EXPERIMENTAL MODELS OF BLOOD COAGULATION

The fibrin gel is the end product of blood coagulation. It is formed as a result of the action of thrombin on fibrinogen, a blood plasma glycoprotein with molecular weight of 340,000 Da. The fibrinogen molecule is composed of 2 Aα, 2 Bß and 2 γ polypeptide chains, bound together by disulfide bridges. Thrombin is a serine protease with high substrate specificity: it cleaves the peptide bond at Arg16 in the Aα chains. As a consequence of this cleavage fibrinopeptide A is released and fibrin I monomer is formed. In solutions of physiological ionic strength the fibrin I monomers (in contrast to fibrinogen molecules) aggregate with high affinity. If their concentration remains relatively low (less than 0.1 g/l), the only consequence of their formation is the reduced stability of the fibrinogen solution. This phenomenon may occur in blood plasma under certain pathological conditions (e.g. DIC*). Experimentally the decreased water-solubility of the fibrinogen- fibrin monomer mixture can be detected with the help of the ethanol gelation test described later (in the past this test was used for the diagnosis of DIC). If the concentration of fibrin I monomer exceeds the above-mentioned value, the molecules polymerise: long double-stranded fibrin I protofibrils are formed via end-to-end aggregation (Fig. 1). The protofibrils are substrates of thrombin: the peptide bond at Arg14 in the Bß chains is cleaved resulting in the formation of fibrinopeptide B (the N-terminal part of the Bß chain) and fibrin II protofibrils. The latter can aggregate not only in an end-to-end pattern, but also side-to-side. So an extensive gel network is formed (that is the fibrin gel).

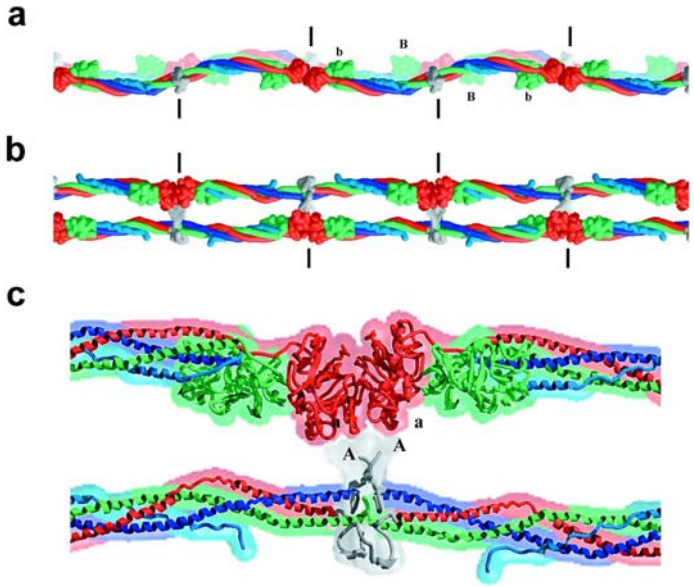

Fig. 1. Fibrin polymerisation. Two perpendicular views of the double-stranded fibrin protofibril (a and b) and magnified view of the junction point of the γ-chains of two fibrin I monomers and the two α-chains of a third fibrin I monomer (c). Straight lines indicate the boundaries of the monomers. The polymerisation sites are labelled with letters; A and B: polymerisation sites available only after the cleavage of fibrinopeptide A and B, respectively; a and b: permanently available polymerisation sites. The A-a and the B-b polymerisation forces lie in perpendicular planes (compare a and b views). The two A sites of the fibrin monomer look in the same direction resulting in double-stranded structure, whereas the two B sites - in opposite directions resulting in extensive spatial structure (fibrin gel).

*DIC, disseminated intravascular coagulation is a severe complication in a number of well defined disease states, in which active thrombin and active plasmin are simultaneously present in systemic circulation resulting in thrombotic and hemorrhagic symptoms.

Because of the non-covalent character of the bonds in the polymer fibrin can be dissolved in concentrated urea solutions. In addition thrombin activates the blood plasma coagulation factor XIII that inserts N^{ε}-(γ-glutamyl)lysine isopeptide bonds between the α and γ chains of the monomers. The cross-linked, covalently stabilised fibrin can not be dissolved in urea. In our experiment we use a model of cross-linked fibrin (fibrin treated with glutaraldehyde that forms Schiff-base crosslinks between lysine residues of different polypeptide chains).

The thrombin activity is a subject of stringent control. Thrombin is formed from its plasma precursor prothrombin via activation by prothrombinase complex (Xa and Va factor bound to phospholipid surface via Ca^{2+}). The active serine protease of the latter complex (Xa) is generated from the inactive zymogen (factor X) only in case of damage in the integrity of the blood vessel wall by two different tenase complexes:

a) extrinsic tenase (factor VIIa, Ca^{2+} and tissue factor that is constitutively expressed on the surface of cells that are not in contact with blood plasma);

b) intrinsic tenase (factor IXa and VIIIa bound to phospholipids via Ca^{2+}) that is formed after the activation of the contact phase proteins- factor XII, XI, prekallikrein, high molecular weight kininogen- on non-membrane surfaces. (Fig. 2.)

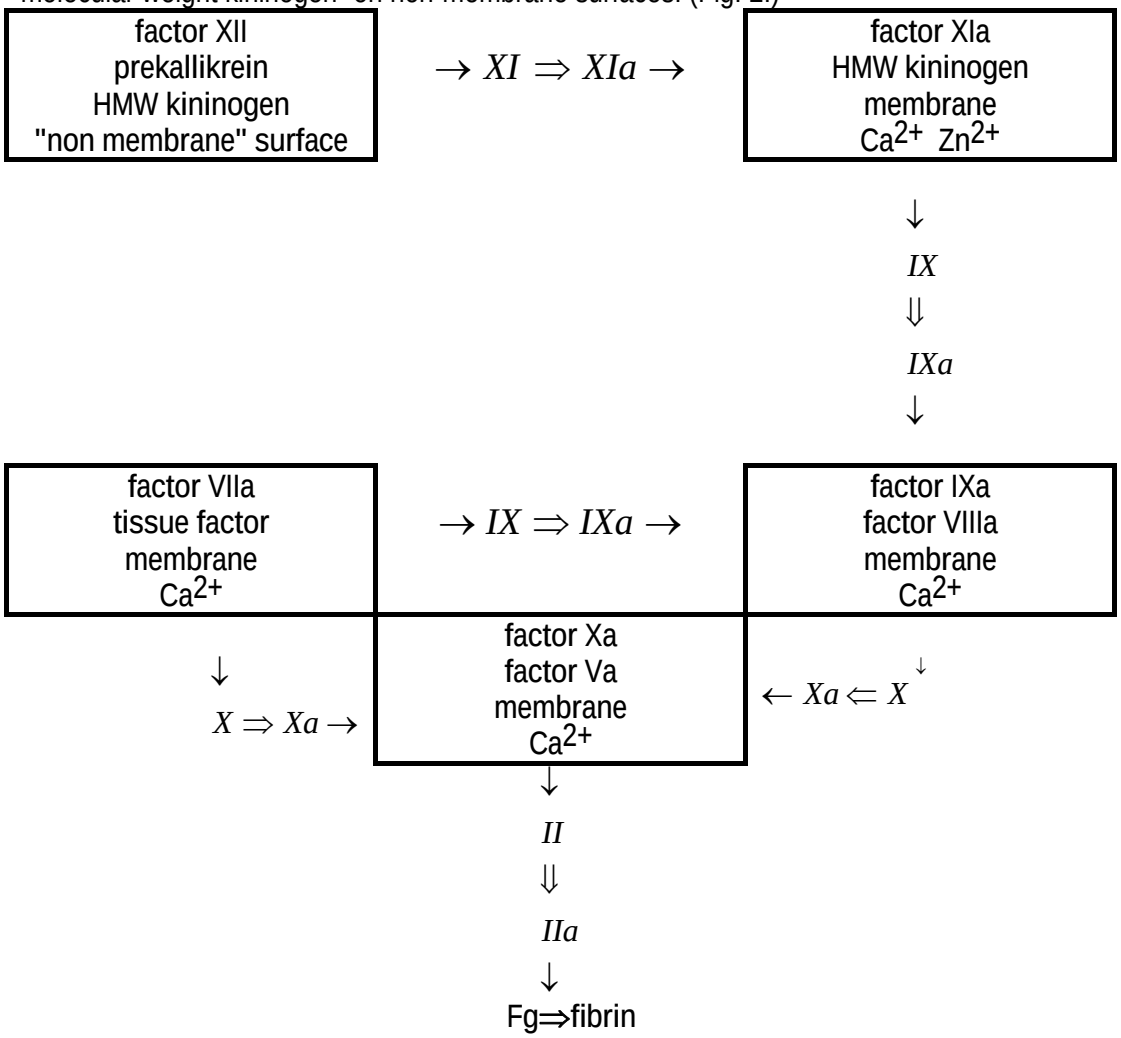

Figure 2. Enzyme complexes, participating in the activation of prothrombin.

Our experimental model of the extrinsic pathway activation contains rabbit brain thromboplastin (composed of tissue factor and plasmamembrane). This experiment represents the tissue factor induced coagulation time test, previously named prothrombin time test that is used in the clinical laboratories. In our intrinsic pathway model the activation is induced on kaolin surface to which partial thromboplastin (containing only lipid-membrane) is added. This

represents the surface induced coagulation time test, previously named activated partial thromboplastin time test that is used in the clinical laboratories.

In addition to the regulated activation of the zymogen the inhibition of the active enzyme is the other way of regulation of thrombin activity. The plasma inhibitors of thrombin (antithrombin, heparin-cofactor II, α_1-protease inhibitor, α_2-macroglobulin) are present at high concentrations (10^{-6} M) in blood plasma. So they inhibit very rapidly the free thrombin, the concentration of which does not exceed 10^{-10} M). The action of the first two listed inhibitors is enhanced by heparin. In our experiment we are going to study the thrombin-antithrombin reaction.

EXPERIMENTAL

1.Tissue factor induced coagulation time (prothrombin time)

Materials:
-citrated normal plasma (N Pl)
-citrated plasma, containing reduced amount of vitamin K dependent factors (pathologic plasma) (P Pl)
-rabbit brain thromboplastin-0,025 M $CaCl_2$ reagent (TP)
-reaction polystyrene tubes
-glass rod
-stopwatch

Experimental procedure:
Incubate the thromboplastin-$CaCl_2$ reagent for at least 2 minutes at 37°C-on. In prewarmed (at 37°C) tubes prepare the following reaction mixtures:

	1	2
normal plasma (N Pl)	0.1 ml	-
pathologic plasma (P Pl)	-	0.1 ml
2 min incubation (37°C)	+	+
pre-warmed thromboplastin- $CaCl_2$ (TP)	0.2 ml	0.2 ml
PT (s)		
PR		

When the thromboplastin- $CaCl_2$ mixture is added, start the stopwatch and gently moving the glass rod measure the time till the first fibrin fiber is seen! That is the prothrombin time (PT).

Measure the PT of citrated normal plasma and of the plasma with reduced concentration of vitamin K dependent proteins! Determine the tissue factor induced coagulation relative time, previously named prothrombin ratio ($PR= PT_{patient}/PT_{normal}$). This test is used

in the clinical practice to monitor the therapy with vitamin K antagonists. The therapeutical range of PR is 2 to 4.5.

2.Surface induced coagulation time, previously named activated partial thromboplastin time (APTT)

Materials:

-a mixture of partial thromboplastin (phospholipid membrane) and kaolin suspension (APTT reagent, APTI R)
-0.025 M $CaCl_2$
-citrated normal plasma (N Pl)
-haemophilic plasma (H Pl)
-plasma containing 1 U/ml heparin (Hep Pl1)
-plasma containing 0.01 U/ml heparin (Hep Pl2)
-reaction polystyrene tubes
-glass rod
-stopwatch

Experimental procedure

In prewarmed tubes (at 37°C) incubate 0.1 ml of thoroughly suspended APTT reactant for 2 minutes at 37°C. Then mix with 0.1 ml plasma and incubate for 2 minutes at 37°C. Start the reaction with 0.1 ml of 0,025 M $CaCl_2$ (prewarmed at 37°C). Measure the time elapsed till the first fibrin fiber is seen (as in the measurement of PT). This value is the APTT of the sample.

	1.tube	2.tube	3.tube	4.tube
pre-warmed APTT reagent (APTI R)	0.1 ml	0.1 ml	0.1 ml	0.1 ml
normal plasma (N Pl)	0.1 ml	-	-	-
haemophilic plasma (H Pl)	-	0.1 ml	-	-
plasma (0.1 U/ml heparin) (Hep Pl1)	-	-	0.1 ml	-
plasma (0.05 U/ml heparin) (Hep Pl2)	-	-	-	0.1 ml
2 min incubation (37°C)	+	+	+	+
0.025 M $CaCl_2$	0.1 ml	0.1 ml	0.1 ml	0.1 ml
APTT (s)				

This test is used in the clinical practice as a screening test of hemostasis and for monitoring the heparin therapy.

3.Study of the stability of fibrin

Materials:

-2.5 g/l fibrinogen in 0.01 M imidazole 0.15 M NaCl pH 7.4 (Fg)
-50 NIH U/ml thrombin in 0.01 M imidazole 0.15 M NaCl pH 7.4 (Th 50)
-8 M urea solution (urea)
-2,5% glutaraldehyde in 0.01 M imidazole 0.15 M NaCl pH 7.4 (Glut)
-reaction glasstubes

Prepare the following reaction mixtures at room temperature:

	1	2
fibrinogen (Fg) (μl)	500	500
50 U/ml thrombin (Th 50) (μl)	100	100
1 min incubation	+	+
glutaraldehyde (μl)	-	100

Incubate for 5 minutes at room temperature. Then try to dissolve the fibrin with 2 ml of urea solution.

4.Ethanol gelation test

Materials:

-fibrin monomer solution (Fn M)
-2,5 g/l fibrinogen in 0.01 M imidazole 0.15 M NaCl pH 7.4 (Fg)
-ethanol- 0.15 M borate buffer pH 8.0 (EB)
-reaction glasstubes

Test:

Add 1.5 ml of ethanol-borate buffer to 1 ml of the sample! Incubate for 1 h at room temperature! Shake the tubes thouroughly and evaluate the results:

-the test is positive (= decreased solubility of fibrinogen), if an extensive jellyfish-like gel is seen in the solution;

-the test is negative (= normal solubility of fibrinogen), if only a small aggregate is seen on the surface of the solution.

Prepare the following reaction mixtures:

	1	2
fibrinogen (Fg)	1 ml	1 ml
fibrin monomer (Fn M)	-	0.1 ml
ethanol-borate buffer (EB)	1.5 ml	1.5 ml

This test illustrates the effect of fibrin monomer on the stability of the fibrinogen solution. It provides information about the presence of fibrin monomers in human plasma. That was the basis for its application in the diagnosis of DIC.

5. Antithrombin- thrombin reaction

Materials:

-0.01 M imidazole 0.15 M NaCl pH 7.4 buffer (Imi)
-antithrombin solution (AT)
-20 NIH U/ml thrombin
-2.5 g/l fibrinogen in 0.01 M imidazole 0.15 M NaCl pH 7.4 (Fg)
-0.2 g/l (approx. 30 U/ml) heparin (Hep)
-reaction glasstubes and Eppendorf-tubes
-glass rod
-stopwatchr

Experimental procedure:

In the course of the antithrombin-thrombin reaction the concentration of thrombin decreases. The actual concentration of thrombin can be detected by measuring the fibrinogen clotting time: at appropriate intervals 100 µl samples are taken from the antithrombin-thrombin reaction mixture and these are added to 200 µl fibrinogen solution. The time elapsed till the appearance of the first fibrin fibre (as in the PT test) is the clotting time (CT). Prior to the initiation of the thrombin-antithrombin reaction prepare 6 glass tubes with 200 µl fibrinogen solution in each of them.

Prepare the following reaction mixtures in Eppendorf-tubes:

tube No.	1	2	3
antithrombin (AT) (µl)	--	110	110
heparin (Hep) (µl)	--	--	20
imidazole buffer (Imi) (µl)	130	20	--

Start each reaction separately (after all measurements with the previous one have been completed) by the addition of 120 µl thrombin.

Take samples (100 µl) at the 1st and 7th min. of incubation and measure CT. If the CT is longer than 120 s, consider it to be infinite. Consider the mean of the two values in the 1st tube to be the initial state (at time 0) of the thrombin-antithrombin reaction.

Results:

reaction time	1'	7'
clotting time t(s) 1.tube		
1/t 1.tube		
clotting time t(s) 2.tube		
1/t 2.tube		
clotting time t(s) 3.tube		
1/t 3.tube		

Plot the reciprocal value of the CT as a function of the reaction time.