

INTRODUCTION TO HEMOSTASIS

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Introduction

People expect normally to be able to walk around, bang into things, cut themselves, play rugby, get into all sorts of divers scrapes, and let the hemostatic system take care of things. The word *hemostasis* means everything that contributes to keeping blood contained with the blood vessel, or *vascular system*. For most of the injuries of everyday life hemostasis is achieved with little or no help. Broken blood vessels will be plugged, stopping the leakage of blood; and eventually they will be put back in use, and/or new vessels will grow into the injured area. Most everyday damage—short of massive injury—is to the capillaries and small arterioles and venules, and occasionally the larger veins, and all these are under fairly low pressure. It is fortunate that most arteries—at least the larger ones—live a bit deeper in tissue because the arterial system is under considerably higher pressure and it is difficult to fix a leak in an artery without help, e.g. firmly holding your finger on it. Like Gaul, hemostasis *in tres partes divisa est*, and they are closely interrelated.

Vasoconstriction. The obvious benefit of constriction of an artery or vein is the resulting reduction in blood flow. Another major role of vasoconstriction is not so obvious. Pressure is force per unit area, and it follows that a small tube is more resistant to a given pressure than a large one (e.g. reducing the diameter reduces the total *force* on the wall in proportion). So when a blood vessel contracts it both reduces the blood flow and renders itself less liable to further rupture. Vasoconstriction occurs immediately upon vessel injury under control of the nervous system and is done by smooth muscle cells in the vessel wall.

Platelets. Platelets form an aggregated clump where there is a hole in a blood-vessel wall. Platelets are small fragments of cells that circulate all the time in the blood, numbering about 2–300,000 per μl ($1 \mu\text{l} = 1 \text{ mm}^3$; cf. red cells are about 5 million per μl). Platelets are ellipsoid discs, with a diameter of about $2 \mu\text{m}$ (cf. red cells are about $5 \mu\text{m}$). They are made by the fragmentation of enormous cells in the bone marrow, the megakaryocytes. These precursor cells of course have a nucleus, but platelets, like red cells, do not. Unlike red cells, they do have large numbers of mitochondria and they have an active metabolism, but they cannot synthesize new proteins, either structural or enzymes, and they cannot divide (i.e. multiply). It is not surprising that they have a fairly short life in the circulation: around 7 days. They play three major roles in hemostasis: (i) they *adhere* to places where the lining of the blood vessel is broken and they activate; (ii) they then stick to each other, forming *aggregates* or *clumps* that make up a substantial part of the final clot; and (iii) when *activated* they provide things that are needed for the reactions of the clotting system and the generation of fibrin. Fibrin is an insoluble protein that polymerizes into a strong gel and it provides further architecture and strength to the platelet plug as it builds. Platelet activation is mainly initiated by the adhesion of platelets to the collagen that is an essential part of every vessel wall.

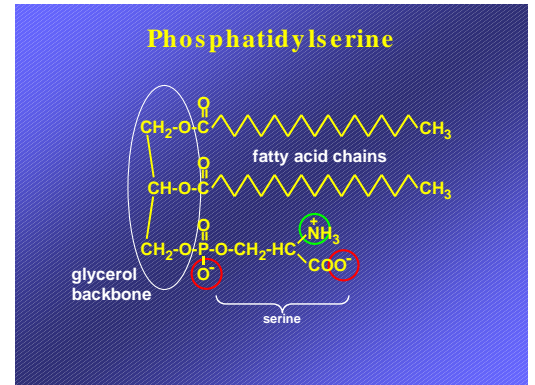
Coagulation. The function of the clotting system is to form the insoluble protein fibrin. Coagulation is initiated by a specific protein on the membrane of cells that are in the vessel wall, chiefly *fibroblasts* and *smooth muscle cells*. The protein is called *tissue factor*. It is often said that the coagulation, or clotting, system exists solely to produce *fibrin*, implying some sort of isolated edifice separate from the platelets and everything else. This is the wrong way to think of it. Activated platelets are essential in the clotting system, and, conversely, we will see that the clotting system is itself intricately involved in platelet function, because it produces one of the major stimulators of the platelets—*thrombin*. Except for tissue factor, the initiator, all the proteins of the clotting system are soluble plasma proteins.

PLATELETS

Membrane phospholipids

The phospholipids of the platelet membrane bilayer play a major role in hemostasis, particularly in providing a suitable surface for several reactions of the coagulation system.

Phospholipid headgroup and charge. The pair of long fatty-acid tails of a phospholipid point away from the membrane surface into the middle of the phospholipid bilayer. The *headgroup*—at the bottom of the picture—lies in the surface of the membrane. It consists of a phosphate that is linked each side by ester bonds: (i) on its "left" side, to the end hydroxyl of the glycerol backbone and (ii) on its "right" side, to the hydroxyl of the terminal, variable, part of the headgroup. All phospholipids have a phosphate, but the nature of the terminal part differs. In this example, the group is the aminoacid serine, which is linked via its hydroxyl to the phosphate (N.B. the figure is diagrammatic: headgroups do not lie beside fatty acid chains).



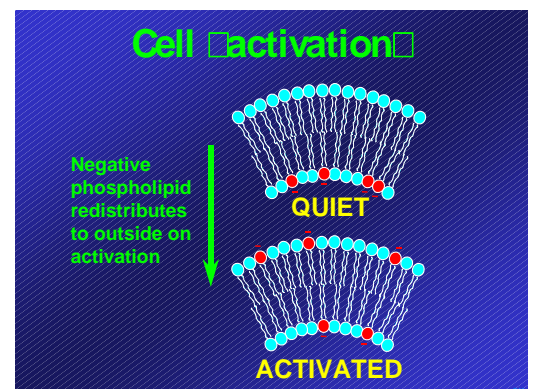
Only one thing is important to this discussion: the *charge* on the headgroup. The phospholipid shown, phosphatidylserine (PS), has a *net negative charge*, from (i) one negative charge on the phosphate, and (ii) both a positive and negative charge on the aminoacid. There are several other negatively charged, or anionic, phospholipids (e.g. phosphatidylglycerol, phosphatidylinositol, phosphatidic acid), but PS contributes most of the negative phospholipid in cell membranes. One of the major properties of negative phospholipids is that *they bind Ca²⁺ ions*, and this becomes very important when we get to the coagulation system. Negative phospholipids generally account for 25-30% of a membrane's phospholipid, the remaining majority being neutral. The major neutral one is *phosphatidylcholine*: in the figure, replace the serine, -O-CH₂-CH(N⁺H₃)COO⁻, with choline, -O-(CH₂)₂N⁺(CH₃)₃.

Platelet activation

Ca²⁺ and intracellular function. Ionized calcium in the cytoplasm of a normal resting cell is held at a very low concentration—typically 10⁻⁸ M—by Ca²⁺ pumps. Platelets possess a very potent membrane Ca²⁺ pump that drives Ca²⁺ ions from the cytoplasm into small Ca²⁺-storage vesicles, sealed from the cytoplasm by a membrane. These Ca²⁺-rich vesicles are known as *dense granules*. (They are not granular in the ordinary sense of the word: they just look like that under the microscope. The "dense" refers to their dark staining in electron microscopy, which is caused by the very high Ca²⁺ concentration inside.) Upon stimulation of a platelet, Ca²⁺ ions are released from the dense granules into the cytoplasm, raising the Ca²⁺ concentration at least two orders of magnitude. This makes all sorts of major things happen.

Shape change. Activation of the platelet causes a massive shape change. (i) The platelet outer membrane is connected to a large membrane system inside the cell called the open canalicular system. On activation a large part of this is moved to the outside, and there is a big increase in surface area. (ii) Actin polymerizes inside the cytoplasm, extending out and forming pseudopods (actually they are more like legs than feet). The result of all this is a large increase in the ability of the platelet to bump into another platelet and stick to it.

Phospholipid translocation. The resting platelet has predominantly *neutral phospholipid on the outer surface*. (As far as we know this goes for all quiescent cells, i.e. those not activated or transformed, etc.) Just about all (>98%) of the *negative phospholipid* of the membrane is on the *inner* leaflet of the membrane bilayer. Maintenance of this distribution is a result of the action of ATP-dependent *phospholipid translocases*. Upon activation of the cell and the rise in the cytoplasmic Ca²⁺ concentration, negatively charged phospholipid—mainly phosphatidylserine—moves from the inner to the outer leaflet of the bilayer. The mechanistic details are not quite clear yet. In any case, the



activated platelet now has negative phospholipid on its surface, and it can thereby bind Ca^{2+} ions and some specific proteins of the clotting system.

α -Granules. Just like the Ca^{2+} -containing dense granules above, the platelet's α -granules are membrane-bound vesicles within the cell. When the platelet is activated they fuse with the outer membrane and dump their contents to the outside. Included in those contents are (i) a number of *platelet stimulants* (agonists) such as ADP, which further accelerate the process of platelet aggregation and the formation of the platelet clump, and (ii) some proteins, included among them two that are relevant to adhesion and aggregation: von Willebrand factor (vWf) and fibrinogen.

Adhesion and Aggregation

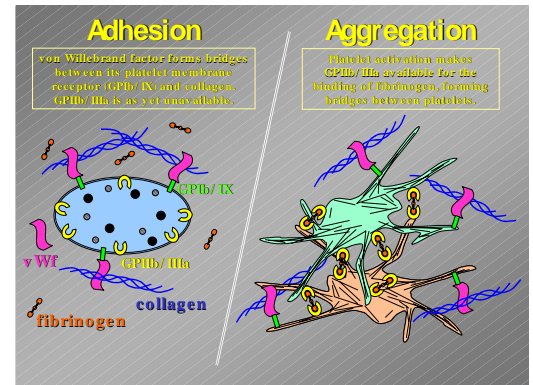
Adhesion to collagen; von Willebrand factor. vWf is a very large multimeric protein found in the blood plasma, and at high concentration in vesicles in both the *platelets and endothelial cells*. It is therefore always available. Endothelial cells are the cells that line all blood vessels. vWf consists of multimers of a protein of about 220,000 daltons, the multimers going up well into the 32 \times and 64 \times range, or more than 10 million daltons. In platelet *adhesion* two interactions are important: (1) binding of vWf to collagen in the wall of the vessel; (2) binding of vWf to a receptor protein on the platelet membrane: glycoprotein Ib/IX (GPIb/IX). vWf thus enables platelets to *adhere* to collagen. There are a number of complications (particularly the effect of blood flow) that we won't go into here. *Adhesion to collagen via vWf causes the initial activation of platelets*, initiating a signalling pathway that results in the release of Ca^{2+} from the dense granules and platelet activation.

Aggregation: adhesion of platelets to each other. Upon platelet activation, another major receptor of the platelet membrane becomes functional. It is glycoprotein IIb/IIIa (GPIIb/IIIa). *Unlike GPIb/IX, which is always functional on the membrane of even the unactivated platelet, GPIIb/IIIa is not functional until the platelet is activated.* This is sensible, since its function is to enable platelets to stick to each other; in the normal circulation you certainly do not want your unactivated platelets to be aggregating. Once platelets are activated, GPIIb/IIIa becomes functional on the surface, and it binds fibrinogen. Fibrinogen, which is present all the time in solution in the plasma, is a long dimeric molecule (2 \times 3 polypeptide chains—see Coagulation, below), and it has two GPIIb/IIIa-binding sites, one at each end. This means that *one fibrinogen molecule can bind to two GPIIb/IIIa molecules*, and act as the bridge between two platelet membranes. Activated platelets thus adhere to each other and form aggregates.

A major class of new anti-platelet drugs, including one invented at Stony Brook (ReoPro), block GPIIb/IIIa's binding to fibrinogen, and are nowadays nearly standard treatment immediately after heart attacks. They prevent, at least temporarily, the formation of new platelet aggregates at the initial site of thrombus formation.

Activation of platelets by platelets; thromboxanes. Upon initial activation, platelets work hard to stimulate their neighbors into action as well. To do this they release a number of platelet agonists. ("Agonist" is a back-construction: the opposite of an antagonist, i.e. something that stimulates.) The contents of the α -granules have already been mentioned, but they are pretty minor. A more potent class of agonists are the thromboxanes. These are formed by a complex but rapid pathway from a particular fatty acid chain of membrane phospholipids (see Phosphatidylserine figure), *arachidonic acid* (a poly-unsaturated C_{16} acid). The chief thromboxane is A_2 (TXA_2), and it is the main means (though far from the only one) by which platelets stimulate each other.

Aspirin. An essential step in the pathway of thromboxane formation is a cyclization of the arachidonic acid fatty acid chain that is catalyzed by the enzyme *cyclo-oxygenase*. This enzyme is inactivated by several drugs, the most important being aspirin (acetylsalicylate). Aspirin inactivates the enzyme permanently, and renders platelets aggregationally challenged for the rest of their (short) lifetime. They can still aggregate by some other signalling pathways—particularly when thrombin is the agonist—but they have lost an important piece of their function. This is the main reason that small doses of aspirin (e.g. 1 children's aspirin, 75 mg,



per day) substantially reduce the incidence of heart attacks (clots in coronary arteries) and transient ischemic attacks (clots in the arteries of the brain).

Thrombin: platelet agonist. Thrombin is the major enzyme produced by the clotting system (more on that below), and is responsible for fibrin (clot) formation. However it plays another very major role, being a prime agonist of platelets. Were I to order platelet agonists in importance—whatever that might mean, and I'm not a platelet expert anyway—thrombin would be at the top of my list (though others might put TXA₂ there instead). As you might expect, thrombin acts on the platelet via a specific thrombin receptor in the platelet membrane. Its mechanism is interesting and unusual, but we won't go into that here.

Summary. Platelet function in hemostasis—i.e. platelet activation—is *initiated* by the interaction of platelets (resting, quiescent, ellipsoid) with material behind the endothelium, chiefly collagen. This requires the huge multimeric protein von Willebrand factor (vWf), which is provided by the endothelial cells and the platelets themselves. vWf binds to both collagen and the receptor protein GPIb of the platelet membrane. The process so far is called *adhesion*, and it starts the internal processes of platelet activation.

The second stage is adhesion of platelets to each other—the process of *aggregation*, which is responsible for formation of the platelet plug. This involves the activated platelets' production of platelet agonists, and the binding of platelets to each other. This binding is a result of (i) the receptor protein GPIIb/IIIa becoming functional when platelets are activated and (ii) the binding of fibrinogen to it. Because fibrinogen is dimeric it can bind two GPIIb/IIIa molecules, and thereby bridge platelets.

At exactly the same time as the platelets first see collagen behind a damaged endothelium and initiate platelet activation, the clotting proteins of the plasma see a cell membrane protein in the fibroblasts and smooth muscle cells called tissue factor, and the clotting pathway is initiated. Its products are thrombin and fibrin.

Pathology

Bleeding due to platelet problems is quite common. However, although platelets do, rarely, come with congenital defects (e.g. lack of a major receptor), it is reduction in the *number* of platelets that is the much more common cause of bleeding. Particularly important causes include autoimmune disease, in which an antibody is directed against the platelets, leading to their clearance from the circulation; and chemotherapy (particularly for leukemia), which shuts down blood cell—including platelet—production in the bone marrow. Because platelets have only a 10-day life in the circulation, it is common that chemotherapy will involve multiple platelet transfusions. The normal platelet number is 200-300,000/ μ l of blood. Anything above 100,000/ μ l is fine for all purposes. When you get below about 50,000, easy bruising and bleeding start to be seen; while at 5-10,000 there is a severe risk of major, sometimes fatal, bleeding.

Platelet adhesion and activation commonly occur when platelets contact foreign materials. An example is the semipermeable membranes of the dialysis machines that patients with severe kidney disease are hooked up to. Since it is difficult to measure directly a material's propensity for platelet activation, selection of these materials relies largely on animal testing and clinical experience rather than studies of platelets themselves.

Overactive platelets, which lead to the formation of platelet aggregates, or thrombi, are common in vascular disease, such as the formation of sclerotic plaques in the blood vessel wall. This is particularly common in the coronary and the carotid arteries. A major cause of platelet activation here is likely the abnormal flow patterns that exist around these thickened areas. The narrowing of arteries by such plaques is called a stenosis (pl. stenoses). Other sites of both local adhesion and abnormal flow patterns are found in implanted devices like mechanical heart valves. Even the best of these cause a continuous low level of platelet activation, and these patients have to be routinely maintained on anticoagulant therapy. (It might seem odd that the therapy for abnormal platelet activation is directed against coagulation rather than against the platelets; but as we will see, although activated platelets do not *initiate* coagulation, they are central in enabling the reactions to occur.)

COAGULATION

Introduction

Used in a technical sense, *coagulation* or *clotting* is separate from platelet aggregation and means the formation of a fibrin clot, even though the two go completely together in the overall process of hemostasis.

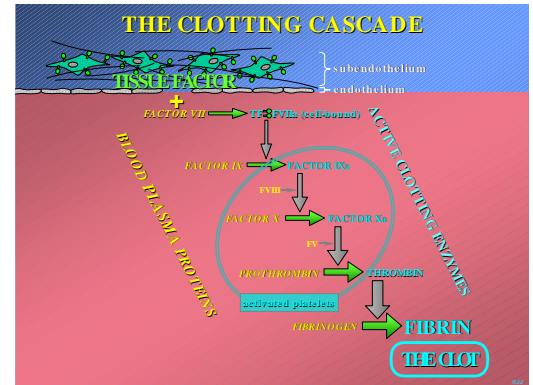
The central architecture, shall we say, of coagulation is a series of conversions of inactive precursor proteins (*zymogens*) to active proteolytic *enzymes*...to use arbitrary names, an initiating enzyme, A, converts zymogen pre-B to enzyme B; B then converts pre-C to enzyme C; and C converts pre-final-product to final product. Hence the name cascade. The precursors, along with a few other necessary proteins, are already present in the blood plasma. There are only two things in the process that are not provided in the plasma: (1) the initiator of the whole performance, *tissue factor*; (2) the negative phospholipid membrane that is required for several reactions in the pathway, which, as we have seen, is provided by the *activated platelets*; and they are right now starting to form aggregates at the site of damage to the vessel wall.

Initiation. When the endothelium is broken, factor VII, a protein present in the plasma at extremely low concentration (ca. 0.5 µg/ml, or 10 nM), is able to see tissue factor (TF) on the surface of the cells of the vessel wall: fibroblasts and smooth muscle cells. Tissue factor does not exist in the platelets and is at only very low levels—if at all—in the endothelial cells. The interaction between TF and factor VII is very tight, essentially irreversible, and it produces the TF:VII complex. Note that because TF is a cell-membrane protein, anchored to the cell surface, TF:VII is also anchored.

Factor VII is a zymogen, not yet an enzyme, so the TF:VII complex is as yet enzymically inactive. The details are complicated, but it is quickly activated, producing the active-enzyme complex, TF:VIIa. (For all the clotting proteins, the conversion of the precursor form in the plasma to the active species is written by adding a small "a" to the Roman numeral name; e.g. the precursor protein factor X becomes the enzyme factor Xa; the TF:VII complex becomes TF:VIIa.)

We now have an active proteolytic complex—TF:VIIa—sitting on the wall of the cells exposed where the endothelium is damaged. Probably less than 20 seconds has passed so far. The main target of this enzyme complex (in terms of function in normal clotting, but not necessarily in the lab) is factor IX, generating the active enzyme factor IXa. This enzyme requires a cofactor protein, factor VIII, which is also present in the plasma. It happens that the genes for both factors IX and VIII are both on the X chromosome, and so deficiencies of either are sex-linked bleeding defects, i.e. mothers carry the defect, but only males get it. For factor VIII or factor IX deficiency, the illness is called *hemophilia*. Factor VIII deficiency—hemophilia A—is the more common one, afflicting about 1 in 10,000 males. Until the 1960s severe hemophiliacs usually died before adulthood from brain hemorrhage, and in considerable chronic pain from hemoarthroses, i.e. bleeding into the joints and associated destruction of joint tissue.

[Historical tangent of interest to trivia collectors... Czar Nicholas and Alexandra's son and heir, Alexei, was a severe hemophiliac. Alexandra was a granddaughter of Queen Victoria, and a carrier of the defective factor VIII gene that first appeared in Victoria. Enter Rasputin, who accrued power largely because his hypnotic powers were beneficial in the care of Alexei's hemophilic problems (hypnosis definitely works to ameliorate symptoms), and he became Alexandra's favored adviser. Unfortunately this was not confined to Alexei's care—Rasputin also advised Alexandra about government—and it was she who was the power in that marriage. There is a credible case that if Alexei had not had hemophilia, and Rasputin had thus had never become involved with the family, events in Russia in 1915-1917 might have gone a little differently. Eventually Alexei died of bullet wounds rather than hemophilia along with the rest of the family in Ekaterinberg in 1918. Their graves in the woods were finally confirmed just a few years ago by DNA analysis of the remains and comparison with living members of related European royal families.]



Localization

To return to the details... An absolutely critical control in coagulation is making sure that the cascade process and the generation of fibrin occur *only at the site of vessel damage*. One means of localization is that TF is a membrane protein. If cells are subject to massive damage, it is certainly possible for them to form small vesicle fragments, which will contain TF in their membranes and may be swept off into the bloodstream; but for smaller damage, i.e. day-to-day hemostasis, we expect that the cells of the vessel wall will mostly remain where they are—exposed to the blood, yes, but still localized.

While the activation of factor IX must occur on the TF-bearing cell, the factor IXa produced is *not* bound to TF, and it must be localized by other means. The key to its localization, and that of factor X and prothrombin as well, is the negative phospholipid of the activated platelets, and here we must consider vitamin K. It is a real vitamin, fortunately not yet discovered by manufacturers of vitamin supplements.

Vitamin K; negative phospholipid. Four clotting factors (and two other clotting-related proteins) require vitamin K for synthesis of the mature proteins: they are factors VII, IX, X, and prothrombin. As the new protein comes off the ribosome during translation and moves into the endoplasmic reticulum of the liver cell, a vitamin K-dependent carboxylase puts extra COOH groups on the first 8-12 Glu residues of these proteins, forming γ -carboxyglutamic acid (known by the abbreviation Gla).

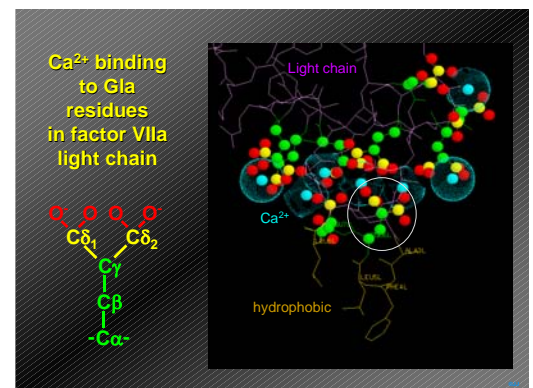
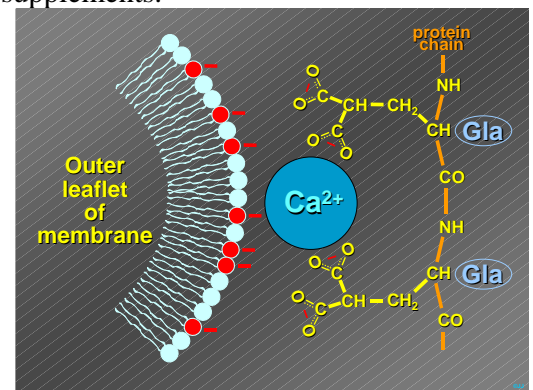
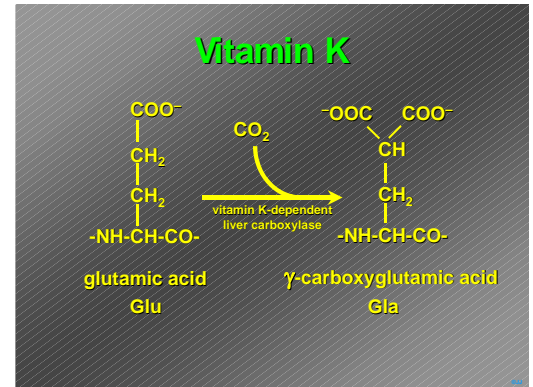
As the both the diagram and the crystal-structure picture factor VII show, multiple carboxyl oxygens—sometimes on the same Gla, sometimes on neighboring ones—bind Ca^{2+} ions. Because negative phospholipid also binds Ca^{2+} , these proteins themselves bind to negative phospholipid with the Ca^{2+} acting as the bridge. (The Gla residue circled below is particularly clear, and shows the color scheme.) And where do we find any negative phospholipid? There's none on the normal surface of the endothelium; red cells don't have it; and nor do any other *normal* circulating blood cells. *Activated platelets provide the negative phospholipid.*

All this is shown by the line drawn around the central block of the Cascade figure, above, and it is the key to restricting the reactions of the clotting system, and the generation of fibrin, to places where a blood vessel has been damaged. Remember that platelet activation and coagulation are initiated, though by different mechanisms, at the same time and in the same place—immediately behind the broken endothelium: so the platelets are already being activated right there.

Thrombin

Thrombin is the last and the most important proteolytic enzyme produced in coagulation. We have already heard how it is a major stimulator of *platelets*, accelerating platelet aggregation, the formation of the platelet clump, and providing yet more negative phospholipid. It also is the enzyme that converts fibrinogen, which is a soluble protein of the blood, into fibrin, which is insoluble.

Fibrinogen is a dimer of a unit of three different chains, the central "knot" being the place where all 6 chains are joined *near their amino termini*. Two very small areas of dense negative charge right at the amino termini in fibrinogen block polymerization sites and are responsible for keeping fibrinogen soluble. Thrombin cleaves short peptides (fibrinopeptides A and B) from the amino termini of two of the chains, thereby removing the blocks, and the molecule (it is now *fibrin*) spontaneously polymerizes. The charac-



teristic arrangement of the initial polymer is a half-staggered overlap, where the central (amino-terminal) "knot" regions interact with the large end domains of *two* neighbouring fibrin molecules. In addition, the large end domains interact with each other. This structure is clearly visible by electron microscopy. Fibrin is deposited throughout the platelet plug and gives it strength. A little later in the process fibrin is also cross-linked to give the clot a strong stable matrix.

Other controls

Although localization of the central part of the cascade on activated platelets is undoubtedly the key means of restriction of the process, it cannot be the only one. One particular problem is *thrombin*, which, because of the way prothrombin is activated, no longer contains the Ca^{2+} -binding Gla domain of prothrombin and does not bind to negative phospholipid. It does bind to fibrin, however, and in fact a large part of the thrombin generated during clotting ends up still active inside the clot. However, the affinity of thrombin for fibrin is rather weak, and leakage of thrombin is almost bound to occur. To prevent downstream coagulation, this *must* be looked after. Two important mechanisms exist, and a bunch of minor ones that I won't go into.

Inhibitors of the clotting proteases are present in the plasma. The main one is *antithrombin III* (ATIII), which inhibits both thrombin and, rather less efficiently, factor Xa. People who are partially deficient in ATIII are at greatly increased risk of thrombosis. Even when the plasma concentration is as high as 50% of normal, and ample in terms of the *mass* of ATIII available, patients will often suffer repeated thrombotic episodes. A total (homozygous) deficiency has never been described, even though statistically from the known frequency of the heterozygous deficiency one would expect a certain observable frequency; the implication is that total ATIII deficiency is fatal to the embryo or fetus.

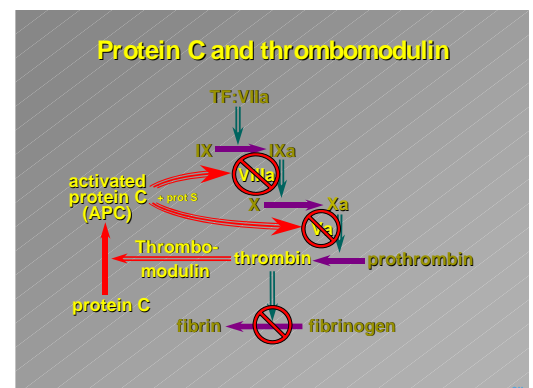
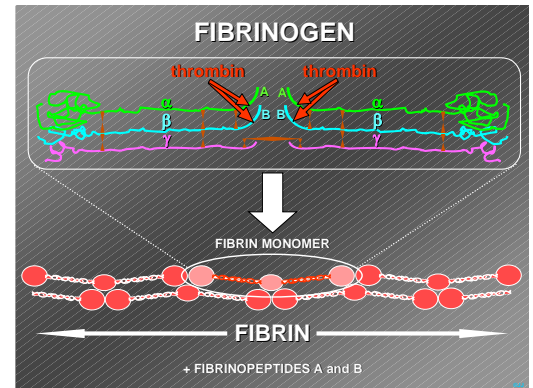
Thrombomodulin and protein C are proteins involved in an *anticoagulant* pathway that is initiated by thrombin.

Thrombomodulin is a membrane protein of endothelial cells and coats the entire endothelium (600-800 m² in area in adults), and it binds thrombin. When thrombin is so bound, it can no longer cleave fibrinogen. No longer able to form a clot, it can now activate protein C, which is involved in the *inactivation* of the two major cofactors of clotting, factor V and factor VIII. A number of things tell us how important this pathway is in switching off the system. (1) Partial deficiency of protein C causes thrombosis, and total deficiency usually leads to death within a few days of birth from massive systemic thrombosis. (2) Thrombomodulin deficiency in humans has never been described. However, transgenic ("knockout") mice with homozygous deficiency die in the embryo stage, at about the time that the vascular system starts to develop. (3) A common defect, not discovered until the 1990s, is caused by a mutant factor V that is resistant to inactivation by protein C. The cofactor therefore remains active longer than it should, enabling thrombin generation and clot formation to continue longer than normal. So far this is the most common specific congenital risk factor for thrombosis, the heterozygous deficiency being present in about 7% of people of Caucasian descent. Its prevalence in other races is much lower.

Pathology

Hemophilia has been mentioned. The more common hemophilia is type A—a deficiency of or defect in factor VIII—which afflicts about 1 in 10,000 men. Hemophilia B—factor IX deficiency—is about 1/5 the incidence of A. Both genes are on the X chromosome and are therefore sex-linked: the mother carries one copy of the defective gene and does not get the disease. She passes on the single gene to half (on average) of her sons. Other hereditary bleeding defects are even rarer.

Problems of serious thrombosis are about 1000 times more common than problems of serious bleeding. Overall, for Western countries (not races: societies), the blockage of a blood vessel by a clot is finally



responsible for *about half of all deaths*. Of these clots, a large number are in the coronary vessels of the heart, and especially at *atheromatous plaques*. Plaques not only close down arteries; they also contain very high levels of tissue factor and can cause explosive activation of clotting when they rupture. Although the prior narrowing of the vessel is far from good, it is clot formation that finally causes the heart attack. A similar process occurs in thrombotic strokes, though here the clot might have formed somewhere else (e.g. in the carotid artery or the heart itself) and travelled some distance before lodging in the brain. Traveling clots are called *thrombo-emboli*. A third class of clotting problems is found in the venous circulation, and these too are usually emboli. Most common are those that form in the large veins of the legs and hips. If they stay there the problem is *thrombophlebitis*, which is generally fairly mild and easy to treat. However, if they break off they travel towards the heart. As the veins get larger towards the heart they don't get stuck. Having been pumped through the right side of the heart they go to the lungs, where the vessels get smaller again, and the clot will finally lodge there. These are called *pulmonary emboli*, and if the blockage is large they can be fatal.

How do we get rid of clots, either as part of the normal physiological process of wound repair, or deliberately, as therapy for patients?

FIBRINOLYSIS

Introduction

So far we formed a clot and plugged up a ruptured blood vessel. If we were unlucky it was a large clot capable of totally blocking a critical coronary or brain artery. More usually, a clot will be small and appropriate to the damage. A normal part of healing is for cells to grow back into the area of a clot, breaking it up, and at the same time growing a new endothelial layer. To dissolve the clot we need fibrinolysis.

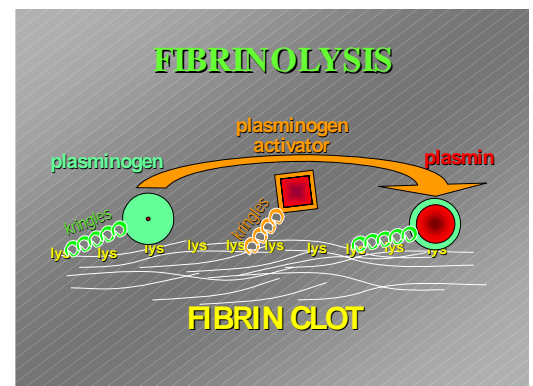
Initiation/initiators

In coagulation we saw that the initiator, TF, is found neither in, nor in contact with, the circulation: the endothelium must usually be damaged to initiate both clotting and platelet activation. In contrast, activators of fibrinolysis are present at low levels in the circulation all the time. There are two activators: *tissue plasminogen activator (tPA)*, which is probably the major one involved in clot dissolution, and *urokinase-type plasminogen activator (uPA)*, which is more significant in other areas that involve activation of the fibrinolytic system (cell migration, tissue invasion, etc.). Although it may turn out that both are fundamentally involved in fibrinolysis—the dissolution of fibrin—this handout concentrates on tPA.

Tie-in with hemostasis. It is clear that the fibrinolytic system is tied in with activation of the coagulation system, i.e. plasminogen activator levels rise in response to coagulation. The major link is probably thrombin, which is capable of stimulating various cell types to secrete plasminogen activators. Whatever the mechanism, activation of the clotting system is invariably accompanied by activation of fibrinolysis.

As in coagulation the key to understanding fibrinolysis is *localization*. Unlike the enzymes of coagulation, which are quite specific, the central enzyme in fibrinolysis, plasmin, is a potent nonspecific proteolytic enzyme and it is essential that its generation and action be restricted to the clot. There are two ways by which this is brought about. (1) Plasminogen and tPA are designed—perhaps I should say evolved—to activate plasminogen (the zymogen precursor of plasmin) *only on fibrin*, so despite there being tPA and plasminogen always present in the circulation, plasmin generation is restricted to the clot. (2) The inhibition of plasmin by its major inhibitor—a very potent brute called α_2 -plasmin inhibitor—is extremely rapid when plasmin is *not* bound to fibrin, but is orders of magnitude slower when plasmin *is* bound to fibrin.

Localization: kringle and lysines. A kringle is a Danish pastry of a particular shape that is similar to the structure of a series of domains in the plasminogen molecule (which has five of them) and in the plasminogen activators. Kringle domains are



capable of binding specific lysine residues in a number of proteins. In other words, kringle contains *lysine-binding sites*. (Note the hyphen there: these kringle sites are *not* lysine; they *bind* lysine.) Chief among the Lys-bearing proteins that they bind to is *fibrin*; not fibrinogen, only fibrin.

tPA and other plasminogen activators activate plasminogen—a single chain of 791 amino acids—by a single cleavage at position 561, but *they need to be bound to fibrin to do this*. Until this cleavage occurs plasminogen has no catalytic activity, i.e. it is a zymogen. Cleavage of the single peptide bond produces the 2-chain enzyme *plasmin* (the chains are held together by two disulfide bonds). The larger chain (1-561) retains all five kringle domains and therefore the lysine-binding sites. The smaller chain (562-791) is the *catalytic domain*, which is responsible for the actual proteolytic activity of plasmin: it contains the enzyme's active site and catalytic apparatus.

As in the clotting system, there is one major potent inhibitor of fibrinolysis that lives in the blood plasma: it is α_2 -plasmin inhibitor (α_2 PI). Its potency depends on where its target—plasmin—is. When plasmin is in solution in the plasma inhibition is extremely rapid (half-life < 1 second). In contrast, when the plasmin is bound to fibrin (as in the figure), inhibition is at least 100-fold slower. Thus any small amount of plasmin that might be produced in solution, or leak into solution from the fibrin clot, is inactivated almost immediately by this inhibitor.

Pathology

The fibrinolysis system doesn't commonly contribute *by itself* to major pathology. Recent studies of knockout plasminogen *-/-* mice show that the mice are born and can actually reproduce, but they are subject to later wasting, weight loss, and early death, and wound healing is severely prolonged. However, I'm not sure that homozygous plasminogen deficiency has ever been observed in humans, who perhaps would be much more severely affected. (It is always good to remember that humans are not just big biped mice.) Deficiency of α_2 -plasmin inhibitor in humans is rare and causes a clear bleeding defect, which is logical if you think about it: plasmin presumably hangs around longer than normal, and the extent of fibrinolysis increases, destroying the clot more quickly than it should. This observation also makes clear the importance of the *balance* between coagulation and fibrinolysis. Interestingly, studies of transgenic mice with knockouts of the *plasminogen activators*, tPA and uPA, show significant physiological defects only if *both* are absent: single (homozygous, total) defects produce no major observable (phenotypic) abnormalities.

Thrombolytic therapy

In a serious heart attack it is essential to dissolve the clot as quickly as possible and reopen the blocked vessel. Heart muscle will not last long without adequate oxygen, and any damage is permanent. 30 minutes is about as long as you can go. Strokes are usually caused by clot formation too, and here any time delay—even a few minutes—is still more serious. However, there are increasing attempts to use thrombolytic therapy for stroke patients.

To dissolve clots you activate plasminogen. (Infusing plasmin obviously wouldn't work because it would be inhibited before it got to the clot.) There are three significant plasminogen activators available for thrombolytic therapy, and some other related "designer" versions. (1) Streptokinase is a plasminogen activator from *Streptococcus* that is off-patent. It is dirt cheap (about \$200 a pop), but it does not bind to the clot, and it causes massive plasminogen activation in the plasma (though most of the plasmin produced is instantly inhibited by α_2 -plasmin inhibitor because it is in solution). Even though it does a pretty good job it is not much used in the U.S., where money is generally no significant object, and drug companies tout the mantra that more expensive must be better: the evidence for that is not very good. Although bleeding can very occasionally be a problem, it works well. (2) Recombinant (genetically engineered) tPA is available at about \$2000 a pop. Even if simply injected into a vein, it is only active when it finds the clot because its kringle needs to bind to lysines on the fibrin, so major systemic plasminogen activation does not occur. (3) Recombinant uPA is now also available at about \$2400 a pop, and is generally given via a catheter threaded by a friendly cardiologist into the artery that is blocked. This is now quite standard. Regardless of which thrombolytic agent your rescuer uses, because tissue factor is still there at the site of damage, she must also treat with anticoagulants, and in fact these must be continued for some time after the clot has been dissolved; otherwise the patient just gets a new clot in the same place. When in reports of "clot-busting" drugs you read of the problem of *reocclusion*, it is re-formation of the clot that is being referred to.