Platelet activation and microparticles in the pericardial cavity during cardiopulmonary bypass
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Citation for published version (APA):
Maquelin, K. N. (2003). Platelet activation and microparticles in the pericardial cavity during cardiopulmonary bypass

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Chapter 1

General introduction
1. CORONARY ARTERY BYPASS GRAFTING
AND CARDIOPULMONARY BYPASS

1.1. Introduction
In The Netherlands cardiovascular disease remains the leading cause of death, although the last few years its mortality and morbidity have been reduced due to improved medication, availability of angioplasty and improved coronary artery bypass grafting performance. Still, in 1999 some 40% of the mortality was caused by cardiovascular disease, and 25% of those cases were due to myocardial infarction.

In 1999, cardiac surgery was performed on 14,709 patients in 13 Dutch hospitals. Of these patients, 85% received coronary artery bypass grafting (CABG), where venous and/or arterial grafts were placed to restore the blood supply behind the coronary artery stenosis. Until recently, all CABG operations were done with the assist of cardiopulmonary bypass (CPB). The CPB circuit is connected to the patient to get a motionless and bloodless operating field during the anastomoses, i.e., the placement of the grafts. In addition, cardioplegia is used to reduce oxygen demand during the interruption of blood supply. Compared to CABG operations in 1970, the 30-day mortality has been impressively reduced from 5.5% to approximately 1% in the low risk group. On the other hand, there is still a morbidity associated with cardiac surgery, which is higher than comparable surgical operations without the use of CPB. Presumably, this is caused by blood activation through the CPB circuit.

1.2. Cardiopulmonary bypass surgery in the Onze Lieve Vrouwe Gasthuis, Amsterdam
There are many ways to reach the situation of a non-beating heart without flow through the coronary arteries to enable a CABG procedure. A short description of a routine CABG in our clinic will be given to provide some background to those not particularly familiar with this procedure.

After administration of the anaesthetics, i.e., induction, the thorax is opened and heparin is administered to the patient to prohibit extensive activation of the coagulation system during the operation. The arterial cannula for the return of the blood from the CPB
circuit into the patient is inserted in the aorta. The venous cannula(s) for drainage of the venous blood is (are) inserted via the right atrium. After the achievement of a proper level of anticoagulation based upon the activated clotting time (ACT > 450 seconds), the CPB is started with subsequent cooling of the patient to 32 °C to slow down the metabolism of the patient and thus to reduce possible energy depletion. To prevent blood coming into the aortic root, an aortic crossclamp is placed between the aortic cannula and the aortic needle. Cardiac arrest is then induced with the so-called cardioplegic solution, which is administered in the aortic root where the ostia of the coronary arteries are situated. This ice-cold solution, containing amongst others high concentrations of potassium, is infused through an aortic needle, which is placed proximal from the aortic cannula. At this time the heart is topically cooled by ice-cold physiologic saline. Once a complete cardiac arrest has been achieved, one side of the grafts is attached to the coronary arteries. At the end of the CPB the patient is rewarmed. After completion of all distal anastomoses the aortic crossclamp is removed and the heart is reperfused with blood and will start to beat. An aortic sideclamp is placed to enable restoration of coronary blood flow, but also to provide a bloodless part of the aorta to connect the proximal ends of the venous grafts to the aorta. Once acceptable hemodynamic parameters are monitored the CPB is stopped. To counteract the infused heparin a sufficient amount of protamine sulphate is administered and the cannulas are removed. The patient is then transported to the intensive care to recover from the operation.

1.3. Blood activation during cardiopulmonary bypass

During CABG the blood is in extensive contact with the synthetic surface of the CPB circuit, which leads to the so-called “whole body inflammatory response”\(^2\). This is a complex phenomenon involving activation of various cells including blood platelets, leukocytes and endothelial cells, and activation of the coagulation, fibrinolysis and complement systems. In the systemic circulation elevated activation products of platelets and coagulation, fibrinolysis and complement systems are detected. This may lead to thrombotic, bleeding and inflammatory reactions. Clinically this is manifested for example in microthrombi as observed in retinal vessels,\(^3\) enhanced postoperative bleeding requiring blood transfusion and increased capillary permeability resulting in compromised
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lung function.

Although improvements in biocompatibility of the CPB circuit, such as the composition of the synthetic materials, the use of membrane oxygenators, and heparin coating of the circuit have resulted in considerably less systemic blood activation, CPB is still associated with the morbidity as described above. This is not entirely surprising. Originally most studies did suggest that blood activation is especially due to its contact with the CPB circuit, but more recent studies suggest that the blood, which collects in the pericardial cavity and is peroperatively returned into the patient, is another possible source of systemic blood activation. When the pericardial blood was discarded and not returned into the patient, postoperative blood loss was reduced. However, it is still common practice to return the pericardial blood to reduce the peroperative blood loss and thus the need for blood transfusion. Compared to systemic blood, pericardial blood contains highly elevated levels of fibrinolysis activation products such as plasmin-α2-antiplasmin complexes, coagulation activation products such as prothrombin fragment F1+2, thrombin-antithrombin complexes, and activated coagulation factor VII, and soluble tissue factor. The question therefore arises whether, upon its return into the patient, the pericardial blood can provide ongoing activation of for instance the coagulation system in the systemic circulation.

2. HEMOSTASIS

2.1. Introduction

Hemostasis, i.e. the process causing the blood to stop from flowing out of the injured vessel, is achieved by three main components. First, vasoconstriction reduces the diameter of the injured vessel(s) and thus the blood flow. This enables the other two components of the hemostasis system, i.e. the platelets and the coagulation system, to stop the blood flow from the injured vessel. The platelets form an initial plug in the process called primary hemostasis and the coagulation system consolidates this plug in the secondary hemostasis. The latter two components are under investigation in this thesis.
2.2. Platelets and their function

Blood platelets are anucleated cells derived from megakaryocytes in the bone marrow. Their mean life span in the systemic circulation is 10 days. The shape of the non-activated platelet is discoid and its diameter approximately 1 – 2 μm. The platelet outer membrane has the regular double phospholipid structure, which also forms the open canalicular system by protruding deep into the platelet. Platelets have several types of intracellular granules (α-granules containing all the proteins which are extruded by the platelet in the so-called secretion reaction, dense bodies containing the low molecular weight substances such as ADP and Ca^{2+} which are also extruded in the secretion reaction, lysosomes containing the regular lysosomal enzymes but with uncertain function, and mitochondria as energy factories). Platelets also contain several contractile systems, such as the microtubules, which keep the non-activate platelet in its discoid shape, and microfilaments that form the membrane and cytoskeleton. The intracellular membrane system is called the dense tubular system in the platelet (Figure 1).

Upon activation the shape of a platelet changes within seconds into a sphere with protruding pseudopods. Activation can be induced by several stimuli including ADP, collagen, thrombin and adrenaline. In a physiological environment the trigger for platelet activation is damage of the vessel wall. Following damage, subendothelial collagen becomes exposed that binds von Willebrand factor, which subsequently changes its conformation. The conformational change allows platelets to adhere to the von Willebrand factor via the glycoprotein (GP) Ib receptors, which are already present on the platelet membrane.\textsuperscript{9} Upon adherence platelets become activated, which results in several reactions. Negatively charged phospholipids such as phosphatidylserine and phosphatidylethanolamine, originally localised in the inner leaflet of the cell membrane, become exposed on the outer leaflet and thus provide an appropriate phospholipid surface to bind (activated) coagulation factors.\textsuperscript{10} Apart from spreading, platelets release their contents of granules during the secretory reaction, with membranes of the various granules fusing with the membranes of the open canalicular system and/or the cell membrane. This leads to both extrusion of the contents of the granules to the outer milieu and to the exposure of neo-antigens on the cell membrane that were originally localised
only in the granular membrane. Examples of such neo-antigens are GP53 (CD63) from the lysosomes and P-selectin from the α-granules\textsuperscript{11-13}. Many intracellular processes take place, including the conversion of arachidonic acid into thromboxane A\textsubscript{2} (TxA\textsubscript{2}). The latter compound, diffusing from the activated platelet, is a most potent platelet activator. TxA\textsubscript{2} causes other circulating platelets to adhere and aggregate to the originally adhered platelets. In this way the platelet plug is extended as an initial attempt to stop the blood flow through the damaged vessel wall. The activated platelets also bud off small parts of their cell membrane, the so-called microparticles, which expose negatively charged phospholipids to promote the activation of the coagulation system (Figure 1).\textsuperscript{14-17} The best-known function of platelets is obviously their role in primary hemostasis, i.e., the formation of the platelet plug. However, via exposed phospholipids and the production of microparticles, and possibly via various secreted coagulation factors, such as factor V, von Willebrand factor and fibrinogen, they also contribute to the consolidation of the

Figure 1. Schematic overview of the resting and activated platelet.
platelet plug by supporting the activation of the coagulation system, which is the central event in the secondary hemostasis.

2.3. Platelet glycoproteins
Platelets have several glycoproteins on their granular and outer cell membranes, which play an important role in platelet function. These glycoproteins, indicated with Roman figures, change their conformation and/or their subcellular distribution upon activation of the platelet. The glycoproteins studied in this thesis as markers of platelet activation, and their behaviour during CPB, will be discussed below (Figure 1).

**Glycoprotein Ib**: GPIb, a receptor that is predominantly found on platelets, consists of an α-chain (140 kD) and a β-chain (24 kD). It forms a 1:1 complex with GPIX and a 1:2 complex with GPV. Platelets contain approximately 150,000 molecules of GPIb. On a resting platelet, approximately 25,000 copies of GPIb are exposed on the cell membrane. Most of the GPIb is stored in an internal pool, the open canalicular system (approximately 110,000 copies) and the membrane of the α-granules (probably 15,000 copies).\(^{18,19}\) GPIb is an important adhesion receptor because of its ability to bind von Willebrand factor, but it also acts as a high affinity receptor for α-thrombin.\(^{20}\) Upon platelet activation, GPIb can partly disappear from the platelet cell membrane. In vitro experiments show that GPIb internalises from the cell membrane into the open canalicular system, for example upon stimulation of platelets with thrombin.\(^{21}\) These movements of GPIb are dependent on cytoskeletal activity and are reversible.\(^{22}\) On the other hand, in vitro experiments with washed platelets show that GPIb is also sensitive to proteolysis, for example by plasmin or cathepsin G,\(^{23}\) which may also result in ‘disappearance’ of GPIb from the cell membrane. Whether or not this phenomenon occurs in vivo, i.e., in the plasma environment, is unknown. The physiological role of the disappearance of GPIb from the platelet surface can only be speculated upon. Possibly, it makes the activated platelet less prone to interact with the damaged vessel wall and more prone to interact with other-activated-platelets through their activated GPIIb-IIIa complexes in the process of plug formation. Downregulation of the platelet GPIb complex from the platelet surface has not only been demonstrated in vitro, but also in blood oozing from the standardised lesion of a bleeding time wound.\(^{24}\)
The clinical relevance of GPIb in hemostasis is demonstrated in patients with the Bernard-Soulier syndrome. These patients completely lack or have a dysfunctional GPIb and thereby suffer from a bleeding disorder. The most pronounced effect of CPB on GPIb was presented by van Oeveren et al., who observed a 50% decrease of the number of GPIb molecules on the platelet surface at the start of CPB. Those authors used an ELISA method to quantify the GPIb antigen. The finding was confirmed by other investigators who used flow cytometry to detect GPIb. Some authors substantiated the findings of a reduced platelet surface exposure of GPIb by demonstrating a reduced ristocetin-induced - and thus GPIb-dependent- platelet agglutination, but other investigators could not confirm this.

Glycoprotein IIb-IIIa: A platelet contains approximately 100,000 copies of the GPIIb-IIIa complex. In a non-activated platelet, half of the total number of GPIIb-IIIa complexes is located in the cell membrane, and the other half is located in the membranes of the α-granules and the open canalicular system. Upon activation, the intra-platelet pool becomes surface exposed due to fusion of the membranes of the α-granules with the cell membrane. Furthermore, the conformation of the GPIIb-IIIa complex changes and becomes capable of binding its ligand. In the absence of shear stress the ligand for GPIIb-IIIa is fibrinogen, which cross-links platelets (platelet aggregation). Under flow conditions the ligand is von Willebrand factor, and thus not only GPIb but also the GPIIb-IIIa complex can function as von Willebrand factor receptor. The fibrinogen-binding conformation of the GPIIb-IIIa complex can be detected with a monoclonal antibody, called PAC-1.

The clinical relevance of GPIIb-IIIa in relation to hemostasis is demonstrated in patients with absent or dysfunctional GPIIb-IIIa, i.e., Glanzmann thrombasthenia, who suffer from a bleeding disorder, but also the reduction of thrombotic complications by GPIIb-IIIa antagonists, such as abciximab, in patients with refractory unstable angina who underwent percutaneous coronary intervention. During heart surgery, no or only a slight increase of platelets binding PAC-1 were reported. In a study with dogs undergoing CPB, temporary platelet impairment during the operation was created with Integrerin, a short acting inhibitor of GPIIb-IIIa. Integrerin preserved platelet function and reduced postoperative bleeding, which indicates that GPIIb-IIIa in some way plays a role in the
platelet response during CPB.

P-selectin: P-selectin, also called platelet activation-dependent granule-external membrane protein (PADGEM) or GMP-140, is a protein synthesized in the megakaryocyte and stored in the membrane of the α-granules of platelets. It is also synthesized by endothelial cells where it is stored in their Weibel-Palade bodies. Upon activation of the platelet by agonists such as thrombin, P-selectin becomes exposed on the surface during the secretion reaction, i.e., the fusion of the α-granular membrane with the cell membrane.¹¹,¹² Platelets that express P-selectin are able to adhere to neutrophils and monocytes. The interaction of purified P-selectin or P-selectin on microparticles and platelets promotes the expression of tissue factor by monocytes.³⁹,⁴⁰

So far no patients with a reduced presence or complete absence of P-selectin have been described. In blood obtained from a bleeding wound an increase of P-selectin on the platelet membrane was detected.²⁴ An increased percentage of P-selectin positive platelets during heart surgery has been reported by some authors,⁴¹ but not by others.⁴²

2.4. Coagulation

Originally, thrombin formation was thought to be initiated via two cascades: the extrinsic (tissue factor-factor VII dependent) and the intrinsic (factor XII dependent) pathways. The cascades involve a series of proteolytic reactions in which pro-enzymes are converted into active enzymes, all serine proteases. As the word cascade suggests, one enzyme can generate many other enzymes at the next level, although this process is downregulated by natural inhibitors, such as antithrombin and α₂-macroglobulin. Both cascades lead to activation of a common pathway, through which thrombin and finally fibrin are formed (Figure 2).

Intrinsic pathway: Activation through the intrinsic pathway is dependent upon the exposure of blood to a negatively charged surface. Factor XII binds to the negative surface and is capable of auto-activation in the presence of kallikrein (K) and high molecular weight kininogen (HMWK). In the presence of HMWK, the activated factor XII (factor XIIa) activates prekallikrein (PK) to kallikrein to increase factor XII activation. In the intrinsic pathway factor XIIa activates factor XI to XIa. Factor XIa subsequently activates factor IX to IXa in a Ca²⁺-dependent manner. Factor IXa, once
complexed to thrombin-activated factor VIII on a phospholipid surface and thus forming the tenase complex, activates factor X to Xa in a Ca\(^{2+}\)-dependent manner.

**Extrinsic pathway:** Activation of the extrinsic pathway starts with auto-activation of factor VII in the presence of tissue factor (TF) if the latter is properly embedded within a lipid surface. TF is normally not expressed by blood cells and endothelial cells lining the vessels. However, TF is constitutively expressed by extravascular cells and its expression by monocytes, endothelial cells and possibly granulocytes is inducible. The TF-factor VIIa complex activates factor X to Xa in a Ca\(^{2+}\)-dependent manner.

**Common pathway:** The factor Xa forms the prothrombinase complex with thrombin-activated factor V (factor Va) on a suitable phospholipid surface. This complex Ca\(^{2+}\)-dependently cleaves prothrombin (factor II) to produce thrombin and the prothrombin activation fragment F\(_{1+2}\). Thrombin subsequently converts fibrinogen into fibrin monomers, which spontaneously form polymers. The polymers are finally crosslinked into

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**Figure 2.** Pathways of coagulation.
the consolidated fibrin network by the action of thrombin-activated factor XIII (factor XIIIa) (Figure 2).

Recent insights in coagulation activation: The present view is that mainly the TF-factor VII pathway is important for the activation of the coagulation system in vivo. At low concentrations, the TF-factor VIIa complex activates factor IX (the so-called Josso-loop), at higher concentrations it directly activates factor X in a Ca$^{2+}$-dependent manner (Figure 3). The significance of the Josso-loop is clear from the clinical findings in patients deficient in either factor VIII (hemophilia A) or IX (hemophilia B). These patients suffer from severe bleeding complications, and the contribution of both coagulation factors to coagulation in vivo is clearly reflected by the reduced levels of factor VIIa when compared to healthy individuals.

The traditional two activation pathways for the coagulation system, linked together via the Josso-loop, did not provide an explanation for the fact that factor XII-deficient patients frequently have a thrombo-embolic tendency instead of a bleeding disorder, similar to the findings in patients with a HMWK or prekallikrein deficiency. This controversy was explained by direct activation of factor XI by thrombin, which enforces thrombin formation without factor XII, prekallikrein and HMWK involvement (Figure 3). The clinical relevance of factor XI is clear, because

Figure 3. Recent insight in the coagulation system.
some patients with factor XI deficiency (hemophilia C) suffer from bleeding, usually after trauma or surgery, especially from tissues with a high local fibrinolytic activity.

Contact activation has been claimed to play a role in the CPB situation, when extensive contact of the systemic blood and the foreign surface of the extracorporeal circuit occurs. In more recent studies this contact activation was also shown to be of less importance, which may be partly due to the improved biocompatibility of those circuits in the last few years.  

As described above, thrombin plays several roles. It activates factors V, VIII, XI and XIII, and it is a potent platelet activator. However, at low concentrations thrombin may actually have an anticoagulant effect by binding to thrombomodulin, which is on the endothelial cell membrane. Thrombomodulin bound to thrombin, converts protein C into activated protein C. Activated protein C, assisted by its cofactor protein S, inactivates factors Va and VIIIa and thereby inhibits coagulation. At higher concentrations, the coagulation-promoting capacity of thrombin prevails, and at even higher concentrations thrombin inhibits fibrinolysis via thrombin activated fibrinolysis inhibitor.

For this thesis it is important to summarise that the coagulation system can be activated through the TF-factor VII complex and through contact activation of factor XII plus subsequent factors of the intrinsic pathway, whereas coagulation enforcement can frequently be ascribed to thrombin-mediated activation of factor XI.

2.5. Tissue factor

Under physiological, non-activated conditions, TF is not present on the surface of cells within the circulation or the endothelial cells lining the vessels. However, upon activation endothelial cells, monocytes and possibly granulocytes synthesize TF and expose it on their cell surface to initiate the activation of the coagulation system. Monocytes and endothelial cells, upon appropriate stimulation with e.g. bacterial endotoxins or the cytokine TNF-α, also release microparticles (see part 3), which expose functional TF. Finally, increased concentrations of the so-called soluble TF have been reported in e.g. patients with cancer, acute myocardial infarction, angina pectoris or disseminated intravascular coagulation. The exact nature of this soluble TF, i.e. whether it is partly
bound to microparticles and whether it is procoagulant in vivo, was not known until we performed the studies as described in chapters 3, 5 and 6.

3. MICROPARTICLES

Upon activation many cells can bud off small parts of their cell membrane, presently called microparticles. These cells are for instance monocytes, granulocytes, erythrocytes, endothelial cells and platelets. Similar structures arise if a cell undergoes apoptosis. Isolated platelets produce microparticles, platelet-derived microparticles (PMP), upon stimulation with ADP, collagen, thrombin, complement complex C5b-9, or the calcium ionophore A23187.\(^{66-68}\) The microparticles expose negatively charged phospholipids, i.e., phosphatidylserine and phosphatidylethanolamine, which are normally localised on the inner leaflet of the cell membrane. These phospholipids provide a surface for formation of tenase and prothrombinase complexes involved in the coagulation cascade. Thus, PMP can facilitate the activation of the coagulation process, a phenomenon called “platelet factor 3 activity” in the older literature.\(^{69}\)

Already in 1967, PMP, then called “platelet dust”, were described in blood of healthy individuals.\(^{69}\) Subsequently it became clear that PMP numbers are increased in the circulation of patients suffering from a variety of diseases that are all associated with a thrombo-embolic tendency. These include idiopathic thrombocytopenic purpura, paroxysmal nocturnal hemoglobinuria, uremia, diabetes, and sickle cell disease.\(^{70-74}\) Conversely, patients with the Scott syndrome have an impaired microparticle formation and a bleeding tendency.\(^{75,76}\) This indicates that PMP are likely to play a role in the hemostatic process.

PMP are the most numerous microparticles in plasma, both in healthy individuals and in various patients studied so far.\(^{77-80}\) Erythrocyte-derived microparticles are usually also present at substantial numbers. In healthy individuals PMP and erythrocyte-derived microparticles made up 70% of the total number of microparticles, but microparticles from granulocytes and endothelial cells could also be detected.\(^{77}\) In patients with meningococcal sepsis, especially microparticles from platelets and granulocytes were
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present at increased numbers. In those patients evidence was obtained that TF, present on the surface of monocyte-derived microparticles, was active in thrombin generation and possibly involved in the diffuse intravascular coagulation occurring in such patients. The presence of microparticles from endothelial cells in the circulation was first described in patients with SLE.

The detection and quantification of microparticles, including PMP, is possible by the use of flow cytometry. Microparticles retain antigens of their parent cell during formation. Those antigens can be detected using monoclonal antibodies to establish their cellular source. In double-label experiments the presence of other antigens on particular subpopulations of the total microparticle population can be established, such as P-selectin or TF. The exposure of negatively charged phospholipids on the microparticles can also be established by labelling the microparticles with annexin V, a protein that in a Ca\(^{2+}\)-dependent fashion binds to phosphatidylserine.

Evidently, microparticles can play a role in coagulation, but other functions have also been reported. In vitro, PMP can transport arachidonic acid - the precursor for the synthesis of prostacyclin - to endothelial cells. Also in vitro, PMP exposing P-selectin can bind to monocytes and induce them to produce and expose TF. Thus, carrier functions and cell-activating functions may also be attributed to microparticles. Also recent findings by Boulanger et al. are noteworthy, who reported that microparticles from the circulation of patients with a myocardial infarction induced endothelial dysfunction in vitro, i.e., they impaired acetylcholine-induced endothelium-dependent relaxation of aortic rings.

In this aspect it is also interesting to note that the GPIIb-IIIa antagonist abciximab, nowadays frequently used in patients undergoing a PTCA, not only inhibits the binding of fibrinogen to this receptor but also the formation of PMP.

A possible prothrombotic effect of microparticles had not been investigated prior to the studies described in chapter 6.
Aprotinin is a small (6.5 kDa) serine protease inhibitor derived from bovine organs. It has originally been used as an antifibrinolytic agent to control hyperfibrinolytic states, such as pancreatitis and overdose of thrombolytic agents. In vitro studies and clinical applications on aprotinin were reported as early as 1963.\textsuperscript{90} Already in 1987 Royston et al. described an unexpected reduction in postoperative blood loss after heart surgery.\textsuperscript{91,92} That study actually investigated an anticipated reducing effect of aprotinin on complement activation, but surprisingly the postoperative blood loss was extremely reduced. Its routine use in Europe came after the study of van Oeveren et al. in 1987.\textsuperscript{93} Many other investigators confirmed a reduction of blood loss between 40 and 80\% with aprotinin, combined with the expected reduced need for blood transfusions. The extent of reduction seems dose dependent. The specific activity of various aprotinin preparations is usually designated in terms of kallikrein inhibitor units (KIU). The most commonly used dosages are the high dose or the "Hammersmith regimen", which consist of a bolus loading dose (2 million KIU), a pump-priming dose (2 million KIU) and a continuous infusion (0.5 million KIU/hr) which leads to a total of 4 to 5 million KIU equivalents. The low dose regimen consists of only the pump-priming dose of 2 million KIU.

Although the extensive literature on aprotinin would indicate otherwise, the working mechanism has not yet been elucidated. As a non-selective serine protease inhibitor it forms a reversible stoichiometric complex with a protease. Structure and biophysical characteristics of aprotinin are related to other protease inhibitors. This type of inhibitor is named after one of its first describers, Kunitz. Another Kunitz type protease inhibitor is tissue factor pathway inhibitor. On the other hand, the structure of aprotinin resembles that of hirudin, which is a highly specific inhibitor of thrombin. Aprotinin has a number of substrates. In decreasing potency, it inhibits plasmin ($K_i = 0.07 \text{ nM}$), plasma kallikrein ($K_i = 36 \text{ nM}$), activated protein C ($K_i = 1100 \text{ nM}$), factor Xla ($K_i = 1100 \text{ nM}$), TF-factor VIIa complex ($K_i = 9800 \text{ nM}$), two-chain urokinase ($K_i > 2500 \text{ nM}$), factor Xa ($K_i > 9800 \text{ nM}$) and thrombin ($K_i > 61,000 \text{ nM}$).\textsuperscript{94,95} Aprotinin also has the ability to bind to enzymes that are already complexed to a third partner. For example, aprotinin complexes with plasmin at the fibrin surface, so that plasmin cannot interact with its
natural plasma inhibitor. Taking this into account, aprotinin (i) enhances the total anti-protease capacity of plasma due to its direct enzyme inhibiting capacity, and (ii) protects plasma inhibitors from proteolytic degradation. Evidently, aprotinin affects fibrinolysis, coagulation, complement and platelet activation.

As for fibrinolysis, aprotinin inhibits the activation of plasmin at various levels. Generally accepted is the attenuation of increased concentrations of plasmin-\(\alpha_2\)-antiplasmin complex, split products of cross-linked fibrin (D-dimer) and fibrin(ogen) degradation products during CPB. The intrinsic activation of fibrinolysis through the contact activation is inhibited as shown by a pronounced decrease in concentrations of kallikrein-inhibitor complexes. The endogenous activation by plasminogen activators is inhibited as shown by less increased tissue plasminogen activator concentrations, although others could not confirm this. No effect on plasminogen activator inhibitor-1 concentrations could be found.

As for coagulation, the use of aprotinin does not affect the formation of thrombin-antithrombin complexes and prothrombin fragment F\(_{1+2}\) in the circulation of patients undergoing CPB. The intrinsic, factor XII-dependent, coagulation activation pathway is inhibited by the effect of aprotinin on kallikrein.

Aprotinin has no effect on the alternative and classical complement pathways, as reflected by C3b/c and C4b/c levels.

As for the platelet, the most prominent effect of aprotinin is the apparent absence of the 50% decrease in GPIb from the platelet surface during CPB. Also, a low dose of aprotinin was reported to prevent \(\alpha\)-granule secretion and microparticle formation, and to maintain the platelet aggregation capacity induced by thrombin. Others, however, could not confirm the effects on GPIb disappearance, \(\alpha\)-granule secretion or activation of GPIIb-IIIa into its fibrinogen binding conformation.

Some side effects related to the use of aprotinin have been reported, but were not confirmed by others. These side effects are renal dysfunction, myocardial infarction based on early graft occlusion, anaphylactic reaction and thrombus formation, but in those articles an insufficient systemic anticoagulation cannot be ruled out as a cause. These side effects are too infrequent to abandon the use of aprotinin during cardiac surgery considering its benefit in decreasing blood transfusions.
5. AIM AND OUTLINE OF THE THESIS

Many studies have been performed on the activation status of several cell populations and protein systems in the systemic circulation of patients undergoing CPB, whereas much less is known of the blood collecting in the pericardial cavity. Pericardial blood originates from blood oozing from vessels cut by the surgical procedure or leakage along sutures. The quantity of pericardial blood differs extensively between patients undergoing cardiac surgery, but can easily reach more than one litre in some patients. It is removed from the pericardial cavity by suction and periodically returned into the patient during the operation. The aim of the thesis is to investigate the changes in platelet activation, and the occurrence and possible procoagulant role of microparticles in this pericardial blood, as compared to the systemic blood.

Chapter 1 gives a general introduction of a coronary bypass operation, and an overview of platelets, the coagulation system and microparticles. Also some background information on aprotinin is given for a better understanding of chapter 4.

Several investigators have already reported unfavourable platelet activation during CPB as a probable cause of impaired hemostasis and thus blood loss after the operation. This platelet activation was determined by the disappearance or appearance of receptors on the platelet surface. However, the situation in the pericardial blood is unknown. In the study in chapter 2 blood is collected at various time intervals during the operation from the pericardial cavity and compared with systemic blood. The important platelet adhesion receptor glycoprotein Ib (GPIb) disappears largely from the platelet surface in pericardial blood, compared to only a small amount in systemic blood at the end of the operation. No other evidence for platelet activation, e.g. granule secretion, was found in the pericardial blood.

In the study presented in chapter 2, flow cytometry was used to establish the platelet activation status and surface exposure of GPIb. In this study it was noted that material with a size smaller than platelets, but obviously derived from platelets as it stained with the appropriate antibodies, was present at increased concentrations in pericardial blood. These were the so-called platelet microparticles. Microparticles originating from various cells were isolated from pericardial blood and characterised as
described in chapter 3. The microparticles were able to generate thrombin in an in vitro thrombin generation assay and this involved the TF-factor VII dependent pathway.

Aprotinin is frequently used in cardiac surgery to reduce the postoperative blood loss and thus the requirement for blood transfusions. One of its presumed mechanisms is the prevention of GPIb disappearance from the platelet surface. We hypothesised that per-operative local administration of aprotinin at the site of blood activation, i.e., in the pericardial cavity, might have a protective effect on platelet activation. As described in chapter 4, no effect on platelet activation and microparticle formation was established. A discussion of the presumed effect of this local administered aprotinin, raised by Landis and Taylor in a letter to the Editor (chapter 4a), and our answer to this letter, are presented in chapter 4b.

Phillippou et al. observed increased concentrations of soluble TF in pericardial blood of patients on CPB and they suggested in the Discussion section of their article that the soluble TF might partly be microparticle-associated. In chapter 5 it is shown that a major part of this soluble TF is indeed microparticle-associated and capable of thrombin generation in vitro through the TF-factor VII pathway.

So far, the thrombin generating capacity of microparticles was established in in vitro studies. The last study in this thesis, chapter 6, shows the conclusive evidence of the thrombogenicity of human (pericardial) microparticles via the TF-factor VII dependent pathway, in a venous stasis thrombosis model in the rat.

The results presented in this thesis are summarized in chapter 7.

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