrations of fibrinogen, PAI-1, the t-PA/PAI-1 complex, and VWF were significantly associated with the risk of MI. The group studied two alleles of PAI-1 and found that individuals homozygous for the 4G allele had increased levels of PAI-1, associated with MI [28,29]. They also found that the platelet PAI-1 fraction of healthy 4G carriers was about 10% active. This is quite high compared to what is normally found in plasma. Moreover, they developed a method to study the t-PA/PAI-1 complex and found that measurements of this complex might be of value in assessing the risk of MI [30]. Patients with stroke had an elevated PAI-1 level and a decreased fibrinolytic profile in their plasma, as determined by a new method [31]. Thus, it has become increasingly evident that inhibition of fibrinolysis is a highly important aspect to take into consideration with regard to the pathogenesis of arterial cardiovascular disorders.

5. Other research results

Using a specific method for determination of active, two-chain factor VII, Hamsten showed that triglyceride-rich lipoproteins activate factor VII in the postprandial period. In vivo data indicated that factors IX and XI were involved in the process, whereas data in a purified system showed that mainly large and small VLDL supported factor Xa- and factor Xa/Va-mediated activation of factor VII. Increased generation of factor VIIa in the postprandial state strengthens the potential for thrombin production in the event of plaque rupture, with exposure of tissue factor, suggesting alimentary lipemia as a clinically important procoagulant state [32]. Hamsten has later worked on the characterization of regulatory genetic variants affecting plasma fibrinogen concentrations Furthermore our group has shown that PAI-1 is increased in preeclamptic mothers and coupled to increased resistance in the placental circulation. Similarly, the level of PAI-2 (formed in placenta) was found to be correlated to the function of placenta, its weight and to the growth of the fetus [33]. The influence of hormones on hemostasis in women was studied in many respects [2] and also in prostate cancer [34,35].

Kallikrein isolated from pig plasma and infused into minipigs produced an immediate circulatory response similar to that of bradykinin, a transient rise of the pulmonary artery pressure, and a transient fall of the systemic blood pressure [36]. Factor XII, prokallikrein, and fibrinogen levels and platelet counts showed a progressive decrease over a 3 h period. A similar fall was observed for the C1s inhibitor and antithrombin, suggesting that the kallikrein infusion induced a low-grade intravascular coagulation. Bradykinin gave an immediate and kallikrein a slow but pronounced increase of tissue plasminogen activator. Both bradykinin and kallikrein infusions caused a several-fold increase in the urinary excretion of the major metabolites of thromboxane and prostacyclin [37].

During the many years of research at the Coagulation Unit, discoveries have been brought into clinical practise and many produced theses at the Karolinska Hospital. Most clinical work has focused on thrombotic disorders [2]. Two large multicentre, randomized trials on the optimal duration of anticoagulation in patients with the first or second event of venous thromboembolism were started in 1988. The group showed that the presence of cardiolipin antibodies of IgG type was associated with an increased risk of recurrent venous thromboembolism and death [38]. Wiman analyzed PAI-1 activity and t-PA, demonstrating that reduced fibrinolytic activity was of only minor importance for the risk of recurrence, as were gene mutations in factor V Leiden and prothrombin.

6. Future basic research

Recent work from Thomas Renné, professor at the Unit since 2008, focuses on the factor XII-driven plasma contact activation system, a procoagulant and proinflammatory protease cascade operating on vessel walls and blood cells. This work has demonstrated that polyphosphate, an inorganic polymer secreted from platelets, provides the long-sought link between primary (platelet activation) and secondary (fibrin formation) hemostasis [39]. Smith and collaborators at the Huddinge branch of Karolinska, have developed a non-viral gene transfer method [40]. By designing plasmids, or oligonucleotides, to contain the corresponding target sequence for a specific anchor molecule, complexes with desired biological functions can be generated. This is of interest for possible future gene transfer and might be a step towards the dream of gene therapy in hemophilia A. Even a small increase in the factor VIII level would have a significant clinical effect.

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