Plasma Fibrinopeptide A and Betathromboglobulin as Markers for Thrombosis in Clinical Disease

by

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A thesis submitted for the degree of
Doctor of Philosophy to the
University of Glasgow
October, 1983

II

TO THE MEMORY OF MY MOTHER

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Acknowledgements

It is a great pleasure to thank Drs C D Forbes and G D O Lowe for the encouragement and guidance during the three years spent working for this project. The author would also like to thank Professor A C Kennedy for access to laboratory facilities in the University Department of Medicine at Glasgow Royal Infirmary and Berk Pharmaceuticals for financial assistance. The advice of Professor C R M Prentice during the early stages of the project is gratefully acknowledged.

The author would like to thank Dr Jill Belch, Dr M Small, Dr M Shah, Dr G Lang, Mr S Blamey, Dr S Bowry and Dr M Stewart for providing the relevant clinical material used in this project. She thanks Mr S Miller for determining fibrinogen concentrations.

The illustrations for this thesis were prepared with great skill by Mr J. Douglas. The author would like to thank Miss D Lindsay, Librarian, for checking the references and Mrs J Kennedy for typing the text with such patience and accuracy.

Declaration

The work described in this thesis was performed in the University Department of Medicine, Glasgow Royal Infirmary, from October 1980 until September 1983. The detailed planning of the work and its execution was performed entirely by the author. Several joint studies were carried out in this thesis and the author would like to thank Dr G D O Lowe for permission to include the results obtained in long-standing retinal occlusion (Chapter 7.3) in this thesis, Dr M Small for the results obtained in the administration of stanozolol to volunteers (Chapter 7.4) and Dr M Stewart for the results in the transient cerebral ischaemia study (Chapter 8.6).

Some of the work described in this thesis has already been published:-

(1) Douglas, J T, Lowe, G D O, Forbes, C D and Prentice, C R M (1982). Beta-thromboglobulin and platelet counts - effect of malignancy, infection, age and obesity.
Thrombosis Research, 25, 459-464.

(2) Douglas, J T, Shah, M, Lowe, G D O, Belch, J J F, Forbes, C D and Prentice, C R M (1982)
Plasma fibrinopeptide A and beta-thromboglobulin in pre-eclampsia and pregnancy hypertension.
Thrombosis and Haemostasis, 47, 54-55.

(3) Small, M, Douglas, J T, Lowe, G D O and Forbes, C D (1983)
Increased plasmin activity during stanozolol administration.
Lancet 1, 1114 (Letter)

(4) Trope, G E, Lowe, G D O, McArdle, B M, Douglas, J T, Forbes, C D, Prentice, C M and Foulds, W S (1983)
Abnormal blood viscosity and haemostasis in long-standing retinal vein occlusion.
British Journal of Ophthalmology, 67, 137-142.

(5) Stewart, M E, Douglas, J T, Lowe, G D O, Prentice, C R M and Forbes, C D (1983)
Prognostic value of beta-thromboglobulin in patients with transient cerebral ischaemia.
Lancet, 11, 479-482.

(6) Douglas, J T, Lowe, G D O, Forbes, C D and Prentice, C R M (1983)
Plasma fibrinopeptide A and beta-thromboglobulin in patients with chest pain.
Thrombosis and Haemostasis, 50, 541-542.

Copies of these papers can be found inside the pocket in the back cover of this thesis.

The author would like to thank the editors of the journals concerned for permission to reproduce the following tables and figures from these papers in this thesis:-

Figure 6.1.	Thrombosis and Haemostasis
Figure 6.2.	Thrombosis and Haemostasis
Table 7.1.	British Journal of Ophthalmology
Figure 7.2	British Journal of Ophthalmology
Figure 7.3.	British Journal of Ophthalmology
Table 7.3.	Thrombosis Research
Figure 8.1.	Thrombosis and Haemostasis
Figure 8.2.	Thrombosis and Haemostasis
Figure 8.10	The Lancet
Figure 8.11	The Lancet
Figure 8.12	The Lancet

The following papers based on work in this thesis have been personally presented by the author:-

1. Fibrinopeptide A and beta-thromboglobulin levels in pre-eclampsia and hypertensive pregnancy.
   VIIIth International Congress on Thrombosis and Haemostasis
   12th July, 1981. Toronto.
2. Fibrinopeptide A and beta-thromboglobulin levels in pre-eclampsia and pregnancy hypertension.
   British Society for Thrombosis and Haemostasis
   28th and 29th September, 1981. Leeds.
3. Plasma fibrinopeptide A and B-thromboglobulin in pre-eclampsia and essential hypertension in pregnancy.
   Scottish Society for Experimental Medicine
   9th October, 1981. Aberdeen.
4. Plasma fibrinopeptide A and beta-thromboglobulin in patients with acute chest pain.
   VIIIth International Congress on Thrombosis
   13th-16th October, 1982, Valencia.
5. Relationship of BTG, FPA and B β 15-42 to postoperative DVT, malignancy and stanozolol treatment.
   British Society for Thrombosis and Haemostasis
   22nd and 23rd September, 1983, London.

Summary

This thesis investigates the value of plasma fibrinopeptide A and beta-thromboglobulin as markers for thrombosis. The role played by intravascular fibrin deposition and platelets in thrombosis has recently aroused great interest as well as controversy. Recently developed tests measuring in vivo thrombin generation (fibrinopeptide A) and platelet release (beta-thromboglobulin) might therefore be of value as markers for thrombosis.

Because of the highly specialised area of this research I was required by my supervisor to extensively review the different mechanisms which have been postulated as causes of thrombosis. In order to understand the problems surrounding these proposed mechanisms a detailed discussion on the biochemistry of the haemostatic mechanism is given in Chapter 1. The proposed mechanisms surrounding thrombosis are then discussed in detail in Chapter 2. The methods currently available for the measurement of platelet activation and release (beta-thromboglobulin) are discussed in Chapter 3.

Intravascular fibrin deposition has recently been suggested to be the result of an imbalance in thrombin or plasmin generation as discussed in Chapter 2. Chapter 4 therefore discusses in detail the development of techniques for measuring fibrinopeptide A and the recently developed assay of plasmin activity B β 15-42, as well as their application to previous clinical studies. Details of the methods used for the studies to be described in this thesis are given in Chapter 5.

The first clinical studies undertaken in this thesis examined plasma levels of BTG and FPA in patients with pre-eclampsia and pregnancy hypertension (Chapter 6). Abnormalities in platelet activity, blood coagulation and fibrinolysis may in part be responsible for the increase in fibrin deposition seen in the placental bed in pre-eclampsia as well as the pathogenesis of this disease. In order to assess the results in pre-eclampsia several other obstetric groups were studied.

Normal values of BTG and FPA levels were obtained in normal, non-pregnant women, normal non-pregnant women taking oral contraceptives and pregnant women in the first trimester of pregnancy suggesting no evidence of intravascular coagulation occurs in this group.

Increased BTG levels but normal FPA levels were observed in the 2nd and 3rd trimester of normal pregnant women, suggesting increased platelet activity is occurring in this group. Increased BTG levels and FPA levels were observed in essential hypertension of pregnancy suggesting activation of coagulation in this group. Normal BTG levels but increased FPA levels were observed in patients with intra uterine growth retardation (IUGR), possibly reflecting an increase in fibrin deposition in the placental bed in IUGR. Similar results were observed in patients with mild to moderate pre-eclampsia. Increased plasma BTG and FPA levels were observed in patients with severe pre-eclampsia suggesting activation of coagulation is occurring in these patients. BTG and FPA measurements did not distinguish the severe pre-eclampsia patients from the essential hypertensive group.

Measurements of plasma levels of BTG, FPA and B β 15-42 were then studied in patients with venous thrombosis as described in Chapter 7. An increase in plasma fibrinogen, BTG and FPA levels were observed in patients with longstanding retinal vein occlusion (RVO) when compared

to control subjects. These measurements did not distinguish between patients with and without RVO or RVO patients with and without complications.

Increased plasma BTG levels were observed in patients with the nephrotic syndrome when compared to control subjects suggesting an increase in platelet release occurs in this syndrome.

An increase in plasma B β 15-42 levels in normal volunteers after the administration of stanozolol suggests direct evidence of increased plasmin activity occurs for at least seven days after administration of this drug. The next study therefore evaluated a double blind trial of the administration of stanozolol in the prevention of deep venous thrombosis after major gastrointestinal surgery. An increase in plasma BTG levels was observed one day postoperatively in each of three sub groups of patients ie (a) those not receiving or those receiving stanozolol treatment (b) those patients who did not and who did develop a postoperative DVT, and (c) those patients with and without early operable malignancy. This observed increase in platelet activity may be the result of tissue damage and trauma during surgery. In patients who then developed a postoperative DVT a significant increase in plasma BTG levels was observed suggesting an increase in platelet activation occurs before the onset of thrombosis. The increased values did not distinguish those with and without thrombosis. Plasma FPA levels rose on the first day after surgery and were significantly elevated on day 7 in those patients who develop a DVT, thus suggesting that fibrin formation occurs in patients who develop a DVT.

Higher preoperative FPA levels were observed in patients with operable malignancy. Plasma B β 15-42 levels did not fall on the first

day of surgery however elevated values were observed after surgery and after stanozolol administration. Higher Bβ15-42 levels were also observed on the morning of surgery in patients who develop DVT.

The last study in Chapter 7 measured plasma BTG levels in an older patient group, patients with malignancy and patients with non-vascular illnesses such as chest infection and compared these groups to a young control group. Increased BTG levels were observed in the elderly controls and the patients with chest infection but not in early malignancy when compared to the normal control group. Platelet activation therefore occurs with non-vascular illness and hence such activation is not specific to 'vascular' illness such as venous thrombosis.

Plasma levels of BTG, FPA and Bβ15-42 were subsequently measured in arterial thrombosis as described in Chapter 8. No evidence of increased platelet release or fibrin formation was found in patients with acute chest pain such as myocardial infarction, unstable angina or non-cardiac chest when compared to control patients. Hence BTG and FPA levels are of no value in the separation of patients with acute pain into those with and without myocardial infarction.

The next study in Chapter 8 found little evidence of increased plasma levels of BTG, FPA or Bβ15-42 in patients with coronary disease and no relation between these parameters and the extent of the coronary artery disease.

The following study found no evidence of platelet activation or fibrin formation in patients with type II or IV hyperlipoproteinaemia.

The next study observed ongoing platelet release and fibrin formation in diabetic patients with retinopathy and this appeared to be higher in patients with proliferative retinopathy than in those without.

The final study in arterial thrombosis examined plasma BTG and FPA levels in patients with transient ischaemic attacks (TIA) and control patients. Platelet release rather than fibrin formation appeared to occur in TIA and plasma BTG levels appeared to be an index of patients having an increased risk of further vascular events.

The measurement of BTG and FPA as tests for in vitro blood compatibility of artificial surfaces are described in Chapter 9. BTG was found to be a marker of blood compatibility as observed by the finding that silicone was more thrombogenic than polypropylene, and polyvinyl chloride was more thrombogenic than silicone.

As discussed in Chapter 10 the results obtained in this thesis have suggested that abnormalities are suggested but not necessarily proved to occur in both venous and arterial thrombosis. The measurement of BTG, FPA and B β 15-42 has provided some insight into the possible mechanisms of thrombosis and are useful tools in further clinical investigations.

Chapter 1

Historical Introduction

In the past few decades much interest has been expressed in the role played by intravascular fibrin deposition and platelets in thrombosis. Recently developed tests measuring in vivo thrombin generation (fibrinopeptide A) and platelet release (beta-thromboglobulin) might be of value as markers for thrombosis. It is the purpose of this thesis to investigate the value of these tests.

Before discussing the literature available on these a general review will be given on haemostasis, thrombosis and intravascular coagulation in order to outline the problems involved in interpreting results of these complex interacting systems in clinical studies.

1.1. Haemostasis

The function of haemostasis is to prevent blood loss from intact vessels and to stop excess bleeding when vessels are damaged either by cutting or other trauma. This is achieved by deposition of platelets and fibrin. A further function of haemostasis is to limit the extent of such platelet and fibrin deposition to ensure maintenance of the patency of the vascular tree.

The prevention or arrest of haemorrhage represents one of the most basic homeostatic mechanisms of the body; platelet function, vessel contraction and blood coagulation protect the body by reduction of blood loss and fibrinolysis ensures vessel patency. Failure of any component of these complex systems results in excess bleeding which may vary from leakage of a few red cells from capillaries to the

more dramatic haemorrhage seen in severe haemophilia, thrombocytopenia or consumption coagulopathy in which the patient may bleed spontaneously from a multitude of sites and eventually die of uncontrollable haemorrhage. While it is convenient for descriptive purposes to consider them separately, the reactions involved are intimately interlinked and interdependent.

1.2.1. The Platelet - Historical aspects

Platelets are the smallest formed elements of the blood and are 2-3 μm in diameter. They were observed only after development of the compound microscope in 1830. Bizzozero in 1882 defined these "new cells" as independent of leukocytes and erythrocytes and named them "Plättchen". He demonstrated in severed or damaged blood vessels that the "plattchen" accumulated to form a mass which became the effective haemostatic plug. The early literature on platelets has been summarised in detail by Tocantins (1949) and Robb-Smith (1967).

1.2.2. Platelet morphology

In blood normal platelets are found in the form of discs and they are formed in the bone marrow from megakaryocytes. Platelets are also found in lungs, kidneys, liver and in the circulating blood (Bessis, 1977). The platelet count ranges normally from 15×10^9 /l to 40×10^9 /l of blood and they normally survive between 8-14 days in the blood. Platelets are enclosed by a membrane and contain organelles including mitochondria, lysosomes, tubular systems and granules. The platelet granules contain a variety of stored substances which are released from the platelet when they are activated (see Table 1.1). Platelet αgranule release will be discussed in detail in Chapter 3.

TABLE 1.1

Constituents of Human Platelet storage Organelles

Platelets contain three types of storage organelles, which can be distinguished by density. These are dense granules or bodies, α-granules and lysosomal enzyme storage organelles.

a) Dense bodies contain:

Adenosine diphosphate (ADP)

Adenosine triphosphate (ATP)

Guanosine triphosphate (GTP)

Serotonin (5HT)

Calcium

Pyrophosphate

b) Alpha granules contain:

Fibrinogen

Platelet Factor 4 (PF4)

β-Thromboglobulin (BTG)

Albumin

Factor VIII-related antigen (FVIII RAg)

c) Lysozomes contain:

β-N-Acetylglucosaminidase

β-Galactosidase

β-Glucuronidase

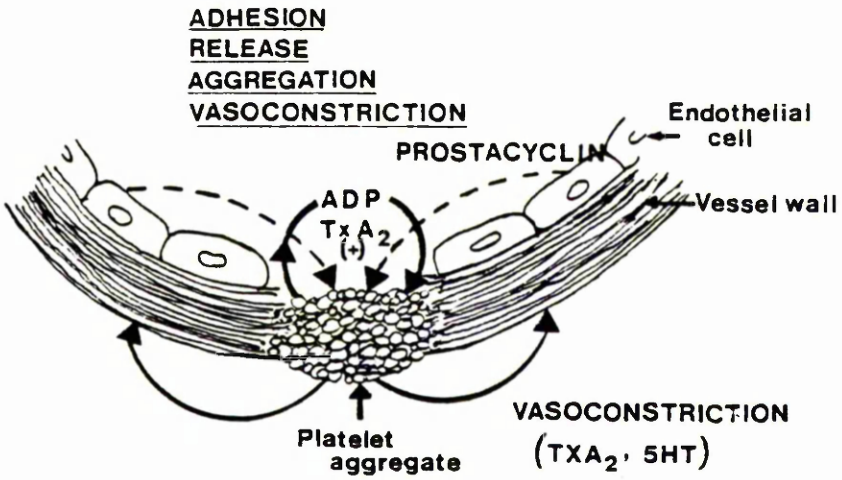
β-Glycerophosphatase

1.2.3. Platelet function

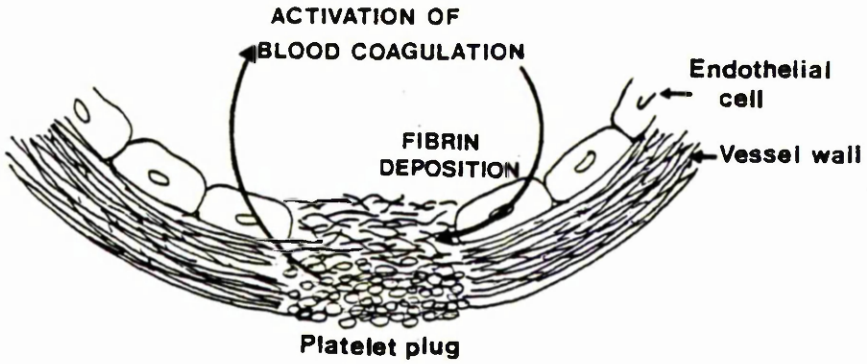
Transection of a vessel results in adhesion of a monolayer of platelets to the exposed subendothelial collagen, basement membrane and elastic fibres. Adhesion causes the platelets to release constituents of their granules, in particular beta-thromboglobulin (BTG) and platelet factor four (PF_4) from the αgranules and amines such as adenosine diphosphate (ADP) adenosine triphosphate (ATP) and the vasoactive material, serotonin (5-hydroxytryptamine, 5HT) and Ca^{++} from the dense granules. These amines are liberated into the "plasmatic atmosphere" of the adherent platelets and cause other platelets in the local circulation to change shape from disc to spheroid forms with pseudopodia within a second. These altered platelets aggregate together and form a mass with the already adherent platelets which plugs the defect in the vessel. This reaction is rapid and occurs within a few seconds of vessel injury (see Figure 1.1a).

In addition to the above mechanism, platelet membrane phospholipase A_2 is activated during aggregation and hydrolyses an ester bond in platelet phospholipid to produce arachidonic acid (Bills, Smith and Silver, 1972) (see Figure 1.2). This arachidonic acid is then acted on by cyclooxygenase which converts it to unstable prostaglandin endoperoxides (PGG_2 and PGH_2). The prostaglandin endoperoxides are also transformed by other enzymes into thromboxane A_2 (TXA_2) in platelets and prostaclyclin (PGI_2) in endothelium. Both TXA_2 and PGI_2 are chemically unstable, breaking down to thromboxane B_2 and 6 oxo-$PGF1_\alpha$ (see Figure 1.2).

Thromboxane A_2 was discovered by Piper and Vane in 1969 in effluents of mammalian lung during anaphalaxis i.e.



PLATELET PLUG FORMATION (a)



STABILIZATION OF PLATELET PLUG BY FIBRIN FORMATION (b)

Figure 1.1

(A) An illustration of the formation of the platelet plug.

(B) The stabilisation of the platelet plug by the formation of fibrin.

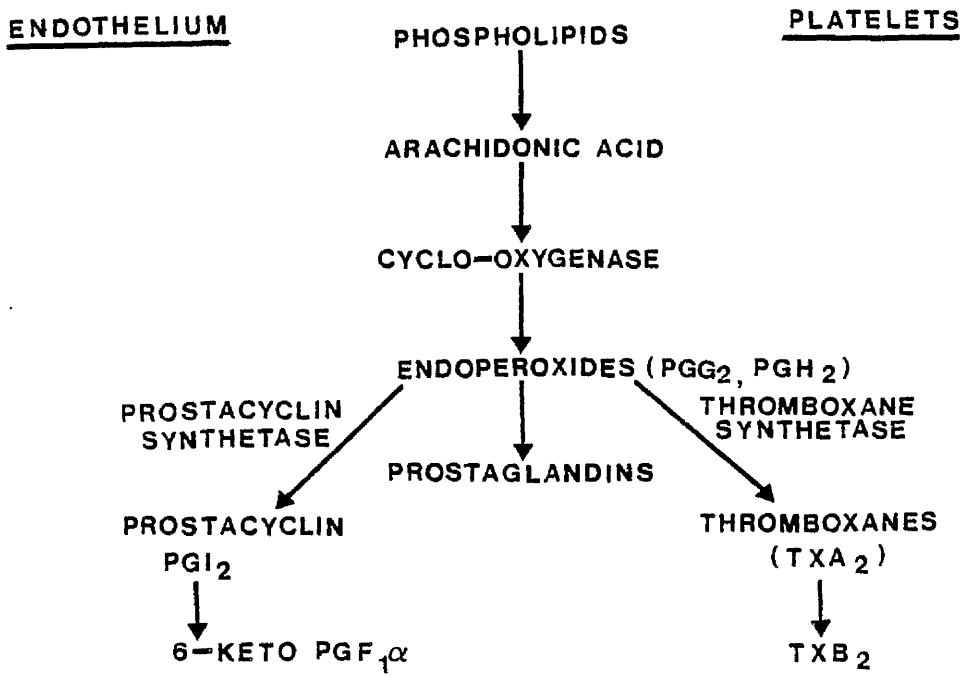


Figure 1.2

The cyclo-oxygenase pathway of arachidonic acid metabolism.

susceptibility and increased sensitivity to a foreign substance, usually a protein. In allergic patients this leads to the formation and the release of biologically potent substances such as histamine and prostaglandins which then mediate the symptoms of anaphalaxis. It was then named "rabbit aorta contracting substance" (RCS), due to its potent action on this tissue. Later its structure was elucidated and named thromboxane A_2 (Svensson, Hamberg and Samuelsson, 1975; Needleman, Moncada, Bunting et al., 1976).

Extensive research studies have shown that TXA_2 has two prominent physiological functions: it is a powerful vaso-constrictor and a powerful aggregating agent which enhances rapid accumulation of platelets at the site of vessel injury (Hamberg, Svensson and Samuelsson, 1975; Malmsten, Hamberg and Svensson et al., 1975; Moncada and Vane, 1979). Moncada and Vane (1977) and others have suggested that a balance is established between prostacyclin production by the endothelium (see Chapter 1.2.4) and thromboxane A_2 by the platelets. Prostaclyclin inhibits platelet aggregation by stimulating adenylate cyclase, leading to an increase in adenosine 3':5' - cyclic-monophosphate (cyclic AMP) levels in platelets (Tateson, Moncada and Vane, 1977; Gorman, Bunting and Miller, 1977). Prostglandin endo-peroxides and TXA_2 reduce a raised cyclic AMP level in platelets (Miller and Gorman, 1976) (see Figure 1.3). Because of opposing effects this balance regulates the level of cyclic AMP in platelets in vivo and, therefore, the ability of platelets to to aggregate. This proposition has been reinforced by the finding that prostacyclin is a circulating hormone (Gryglewski, Korbut and Ocetkiewicz, 1978, Moncada, Korbut, Bunting et al., 1978) with a local effect due to its short half-life of two minutes.

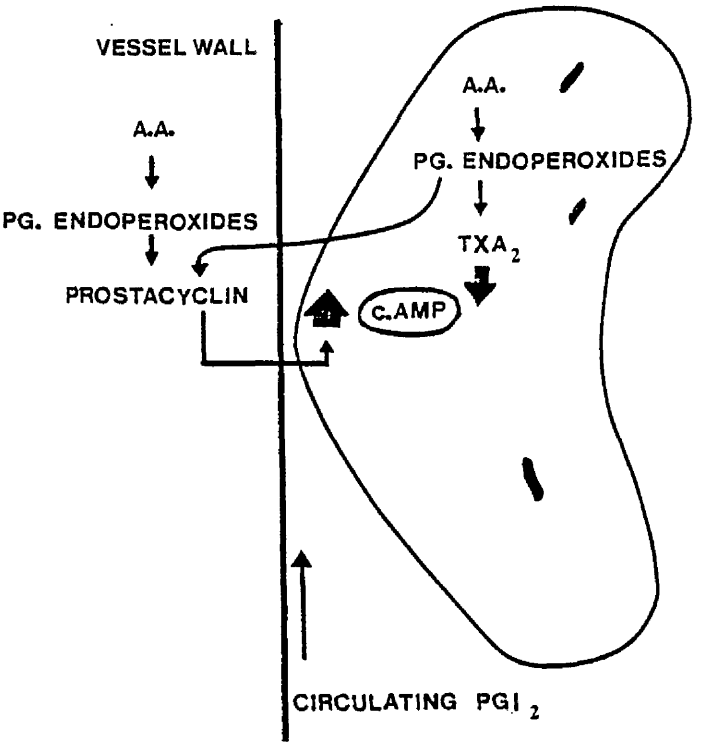


Figure 1.3.

Regulation of platelet cyclic-AMP levels by metabolites of arachidonic acid produced locally or present in blood.

AA-denotes arachidonic acid, PG-prostaglandin, c.AMP-cyclic AMP, TXA_2-thromboxane A_2, and PGI_2- prostacyclin.

Collagen fibres in the subendothelium adsorb plasma factor XII which activates the coagulation pathway by a series of rather slow reactions resulting in thrombin and fibrin generation (see Figure 1.1b). Thrombin is a powerful platelet aggregating agent. Thus at least three separate mechanisms act synergistically to produce platelet aggregation at the site of injury; amine release (ADP and 5HT) from platelets, TXA_2 generation and thrombin formed by the coagulation mechanism.

Simultaneously with the above events a phospholipid becomes available on the platelet surface (platelet factor 3 (PF_3)) and this plays a key role in promoting the activation of factors in the blood coagulation pathway as shown in Figure 1.4. It is not released into the plasma but acts as a surface onto which coagulation factors bind and subsequently react.

Serotonin (5HT) is also liberated by adherent and aggregated platelets and has a powerful vasoconstrictive function, altering flow and causing constriction of the vessel at the site of formation of the platelet aggregate. A similar action is produced by thromboxane A_2. At this stage the platelet aggregate is unstable and may be washed away by the force of blood flow. Stabilisation occurs as fibrin is formed in and around the aggregated platelet mass (see Figure 1.1b).

Thus haemostasis is initially dependent on platelet and vessel function with formation of a platelet plug. At this early stage, coagulation seems to play little part but if there is a deficiency of clotting factors as in haemophilia, excess bleeding will occur later due to failure of stabilisation of the platelet plug, which requires the formation of fibrin, and is washed away as the vasoconstrictor effect of 5HT TXA_2 declines.

1.2.4. Vascular structure and function

From clinical studies there is clear evidence that normality of the endothelial cell lining, basement membrane and supporting collagen and elastic fibres are essential for haemostasis as well as the components in blood.

Injury to an arteriole or venule leads to immediate vasoconstriction probably due to contraction of the muscle layer, by the action of serotonin and thromboxanes generated from aggregated platelets as well as by reflex autonomic action. This response is transient and wears off over a period of 10-20 minutes. In capillaries no such muscular mechanism exists but flow into a capillary bed is controlled by a "pre-capillary sphincter" which may serve the same function. In addition, from experimental work there is evidence that occlusion of capillaries may result from endothelial adhesion. This period of vasoconstriction allows time for platelet and fibrin deposition to occur and secure haemostasis.

The normal capillary is a simple structure consisting of a tube lined with endothelial cells on a basement membrane supported by pericytes and collagen fibres. The endothelial lining of the vascular tree consists of a single layer of these deformable cells, 30 μm by 10 μm, the long axes of which are aligned to the direction of blood flow. The cells are joined together by intracellular bridges and are coated by a thin layer of mucopolysaccharide which extends into the potential space between cells. This continuous sheet of cells retains blood within the vascular tree, stops platelets and fibrin forming in excess but still allows the passage of gases, nutrients and fluids into the tissues of the body. These endothelial cells produce prostacyclin (PGI_2), factor VIII related antigen (VIII: RAg) and a fibrinolytic activator.

In 1976 it was discovered that a microsomal enzyme obtained from blood vessels transforms PGG_2 and PGH_2 into an unstable substance (originally called PGX) which is a potent vasodilator and inhibitor of platelet aggregation (Moncada, Gryglewski, Bunting and Vane, 1976). This substance was subsequently shown to be an intermediary between PGH_2 and 6-keto-$PGF_{1\alpha}$ (Fig.1.2) and was renamed prostacyclin (PGI_2). PGI_2 is formed by vascular tissues from all species examined so far and is the predominant metabolite of arachidonic acid in isolated hearts of the rat, guinea-pig and rabbit. It also appears to be a major prostaglandin product of the gastric mucosa of several species. It is a powerful inhibitor of platelet aggregation and its production may be inhibited by cyclo-oxygenase inhibitors such as aspirin. In addition prostacyclin causes already formed platelet aggregates to disaggregate and this may be a further protective function. The half-life of prostacyclin in the blood is 2-3 minutes which implies that local stimulation of production has only a local effect.

Moncada and Vane (1981) have proposed that prostacyclin protects the vessel wall against deposition of platelet aggregates, so providing at least a partial explanation of the long recognised fact that contact with healthy vascular endothelium is not a stimulus for platelet clumping. Damage to a vessel is followed by platelet adhesion to the affected site with the degree of injury an important determinant. Such observations are in accordance with the distribution of prostacyclin synthetase, for it is abundant in the intima. Indeed, the endothelial cell

itself is the most active in generating prostacyclin. There is then a progressive decrease in concentration of the enzyme from the intima to the adventitia (Moncada, Herman, Higgs et al., 1977). Moreover, the pro-aggregating elements increase from the sub-endothelium to the adventitia. These trends render the endothelial lining anti-aggregatory and the outer layers of the vessel wall much more thrombogenic.

Prostacyclin inhibits aggregation (platelet-platelet interaction) at much lower concentrations than those needed for adhesion (platelet-collagen interaction) suggesting that prostacyclin allows platelets to stick to damaged vascular tissue and begin the repair process while at the same time preventing or limiting thrombus formation (Higgs, Higgs, Moncada, et al., 1978). Thus in such a situation of close physical proximity, the platelet could contribute endoperoxide intermediates to the vessel wall, thereby increasing prostacyclin generation. Prostacyclin inhibits platelet aggregation by increasing cyclic AMP in platelets (see Chapter 1.2.3).

There is also evidence that normal circulating platelets are required to maintain the functional integrity of the endothelial layer. Atrophy of the endothelium results when platelets are quantitatively or qualitatively deficient and it seems likely that they act in a nutritional role. The number of normal platelets required to maintain normality of endothelium is small and in patients with thrombocytopenia an infusion of fresh normal platelets can correct the abnormality without raising the count in the systemic circulation. The exact mechanism by which these platelets interact with endothelial cells is not clear but evidence is now emerging of transfer of platelet constituents to the metabolic pathways of endothelial cells.

1.3 Blood Coagulation

1.3.1 Development of knowledge

In 400 B.C. Plato suggested in the Timaeus that blood contained fibres which caused it to congeal when it left the warmth of the body. William Hewson (1772) localised the source of the fibres to the "coagulable lymph" and suggested that solidification of such lymph was initiated by air and this view was extended by Thackrah (1819) who postulated a "vital influence" in the vessel wall which prevented clotting. This was confirmed experimentally by Brücke (1857) using turtle blood which clotted rapidly in a glazed basin but remained fluid if kept in the isolated heart. Lister (1863) confirmed the importance of the type of surface in contact with blood by allowing blood to clot in India rubber and glass tubes. He then extended these observations in vivo and was able to show that in the intact circulation exposure to a foreign surface initiated a "contact" mechanism which rapidly lead to clotting on the foreign surface. In 1905 Morawitz postulated his now classical theory of coagulation; thrombin, formed from prothrombin in the presence of thromboplastin and calcium ions converts fibrinogen to insoluble fibrin. In the intervening years many other clotting factors have been demonstrated as a result of discovering patients with genetically determined haemorrhagic defects. The cascade (waterfall) concept of blood

coagulation was formulated in 1964 to encompass all the known clotting factors at that time. Since then additional factors have been found and a current scheme for the cascade is shown in figure 1.4. It is postulated that most of the clotting factors circulate as inert pro-enzymes (zymogens) which are converted to an active form and this in turn activates the following zymogen. It is the recommendation of the International Committee on Blood Clotting Factors that the factors be given Roman numerals and the activated form be indicated by the addition of the suffix (a) (see Table 1.2).

1.3.2. The Coagulation Mechanism

The coagulation mechanism may best be described in five phases although it should be appreciated that important feed-back inhibition and acceleration mechanisms do exist.

(A) The contact phase of blood coagulation

There are at least four separate clotting factors in this part of the scheme, factors XII and XI, Fletcher factor (pre-kallikrein) and Fitzgerald factor (high molecular weight kininogen).

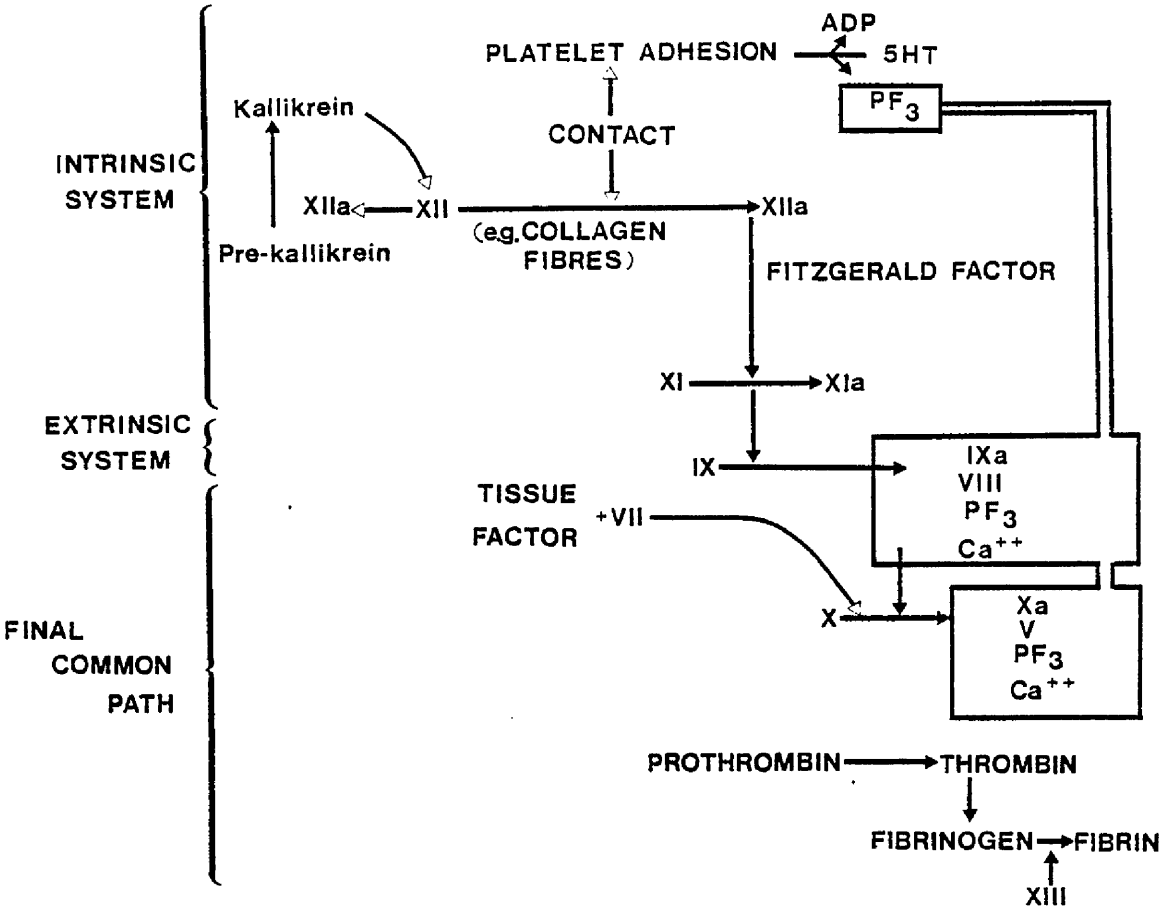


Figure 1.4.

A model of the coagulation cascade based on that proposed by Macfarlane (1964).

TABLE 1.2.

FACTORS INVOLVED IN COAGULATION TO BE READ IN CONJUNCTION WITH FIGURE 1

FACTOR	NAME	FATE OR ROLE IN COAGULATION
I	Fibrinogen	Converted to fibrin monomer (Ia) and then to fibrin polymer (Ib). Absent from serum.
II	Prothrombin	Converted to thrombin (IIa); an enzyme that converts fibrinogen (I) to fibrin (Ia). Absent from serum.
III	Tissue thromboplastin	A lipoprotein complex released by tissue damage which acts in conjunction with Factor VII to activate Factor X
IV	Calcium ions	A cofactor for various enzymatically catalysed reactions in coagulation.
V	Proaccelerin or Labile factor or Accelerator globulin	A cofactor for prothrombin activator (Xa). Absent from serum.
VII	Proconvertin or Stable Factor	A cofactor for tissue thromboplastin (III). Present in serum.

TABLE 1.2 (continued)

FACTOR	NAME	FATE OR ROLE IN COAGULATION
VIII	Antihaemophilic Factor (AHF)	Modified to Factor VIIIa; by thrombin which accelerates the reaction. This factor is deficient in classic haemophilia.
IX	Christmas factor or Antihaemophilic factor B or Plasma thrombo-plastin component (PTC)	Converted to Factor IXa; an enzyme that complexes with Factor VIII to activate Factor X. Present in serum.
X	Stuart-Prower factor	Converted to prothrombin activator (Xa) an enzyme that converts prothrombin (II) to thrombin (IIa). Present in serum.
XI	Plasma thromboplastin anticedent (PTA) or Antihaemophilic factor C.	Converted to Factor XIa; an enzyme that activates Christmas factor (IX). Present in serum.
XII	Hageman factor	Activated by contact with foreign surfaces (e.g. glass) damaged vascular endothelium or low pH. Factor XIIa is an enzyme that activates Factor XI. Present in serum.
XIII	Fibrin-stabilising factor	An enzyme which stabilises fibrin polymer (Ib) by formation of cross-linkages. Absent from serum.
PF3	Platelet factor 3	A phospholipid released from platelets which acts as a cofactor for several enzymes concerned with coagulation

Human factor XII is a single chain polypeptide which migrates in the β -globulin band. The sedimentation constant of purified factor XII has been variously estimated between 4.5s and 5.5s with the iso-electric point between 5.8 and 7.5 (Spragg, Kaplan and Austen, 1973). The molecular weight measured by a variety of techniques is about 80,000 (Schoenmakers, Matze, Haanen, et al., 1965; Revak, Cochrane, Johnston, et al., 1974).

Human factor XI (plasma thromboplastin antecedent) has an apparent molecular weight of about 160,000, a sedimentation constant of 6.9-7.5s and an iso-electric point of 8.0-8.2. It migrates in the γ -globulin band (Forbes and Ratnoff, 1972; Schiffman and Lee, 1974). It has been suggested that the structure consists of two identical polypeptide chains with similar properties joined by disulphide bridges (Wuepper, 1972).

Fletcher and Fitzgerald factors are extremely rare coagulation abnormalties. Their discovery resulted from the investigation of patients with a defect in the initial, surface mediated reactions of blood clotting. A coagulation abnormality has been described in the plasma of a family resulting from a consanguineous union (Hathaway, Belhasen and Hathaway, 1965). In affected members the partial thromboplastin time was abnormally long (with a normal prothrombin time) and prolonged exposure to surface activating agents corrected this defect (Hathaway and Alsever, 1970). The deficient protein, Fletcher factor is identical to plasma prekallikrein (Wuepper, 1972). It may also be called Factor XII-dependant plasminogen proactivator. The protein has a single chain structure of M.W. 85,000 and migrates in the γ -band.

Deficiency of yet another contact factor, Fitzgerald factor (high molecular weight kininogen) was described in an asymptomatic patient who was found to have a prolongation of the activated partial thromboplastin time (APTT), which, unlike Fletcher factor deficiency, was not corrected by prolonged incubation with kaolin. Since the original case several patients have been described with rather different deficiencies but the common factor is probably an abnormality in the level of H.M.W. kininogen, a single chain protein of M.W. 110,000 which migrates in the α band.

Activation of factor XII may occur after exposure to a wide variety of foreign surfaces such as glass, kaolin, bentonite, diatomaceous earths, dacron, nylon, and homocystine. Of more interest is the wide range of physiological substances which may also cause activation; these include, collagen, sebum, basement membrane, uric acid crystals, endotoxins and the soaps of saturated fatty acids.

The mechanism of activation seems to be related to the negative surface charge of the material and inhibition of activation follows exposure of the surface to a positively charged surface such as protamine sulphate, hexadimethrine bromide and cytochrome C (Nossel, Rubin, Drillings, et al., 1968). It has been suggested that activation of factor XII results from conformational changes in the molecule following its adsorption onto rigidly spaced negatively charged sites (Griffin, 1978). Additional evidence of this comes from studies suggesting that the molecular weight is unaltered by activation (Cochrane, Revak and Wuepper, 1973; McMillan, Saito, Ratnoff, et al., 1974). It is probable that activation is associated with unfolding of the molecule which in the process exposes functional sites.

The end result of such activation is the development of active sites with esterase and proteolytic activity as shown by the ability to hydrolyse synthetic esters. In addition, activated factor XII initiates the clotting, fibrinolytic, kallikrein-kinin systems and enhances vascular permeability and chemotaxis. In the coagulation cascade activated factor XII acts on factor XI (plasma thromboplastin antecedent, P.T.A.). It is also possible to split factor XII into two fragments with trypsin or plasma kallikrein to give fragments of MW 52,000 and 28,000, this latter being associated with functional activity.

Surface activation of factor XII is associated with a positive feedback mechanism in which HMW - kininogen enhances the activation of prekallikrein to kallikrein. This in turn activates more factor XII. Further degradation of factor XIIa occurs with production of fragments, one of which, fragment E (MW 28,000) retains esterolytic activity.

The activation of factor XI by factor XIIa does not require the presence of ionised calcium and the process consists of cleaving internal peptide bonds in the two chains resulting in two subunits (heavy chains) with a molecular weight of 50,000 each and two subunits (light chains) with a molecular weight of 30,000 each. The active site is located on the light chains. Trypsin, but not plasmin, kallikrein or thrombin, will also activate factor XI in a similar fashion (Saito, Ratnoff, Marshall, et al., 1973).

Platelets treated with collagen or kaolin, may also activate factor XI in the absence of factor XII (Walsh, 1972b). Factor XII requires the presence of an additional factor to activate purified factor XI, this is Fitzgerald factor (Saito, Ratnoff, Waldman, et al., 1975). Activated factor XI is a hydrolytic enzyme which converts factor IX to its activated form. Factor XIa is inhibited by a variety of agents including di-isopropyl-fluorophosphonate (DFP), phenylmethyl sulphonylfluoride (PMSF) heparin, apyrase C1 inactivator and α_1-anti-trypsin (Forbes, Pensky and Ratnoff, 1970; Heck and Kaplan, 1974).

It has been known for many years that the clotting time of platelet rich plasma from patients with factor XII deficiency is accelerated by any contact procedure (Ratnoff and Rosenblum, 1959). This is probably due to the presence of factor XI adsorbed to the platelet membrane (Horowitz and Fujimoto, 1965). It has also been suggested that small amounts of factor XII are adsorbed on the platelet membrane (Iatridis and Ferguson, 1965). The addition of ADP to platelets releases a clot-promoting agent into the plasma, but only if factor XII is present (Walsh, 1972a). The addition of collagen appears to generate activity attributable to activated factor XI (Walsh 1972b). While it it seems clear that platelets may produce a clot-promoting contact product, the exact mechanism is unclear.

(B) Formation of factor 1Xa

Factor IX (Christmas factor (CF), plasma thromboplastin component (P.T.C.) is a vitamin-K-dependant glycoprotein containing carboxyglutonic acid residues necessary for the binding of calcium. A molecular weight of about 55,000 has been suggested. Vitamin K is necessary for synthesis of the molecule and its absence due either to malabsorption of vitamin K or anticoagulant therapy, results in the appearance of a circulating competitive inhibitor (protein induced by vitamin K absence antagonism (PIVKA) (Hemker, Keltkamp, Hensen et al., 1963)). This protein which is functionally inert may be detected immunologically (Reekers, Lindhout, Kopklaasen et al., 1973). Such PIVKA's also exist for factors II, VII and X and they may interfere with the coagulation process by competitive inhibition. Factor IX is the factor deficient in patients with Christmas disease, a sex linked recessive condition which produces life-long haemostatic difficulties.

Factor IX is activated by factor XIa in the presence of calcium ions in a reaction which occurs in two steps.

In the first (slow reaction) an arginine-alanine bond is cleaved giving rise to a two chain intermediate held together by a disulphide bond. This intermediate which is inert is then cleaved in a second step at an arginine-valine bond to give rise to activated factor IX and a polypeptide residue (fast reaction). Activated

factor IX consists of a heavy chain (MW 38,000) and a light chain (MW 16.000) held together by disulphide bonds. The light chain contains the γ -carboxyglutomic acid residues, necessary for binding calcium, and the heavy chain contains the active site which functions as a serine esterase. In addition there is evidence that factor IX may be activated by factor Xa and by kallikrein (Kalousek, Koningsberg and Nemerson, 1975), and a protease in Russell's viper venom may also cleave the molecule at the arginine-valine bond.

(C) Formation of factor Xa

There are two separate pathways for the generation of factor Xa. Both are necessary for normal haemostasis.

The intrinsic pathway - slow factor Xa generation

This step involves the interaction of factor IXa, factor VIII, phospholipid and ionic calcium to form a complex which then activates factor X (Hemker and Kahn, 1967).

Factor X as it normally circulates in the plasma is a glycoprotein of molecular weight 55,000 (Jackson and Hanahan, 1968, Fujikawa, Legaz and Davie, 1972; Jesty and Esnouf, 1973) the heavy polypeptide chain of factor X is homologous to the prethrombin-2 portion of prothrombin (Titani, Hermodson, Fujikawa et al., 1972).

Factor VIII (antihaemophilic factor, AHF) is thought to have a molecular weight of 2×10^6 or higher (Ratnoff, Kass and Lang, 1969). Factor VIII is usually purified from a precipitate of plasma which is then chromatographed on large pore agarose gels (Kass, Ratnoff and Leon, 1969;

Bennett, Forman and Ratnoff, 1973). By such means purification up to 10,000 fold may be achieved and the material is homogeneous by ultracentrifugation and zone electrophoresis. Sedimentation analysis of this material reveals a symmetric peak with a sedimentation coefficient of 27s.

Factor VIII is the protein which is deficient in its activity in patients with classic haemophilia (haemophilia A). In addition to its activity in the coagulation cascade factor VIII is necessary for aggregation of human platelets by the antibiotic ristocetin, for platelet adhesion and correction of a prolonged bleeding time in patients with von Willebrand's disease. Separation of the coagulation activity (FVIII:C) and the platelet aggregating activity (FVIII:vWF) can be achieved with high ionic strength solutes or in the presence of calcium ions (0.25M) (Rick and Hoyer, 1973; Wagner, Cooper and Owen, 1973; Austen, 1974). This reversible dissociation produces a low molecular weight fragment of approximately 120,000 (Legaz, Schmer, Counts et al., 1973). Recombination of the small fragment with the large carrier molecule occurs in the absence of calcium ions. It is not clear whether in native plasma the two molecules are in combination or exist separately. In classic haemophilia the evidence is that factor VIII molecule is deficient at the site of coagulant activity (Zimmerman, Ratnoff and Powell, 1971) but the FVIII RAg is identical to normal (Bennett et al., 1973; Hoyer and Breckenbridge, 1970).

Factor VIII:vWF is probably synthesised in the vascular endothelium as antigenic material has been demonstrated in endothelial cells and platelets (Bloom, Giddings and Wilks, 1973; Howard, Montgomery and Hardisty, 1974).

The phospholipid micelles are probably produced by platelets and both factors VIII and IXa require to be absorbed onto the micelle surface for the reaction to occur. There is also evidence of a feedback mechanism in that the reaction may be accelerated by alteration of the physical structure of the factor VIII molecule by thrombin (Rapaport, Schiffman, Patch et al., 1963) which may be due to conformational changes in the molecule rather than splitting of it (Davie and Fujikawa, 1975). Factor VIII acts as a regulatory protein enhancing the rate of factor X activation and this is similar to the role of factor V in the activation of prothrombin

The extrinsic pathway - rapid Xa generation

This pathway involves the participation of a tissue factor (extrinsic to the blood), factor VII and calcium ions.

It has been known for many years that addition of certain tissue extracts activate and accelerate the clotting of plasma. Tissue factor has been identified as a cell membrane component in a variety of cells and has been found in the brain, lung, kidney, liver and large vessels (Astrup, 1965). Attempts have been made to purify it and it seems to be a lipoprotein complex with a molecular weight between 220,000 and 300,000. The apoprotein has been partially characterised and that from human brain has a molecular weight of 52,000. The lipid-protein

complex is required for coagulant activity and although it also has peptidase activity this does not appear to be associated with its procoagulant activity.

Factor VII is a single chain glycoprotein of MW 45,000. It is produced by the liver and requires the presence of vitamin K for synthesis. The liver pathway may be inhibited by oral anticoagulants and this results in an immunologically identical but inert protein (PIVKA).

Tissue factor, factor VII and calcium ions form a complex during which factor VII is activated and in turn activates factor X.

Factor VII is activated by tissue factor, by exposure of plasma to cold and by prolonged contact with glass, celite or kaolin. Other factors which modify its function are factor Xa, thrombin, factor XIIa, kallikrein, factor IXa and plasmin (Cochrane, Revak and Wuepper, 1973; Alexander and Landwehr, 1949; Colman, Mattler and Sherry, 1969; Osterud, Berre, Otnaess et al., 1972).

Factor VII as it normally circulates in plasma has weak proteolytic activity. When activated by thrombin or Xa this activity may be increased several hundred fold.

The role of tissue factor appears to be that of a co-factor similar to the role of factor VIII, factor V and high molecular weight kininogen (Rezvan and Howell,1977).

Factor X-activation

Factor X may be activated either by a complex formed by factor VIII, factor IXa, phospholipid and ionized calcium or by tissue factor and factor VIII, or

in vitro by Russell's viper venom or trypsin. During activation there is a cleavage of a single specific arginyl-isoleucine bond in the heavy chain of factor X, with release of one or more polypeptide fragments. This gives rise to the formation of a glycoprotein of molecular weight 45,300 (αXa) and a peptide chain of MW 10,000 which is split from the amino terminal end of the heavy chain. The result is the formation of the active enzyme.

A further degradation may occur either in Factor Xa or in the zymogen as a result of cleavage of a peptide of molecular weight 2,700 from the carboxy terminal portion of the heavy chain. For this second degradation, thrombin is necessary and the product is Factor Xa. The activity of activated factor X does not depend on this reaction.

D. Formation of thrombin

The enzyme thrombin is formed by activation of the inert precursor, prothrombin (factor II) by a complex formed by factor Xa, factor V and phospholipid in the presence of ionic calcium.

Prothrombin is a single chain glycoprotein of MW 70,000 and is produced like factors VII, IX and X in the liver. The rate of synthesis is dependent on vitamin K and may be inhibited by oral anticoagulants.

A variety of abnormal prothrombins have now been described in patients with reduced levels of immuno-reactive material (Shapiro, Martinez and Holburn, 1969). In these patients, activation of prothrombin was

abnormally slow and abnormal products of splitting were found in the serum. Several variants of the molecule have been found subsequently by immunological means (Girolami, 1975).

Factor V was postulated to occur in plasma when it was appreciated that partially purified prothrombin required an additional factor to rapidly convert it to thrombin (Nolf, 1908). It was then observed that the clotting time of stored plasma grew increasingly long suggesting the presence of a labile factor (Quick, 1943). A patient with such a congenital deficiency was then described (Owren, 1947) but this is a rare disorder which is associated with only a minor to moderate bleeding tendency. There is evidence that factor V is synthesised in the liver. There is dispute over the molecular weight of the protein and various estimates range from 70,000 350,000. It is probable that the basic active unit is of the order of 60,000-70,000 and the functional protein exists as multiples of this with calcium ions maintaining the integrity of the polymer (Day and Barton, 1971).

The function of factor V is to accelerate the conversion of prothrombin to thrombin in the presence of factor Xa, the evidence is that it has no other activity.

Activation of prothrombin is complex and has been defined by Magnusson, Peterson, Settrup-Jenson et al., 1975. It is probable that activation occurs in several steps and the reaction is further complicated by digestion of the molecule by forming thrombin. It is probable that in vivo more than one pathway of thrombin production exists.

In the factor Xa mediated pathway prothrombin is cleaved to give two products: pre-thrombin 2 (intermediate II) and fragment 1:2. Prothrombin 2 is then cleaved at an arginine-isoleucine bond to give thrombin. This is the pathway when pure systems are used and the reaction takes place in the presence of a thrombin inhibitor. If thrombin is not inhibited at the time of formation it acts on the prothrombin molecule itself and cleaves off fragment 1 to give prethrombin 1 (intermediate 1) which is then slowly converted to thrombin by the factor Xa complex. In addition thrombin acts on fragment 1:2 to give fragment 1 and this competitively inhibits prothrombin conversion (Prowse, Mattock, Esnouf et al, 1976). Also the calcium binding sites of prothrombin are in fragment 1:2 and fragment 1 and the removal of fragment 1 by thrombin generation does not allow the factor Xa complex to bind properly and results in slow generation of further thrombin. Factor V competes with fragment 1 for a common binding site on the prothrombin molecule and reduces this inhibitory action and thus accelerates the reaction. Phospholipid also increases the reaction rate by about tenfold perhaps by binding the reactants to the lipids surface and so raising the local concentration of the molecules or by inducing conformational changes in the protein.

Thrombin is the product of prothrombin activation. Thrombin is a two-chain (A and B) serine protease with a molecular weight of approximately 38,000. The larger B chain has sequence homology with other serine proteases particularly those from the pancreas. The

short A chain restricts the specificity of the enzyme. Thrombin has numerous activities in coagulation, the two most important functions are to convert fibrinogen to fibrin and to induce platelet aggregation and release. Thrombin also activates factor VIII, digests fragment 1:2 of prothrombin, alters the structure of factors V and VII, and in vitro it also digests a variety of synthetic substrates.

E. Conversion of fibrinogen to fibrin

Fibrinogen is a glycoprotein synthesised in the liver parenchymal cells. It is the inert circulating precursor of fibrin and as such it plays a key role in haemostasis.

Fibrinogen is a β-globulin with a molecular weight of approximately 340,000 of which 3% is carbohydrate. It is thought to have an elongated form (Shulman, 1953; Caspary and Kekwick, 1957) but its detailed shape is unknown. Hall and Slayter, 1959, obtained electron micrographs of individual molecules, revealing a protein about 475Å long consisting of a linear array of three globulin domains (see Figure 1.5(A)). Koppel (1970) proposed a sphere of 24 nm which he suggested had the form of a pentagonal dodecahedron (see figure 1.5(B)). Despite considerable controversy, the 'trinodular' model of Hall and Slayter is now generally accepted. More recently a precise length of 450Å has been obtained for the trinodular model, with a more detailed substructure (see Figure 1.5(C))(Weisel, Phillips and Cohen, 1981).

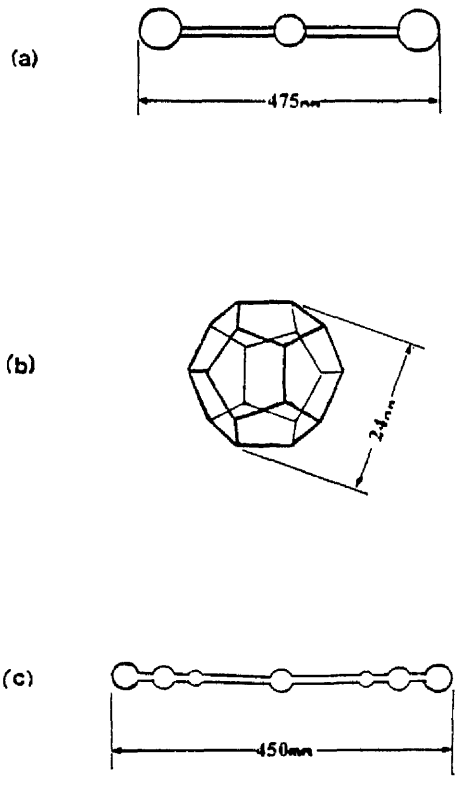


Figure 1.5.

Three proposed slopes for the fibrinogen molecule (a) the trinodular representation of Hall and Slayter (1959)

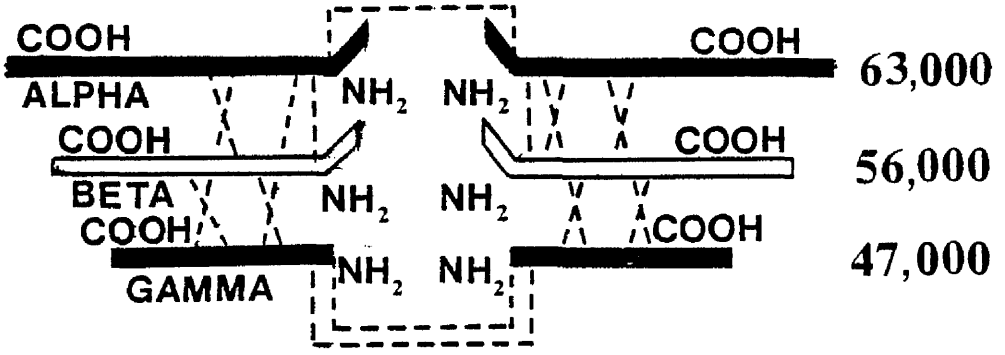
(b) the pentagonal dodecahedron of Koppel (1970)

(c) the multi-nodular structure proposed by Weisel, Philips and Cohen (1981).

The fibrinogen molecule is a dimer, each half consisting of three dissimilar polypeptide chains ($A\alpha$, $B\beta$, γ) with molecular weights of 63,000, 56,000 and 47,000 joined by disulphide bridges (McKee, Mattock and Hill, 1970; Gaffney, 1977). These chains are linked by disulphide bonds near the N-terminal end of the α and γ chains (see Figure 1.6).

In addition to its function in coagulation fibrinogen, because of its molecular size and shape, has important effects on red cell aggregation and is the most important protein determinant of plasma viscosity. It, therefore, plays an important part in determining blood flow (Dintenfass 1971, Lowe, Drummond, Forbes et al., 1981). There is also some evidence which links the fibrinogen level as a risk association in atherosclerosis, immunity, in the inflammatory response, in defence mechanisms against invasion by bacteria and by malignant cells and it is of importance in providing a scaffold for fibroblast growth in wound healing.

The conversion of fibrinogen to fibrin involves proteolysis, polymerisation and stabilisation (see Figure 1.7). Fibrinogen cleavage is confined to the 'amino-terminal ends of the $A\alpha$ and $B\beta$ chains. Thrombin first splits the arginine ($A\alpha$16)-glycine ($A\alpha$17) bond on the $A\alpha$ chain to provide fibrinopeptide A (FPA-16 amino acids). This reaction proceeds rapidly and leads to conformational change with end to end associations of the intermediate fibrin monomer molecules. Thrombin then



Six chain structure of FIBRINOGEN

--------Interchain disulphide bonds

FPA

FPB

Figure 1.6

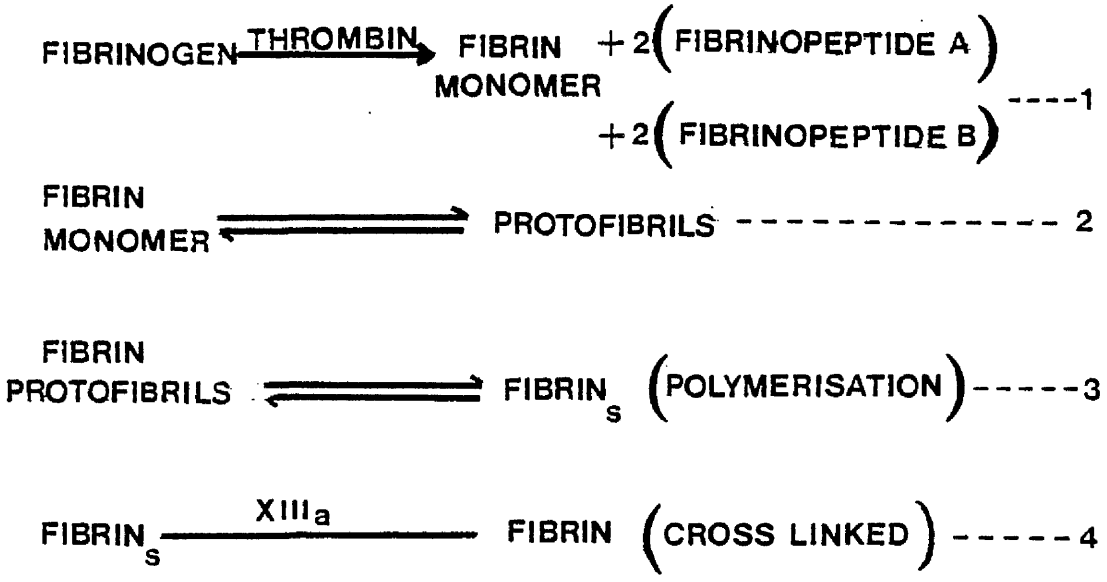


Figure 1.7

The conversion of fibrinogen to fibrin.

(1) Activation by thrombin, removal of FPA and FPB, monomeric fibrin.

(2) End-end polymerisation, protofibrils.

(3) Lateral association of protofibrils , fibrin fibers.

(4) Covalent cross-linking by Factor XIIIa , cross-linked fibrin.

cleaves the arginine (Bβ 14)-glycine (Bβ 15) bond on the Bβ chain to produce fibrinopeptide B (FPB-14 amino acids) after a lag phase.

The subsequent process of polymerisation is poorly understood. It would appear to involve the formation of weak reversible bonds between the polymerising units. A schematic model of the initial events involved in the polymerisation of fibrin has been proposed by Doolittle 1981 (see Figure 1.8) and is consistent with electron-micrographic and structural data.

The fibrinogen molecule (a) is converted into a fibrin monomer (b) by the release of fibrinopeptides A and B as described previously. The newly exposed ends of the alpha chains serve as "knobs" that interact with "holes" in the terminal domain, linking fibrin monomers with the observed half-monomer overlap (c). The assembly can be extended into a long "intermediate polymer" two molecules thick (d) which is stabilised by the formation of covalent cross-links between adjacent terminal domains. Intermediate polymers are then interwoven laterally to form a fully developed fibrin strand. The initial polymerisation seems to depend specifically on the alpha knobs. The end-to-end covalent cross-linking is between two gamma chains. Lateral growth may be mediated by the beta knobs and then strengthened by alpha-chain cross-linking.

It is important to keep in mind that the raison d'etre of the fibrinogen-fibrin transformation lies in

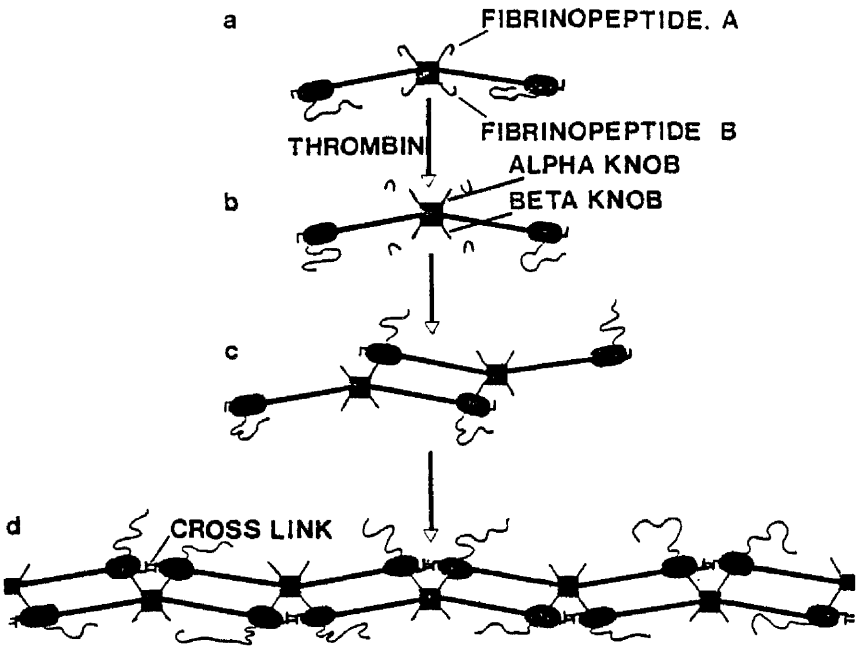


Figure 1.8.

Polymerisation of Fibrin

The fibrinogen molecule (a) is converted into fibrin monomer (b) by the removal of fibrinopeptides A and B. Fibrin monomers then form by half monomer overlaps (c). Long intermediate polymers are then formed by extension of the monomers (d), stabilisation occurs by the formation of covalent cross links between terminal domains.

its ability to produce a gel that can prevent blood loss from a closed circulatory system. Fibrin clots are gelled and semi-solid masses formed by an all-pervading network of fibres. Ordinarily the propagating fibres become entangled in the formed elements of the blood, including the red and white cells, but especially the platelets with which preferred interactions occur.

Factor XIII (fibrin stabilising factor)

In the final stage of blood coagulation fibrin becomes insoluble as a result of the cross-linking of fibrin monomer by the action of a transpeptidating enzyme (FXIII) first described by Lorand and Jacobsen (1958).

Plasma factor XIII is composed of two A chains (MW 75,000) and two B chains (MW 88,000); the whole molecule has an $A_2 B_2$ structure with a molecular weight of 320,000 (Schwartz, Pizzo, Hill et al., 1973). Factor XIII circulates as an inactive precursor in plasma, and is activated to XIIIa by the action of thrombin in the presence of ionic calcium. This enzyme is responsible for the strengthening of fibrin-to-fibrin bonds by inter-glutamyl-e-lysine bridges to form γ - γ dimers and more slowly across α chains (Lorand, 1972). Covalently stabilised fibrin is mechanically stronger than non cross-linked fibrin and is more resistant to fibrinolysis.

1.3.3 Physiological inhibitors of blood coagulation

It is probable that every activated coagulation factor in the cascade has one or more inhibitors which regulate the rate of generation of the active product or determines its destruction. The purpose of these would seem to be the limitation of activation of coagulation and protection against inadvertent deposition of fibrin in the vascular tree.

The most important of such inhibitors is antithrombin III (ATIII). The presence of an inhibitor of thrombin has been postulated since Morawitz (1905) showed a gradual decline in coagulant activity of clotted blood. It was initially suggested that this might be due to either some specific antithrombin or to adsorption of thrombin to fibrinogen. It was soon appreciated that using heparinised plasma acceleration of this inhibitory effect occurred and it was thought to be due to the presence of a heparin co-factor in plasma, Clark (1938). Deficiency of antithrombin activity and low heparin co-factor activity in plasma was associated with a genetic predisposition to thrombosis (Egeberg, 1965) and later the antithrombin associated with heparin co-factor activity was prepared (Abilgaard, 1968).

ATIII is the most important of at least six different antithrombins three of which (α_1-antitrypsin, α_2-macroglobulin and ATIII) play a physiological role. It is estimated that 75% of antithrombin activity is due to ATIII.

ATIII is an α_2-globulin with a molecular weight of about 65,000. ATIII reacts with thrombin to produce a complex in which both components are inactivated. The rate of the reaction depends on the concentrations of thrombin and ATIII, and there is some evidence that the mechanism of antithrombin activity is made more efficient by activation of the system by the early stages of coagulation.

ATIII has inhibitory actions on factor Xa (Seegers, Cole and Harmison, 1964) except when associated with platelets, factor V - phospholipid complex or phospholipid it is largely protected. Neutralisation of factor Xa generated in plasma is rapid and complete within 15 minutes (Odegard, Lie and Abildgaard, 1975). This is analogous to the situation with thrombin and suggests the inhibitory system is primed by the earlier stages of the cascade.

There is also evidence that ATIII plays a part in neutralisation of factor IXa (Rosenberg, McKenna and Rosenberg, 1975; Osterud, Miller-Andersson, Abildgaard et al., 1976), XIa (Damus, Hicks and Rosenberg, 1973), XIIa (Stead, Kaplan and Rosenberg, 1976) and plasmin and the rate of inactivation of all these factors is accelerated by the addition of heparin.

Recently protein C, a new anticoagulant has been discovered. Protein C, a vitamin K dependent factor, is the zymogen of a serine protease, previously known for many years as auto-prothrombin II-A (Mammen, Thomas and Seegers, 1960; Seegers, Novoa, Hendry et al., 1976). Isolated protein C consists of a two-chain molecule that can be converted into a

serine protease by the proteolytic cleavage of a peptide bond in the amino-terminal region of the heavy chain (Fernlund and Stenflo, 1979). Both thrombin (Kisiel, Ericsson and Davie, 1976; Kisiel,. 1979; Kisiel, Canfield, Ericsson et al., 1977) and Russel's viper venom (Kisiel et al., 1976) have been reported to catalyse such a reaction. Thombin activation of protein C has recently been reported to be greatly accelerated by an endothelial cell surface factor (Esmon and Owen, 1981).

The potent anticoagulant action of protein C (Mammen et al., 1960; Seegers, Marlar and Walz, 1978) is probably related to the inactivation of activated factor V and activated factor VIII, thus limiting Xa and thrombin generation (Kisiel et al., 1976; Marlar, Kleiss and Griffin, 1981). A stimulatory effect on fibrinolysis has also been reported but it is not clear whether this is a direct or indirect effect.

Recently congenital deficiencies in protein C have been reported with the affected families having a thrombotic tendency (Griffin, Evatt, Zimmerman et al., 1981; Bertina, Broekmans, van der Linden et al., 1982).

1.4 Fibrinolysis

1.4.1 Development of knowledge

It has been known for more than a century that blood clots slowly liquify (Hunter 1794, Denis 1838,

Dastre 1893). Early investigators concluded that a physiological process occurred, the function of which was the removal of fibrin after it had fulfilled its function in haemostasis. As a result of intense investigation over the past 30 years, fibrinolysis and thrombolysis have been shown to be the processes by which fibrin, in the form of either a clot or a thrombus, forms degradation products (FDP). The dominant mechanism is the plasminogen-plasmin system; in this, the inactive protein precursor (plasminogen) is activated to the enzyme plasmin by an activator the reaction being controlled by the presence of inhibitors. Such activators may be of tissue, plasma or synthetic origin and this step may be inhibited by a variety of physiological or therapeutic inhibitors (see Figure 1.9). It is known that alternate mechanisms of fibrinolysis exist but these seem to be of minor importance (Plow and Edgington, 1975).

It is probable that the fibrinolytic process functions as a major homeostatic mechanism in the maintenance of patency in the vascular tree by controlling deposition of fibrin. However, the physiological control of the system remains controversial. In addition, the importance of deficiencies of part or the whole of the system in the production of vascular occlusion is not yet clear. Activation of fibrinolysis for therapeutic purpose is possible and in selected situations is of proven value (Forbes, Lòwe and Prentice, 1978).

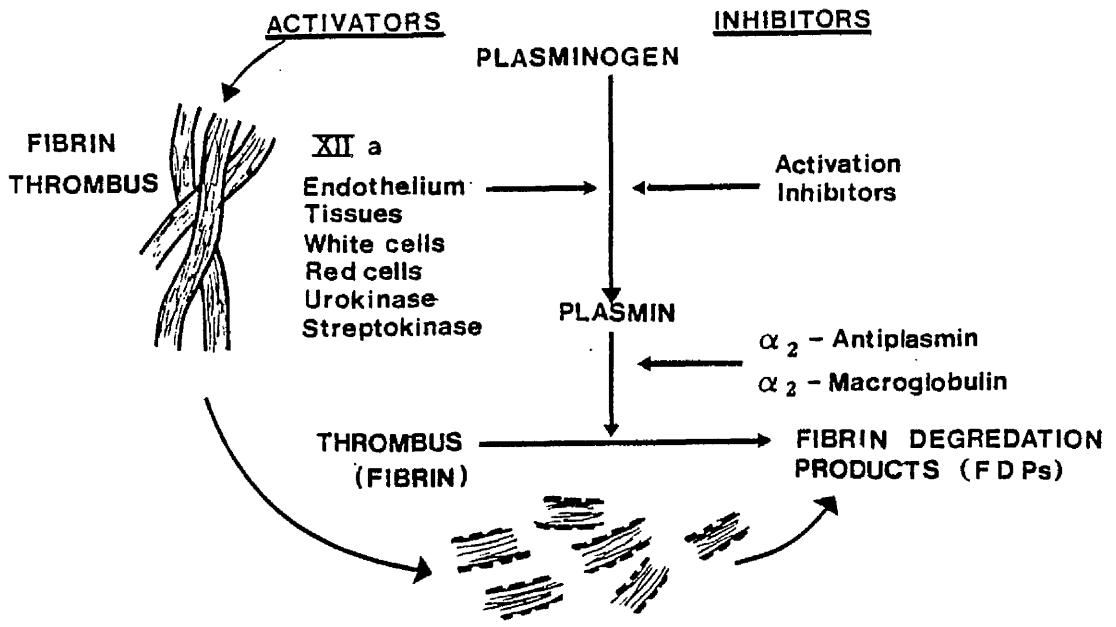


Figure 1.9

The basic fibrinogenolytic/fibrinolytic pathway is shown.

A variety of theories have been proposed to explain the selective digestion of fibrin intravascularly. Plasminogen may be bound selectively to fibrin (Alkjaersig, Fletcher and Sherry, 1959); plasmin may be dissociated from anti-plasmin in contact with fibrin (Ambrus and Markus, 1960); plasminogen activator may be bound to fibrin (Chesterman, Allington and Sharp, 1972).

1.4.2. The Plasminogen - Plasmin System

The four components of this system are plasminogen, plasmin, plasminogen activator and inhibitors.

(a) Plasminogen

Human plasminogen is a single-chain beta-globulin of approximate molecular weight 90,000. It is present in normal adult plasma in a concentration of 200 ng/ml and in the infant at a lower level and is present in all body fluids. Two forms of the molecule have been prepared and their biochemical and physiological activities intensely studied. They differ in the composition of their amino terminals. Native human plasminogen has glutamic acid in the N-terminal position (glu-plasminogen) (Wallen and Wiman, 1970), and the other main form has lysine as the N-terminal amino acid (Lys-plasminogen) (Robbins, Summaria, Hsieh et al., 1967). The biological importance of this biochemical difference is controversial. Both types occur in multiple but electrophoretically distinct forms (Collen and de Maeyer, 1975). The molecular weights of glu- and lys-plasminogen have been estimated variously as being of the order of 97,000

and 84,000 (Collen, Ong and Johnson 1975) and 85,000 and 82,000 respectively (Robbins, Summaria and Barlow, 1975). It seems likely that the lys-form results from limited proteolysis of the glu-form by plasmin formed during the purification procedure (Collen and de Maeyer, 1975; Collen et al., 1975; Markus, Eversand Hobika, 1976) Lys-plasminogen is more readily absorbed to fibrin and more readily activated than glu-plasminogen (Thorsen, 1975) and for these reasons may be of greater potential value in thrombolysis (Kakkar, 1978).

(b) Plasmin

Plasmin is a proteolytic enzyme formed from the inactive precursor plasminogen by any of a variety of activators. The molecule consists of two amino-acid chains of different lengths connected by a disulphide bond. These two chains result from splitting of an arginine-valine bond during activation to produce a heavy (A) chain M.W. approximately 68,000 and a light (B) chain, M.W. approximately 25,000. The A-chain contains the N-terminal end and the B-chain contains the C-terminal end and the active serine centre. The action of plasmin is not restricted to fibrin and it splits peptide bonds in practically all protein substrates and a wide variety of esters of arginine and lysine. It is not normally detected in human plasma as it is rapidly neutralised by inhibitors.

(c) Activation of plasminogen

The principal activator of plasminogen in blood is presumed to be present in the endothelium of blood

vessels and is released 'on demand' by, for example, exercise, hypercoagulability, and drugs (Todd, 1959). At least two forms of this type of activator, extrinsic and intrinsic, are present and the former is enhanced by exercise while the latter is not (Marsh and Gaffney, 1980). The relationship of these two activators to each other is not clear. Plasminogen activators have been found associated with the endothelial cells of vessels, in epithelial cells, synovium, mesothelium, red cells, and platelets, and in such diverse tissues as uterus, ovary, lung, thyroid and prostate (Plow and Edgington, 1975; Semar, Skoza and Johnson, 1969; Ekert, Friedlander and Hardisty, 1970). It is of interest that a variety of tumours may produce plasminogen activator which may interfere with local haemostasis (Davidson, McNicol, Frank et al., 1969) but may also be implicated in the metastasis of malignant cells (Christman, Acs, Silagi et al., 1975).

Despite the interest in activator-plasminogen interactions to produce active plasmin, most work on the activation mechanism of plasminogfen has been performed using the exogenous activators streptokinase (SK) and urokinase (UK).

Circulating blood activator

The walls of the blood vessels are the source of circulating blood plasminogen activator (Todd, 1959; Chakrabarti, Birks and Fearnley, 1963). Normally

the plasminogen activator content of circulating blood is low but it is increased after physical exercise and stress (Biggs, Macfarlane and Pilling, 1947; Marsh and Gaffney, 1980), by venous occlusion (Bennett, Ogston and Ogston, 1968) and in response to a number of diverse stimuli.

While studies on blood plasminogen activators have been hampered by its extreme lability it has been suggested that the isolated form of plasminogen activator is relatively stable (Ogston, Bennett and Mackie, 1976). Thus other enzymes in blood may contribute to the destruction of plasminogen activator in blood obtained after occlusion. It has been recently suggested that the two forms of plasminogen activator, intrinsic and extrinsic, may be merely expressions of such enzymic conversions (Marsh and Gaffney, 1980) though the majority opinion favours the view that they are two distinct enzymic systems (Kluft, 1978). Furthermore, the post-exercise (or other forms of stress) extrinsic plasminogen activator is a serine protease of M.W 60,000 while the intrinsic (factor XII-associated) plasma plasminogen activator has been shown not to be a serine protease and to be immunologically distinct from the extrinsic form (Mackie, Booth and Bennett, 1979).

Plasminogen activator is made up of two chains (M.W 31,000 and 38,000) connected by disulphide bridges, while the smallest chain has been shown to contain a serine-type active site (Rijken, Wijngaards, Zaal-De-Jong et al., 1979). It is, however, becoming clear that

these two chains are formed by a proteolytic cleavage of a single-chain protein.

Exogenous activators

Bacterial activators

Although many bacteria produce plasminogen activators the only one of therapeutic importance is that produced by B-haemolytic streptococci, streptokinase (SK). This is an $\alpha 2$-globulin with a molecular weight of 46,000. For therapeutic purposes it may be produced relatively free of other streptococcal proteins but it remains antigenic and administration may be associated with allergic and immune reactors. Streptokinase does not possess any direct enzymatic activity and it requires the presence of a pro-activator (see later).

Urinary activator

Fibrinolytic activity has been shown to be present in urine for almost a century and crude concentrates of human urine have been used in the laboratory and in patients with a variety of thrombotic disorders (Sobel, Mohler, Jones et al., 1952). Such preparations of urinary activator (urokinase, UK) contain at least two components with molecular weights of approximately 31,000 and 55,000. They are partially identical as shown by cross reactivity with specific antisera and it is thought that the higher molecular weight material is degraded during purification. Activator produced by renal cell cultures has been shown to have corresponding properties and immunological identity (Bernik and Kwaan, 1969; Barlow, Rueter and Tribby, 1975).

Other body fluid activators

Epithelial cells of other tubular systems commonly produce activators to dissolve fibrin which may be potentially produced in their lumens. Plasminogen activators are found in human milk, seminal fluid, cerebo-spinal fluid, saliva, tears and bile.

Activation mechanisms

1. Streptokinase (SK)

Human plasminogen (plgn) and SK form an equimolar complex which undergoes an alteration leading to the evolution of an active site in the plasminogen part of the SK-plgn complex (Castellino, 1979). This activated complex (SK - plgn*) can either directly convert plasminogen to plasmin or can itself be intramolecularly converted to an SK-plasmin complex (SK-pl). The SK moiety of this latter complex is degraded but other tissues contain inhibitors of plasmin but the function and importance is not clear.

(d) Inhibitors of plasminogen activation

A variety of poorly defined inhibitors of plasminogen activation have been described in plasma (McNicol, Gale and Douglas, 1963; Lauritsen, 1968). At least three have been partially characterised; they have no anti-plasmin activity. They range in M.W from 20,000-75,000 and inhibit vascular, and tissue activators as well as urokinase (Markwardt, 1978; Hedner, 1973; Bennet, 1967; Aoki and Moroi, 1974; Beattie, Ogston, Bennett et al., 1976.

1.4.3 Fibrinogen - plasmin interactions

The first detailed examination of the fragments obtained from fibrinogen following plasmin attack was carried out by Nussenzweig, Seligman, Pelmont et al., in 1961. They found that plasmin-induced fibrinogen digests, when fractionated on DEAE ion-exchange columns, separated into five major fractions, which they called A, B, C, D and E, and they described fragments D and E as plasmin resistant or terminal-core fragments, having molecular weights of 83,000 and 33,000 respectively. These core fragments had been described earlier as α and β fibrinogen (Seegars, Nieft and Vanderbelt, 1945). Fragments D and E were 50% and 20% by weight of the original fibrinogen molecule and represented individually distinct structural and antigenic regions of fibrinogen. They also observed intermediate plasmin-labile degradation products of fibrinogen, which were subsequently named by other workers as X and Y fragments.

Examination of the subunits of the various fragments of fibrinogen in conjunction with carbohydrate staining and thrombin treatment of the various polypeptide chains has produced a variety of schemes of fibrin degradation which have a number of common features. These are summarised as shown in figure 1.10.

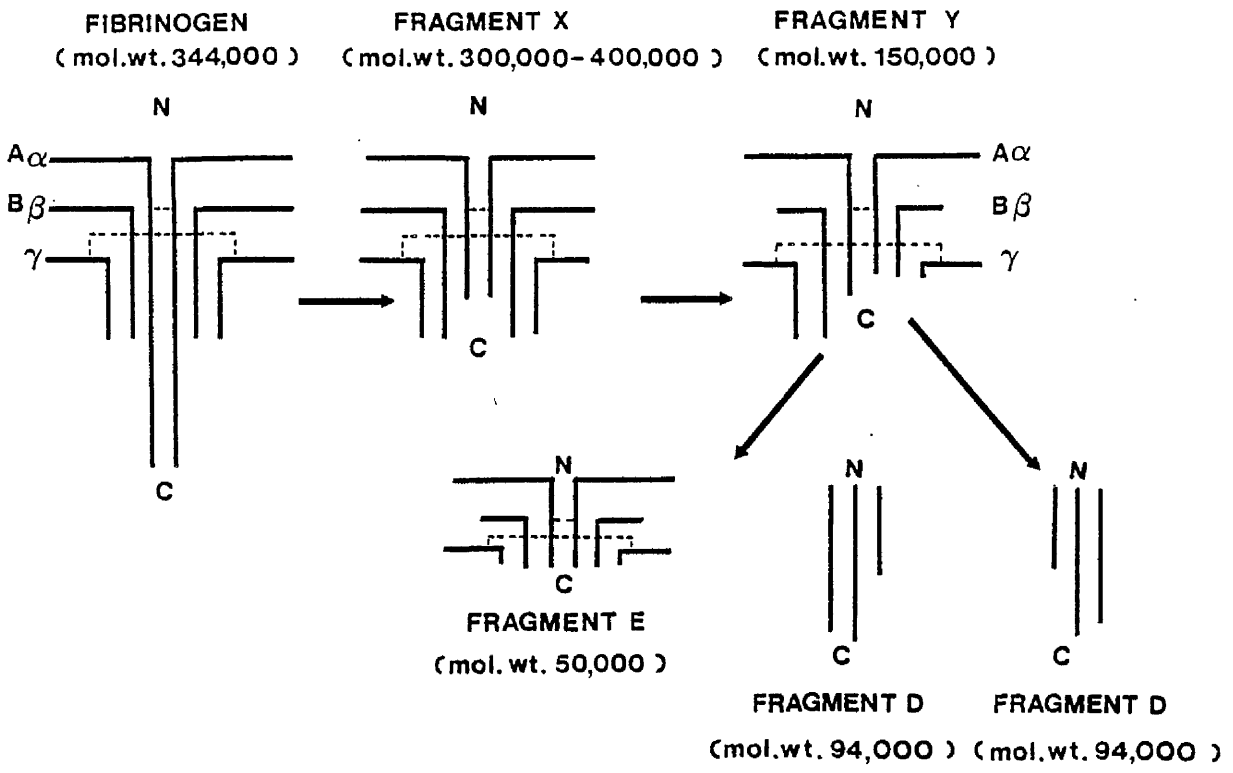


Figure 1.10

Schematic diagram of the plasmin-mediated conversion of fibrinogen to its core fragments D and E, showing the intermediate fragments X and X. (Taken from Gaffney, 1977).

The conservation of both the NH_2 -terminal amino acids and the fibrinopeptides A of the Aα chains suggests early hydrolysis with splitting of a peptide of M.W 40,000 from both carboxyl ends of the Aα chains leaving a high molecular weight fragment X (M.W. 250,000) which consists of the Aα chain remnants disulphide bonded to intact Bβ and γ chains (Gaffney and Dobos, 1971; Marder and Budzynski, 1975). This high molecular weight fragment may still clot under the action of thrombin. In the next step a peptide of M.W 6,000 is split from the N-terminal ends of the Bβ chains (Mosesson, Finlayson and Galanakis, 1973), then all three chains are cleaved on one side of the partially degraded dimer to release fragment D, of M.W 94,000 and leaving fragment Y (M.W 150,000)(Gaffney, 1977; Furlan, Kemp and Beck, 1975; Marder, Shulman and Carroll, 1969). Fragment Y is then rapidly cleaved to produce a second fragment D and a third fragment, fragment E (M.W 50,000). Fragment E is a dimer made up of the N-terminal sections of the three chains of fibrinogen (Kowalska-Loth, Garlund, Egberg et al., 1973). Fragments Y, D and E do not clot on addition of thrombin. They inhibit the conversion of fibrinogen to fibrin by thrombin. The larger of these fragments has a greater effect on inhibition of clotting and purified Y is most active, and it has been shown that they inhibit the enzymatic as well as the polymerisation phase of clotting (Latallo, Budzynski, Lipinski et al., 1964; Lipinski, Wegrzynowicz, Budzynski et al., 1967).

1.4.4. Non-cross linked fibrin and plasmin degradation

From a biochemical viewpoint, it seems reasonable to regard non-cross linked fibrin as being quite similar to fibrinogen. In their chemical structure only the lack of fibrinopeptides A and B distinguishes non cross-linked fibrin from fibrinogen. Thus fibrinogen fragments X and Y differ from fibrin fragments X and Y in that the latter fragments lack fibrinopeptide A. Fibrinogen fragments D and E have been shown to be different from fragments D and E derived from fibrin by their differing ability to bind specific antisera. In radioimmunoassay assay systems these immunologically based differences have been proposed to distinguish between fibrinogenolysis and fibrinolysis in man (Edgington and Plow, 1979).

1.4.5. Cross-linked fibrin and plasmin degradation

Degradation of cross-linked fibrin proceeds at a slower rate, as the A α chain cross-linking confers resistance to the initial steps of plasmin digestion (Gaffney and Brasher, 1973). Unique fragments called D dimer and E are formed. The D-dimer fragments contain the cross-linked Y-chain remnants of the original fibrin (Pizzo, Schwartz, Hill et al., 1973; Kopec, Teisseyre, Dudek-Wejciechowska et al., 1973). It has been suggested that detection of D-dimer may provide a means of monitoring thrombolytic treatment (Gaffney and Brasher, 1973). The molecular weight of the D-dimer is between 63,000 and 80,000 and there is evidence of D-dimer and fragment-E complexing (Hudry-Clergeon, Paturel and Suscillon, 1974; Gaffney, Lane and Kakkar, 1975). See figure 1.11.

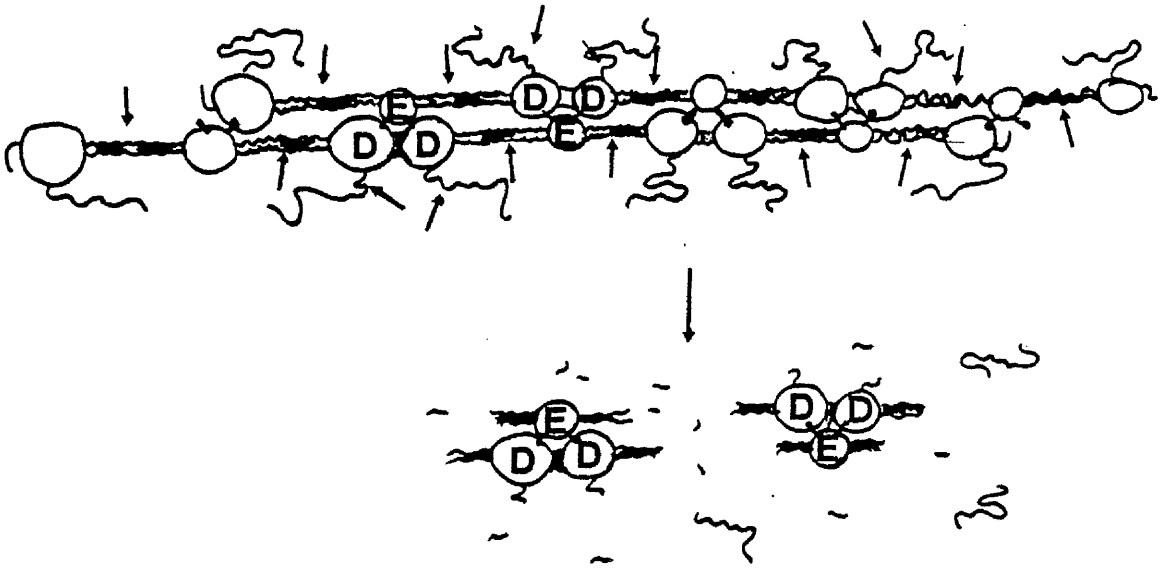


Figure 1.11.

The stable fragment "D_2E" that survives the plasmin digestion of cross-linked fibrin.
(Arrows denote plasmin attack points)

Biological functions of fibrin(ogen) degradation products (FDP)

As well as inhibiting polymerisation of fibrin and the action of thrombin, FDPs have the following actions.

1. Effect on platelet function

FDPs inhibit platelet aggregation, adhesion and the release reaction, the high M.W products being more active than low M.W products (Kowalski, Kopec and Wejrzynowicz, 1964; Jerushalmy and Zucker, 1966; Stachurska, Latallo and Kopec, 1970). Their action is probably due to adsorption of fragments onto the platelet membrane (Kopec, Budzynski, Stachurska et al., 1966) and is found whether the production of fragments is due to SK, defibrase or ancrod (Kowalski et al., 1964; Olsson and Johnsson, 1972; Prentice, Turpie, Hassanein et al., 1969).

2. Effects on heart and blood vessels

These are due to low M.W peptides which potentiate the effects of bradykinin, angiotensin, adrenaline and serotonin (Buluk and Malofiejew, 1969; Takaki, Yamaguchi and Ohsato, 1974). Low M.W fragments also increase the permeability of skin capillaries (Malofiejew, 1971).

1.4.6. Fibrinolysis associated with the cellular compartment of blood

The interaction of enzymes other than plasmin with fibrin has recently been reviewed (Latallo, Teissyre, Ardelt etal., 1978). The most important progress in our knowledge of the role of non-plasmic proteases in

the pathophysiology of thrombosis should be ascribed to the neutral proteases of certain blood cells. The participation of polymorphonuclear neutrophils has been demonstrated in the process of fibrin dissolution (Riddle and Barnhart, 1964). It has been suggested that plasmin may play only a minor role in spontaneous fibrinolysis (Moroz and Gilmore, 1976), while granulocytes have been shown to contain the components of an alternative fibrinolytic pathway in man (Plow and Edginton, 1975). Neutrophil granules have been shown to degrade both fibrinogen and fibrin (Kopec et al., 1973). Platelets contain elastase, thus suggesting a possible role in thrombus dissolution (Legrand, Pegnaud, Caen et al., 1975). Lysis of fibrin has been achieved by lytic agents secreted by eosinphils (Kwaan and Hatem, 1978). While cellular enzymes have been viewed with some scepticism in fibrinolysis, they might explain the clearance of thrombi from vessels through which there is no measurable blood flow.

Whether the mechanisms and components involved during the formation of fibrin in vivo are part of an ongoing systemic process is unknown, but it is probably highly unlikely. That fibrin, as it forms, mediates its own destruction seems certain and the role of a competent fibrinolytic system in this destruction seems essential. The reactions which have been discussed are relevant to most naturally and drug-induced defibrination episodes. The question arises as to their significance in the destruction of fibrin in thrombi and this will be discussed in Chapter 2.

CHAPTER 2

INTRAVASCULAR BLOOD COAGULATION

2.1. Introduction

Intravascular blood coagulation will be defined in this thesis as activation of the coagulation mechanism with the formation of thrombin in the circulating blood and of thrombi within the blood vessels. As a thrombus (or microthrombus) has a definite histological structure composed of platelets, red blood cells, leukocytes as well as insoluble fibrin, it is appreciated that many inter-related factors, besides coagulation, are involved in its development.

Intravascular coagulation may be classified into several types:

1. Pre-thrombotic state (Synonyms: hypercoagulable state, hypercoagulability, thrombotic tendency)

This term describes a clinical state in which the patient is at increased risk of developing thrombosis or intravascular coagulation in which insoluble fibrin deposition occurs although actual thrombi or microthrombi are not present at this stage. It may be associated with an increased level of coagulation factors in the blood or with the presence of activated coagulation factors or platelets.

2. Local thrombosis

This term describes the development of a macroscopic, insoluble fibrin-platelet thrombus in a segment of an artery or vein. This often occurs secondary to local vascular disease.

3. Intravascular coagulation - local and disseminated

Local intravascular coagulation is the development of microthrombi or insoluble fibrin deposits in the small blood vessels of a single organ.

Disseminated intravascular coagulation (Synonyms: DIC,defibrination, consumption coagulopathy, intravascular with fibrinolysis or abnormal proteolytic activity).

DIC has been defined as a pathological process which results in the development of micro-thrombi or insoluble fibrin deposits in the blood vessels of several organs and those areas of the vasculature not involved in primary disease. DIC is not a separate entity but is a condition that may occur in association with any disease or clinical state.

4. Atherosclerosis

Atherosclerosis is the most frequent disorder of blood vessels. It occurs as a result of atheromatous plaques in the endothelium of the blood vessel. Such atheromatous lesions are found only in the arterial circulation, gradually developing from a previously normal arterial wall. The lipid infiltration theory explains atheroma on the basis of the progressive accumulation of lipids originating in the blood (Anitschow and Chalatow, 1913). Another view is that the atheromatous plaque is the end result of the incorporation of microthrombi formed on the endothelium (Duguid, 1976) or of the stimulation of smooth muscle cells by adherent platelets (Ross and Glomset, 1976). Whatever the explanation, the plaque may then be the focus

for rapid thrombin generation, possibly as a result of haemorrhage into or near the plaque. Platelet aggregation and fibrin formation occurs. Platelet aggregation probably depends on the balance between the TXA_2 and PGI_2 levels in the platelet itself and the vessel wall (Moncada and Vane, 1978). If the resulting thrombus is large enough, blood flow to the myocardium is impaired and infarction or sudden death may ensue.

2.2. Development of knowledge

That blood can coagulate within the vascular system during life has been known for two thousand years (Anning, 1957). During the seventeenth century pathologists were familiar with the presence of clotted blood within the heart, aneurysms and blood vessels (Wiseman, 1686; Malpighi, 1686; Petit, 1731; Benivieni, 1928), but at first they did not distinguish between thrombosis, the process by which liquid, flowing blood turns into a solid mass in the living blood vessels (Poole and French, 1961) and embolism, the process through which thrombi break off and are carried in the blood stream to obstruct the flow of blood at a point far removed from the thrombosis. Wiseman (1686) and Hunter (1817) first suggested such clotting within the vessels might be as a result of the slowing of blood flow. Richardson (1858) also suggested abnormal coagulation was involved. Virchow (1863) proposed that three possible factors determine the site and extent of a thrombus.

1. Damage to the vessel wall
2. Alterations in the flow of the blood
3. Changes in the circulating blood

These three factors still form the central theme of modern views on the mechanism of thrombosis. Despite the discovery of platelets by Bizzozero (1882) with their accumulation on exposed subendothelial structures following injury to the vessel wall and the gradual elucidation of the coagulation mechanism over the last century, it has only been in the last two decades that our knowledge of thrombosis has begun to develop.

2.3. The Prethrombotic State

A great diversity in the manifestations of human thrombotic disease occurs throughout the human body. The mechanism of occlusion of a peripheral artery with atheroma probably has little similarity to the process of thrombus deposition in the deep calf veins of an immobile leg. The structure of such thrombi is different as is the natural history of the disease process and the recognised precipitating factors. It is improbable that similar changes would appear in blood as a prelude to two such dissimilar clinical events. Thus the successful prediction of the onset of thrombosis is associated with controversy and frustration. Among the many difficulties in the

prediction of the onset of thrombosis is in deciding which changes in haemostatic function indicate hypercoagulability. Reduction in the concentration of coagulation factors may be a reflection of active consumption of coagulant protein, as occurs in severe intravascular coagulation (Denson, 1977). However, low levels may arise due to failure of synthesis e.g. in liver failure (Bloom, 1975), a condition in which alterations in coagulant protein concentrations is easily misinterpreted. Only a minute proportion of zymogen need be activated for fibrin generation to ensue (Wessler and Yin, 1968) and it therefore seems likely that in most instances of hypercoagulability the fraction of coagulant protein consumed will be too small for reliable measurement. The thrombogenic potential resulting from increase in the concentration of coagulation factors is equally open to argument. Fibrinogen, factors V and VIII and fibrin-degradation products, may behave as acute-phase reactants and rise non-specifically in disease (Isacson and Nilsson, 1972),

Measurements for the activation of thrombin in the circulation may be of value in predicting patients susceptible to thrombosis. The assay of soluble fibrin monomer complexes (Bang and Chang, 1974) and fibrinopeptide A (Nossel, Yudelman, Canfield et al., 1974) have indicated that these derivatives of fibrinogen are present in the blood of normal individuals. Whilst the distribution of values in patients prone to thrombosis overlaps that found in the healthy population, this has

still to be ascertained for several clinical conditions associated with or resulting in future thrombotic events.

Measurements of the activation of platelets may also be of value in predicting thrombosis. Platelets might be more reactive than normal, a property that could be identified as a lowered threshold for aggregation or adhesion. Platelets which have taken part in the formation of a platelet thrombus might show a measurable increase in the release of platelet specific proteins eg BTG, which should be increased in the plasma and decreased in the platelet after platelet adherence and aggregation have occurred. The measurement of platelet release as an index of a prethrombotic state in several clinical conditions has still to be determined.

2.4. Thrombosis

One of the fundamental problems in elucidating the mechanism of thrombosis is that events occurring in thrombosis are similar to those which occur in the formation of a haemostatic clot. However a clot formed in vitro differs from a thrombus. A clot is a structure with the fibrin network uniformly mixed with the platelets and red cells evenly distributed within the fibrin network. An arterial thrombus consists of two parts, the white head (platelet thrombus) and the red tail (composed of red blood cells and fibrin). A venous thrombus is formed by an extensive fibrin network in which red blood cells are enmeshed. Thrombi may form anywhere in the circulation, in the arteries, veins, capillaries or chambers and valves of the heart. An occlusive thrombus occupies the whole lumen of the vessel and obstructs flow, whereas a mural

thrombus adheres to a localised area of the wall (usually of the heart) and blood continues to flow past the thrombus with microthrombi being released into the bloodstream. Occlusive thrombi do not often form in the aorta but are found more commonly in medium arteries and smaller vesels.

2.4.1. Mechanisms of Thrombosis

1. Damage to the vessel wall

Vasoactive substances

Tissue trauma, by exposing the blood to various extravascular structures, and by the release of active substances, may have an important role in thrombosis. In general, accumulated metabolic products and material released from damaged cells (e.g. lactate, potassium, hydrogen ion, adenosine) produce relaxation of vascular tone, restoration of flow to anoxic areas being paramount. A number of vasoactive substances attract particular interest because of their presence in platelets (prostaglandins, serotonin (5HT) and adenosine diphosphate (ADP). TXA_2, an unstable intermediate of platelet prostaglandin biosynthesis is a possible thrombogenic agent, being both a powerful platelet aggregating agent and also a potent vasoconstrictor (Needleman et al., 1976; Moncada and Vane, 1978). Serotonin released during the platelet release reaction, promotes further platelet aggregation and is a potent constrictor of peripheral vessels when released into the arterial circulation (Roddie, Shepherd and Whelan, 1955). ADP can promote vasoconstriction and platelet microthrombus formation in arteries (Begent and Born, 1970).

Inhibitor Systems

Loss of physiological defence mechanisms in the vessel wall may contribute to thrombosis. At least two inhibitor systems are known which protect the endothelium from platelet-aggregate formation. Vascular cells show strong ADPase activity (Lieberman, Lewis and Peters, 1977) which would tend to disperse platelet aggregates. However, a much more important defence mechanism is provided by prostacyclin (PGI_2), a prostaglandin synthesised in the vessel wall (see chapter 1.2.4.). PGI_2 is a vasodilator and powerfully inhibits both platelet aggregation and adhesion to the subendothelium and also promotes platelet disaggregation (Cazenave, Dejana, Kinlough-Rathbone et al., 1979). In the light of current knowledge PGI_2 is the most active of the agents which confer on the vascular endothelium a uniquely non-thrombogenic surface.

A marked reduction in PGI_2 production by the heart and vessel wall is found in rabbits made atherosclerotic (Dembinska-Kiec, Gryglewska, Zmuda et al., 1977). Similarly human atherosclerotic tissue does not produce PGI_2, whereas tissue obtained from an adjacent vessel does (Angelo, Villa, Mysliwiec et al., 1978). Production of prostacyclin seems to be the important arterial mechanism. Small fibrin thrombi formed on the vessel wall are probably normally cleared by fibrinolytic mechanisms. Plasminogen activator is produced by endothelial cells and released continuously into the bloodstream (Pandolfi, Nilsson, Robertson et al., 1967). In patients with venous thrombosis, fibrinolytic activity of the vessel

walls is impaired even at sites not actively involved in the thrombotic process (Pandolphi, Isacson and Nilsson, 1969). Failure of these normally protective functions of the vessel wall may promote thrombosis. Fibrinolysis seems to be the most important mechanism on the venous site.

Response to injury

Some indication of a possible role of the vessel wall in thrombogenesis comes from observations of the effects of injury. Endothelial cells may be damaged during intercurrent illness by circulating substances such as thrombin, bacterial endotoxin, and neuraminidase, all of which have been shown experimentally to injure endothelial cells and promote platelet and fibrin deposition (Barnhart and Chen, 1978). When the injury is minor, platelet thrombi form and parts break off to be washed away by the circulation. The degree of injury is an important determinant of the size of the thrombus: for the development of a large thrombus, severe damage or physical detachment of the endothelium must occur. This is in accord with the distribution of prostacyclin synthetase (see chapter 1.2.4.) from the intima to the adventitia, resulting in the vessel wall being thrombogenic. Homocystine, the amino acid present to excess in homocystinuria, has been shown to promote atherosclerosis-like lesions and thrombosis as a result of primary damage to the endothelium (Harker, Ross, Slichter et al 1976). In patients with clinical thrombosis uncertainty arises as to whether vascular damage has preceded or followed

the thrombotic process. However, endothelial damage has been observed both in the arteries and veins of women dying from pulmonary embolism, possibly induced by administration of oestrogen-containing contraceptive pills (Iray, Manion and Taylor, 1970) and in the coronary arteries of healthy young men who were the victims of violent death (Enos, Beyer and Holmes, 1955); evidence suggesting that endothelial damage precedes thrombosis.

2.42 Alterations in the Flow of Blood

In normal haemostasis, reduction in blood flow allows the haemostatic plug to withstand the disrupting effect of blood flow, thus sealing the damaged vessel wall until fibroblast growth ensures a permanent repair system. In thrombosis, disturbances in blood flow are believed to enhance the formation of a thrombus. Experiments using branched extracorporeal shunts have illustrated the importance of blood flow in thrombogenesis. Branched plastic tubes inserted between the carotid artery and jugular vein in pigs, resulted in the formation of platelet microthrombi downstream of flow dividers on the walls of the tube (Mustard, Murphy, Rowsell et al., 1962). Platelets were deposited preferentially at these sites of turbulent flow and did not attach elsewhere to the tube surface. There appears to be a notable similarity between the distribution of thrombi in this model and the siting of human thrombo-atherosclerotic deposits. Shear rate and the presence of red cells are critically important haemodynamic factors in thrombogenesis, at least in relation to platelet deposition. Platelet

deposition and platelet microthrombus formation have been shown to increase linearly with increasing shear rate (Begent and Born, 1970; Friedman, Liem, Grabowski et al., 1970; Turitto, Muggli and Baumgartner, 1977). Glass models of branched and curved arteries have shown that platelet microthrombus formation occurs selectively where turbulence and vortex formation can lead to stagnation point flow (Baldauf, Wurzinger and Kinder, 1978). Thrombosis may also be promoted by the effect of shear stress in sensitising blood platelets (Brown, Leverett, Lewis et al., 1975).

Experiments on platelet thrombus formation have also emphasised the importance of red cells. Red cells provide a major force contributing to the movement of platelets from the axial stream towards the vessel wall (Grabowski, Friedman and Leonard, 1972; Goldsmith, 1971). The presence of red cells increases diffusion of platelets to the vessel surface and increases platelet-surface collision energy (Brash, Brophy and Feuerstein, 1976), resulting in an increased rate of platelet microthrombus formation at the vessel surface (Turitto and Baumgartner, 1975; Cazenave, Packham, Davies et al., 1978).

Evidence which relates experimental obsevations directly to human disease is difficult to obtain. There is better evidence for the relevance of factors which by reducing blood flow may contribute to thrombogenesis as a

result of stasis in the vessel lumen. Patients with diabetic vascular disease (McMillan, 1976; Barnes, Locke, Scudder et al., 1977; Lowe, Morrice, Forbes et al., 1979) and severe peripheral vascular disease (Dormandy, Hoare, Colley et al., 1973) have increased blood viscosity compared to normal subjects. Thrombotic episodes in patients with polycythaemia are more frequent in those patients with the highest haemotocrits (Pearson and Weatherley-Mein, 1978), an effect probably mediated by slowing of flow since small changes in haematocrit lead to proportionally much larger changes in blood flow (Thomas, Du Boulay, Marshall et al., 1977). It seems all the more likely that reduction in blood flow may contribute to thrombosis, since it has been shown in vitro that following contact activation, thrombi form only in static blood (Botti and Ratnoff, 1964). Stasis may particularly encourage thrombosis on the venous side of the circulation. Pathological studies have shown that deep venous thrombi form preferentially at sites where ligaments or tendons may compress the vein, or valve cusps interrupt laminar flow (Sevitt and Gallagher, 1961).

Evidence for alterations in the flow of blood as a component in the formation of a thrombus remains circumstantial, until better methods become available for studying flow in the intact circulation. It is reasonable to infer that turbulence contributes to thrombosis in the arterial circulation, while stasis may be a more potent factor in the genesis of venous thrombi.

2.43. Changes in the circulating blood

(A) Platelets

There is substantial evidence that blood platelets are involved in the development of thrombosis, particularly in the arterial circulation (Mustard, Kinlough-Rathbone and Packham, 1974; Paton and Douglas, 1976; White and and Heptinstall, 1978; Mustard, Packham and Kinlough-Rathbone, 1978).

While platelets do not adhere to normal endothelial cells (Stemerman, 1974), they have been shown to adhere to exposed subendothelium (see Chapter 1.2.4.). Following attachment of the initial platelet monolayer, there is accretion of further platelets, a process probably controlled through the release of ADP (Cazenave, Packham, Guccione et al., 1975; Tschopp and Baumgartner, 1976). TXA_2 probably strongly augments this process by inducing further platelet aggregation and vasoconstriction (Moncada and Vane, 1978). Under certain circumstances a platelet microthrombus may form. Alternatively white cells and fibrin may add to the platelet mass building up a stabilised mature thrombus (Mustard et al., 1978).

Whether platelet aggregates can be present in the intact circulation is not known, although there are observations to suggest that they do occur (Begent and Born, 1970) and that such aggregates are found more frequently in patients with thromboembolic disorders (Dougherty, Levy and Weksler, 1977).

Adhesion or aggregation leading to secretion of platelet granule contents in the immediate vicinity of the blood vessel wall may produce vascular effects.

Platelets can release acid hydrolases (see Table 1.1) and also a cationic protein capable of altering vascular permeability (Nachman, Weksler and Ferris, 1972). Such substances have potentially destructive effects on the intima which may augment minor localised thrombotic activity.

Changes in the PGI_2 /TXA_2 balance (see chapter 1.2.3) might predispose towards thrombosis. Increased production of TXA_2 in vivo by platelets has been found in patients with arterial thrombosis and in recurrent venous thrombosis (Lagarde and Dechavanne, 1977). In addition, increased sensitivity to aggregating agents and increased release of TXA_2 -like activity has been described in patients who have survived myocardial infarction (Szczeklik, Gryglewski, Musial et al., 1978). Increased levels of TXB_2 (a stable end product of TXA_2) have also been observed in patients during attacks of angina (Lewy, Smith, Silver et al., 1979). Moreover, platelets from rats made diabetic, release more TXA_2 and their vessel walls produce less prostacyclin (Harrison, Reece and Johnson, 1978) and there are reports of similar results in man Johnson, Harrison, Raftery et al., 1979). A lowered prostacyclin production by cultured endothelial cells in the presence of β -thromboglobulin has been observed (Hope, Martin and Chesterman et al., 1979). This might occur in localised vascular segments during the platelet release in vivo, suggesting that the inhibitory effect of a platelet release product on PGI_2 synthesis might be a possible mechanism for promoting thrombosis, however this was not shown in a later study (Poggi, Niewiarowski, Holt et al., 1981).

An increased prostacyclin production has been reported in uraemia (Remuzzi, Cavenaghi, Mecca et al., 1977) and in the blood vessels of the spontaneously hypertensive rat (MacIntyre, Pearson and Gordon, 1978). Thus it seems that diseases which favour the development of thrombosis are associated with an increase in TXA_2 and a decrease in prostacyclin formation, whereas an increased prostacyclin plus decreased TXA_2 is present in some conditions associated with an increased bleeding tendency.

Interaction of platelets with blood coagulation may provide an additinal pathway by which slight vascular injury results in thrombosis. Platelets can initiate both the intrinsic pathway (Walsh, 1972a) and by stimulation of the extrinsic pathway accelerate the local rate of thrombin formation about one thousand-fold (Miletich, Jackson and Majerus, 1977). This combination of pro-thrombotic properties makes the platelet micro-environment an ideal nidus for initiation of thrombosis (Walsh, 1973).

(B) Coagulation

It seems inevitable that since fibrin deposition occurs in the formation of a thrombus, disturbance of the blood-coagulation mechanism should be a prime element in thrombogenesis. Activation of the intrinsic system has been shown by the formation of fibrin in static blood exposed to activators of factor XII (Botti and Ratnoff, 1964). Flow normally helps to prevent this by dispersal of the stimulus and mobilisation of plasma inhibitors. Most recognised activators of factor XII are charged

particles (Ratnoff, 1977) which rarely occur in the circulation. However, this may be part of the mechanism for intravascular thrombosis which follows amniotic fluid or fat embolism (Sharp, 1977). Contact activation may also result from exposure of the blood to free fatty acids (Nossel, 1976) and collagen (Wilner, Nossel and Leroy, 1968), bacterial endotoxin (Morrison and Cochrane, 1974), proteolytic enzymes (Kaplan and Austen, 1972), isoimmune 1gG (Lopas,Birndorf, Bell et al., 1973) and homocystine (Nossel, 1976). The circulation might be exposed to such compounds during illness or following trauma, leading to thrombus formation in stagnant flow. Purified preparations of factor XII and XI do not generate coagulant activity when exposed to kaolin (Schiffman and Lee, 1974) and it seems probable that contact activation of factor XII has a limited role both in haemostasis and in thrombosis because alternative pathways adequately compensate for deficiency of factor XII. Hageman (factor XII) deficiency is usually asymptomatic (Ratnoff, 1977) and John Hageman died of a pulmonary embolism. Most of those stimuli which might induce activation of factor XII in vivo also stimulate blood platelets and possibly generate intrinsic coagulation activity through the inherent pro-coagulant function of platelets (Walsh, 1974).

Involvement of the coagulation mechanism in thrombosis might more feasibly arise via the extrinsic pathway. This might occur by the activation of factor VII. Addition of tissue factor causes rapid generation of factor Xa which autocatalyses the reaction by conversion of native

factor VII to a more potent two-chain form (Radcliffe and Nemerson, 1975). Tissue factor, present in the plasma membrane of endothelial cells is likely to be released into the circulation not only by trauma but by superficial damage to the endothelium. Additionally factor VII may be activated by factors IXa and XIIa, plasmin and kallikrein (Lǽake and Osterud, 1974) which may prime the system for rapid generation of factor Xa after slight vascular injury.

It seems feasible that as fibrin deposition occurs in the formation of thrombi, thrombin generation might be involved in the earliest stages of thrombogenesis. Recent technical advances have resulted in sensitive assays for the demonstration of thrombin activity in the circulation eg fibrinopeptide A. The application of such assays to thrombosis will be discussed in greater detail in Chapter 4.

Thrombotic episodes have been reported to occur in patients with congenital deficiencies of clotting factors. Venous thrombosis occurred in patients with deficiency of factor VII (Godal, Madsen and Nissen - Meyer, 1962), and, as previously noted, factor XII (Ratnoff, Busse and Sheon, 1968). Also recent reports of acute myocardial infarction in severely affected haemophilic patients (Small, Jack, Lowe et al., 1983) raise the question of a central role for coagulation disturbance in the pathogenesis of thrombosis. Congenital deficiencies in the fibrinolytic system or in inhibitors such as anti-thrombin III may be associated with an increased susceptibility to thrombosis (Egeberg, 1965; Mackie,

Bennett, Ogston et al., 1978; Barrowcliffe, Johnson and Thomas, 1978). More recently a congenital deficiency of protein C has been discovered in families susceptible to thrombosis (Griffin, et al., 1981, Bertina et al., 1982). Protein C is thought to limit Xa and thrombin formation. In addition to a potent stimulatory effect on fibrinolysis it is possible that protein C is involved in the regulation of thrombus growth.

Congenital dysfibrinogenaemia has been associated with thrombotic episodes as described in fibrinogens Baltimore (Beck, Charache and Jackson, 1963), New York (Mondiry, Bilezikian and Nossel, 1975), Marburg (Fuchs, Egbring and Havenmann, 1977) and Copenhagen (Sandbjerg-Hansen and Clemmensen, 1980), but it is not known, except in the case of fibrinogen New York, if the dysfibrinogenaemia is responsible for the thrombosis. In fibrinogen, New York, decreased binding of thrombin by the clot was observed and it was assumed that it might be the cause of the thrombotic disorder (Liu, Nossel and Kaplan, 1979).

It has been postulated that fibrin deposition and subsequent thrombosis might result from the failure of fibrin to absorb onto its surface the required plasminogen and activator needed to mediate its own destruction (Gaffney 1981). Recently a new type of congenital dysfibrinogenaemia with defective fibrinolysis -Dusard syndrome - has been found in a family with recurrent thrombosis (Soria, Soria and Caen, 1983). The thrombosis was attributed to the abnormal polymerisation of fibrin which did not allow the exposure of binding sites for plasminogen on the fibrin surface, resulting in ineffective thrombolysis.

Astrup (1956) postulated that the patency of the vascular system depended on a dynamic equilibrium between constantly active coagulation and fibrinolytic systems. Reviews of this hypothesis concluded that neither thrombin nor plasmin proteolysis makes a major contribution to fibrinogen turnover in normal individuals and that the hypothesis of a dynamic equilibrium between clotting and lysis remains unproven (McNicol and Douglas, 1976). Nossel (1981) proposed an alternative view that the relative rates of proteolysis of the B β chain of fibrinogen by thrombin and plasmin determine the occurrence of thrombosis (see figure 2.1).

If a single fibrinogen molecule is considered, in reaction (1), thrombin cleaves fibrinogen to yield free FPA and fibrin I monomer which polymerises in reaction (2). Thrombin and plasmin then compete for the NH_2 -terminal end of the B β chain. If thrombin cleaves the Bβ chain before plasmin the products are FPB and fibrin II. If plasmin cleavage precedes that of thrombin the products are B β 1-42 and fragment X. The balance between the rates of fibrin II and fragment X formation would then determine the pathophysiological consequences of fibrin I generation. Predominant fibrin II formation results in thrombosis whereas predominant fragment X does not.

Problems associated with the thrombotic mechanism

The findings of an association between an abnormal blood test and thrombosis does not imply that the former abnormality promoted the latter event. Thrombosis may equally well give rise to the blood changes: while such changes may find clinical use as 'markers' of thrombosis, they may not reflect the thrombus per se, but some other factor associated with thrombosis. The

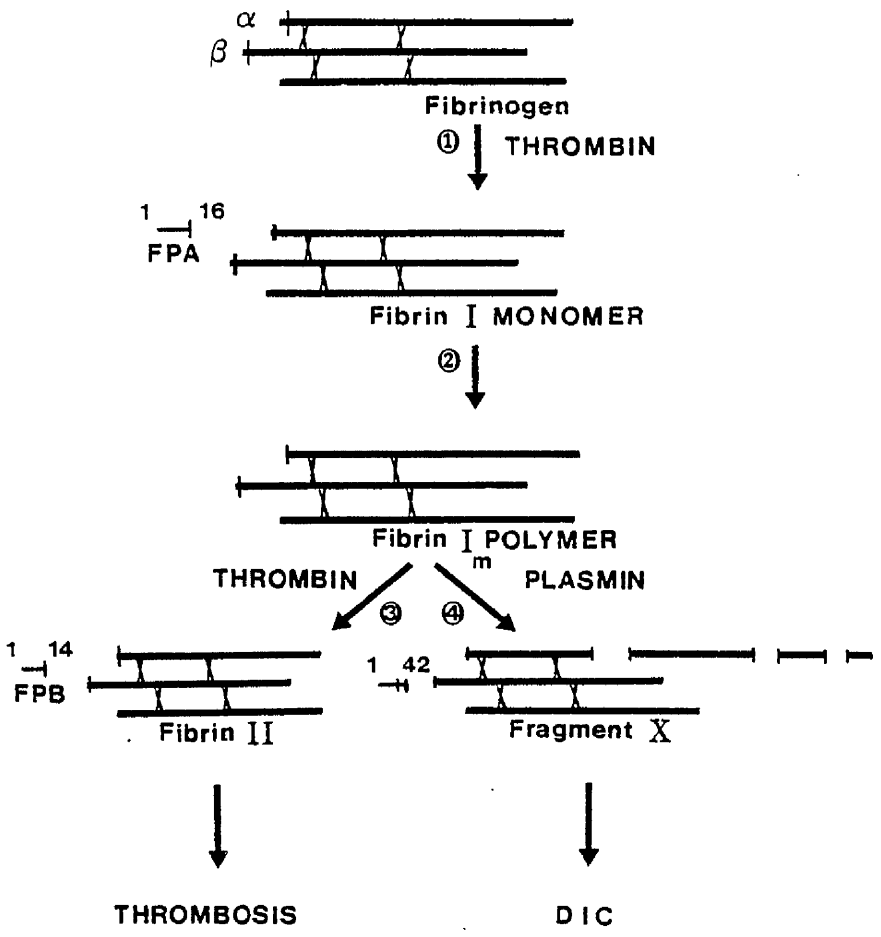


Figure 2.1.

Postulated scheme of fibrinogen proteolysis *in vivo*. Half fibrinogen molecules are depicted. (Taken from Nossel, 1981).

relationship between a blood test and thrombosis may therefore be causal (abnormal blood mechanisms promotes thrombosis), consequential (thrombosis promotes change in blood), or coincidental (thrombosis and blood test are each associated with another factor or factors, known or unknown). Epidemiological differences, as well as histological differences (platelets predominate in arterial thrombi, fibrin in venous thrombi) and differences in therapeutic response (eg anticoagulants), demand that we consider arterial thrombosis and venous thrombosis rather differently. Clinical studies should, involve matching control subjects and thrombotic subjects for such basic factors as age, sex and smoking. As will be discussed throughout this thesis it is only by taking such essential factors into account that appropriate conclusions can be made about the results obtained in clinical studies.

2.5. Disseminated Intravascular Coagulation (D.I.C)

As previously described (2.1) D.I.C. results in the formation of microthrombi and insoluble fibrin deposits throughout the vasculature. The severe disturbance of haemostasis results in abnormal bleeding, small vessel obstruction by fibrin and sometimes by platelets, in tissue necrosis, and in multiple organ dysfunction.

Mechanism of D.I.C.

The initial event in D.I.C. is the activation of the coagulation sequence. This activation causes consumption of coagulation factors and platelets, with fibrin deposition in the microcirculation. Fibrin deposition provokes secondary fibrinolysis; the combination of fibrinolysis with the lack of platelets and coagulation factors leads to a bleeding tendency. At the same time, fibrin deposits occlude small blood vessels and cause ischaemic damage to various organs and may lead to a microangiopathic haemolytic anaemia. This sequence of events is shown in Figure 2.2 and will be discussed below.

Intravascular Blood Coagulation

Three major mechanisms in the activation of coagulation leading to D.I.C. have been suggested by Muller-Berghaus (1977).

1. Direct activation of prothrombin or factor X by a proteolytic enzyme or enzymes.

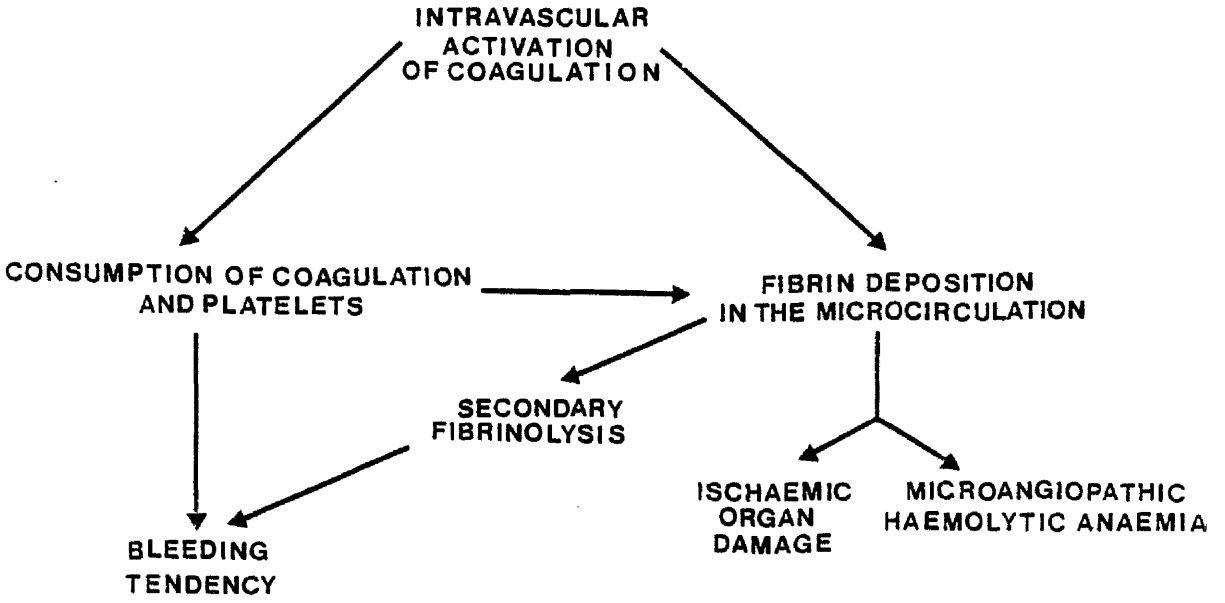


Figure 2.2

Sequence of events in disseminated intravascular coagulation (taken from Brozovic, 1981).

2. Activation via the extrinsic system by the release of tissue thromboplastin.
3. Activation through Hageman factor (factor XII) and the intrinsic system.

Activation of the coagulation system is believed to be initiated by several 'trigger' mechanisms as outlined below.

Erythrocytes

Red cell stroma carries potent thromboplastic activity and in mechanical haemolysis may activate coagulation through the extrinsic pathway (Muller-Berghaus, 1977). Immunologically induced haemolysis probably induces D.I.C. through antigen-antibody complexes.

Platelets

All the triggers of intravascular coagulation can induce platelet aggregation and release. Thrombin generated during the activation will enhance platelet reactions and cause a further fall in the platelet count. However, platelets are not essential for the development of D.I.C., as it can be induced in profoundly thrombocytopenic animals (Muller-Berghaus, 1977).

Leukocytes

Leukocytes are the principal target cells for endotoxin in the circulating blood, both in man and in experimental animals. Administration of endotoxin causes a fall in the white cell count, affecting the neutrophils in particular (Cline, Melmon, Davis et al.,1968).

The damaged cells release procoagulant tissue-thromboplastin-like material. Monocytes can also release procoagulant activity after exposure to endotoxin. Blast cells in acute leukaemia have a high content of procoagulants and D.I.C. commonly occurs in this condition (Gralnick and Abrell, 1973).

Vessel Wall

As previously described, endothelial damage and the exposure of subendothelial structures is associated with the activation of Hageman factor, release of tissue thromboplastin and initiation of platelet reactions, each of which can trigger or accelerate D.I.C. Minimal endothelial damage may interfere with prostacyclin formation and enhance platelet reactions leading to low grade D.I.C.

Complement

The complement system is activated in vivo and in vitro by the same triggers, but a direct functional relationship between the two systems is not clearly established (Brown, 1975; Muller-Berghaus, 1977). Complement activation affects the clinical course of D.I.C. by changing the reactivity and permeability of the vessel wall, neutrophil chemotaxis and platelet aggregation (Pfueller and Luscher, 1972).

Antigen-antibody complexes

Immune complexes are able to activate coaguation in several ways. They may activate factor XII directly (McKay, 1972) or damage the endothelial cells with

subsequent indirect activation of the intrinsic pathway. They may also activate the complement system resulting in release of procoagulants which may induce D.I.C (Götze and Müller-Eberhard, 1971). Immune complexes can cause platelet aggregation and the release reaction, thus accelerating D.I.C.

The role of modifying factors

A number of factors have been suggested as determining the severity and spread of D.I.C. as discussed below.

Blockade of the reticulo-endothelial system (RES)

Normally the RES protects the individual from D.I.C. by removing activated clotting factors and fibrin monomer complexes as well as the trigger itself. Experimental blockage of the RES leads to fulminant D.I.C., as in the Schwartzman reaction (Beller, 1969). Blockage also results in increased frequency of D.I.C. in liver disease and pregnancy (Evensen and Hjort, 1970).

Inhibition of fibrinolysis

The failure to lyse fibrin deposited in the microcirculation leads to severe organ damage and to further acceleration of D.I.C. Antifibrinolytic agents enhance D.I.C. in man (McKay and Muller-Berghaus, 1967) and when given in combination with thrombin cause D.I.C. in experimental animals (Margarettan, Zunker and McKay, 1964).

Stimulation of the adrenergic system

Many experiments point to the importance of catecholamines in D.I.C. (Muller-Berghaus, 1977). For example, stimulation of α receptors affects the peripheral blood flow and plays an important role in the precipitation of fibrin in the skin and the kidney.

Elevated plasma lipids

The combination of lipid infusion and endotoxin injection provokes severe D.I.C. in experimental animals (Huth, Schoenborn and Knorpp,1967). This may result from blockade of the RES, inhibition of fibrinolysis or as a result of the procoagulant effect of lipids (Evensen and Hjort, 1970).

Reduced plasma inhbitors

Reduced plasma levels of antithrombin III are found in liver disease, acute phase reactions, after injury and in congenital deficiency. In all these conditions there is an increased frequency of thrombosis and D.I.C. Consumption of inhibitors at a later stage of D.I.C. perpetuates the process.

Pregnancy

It is known that pregnancy predisposes to D.I.C. Endotoxin administered to pregnant animals may result in the Shwartzman reaction. A marked reduction in fibrinolytic activity has been found in pregnancy (Astedt, Isacson, Nilsson et al., 1970).

Liver disease

Inadequate clearance mechanisms, poor synthesis of coagulation factors and a generalised metabolic disturbance contribute to a high frequency of D.I.C, in liver disease.

Consumption of Coagulation Factors

Activation of the coagulation sequence leads to the consumption of circulating coagulation factors and platelets. The factors involved are those consumed during normal coagulation: factor V, VIII, prothrombin, fibrinogen,

factor XIII and to a lesser extent, factor X. In acute D.I.C. low levels or total absence of factors V and VIII, reduced prothrombin levels and an almost total absence of clottable fibrinogen are commonly found. In sub-acute D.I.C. factors V and VIII are decreased, with prothrombin remaining almost normal and plasma fibrinogen varying from total absence to near normal values. In chronic or low grade D.I.C. the consumption may be minimal, and the intravascular activation may give rise to fallaciously high values for the clotting factor assays. Fibrinogen, factor VIII and V may also be raised, a result of the compensation process (Cooper, Bowie and Owen, 1974) where increased synthesis of coagulation factors in response to low grade D.I.C. leads to true high plasma levels.

Fibrin deposition

The end result of the coagulation sequence activation is the formation of intravascular fibrin. The site and extent of fibrin deposition depend on the relationship between thrombin-fibrinogen and plasmin-fibrin interactions, on the stage of D.I.C., and on various factors that effect localisation of fibrin deposits.

Thrombin-fibrinogen reaction

Thrombin cleaves first fibrinopeptide A, then fibrinopeptide B from fibrinogen (see 1.3, 2e) polymerisation begins before a significant amount of fibrinopeptide B is released (Doolittle, 1977). Soluble fibrin-fibrinogen complexes lacking only one of the four fibrinopeptides may be formed. These complexes can be precipitated by ethanol or protamine sulphate in vitro and their presence in plasma denotes that the circulating

fibrinogen is partly degraded. The complexes are removed by the RES, but precipitation of fibrin may occur as the next step if the activation process accelerates or if localising factors, such as stasis favour it.

Plasmin-fibrin(ogen) reactions

Plasmin cleaves many different fragments from fibrin and fibrinogen. The largest of those (X and larger) are clottable and can, therefore, polymerise with fibrin monomers into soluble complexes or can be precipitated with them. The extent of fibrin deposition and organ damage depends mainly on the efficacy of the fibrinolytic system.

Morphology and localisation of fibrin deposition

In the early stages of D.I.C., spherical fibrin structures can be shown by the electron microscope on the surface of RES cells in the spleen and the liver (Stewart, 1971).

In the later stages micro and macrothrombi are found in the majority of patients dying with D.I.C. (Minna, Robboy and Colman, 1974).

Fibrin thrombi are observed more frequently in the kidney than in the other organs. McKay (1970) suggested that most thrombi form in systemic circulation as fibrin aggregates, and that by a sieving effect they are eventually caught in smaller vessels. Because of its rich microvasculature, the kidney acts as an excellent sieve.

A variety of skin lesions are associated with D.I.C. eg gangrene. Fibrin thrombi are invariably demonstrated

in the capillaries and venules of the papillary dermis and occasionally of the reticular dermis and subcutaneous tissues (Minna et al., 1974).

The pathogenesis of lung lesions in D.I.C. is complex. At the early stages platelet-rich micro-thrombi are commonly observed (Bleyl, 1977) owing to the sieving effect of the lungs; the lungs have a similar role in the venous circulation to that of the kidney on the arterial side. The platelet rich microthrombi may convert to fibrin-rich thrombi after the destruction of platelets. At a later stage of D.I.C. characteristic lung lesions consist of fibrin deposits both intravascularly and extravascularly in the alveoli. Although the formation of intravascular fibrin deposits is easily explained in the presence of D.I.C., the origin of extravascular fibrin is more difficult to interpret. It is possible that it arises as the result of extravasation of soluble fibrin monomer complexes that later precipitates.

Secondary fibrinolysis

Different mechanisms may account for the generalised activation of fibrinolysis in D.I.C. eg (A) the trigger that initiates intravascular coagulation may also activate the fibrinolytic sequence, (B) the activated form of Hageman factor induces fibrinolysis simultaneously with the activation of the intrinsic pathway and the kallikrein-kinin system (Ogston and Bennett, 1977),

(C) proteases other than plasmin, derived from damaged cells may also participate in the fibrinolytic process (Latallo et al., 1978) and (D) the lack of inhibitors, which are rapidly consumed in the early stages of D.I.C. enhances generalised fibrinolysis.

2.6. Atherosclerosis

Recently endothelial cells have been found to generate part of the recognised properties of the factor VIII molecule. Endothelial cells synthesise von Willebrand factor (VIII:vWF) and also the moiety recognised by antisera to factor VIII raised in rabbits (FVIII R:Ag), but the coagulant property (FVIII:C) is not produced there. The physiological role of this site of synthesis is unknown, as yet. Factor VIII has no accurately defined vascular function. However, there is increasing circumstantial evidence to suggest that von Willebrand factor may be involved in platelet-vessel-wall interactions: in the absence of von Willebrand factor, platelet adhesion to subendothelium is reduced (Weiss, Baumgartner, Tschopp et al., 1978) and in subjects with von Willebrand's disease, platelet plug formation at sites of injury is defective (Jorgensen and Borchgrevink, 1964). Pigs with von Willebrand's disease appear to be resistant to the development of atherosclerosis (Fuster and Bowie, 1978; Fuster, Bowie, Lewis et al., 1979); implying that platelet adhesion to the vessel wall mediated by von Willebrand factor may

play a part in atherogenesis. Further evidence of a vascular role in the development of atheroma comes from the finding that the smooth muscle cells proliferating in the fibro-muscular lesions are monoclonal in origin (Benditt and Benditt, 1973), a property conventionally associated with tumour cells. Atheromatous plaques may, therefore, be analogous to benign tumours, smooth muscle cells continuing to proliferate spontaneously following an initiating stimulus at the time of vascular injury. Platelets may also contribute to atherogenesis by the provision of a cell mitogen which stimulates smooth muscle cell proliferation (Ross, Glomset and Harker, 1977). Further evidence to support this hypothesis comes from observations that platelet material can be demonstrated in histological sections from human atheromatous plaques (Woolf, 1978) and that the induction of thrombocytopenia can protect against experimental atherosclerosis in animals (Moore, Friedman, Singal et al., 1976).

There are strong indications that disturbances in the PGI_2 /TXA_2 balance are involved in atherogenesis. Lipid peroxides are potent inhibitors of prostcyclin synthetase (Moncada et al., 1976) and selective inhibition of prostacyclin formation by these substances could lead to increased platelet aggregation, which in turn could play a role in the atherogenesis. Indeed, lipid peroxidation takes place in plasma as a non-enzymic reaction and atherosclerotic plaques contain

lipid peroxides (Slater, 1972; Glavind, Hartmann, Clemmesen et al., 1952). Thus, lipid peroxides could be shifting the balance of the system in favour of TXA_2, and may predispose to thrombus formation. As described previously, reduced prostacyclin formation occurs in the hearts at vessel walls of rabbits made atheroslerotic and also in human atherosclerotic tissue. In addition, increased release of TXA_2-like has been described in rabbits made atherosclerotic by diet (Shimanoto, Kobayashi, Takahashi et al., 1978).

CHAPTER 3

β-THROMBOGLOBULIN: AN INDEX OF IN VIVO PLATELET RELEASE

3.1. Introduction

The adhesion of platelets to the injured vessel wall with release of granule constituents and the resulting formation of platelet aggregates are important initiating mechanisms in thrombosis, as discussed in the previous chapter. The occurrence of arterial thrombi on atheromatous plaques and the predominance of platelets in arterial thrombi suggest that platelet activation and release might be especially relevant to thrombosis in the arterial circulation.

3.2. In Vitro Tests of Platelet Activation

Within the last few decades, several in vitro tests have been developed as markers of platelet activation viz:

1. Bleeding time

The skin bleeding time is thought to assess platelet interaction with the vessel wall to form the primary haemostatic plug. Micropuncture of an arteriole or venule causes bleeding and the time taken to arrest the bleeding is measured.

A shortened bleeding time has been described in patients with myocardial infarction and atherosclerosis (O'Brien, Etherington, Jamieson et al., 1974; O'Brien, Etherington, Jamieson et al., 1975) as well as in subjects at risk of arterial disease due to diabetes or type II hyperlipoproteinaemia (Joist, Baker and Smith, 1978; Joist, Baker and Schoenfeld, 1979).

2. Platelet survival

The lifespan of platelets can be assessed using radiolabelled platelets. Sodium chromate (^{51}Cr) is the most reliable label although it has the technical problem that it binds to plasma proteins, erythrocytes, leukocytes and platelets, hence the platelets require to be isolated and labelled in vitro. Once the radio-labelled platelets are reinfused, approximately 50-70% remain in the circulation. The pattern of survival of ^{51}Cr-labelled platelets is linear in normal subjects (Harker, 1978) but where platelets are subject to abnormal destructive process or interaction with damaged vascular endothelium, an exponential disappearance pattern is seen (Murphy, Francis and Mustard, 1972). A large number of studies have now associated decreased platelet survival with the likelihood of arterial thrombosis (Murphy and Mustard, 1962; Steele, Battock and Genton, 1975) although others have not (Abrahamsen, 1968; Harker and Slichter, 1974).

Decreased platelet survival has also been found in venous thrombosis in the acute stage (Abrahamsen, 1968; Harker and Slichter, 1974), in patients with recurrent thrombosis in the non-acute phase (Steele, Weily and Genton, 1973), and in patients with risk associations for

venous thrombosis such as advancing age, surgery and malignancy (Abrahamsen, 1968). Thromboembolism from abnormal heart valves also results in a decreased platelet survival (Steele, Weily, Davies et al., 1974; Weily, Steele, Davies et al., 1974).

3. Platelet production

An alternative approach to assessment of platelet survival is measurement of platelet production time using platelet formation of malondialdehyde (MDA) which is blocked for the lifespan of the platelet by a single dose of aspirin. The time taken for MDA formation to return to baseline values is measured, and has been found to correlate well with survival of radiolabelled platelets (Stuart, Murphy and Oski, 1975). Shortened platelet production time has been reported in subjects with peripheral arterial disease (Cella, Zahavi, de Haas et al., 1979).

4. Platelet count

The circulating platelet count reflects a balance between production and survival. High platelet counts are associated with increased rates of aggregation as well as with spontaneous aggregation in vitro, increased 'circulating' platelet aggregates, and response to treatment with aspirin in vivo.

Increased platelet counts have been associated with arterial or venous thrombosis. In arterial diseases and in patients with risk associations for arterial disease

studies have generally found platelet counts to be lower, not higher and it has been suggested that these lower platelet counts reflect shortened platelet survival (O'Brien, 1977).

5. Platelet volume

This is the circulating platelet cell mass per volume of blood. Since younger platelets are larger, increased mean platelet volume may reflect increased platelet turnover and hence decreased mean platelet age (Karpatkin, 1978). However, platelet size is effected by platelet isolation methods, hereditary factors, and disturbance of megakaryocyte growth and release. An increased platelet cell volume has been reported in patients after myocardial infarction and with chronic arterial diseases or diabetes (Enticknap, Gooding, Lansley et al., 1969). However, since these patients also have lower platelet counts, their circulating platelet cell mass per volume of blood remains normal (O'Brien, 1977).

6. Platelet adhesion

Platelet adhesion ex vivo has usually been measured by exposure of freshly-drawn blood to glass or collagen-coated surfaces. The most widely used methods are those in which fresh or anticoagulated blood is drawn through plastic tubes containing glass beads: the fall in platelet count due to platelet retention in the column is measured. Such tests in fact measure platelet adhesion as well as aggregation. Results are influenced by many factors including flow rate, haematocrit and von Willebrand factor.

Platelet retention may be increased in acute venous thrombosis but is normal in recurrent venous thrombosis (Isacson and Nilsson, 1972; O'Brien, et al., 1975) and in arterial diseases.

7. Platelet aggregation

Platelet aggregation in vitro has usually been measured in citrated platelet-rich plasma using the photometric technique of Born (1962). A parallel beam of light from a tungsten source is shone through a cuvette containing a sample of platelet rich plasma. Maximal interference of light transmission occurs when the platelets are evenly distributed throughout the plasma. Upon addition of an aggregation stimulus such as ADP, adrenaline, collagen and thrombin, an initial increase in optical density occurs as the platelets change shape. This is quickly followed by a rapid decrease in optical density as the platelets clump together, allowing more light through the cuvette.

Circulating platelet aggregates can also be measured. In the method of Wu and Hoak (1974) blood is drawn into a syringe containing EDTA (which has been shown to disperse platelet aggregates, at least those induced by ADP) or EDTA plus formalin (which fixes aggregates). The ratio of platelet

counts in the two samples, after centrifugation to obtain platelet rich plasma, gives a measure of fixed platelet aggregates which are spun down with the red cells.

In the method of Hornstra (1978), blood is drawn through a microfilter at a constant rate: platelet aggregates obstruct the filter and cause an increased pressure gradient across the filter which is continuously recorded.

Increased aggregation has been found in diabetics and in type II hyperlipoproteinaemia by some workers (Carvalho, Colman and Lees, 1974; Tremoli, Maderna, Sirtori et al., 1979) but not by others (Joist et al., 1979; Lowe, Drummond, Third et al., 1979).

Increased platelet aggregates have been reported in arterial diseases such as acute myocardial infarction, stroke or transient cerebral ischaemia (Wu and Hoak, 1974; Dougherty, Levy and Webster, 1977; Lowe, Reavey and Johnston et al., 1979; Schwartz, Hawiger, Timmons et al., 1980); others have not confirmed this (Prazich, Rapaport, Samples et al., 1977). There are also conflicting reports in chronic arterial disease (Wu and Hoak, 1974; Lowe et al., 1979; Jonker and den Ottolander, 1979). Increased aggregates have been reported in diabetes (Preston, Ward, Marcola et al., 1978; Gensini, Abbate, Favilla et al., 1979) and hyperlipidaemia (Wu, Armstrong, Hoak et al., 1975; Lowe, Johnston, Drummond et al., 1979., Lowe, Drummond, Third et al., 1979). Increased platelet aggregates are not specific for vascular disease as they have been reported in patients with infection or malignancy (Lowe et al., 1979d).

8. Platelet shape change

Platelet shape change following stimulation with aggregating agents is reflected in the photo-aggregometer as a fall in light transmission prior to the increased transmission which occurs on aggregation. Shape change appears also to influence changes in platelet electrophoretic mobility in response to low doses of aggregating agents. Increased sensitivity to such mobility changes has been described in women taking oral contraceptives aa well as patients with arterial disease or hyperlipidaemia: plasma factors, possibly lipids, appear to be responsible (Hampton and Mitchell, 1974).

9. Platelet coagulant activity

Increased platelet factor 3 (PF_3) activity has been found in patients with type II hyperlipoproteinaemia (Nordoy and Rodset, 1971). Collagen induced coagulant activity has been reported to be increased in patients with transient cerebral ischaemia (Walsh, Pareti and Corbett, 1976), acute retinal vein occlusion (Walsh, Goldberg, Tax et al., 1977) and in patients following hip surgery who subsequently developed venous thrombosis (Walsh, Rogers, Marder et al., 1976).

Although some of the tests discussed are relatively simple and involve little interference with circulating platelets: the information they reveal is probably relevant to platelet behaviour in vivo but with little insight into underlying mechanisms. Other tests involve more drastic interference with the platelet

environment, such as anticoagulation, separation from other cells, and exposure to foreign surfaces: they allow study of selective phenomena in relative isolation at the cost of removing other influences normally present as well as the possible introduction of artefacts. The isolation of platelets from whole blood involves risk of activation, deactivation and selection, due to anti-coagulation, mechanical factors (traumatic venepuncture, shaking, haemolysis, centrifugation and changes in pH or temperature (for review see Sixma, 1978). Thus, most of these tests have not proven to be effective in detecting thrombosis or a "predisposition" towards thrombosis with conflicting results often being obtained as discussed.

3.3. β-Thromboglobulin

3.3.1. Introduction

Platelets contain a number of granules in which a variety of substances are stored and which are subsequently released from the platelet when they are activated (see Table 1.1). Proteins are localised in particular in the α-granules. β-thromboglobulin (BTG) was one of the first proteins to be isolated and characterised. Sensitive radioimmunoassays of BTG have been developed allowing its measurement in platlet free plasma. While it is recognised that such assays are also subject to the experimental artefacts discussed previously, plasma concentrations of BTG should provide, in theory, a more sensitive and specific index of in-vivo platelet activation and release.

The present knowledge about BTG with regard to its chemistry, measurement, release and the measurement of BTG levels in clinical studies as an indicator of in vivo platelet release in various disorders will be reviewed.

3.3.2. Isolation and characterisation of BTG and related proteins

Almost 20 years ago, Salmon and Bonameaux (1958) first reported the presence of beta-globulins in platelets. Subsequently, Grette (1962) demonstrated proteins in the soluble material released by platelets during thrombin induced aggregation and it was shown later that this material contained platelet specific alpha and beta globulins (Davey and Lüscher, 1968). It was first isolated by Moore, Pepper and Cash (1975a), and by Moore and Pepper (1976). Because of its β-globulin mobility on cellulose acetate electrophoresis and its localisation in platelets, it was called β-thromboglobulin.

BTG was found to have a molecular weight of 36,000 by sucrose density centrifugation. Amino acid sequencing studies demonstrated the molecule to be composed of four subunits of 81 aminoacids each with a molecular weight of 8851 (Begg, Pepper, Chesterman et al., 1978). When the amino acid sequence of BTG is aligned with that of platelet factor 4 (PF_4) (another alpha granule protein that is chemically and immunologically distinct from BTG) 42 of the 81 residues are identical with PF_4 residues (Begg et al., 1978).

Recently it has been shown that BTG has an iso-electric point of pH 7.0, with another closely related protein, "low-affinity platelet factor 4" (LA-PF_4) having an iso-electric point of pH 8.0 (Rucinski, Niewiarowski, James et al., 1979).

LA-PF_4 appears to differ from BTG only in its four amino terminal amino acids, which are Asn - Leu - Ala - Lys. Residues 5-12 of LA-PF_4 have been shown to be identical to residues 1-8 of BTG (Rucinski et al., 1979). Experimental studies seem to indicate that LA-PF_4 is originally secreted by the platelets and then converted to BTG by a platelet secretory protease (Niewiarowski, Walz, James et al., 1980). The available anti-BTG and anti-LA-PF_4 antibodies do not differentiate between both proteins (Rucinski et al., 1979). It appears that BTG is a proteolytic product of LA-PF_4 , whether the proteolytic conversion occurs in vivo is not clear (Holt and Niewiarowski, 1979).

3.3.3. Content and localisation of BTG

BTG, LA-PF_4 and PF_4 appear to be platelet specific (Moore, Pepper and Cash, 1975a; Moore, Pepper and Cash, 1975b; Niewiarowski, 1977). The concentration of BTG in various washed organ samples was measured by radio-immunoassay and only trace amounts were detected. It is of interest to note that the highest concentration of BTG was found in the spleen and this may reflect their site of destruction (Ludlam, 1979)(see Table 3.1.).

TABLE 3.1.

BTG CONTENT OF VARIOUS TISSUES

	ng/gm net weight tissue
Brain	3.8
Kidney	5.6
Skeletal muscle	4.0
Stomach	4.2
Lung ventricle	9.0
Lung	30.4
Liver	40.0
Spleen	92.0
Platelets	1,240,000

In addition Kaplan found that PF_4 and BTG antibodies did not crossreact with haemolysate, leucocyte extract, human albumin or fibrinogen (Kaplan, Nossel, Drillings et al., 1978). BTG has been estimated to represent at least 0.5% and probably 2.5% of the total platelet dry weight, and at least 10% by weight of the granule content (Moore and Pepper, 1976). LA-PF_4 and PF_4 constitute 1.6% and 0.8% respectively of secreted proteins (Rucinski et al., 1979). The normal platelet content of BTG is 18 ug/10^9 platelets (Weiss, Witte, Kaplan et al., 1979) while that of LA-PF_4 is 24 ug/10^9 platelets (Rucinski et al., 1979).

BTG, LA-PF_4 and PF_4 are localised in the alpha granules of the platelets, together with platelet derived growth factor and fibrinogen. This has been demonstrated by subcellular fractionation with sucrose density gradient centrifugation followed by assay of the fractions obtained (Kaplan, Broekman, Chernoff et al., 1979; Fukami, Niewiarowski, Rucinski et al., 1979) or morphological study with electron microscopy (Ryo, Proffitt and Devel, 1980). Additional evidence comes from studies of in vitro release of BTG and PF_4 in comparison to the release of substances from dense bodies (serotonin, ATP or ADP) and the acid hydrolase containing granules (Kaplan et al., 1979; Ryo et al., 1980; Witte, Kaplan, Nossel et al., 1978).

3.3.4. In vitro release of BTG

In vitro release of BTG only becomes significant after seven minutes as reported by Ludlam and Cash (1976),

with BTG and PF_4 appearing in the serum simultaneously in similar concentrations (Dawes, Smith and Pepper, 1978). BTG and PF_4 can be released from blood anticoagulated with either EDTA, citrate or heparin, with the release being temperature and pH dependent (Ryo et al., 1980; Witte et al., 1978).

In vitro release of BTG and PF_4 induced by various pro-aggregating agents like ADP, collagen and thrombin has also been studied.

ADP or epinephrine do not induce release of BTG and PF_4 with primary aggregation, but during biphasic aggregation both platelet proteins are released in a similar manner (Kaplan et al., 1978; Ryo etal., 1980). ADP induced release of BTG and PF_4 is similar to that of ^{14}C-serotonin but beta-glucuronidase, a lysosomal is not released by ADP (Ryo et al., 1980).

Collagen induces release of BTG, PF_4, ^{14}C-serotonin, platelet derived growth factor (PDGF) and fibrinogen although such release is dose dependent (Kaplan et al., 1978; Witte et al., 1978).

Thrombin also induces release of BTG, PF_4, ^{14}C-serotonin, PDGF and fibrinogen, however, higher thrombin concentrations are required for the release of the dense granule components, serotonin and ADP, and even higher doses are required for acid hydrolase release (Witte et al., 1978).

BTG and PF_4 release is also induced by arachidonic acid and by the endoperoxide analogue U46619 (Kaplan et al., 1979) with a somewhat smaller release of ^{14}C-serotonin.

These studies indicate that the release mechanism for dense granule release, alpha-granule release and lysosomal enzyme release are different (Kaplan et al., 1979; Ryo et al., 1980).

3.3.5. Biologic activity of BTG

The exact biological functions of BTG remain unclear but some biological activities have been recognised. While it was originally thought that BTG had antiplasmin activity (Moore et al., 1975) this activity was later shown to be separable from BTG (Joist, Niewiarowski, Nath et al., 1976). Because of its abundance in platelets, it has been suggested BTG might be a matrix or packing protein which helps to stabilise the active constituents and is readily released by biological stimuli (Moore and Pepper, 1976).

BTG has a weak heparin-neutralising activity (Moore and Pepper, 1976). The specific antiheparin activity of BTG, LA-PF_4 is 1.9, 2.6 and 17 heparin units/mg protein respectively (Rucinski et al., 1979).

It has been suggested that BTG inhibited production of PGI_2 by cultured endothelial cells, suggesting a role for BTG in the process of thrombosis (see Chapter 2.4.3) but this observation was not confirmed in a later study (Poggi et al., 1981).

As recently discussed by Kaplan (1980) whether BTG has cell-growth stimulating properties remains to be established.

3.3.6. Metabolism and clearance of BTG

The clearance of BTG and PF_4 was first studied with the infusion of serum or platelet concentrate into normal individuals (Dawes et al., 1978). The half-life of BTG clearance was estimated to be about 100 minutes, with PF_4 being cleared too rapidly to allow determination of the half-life. The rapid clearance of PF_4 may be related to its binding to endothelial cells (Busch, Dawes, Pepper et al., 1979; Pumphrey, Pepper and Dawes, 1979). BTG is catabolised by the kidney, with such catabolism by the kidney being more important than renal excretion (Bastl, Musial, Kloczewiak et al., 1981). Because of the short half-life of PF_4, the measurement of BTG in plasma and urine might give a more specific index of platelet activation and release.

3.3.7. Radioimmunoassay of BTG

The isolation and purification of BTG enabled specific antibodies to be raised against BTG with the subsequent development of radioimmunoassays for the detection of BTG in plasma or urine as described by several laboratories (Ludlam, Moore and Bolton, et al., 1975; Kaplan et al., 1978; Bolton, Ludlam, Moore et al., 1976; Han, Butt, Turpie et al., 1980). Purified BTG can be labelled with ^{125}Iodine by the method of Hunter and Greenwood (1962) and a rabbit antiserum raised against purified BTG.

Bolton et al., 1976, developed a method where they incubated antiserum, sample or standard and radio-labelled tracer for a specified time. In their standard assay, incubation was for 24 hours, with antibody bound BTG being separated from free antigen with a second antibody over another 24 hours. To increase further the sensitivity of the assay, more dilute antiserum and tracer was used with tracer being added 24 hours after samples had been incubated with antiserum. A more rapid method was developed by the use of an antiserum covalently linked to Sepharose - 4B. After a one hour incubation, bound and free BTG were separated by centrifugation. A commercial kit has been developed along the principles of the rapid assay with bound and free BTG being separated by centrifugation with ammonium sulphate. The detection limits of the methods (in ng/ml) were 0.7 for the standard assay, 0.04 for the sensitive assay and 0.6 for the rapid assay. Similar radioimmunoassays have been developed for LA-PF_4 and PF_4 with detection limits of 0.1 ng/ml (Rucinski et al., 1979) and 0.2 ng/ml (Rucinski et al., 1979; Levine and Krentz, 1977; Handin, McDonough and Lesch, 1978) respectively.

3.3.8. Preparation of platelet poor plasma for assay of plasma BTG

Plasma levels of BTG can only adequately reflect in vivo platelet activation and release when in vitro release is eliminated or minimised. Four steps seem critical in the preparation of platelet poor plasma (1) proper venepuncture, (2) rapid cooling of the blood, (3) ice (0-4 °C), (4) centrifugation at 4 °C. Anticoagulation with antiplatelet agents is also critical. It is recommended that blood is added promptly to a mixture of EDTA, PGE_1, and theophylline, maintained at a temperature between 0 and 4 °C, and centrifuged for 60 minutes at 1,000g, in order to obtain low levels of BTG (Ludlam, 1976).

Because of the potential difficulties in blood collection and processing, it has been suggested that the measurement of BTG in urine might provide a more reliable index of in vivo platelet activation in patients with normal renal function (Dawes et al., 1978). They found a mean urinary BTG level of 0.14 ng/ml in normal subjects, i.e. 0.5% of the plasma level (Dawes et al., 1978; Dawes, Smith, Borsey et al., 1979). Such a low rate of excretion might lower the sensitivity of the assay.

3.3.9. BTG levels in clinical disorders

A. Deep venous thrombosis

A preliminary study of plasma BTG levels in six patients revealed that all patients had raised BTG levels (Ludlam, Bolton, Moore et al., 1975a). While this suggested that BTG levels might be of clinical value in patients with suspected venous thrombosis, a later, more extensive study did not confirm these results (Smith, Duncanson, Ruckley et al., 1978). Elevated plasma BTG levels had a sensitivity of only 50% although the mean BTG concentration was significantly increased in the patients with thrombosis compared with normal subjects. Patients who had a pulmonary embolism in addition to DVT had BTG levels significantly higher than those with DVT alone. They also measured BTG serially in 46 patients screened after major surgery. Thirteen patients developed venous thrombosis as detected by ^{125}I fibrinogen scanning. There was no significant difference in mean BTG levels between patients with or without thrombosis. Increased BTG levels have been reported in patients with recent venous thrombosis (abnormal in 46%) but normal levels were found in patients with chronic venous thrombosis (abnormal in 6%) (Cella, et al., 1979). A later study demonstrated that both plasma and urine BTG were significantly elevated in patients with DVT compared

to symptomatic patients with a negative venogram. Sensitivity (37%) and specificity (80%) of the plasma BTG assay for the diagnosis of DVT were low. The urine BTG assay had a sensitivity of 37% but a specificity of 100%. Serial BTG measurements were made in plasma and urine post-operatively in neurosurgical cases. BTG was elevated post-operatively and returned to normal within two or three days, but rose again in ten patients in association with the development of DVT. The rise preceded the uptake of ^{125}I-fibrinogen and lasted for only a few days (de Boer, Han, Turpie et al., 1981). Similar results have been obtained by other workers (Bolton, Cooke, Lekhwani et al., 1980; Owen, Kvam, Kaplan et al., 1980).

Such studies clearly suggest that platelet activation occurs in venous thrombosis but is maximal or limited to the initial phase of thrombosis development.

B. Coronary artery disease

Elevated levels of BTG and PF_4 were first reported by several workers (Denham, Fisher, James et al., 1977; O'Brien, Etherington and Shuttleworth, 1977; Handin et al., 1978). A number of resulting studies have shown an elevation in plasma

BTG levels in acute myocardial infarction (Rasi, Tortila and Ikkala, 1980), in both acute myocardial infarction and acute angina but normal levels in stable angina (Smitherman, Milam, Woo et al., 1981). A small but significant increase in BTG and PF_4 has been reported in patients with stable angina and coronary artery disease and in patients with myocardial infarction (Files, Malpass, Yee et al., 1981). Another study has reported significantly increased BTG levels in acute myocardial infarction; stable and unstable angina when compared to normal groups (Serneri, Gousini, Abbate et al., 1981). Almost all the patients in the abnormal groups had BTG levels above the upper limit of the normal range, thus raising the question of whether the data are not partly the result of in vitro release due to technical problems. A study using serial measurement of plasma and urine BTG found levels in normal patients and those with non-cardiac chest pain were similar. Mean plasma BTG was increased in patients with myocardial infarction or angina, however, 61% of the patients with coronary disease had normal levels. In the patients with myocardial infarction, the highest values were observed at the time of admission and within four hours of the chest-pain episode. Urine BTG levels were not different from controls in the patients with infarction and angina (de Boer, Turpie, Butt et al., 1981).

A significant difference in plasma PF_4 levels has been reported between patients with and without a confirmed myocardial infarction (White and Marouf, 1981) but this has not been confirmed (Levine, Lindenfeld, Ellis et al., 1981).

Despite the varying levels of BTG reported in the various studies which may in part be the result of in vitro release, platelet activation and release occur in patients with coronary artery disease, in particular in acute ischaemic events such as myocardial infarction or angina. Such platelet release is an intermediate process in coronary atheroma, and only of a measurable magnitude in certain patients. Whether a causal relationship occurs between platelet release and the clinical complications of coronary atheroma remains to be established.

C. Cerebrovascular disease

Elevated plasma BTG levels have been reported in patients with stroke or transient cerebral ischaemic attacks (TIA) (Hoogendijk, Jenkins, van Wijk et al., 1979). PF_4 was also found to be increased in these patients (Matsuda, Seki, Ogawara et al., 1979). In contrast, another study reported normal levels of plasma BTG in patients a few months after a cerebrovascular accident (CVA) (Cella et al., 1979). Increased levels of BTG have been reported in a group of young patients (mean age 35 years) with TIA and stroke, blood sampling being carried out in the non-acute phase, days or weeks after the acute ischaemic event. In another study plasma BTG levels were not significantly different in patients with acute partial stroke when compared to normal patients.

Subsequent measurements a few weeks later gave similar results (de Boer et al., 1981).

Studies of BTG levels in patients with cerebrovascular disease have produced inconsistent results and more studies are required.

D. Peripheral vascular disease

BTG and PF_4 levels were elevated in patients with peripheral vascular disease. After treadmill exercising only BTG showed a significant increase in levels (Baele, Bogaerts, Clement et al., 1981). In contrast normal plasma BTG levels were observed before and after exercise testing in another study (Baker, Fareed, Messmore et al., 1981). Normal plasma and urine BTG levels have been observed in patients with peripheral arteriosclerosis (Johnston, de Boer and Turpie, 1981).

Studies of BTG and PF_4 appear to be inconclusive in patients with peripheral vascular disease and further studies are required to elucidate the association between platelet activation and peripheral arterial disease.

E. Cardiac valve disease

Plasma levels of BTG are increased in patients with rheumatic heart disease and cardiac valve prosthesis. Most of the studies reported no correlation between levels of BTG or PF_4 with the number of valves involved, the type of valve inserted (mechanical or bioprosthesis) or a history of thromboembolism. In a recent sudy higher BTG levels were found in patients with double

valve replacements when compared to patients with single mechanical replacements who had significantly higher BTG values than patients with porcine valves. Patients who had single mechanical valves and who developed emboli had significantly higher BTG levels than those without embolic episodes (Pumphrey and Dawes, 1981). Another study reported increased urine BTG levels in patients with prosthetic valves (Turpie, de Boer, Genton et al., 1981). It appears that platelet activation is a more continuous process in patients with artificial heart valves compared to patients with coronary artery disease.

F. Diabetes mellitus

Platelet abnormalities have been described in patients with diabetes mellitus and are thought to contribute to the vascular complications of the disease, hence plasma BTG concentration has been studied extensively in patients with diabetes mellitus.

Normal plasma levels of BTG were found in the first study reported in patients suffering from diabetes with known complications of the disease including neuropathy, retinopathy and ischaemic skin lesions (Campbell, Dawes, Fraser et al., 1977). Two later studies reported increased plasma BTG levels in patients with diabetes. One study found the BTG in the diabetic patient was increased compared to normal subjects and the highest values were observed in patients with vascular complications (Burrows, Chavin and Hockaday, 1978). In the second study elevated BTG levels were found in diabetic patients both with and without vascular complications

(Preston, Ward, Marcola et al., 1978). It has been suggested that the studies by Preston and Burrows may reflect in vitro release, as their anticoagulant did not contain prostaglandin E_1 (Editorial, Lancet, 1978). Many recent studies have reported elevated BTG levels in patients with diabetes. While most of these studies used an anticoagulant containing prostaglandin E_1 there is a wide overlap with the control groups and a wide variation in individual patient results.

A significant drop in BTG has been reported in patients having their diabetes controlled by diet (Burrows, 1981; Preston et al., 1978; Voisin, Rouselle, Guimont et al., 1981). Another study, however, reported no correlation between BTG and the actual fasting blood glucose concentration, the amount of 24 hours glucosuria or the values of haemoglobin A, (Schernthaner, Sinzinger, Silberbauer et al., 1981). No correlation between BTG and HbA levels has been reported by other laboratories (Matthews, O'Connor, Hearnshaw et al., 1979; van Oost, 1981). It has been suggested that BTG levels are highest in patients with vascular complications such as retinopathy (Burrows et al., 1978; Zahavi, Jones, Betteridge 1979; Janka, Standl and Mehmert, 1981, Schernthaner et al., 1981) although this was not observed in another study (van Oost, 1981).

An interesting study has reported BTG and PF_4 levels were normal in patients with diabetes alone. In patients with diabetes and peripheral arterial disease, increased BTG were observed while PF_4 levels were not different from normal, suggesting that in vitro release

was limited. Increased BTG levels were reported in a group of patients with peripheral arterial disease, but without diabetes. This study supports the active role of platelets in thrombotic occlusive arterial disease, but not in diabetes.

Although the majority of studies suggest in vivo platelet activation occurs in patients with diabetes, no definitive conclusions can be drawn. The elevated levels reported are probably the result of in vitro release. There is evidence that platelets are activated in vivo in patients with diabetes when peripheral vascular disease is also present. However, no causal relationships should be drawn.

G. Miscellaneous conditions

Both BTG and PF_4 levels in plasma were reported to be elevated in hyperlipidemia (Zahavi et al., 1981; Files et al., 1981). Elevated urine BTG levels have been reported in hypertension (Anderton. Fananapazir and Dawes, 1980) but these results are probably due to abnormal clearance of BTG in hypertensive nephropathy than in vivo release since the majority of the abnormal values occurred in patients with renal insufficiency. Increased BTG levels have been reported in myoproliferative disorders (Boughton, Allington and King, 1978; Ireland, Lane, Wolff et al., 1982) and in thrombocytosis (Boughton et al., 1978; Han, Turpie and Genton, 1979). Increased BTG levels have been observed in malignancy (Farrell, Duffy, Duffy et al., 1979; Bidet, Ferriere, Besse et al., 1980; Murakoshi,

Takei, Seya et al., 1981). The study by Murakoshi et al., 1981 also observed elevated plasma FPA levels suggesting in vivo platelet release in cancer patients is associated with low-grade DIC.

BTG levels have also been studied in various diseases associated with DIC. Elevated BTG levels have been reported in patients with established pre-eclampsia (Redman, Allington, Bolton et al., 1977; in the acute phase of haemolytic uraemic syndrome (Cossu, Tantalo, Paracchini et al., 1981) and in cancer (Muraskoshi et al., 1981). BTG, PF_4 , and FPA levels were all increased after intrauterine infusion of hypertonic saline to terminate pregnancy (Nossel, Wasser, Kaplan et al., 1979) suggesting in vivo platelet release occurs in association with in vivo thrombin generation. Increased BTG levels have also been reported in patients with septicaemia, complicated with DIC (Han et al., 1979). Elevated platelet BTG levels have been observed in congenital platelet disorders eg. in May-Hegglin anomaly which is characterised by normal or low platelet count, giant platelets and leukocyte inclusions (Fabris, Casonato, Randi et al., 1980). Platelet release deficiencies have been observed in storage pool disease, which is a bleeding disorder characterised by a decrease in the number and contents of platelet dense granules (Weiss et al., 1979, Pareti, Dawes, Franchi et al., 1979). Increased BTG levels have been reported in pulmonary disease

(Nenci, Berretine, Todisco et al., 1981); after anaesthesia (Zahavi, Price and Kakkar, 1980); and in Raynaud's phenomenon (Zahavi, Hamilton, O'Reilly et al., 1979).

3.3.10. Conclusions

Despite the problems associated with in vitro release, tentative conclusions can be drawn from the various clinical studies published previously. Platelet activation and release occurs in patients with coronary artery disease, in particular, in association with myocardial infarction or angina. This release does not occur in all patients. Platelet activation and release also occurs in patients with cardiac valve prosthesis and in association with the development of venous thrombosis.

The measurement of BTG does not appear to have a role in the clinical management of arterial and venous thromboembolism as no antiplatelet drug has been shown to lower plasma or urine levels in various clinical disorders.

A role does exist for the measurement of BTG in elucidating the role of in vivo platelet activation and release in the pathogenesis of arterial and venous thromboembolic disease.

CHAPTER 4

FIBRINOPEPTIDE A: AN INDEX OF IN VIVO THROMBIN GENERATION AND FIBRIN(OGEN) FRAGMENT, Bβ15-42: AN INDEX OF PLASMIN GENERATION

4.1. Introduction

In normal haemostasis the formation of fibrin from fibrinogen is required to stabilise the platelet plug formed after vessel wall injury as previously discussed (see Figure 1.1(b)). Fibrin is a major constituent of thrombi, particularly those which form in veins and on the myocardial wall. In addition, fibrin and also fibrinogen have been demonstrated as key constituents of arterial wall lesions. The persistence of intravascular fibrin is thought to result from increased fibrin formation or decreased fibrinolysis. Fibrin formation and dissolution is believed to be primarily associated with the activity of thrombin and plasmin as discussed in Chapter 1. The action of thrombin on fibrinogen and plasmin on fibrin(ogen) results in the release of specific soluble fragments whose quantitation, in theory, may be an index of enzymic activity in vivo. Such measurements of fibrin(ogen) degradation products seem relevant to the study of thrombosis associated with activation of either blood coagulation or fibrinolysis.

4.2. In Vitro Tests of Thrombin Derived Fibrinogen Fragments

A. Soluble fibrin-fibrinogen complexes

Soluble intermediate products are produced during the conversion of fibrinogen to fibrin by thrombin (Blombäck

and Laurent, 1957; Blombäck, Hogg, Garlund et al., 1976) (see Chapter 1.3.E). The potential physiological significance of soluble intermediate fibrin-fibrinogen complexes were demonstrated in the blood of rabbits treated with endotoxin (Shainoff and Page, 1960). Such complexes consisted of fibrinogen with fibrin lacking only fibrinopeptide A (FPA) and were also cold precipitable (cryofibrin). When the level of cryofibrin in the rabbits circulation exceeded 26% of the plasma fibrinogen, the cryofibrin dissociated into fibrinogen and fibrin in equimolar proportions, the fibrin then precipitating from solution (Shainoff and Page, 1962). Several assays have been proposted to detect and quantitate these complexes.

1. Paracoagulation assays

Fibrinogen is a large protein of limited solubility in aqueous medium. Alteration of solubility by varying salt concentration (Blombäck, 1967), either through the addition of organic solvents such as ethanol (Blombäck and Blombäck, 1956) and dimethylformamide (Blombäck and Blombäck, 1966) or the addition of charged substances such as protamine sulphate has been used to precipitate fibrinogen and certain of its derivatives (Lipinski and Worowski, 1968). Macromolecular complexes formed between fibrinogen, soluble fibrin and various degradation products will be less soluble than fibrinogen itself and may be quantitated by differential precipitation techniques. The term "paracoagulation" was first used by Derechin (1955) who demonstrated that when human plasma in which plasminogen had been activated by streptokinase was clotted with

thrombin, and the resulting clot dissolved, addition of quantities of protamine sulphate insufficient to precipitate fibrinogen from solution led to reclotting of the mixture.

Protamine sulphate test

This test is thought to be sensitive to the presence of early fibrin X material, as well as fibrin monomer. A good correlation has been found between the results of the serial dilution protamine sulphate test and the soluble complex concentration using agarose gel filtration (Gurewich and Lipinski, 1976). The study examined plasma samples from thrombin and endotoxin treated rabbits, as well as samples from patients with intravascular coagulation.

In clinical studies, most series report a high incidence of positive tests in patients with DIC (Seamen, 1970; Niewarowski and Gurewich, 1971; Gurewich and Hutchinson, 1971). The incidence of positive tests in deep venous thrombosis varies in different series. In one study, an incidence of 8% positive tests in symptomatic deep venous thrombosis patients with positive venograms (Gurewich, Hume and Patrick, 1973). Seamen (1970) detected only two positive tests in 16 deep venous thrombosis patients while another study found one positive test in 38 deep venous thrombosis patients.

A significant incidence of positive tests has also been found in pulmonary thromboembolism, in acute arterial thromboembolism, in disseminated malignant disease, in renal disease (Gurewich and Hutchinson, 1971; Gurewich et al., 1973; Palester-Chlebowczyk, Strzyzewska, Sitkowski et al., 1971) and in systemic hypertension

(Zola-Sleczek and Szczepaniec, 1974). In contrast another study only found one positive test out of 305 samples tested. This was a case of DIC but 15 other similar cases gave negative tests (Hedner and Nilsson, 1972).

It is probable that variables such as incubation time, fibrinogen level, anticoagulant used to collect the blood sample and brand of protamine sulphate are important in this technique.

Ethanol gelation test

The ethanol gelation test is reportedly insensitive to early fibrin degradation products (Gurewich, 1973; Breen and Tullis, 1968) and is said to respond primarily to soluble fibrin (Godal and Abildgaard, 1966; Breen and Tullis, 1968). It appears to be less sensitive than the protamine sulphate test in precipitating fibrin (Yudelman, Spanondis and Nossel, 1974).

Conflicting results have also been obtained by the use of the ethanol gelation test in clinical studies. In one study, only 11.4% of a large number of unselected patients with malignant disease, pneumonia, pulmonary embolism, arterial and venous thrombosis showed a positive test (Kierulf and Godal, 1971). In another study of an unselected group of patients with similar clinical diseases, 10% positive tests were found, but only five out of 17 patients with DIC had positive tests.

Thus this is unreliable and is not recommended for clinical use.

2. Gel exclusion chromatography

In addition to differential precipitation, gel sieving methods may be used for the identification of macromolecular complexes of fibrinogen in circulating blood. Agarose gel chromatography was applied to the identification of complexes of higher molecular weight than native fibrinogen formed in vitro (Sasaki, Page and Shainoff, 1966), then to clinical studies (Fletcher, Alkjaersig, O'Brien et al., 1970). The technique used by Fletcher et al., 1970, employs relatively short (15-30 cm) Biogel A-5M columns; elution conditions are carefully controlled, and the fibrinogen in eluates is determined immunologically by an automated system. The proportion of fibrinogen-related antigen eluting before and after a fibrinogen standard is analysed using a computer programme. Use of longer gel columns increased the time required for analysis but provides substantially greater resolution (Vermylen, Donati and Verstraete, 1971; Donati, Verhaeghe, Culasso et al., 1976; McKillop, Edgar, Forbes et al., 1975).

In studies related to the detection of deep venous thrombosis (DVT) the chromatographic method gave 41 abnormal results out of 72 patients. In 22 of these abnormal patients, fibrinogen scans were normal. In 15 of the patients abnormal results were present pre-opertatively. In another study 12 out of 38 patients showed abnormal chromatography results which were verified by venography (Bang and Chang, 1974). Abnormal results have been

reported in women taking oral contraceptives and in some normal control women (Alkjaersig, Fletcher and Burstein, 1975). Another study reported increased complexes in normal pregnant women and highly elevated levels in women with pre-eclampsia (McKillop, Howie, Forbes et al., 1976). Increased soluble complexes have also been reported in cerebral vascular accidents (Alkjaersig, Laursen and Fletcher, 1972), in myocardial infarction (Fletcher and Alkjaersig, 1973), acute glomerulonephritis (Fletcher and Alkjaersig, 1973), type II hyperlipoproteinaemia (Carvalho et al., 1974) and DIC (Kazama and Abe, 1976).

A critical problem with the chromatographic analysis in clinical samples has been specificity, since the complexes are likely to represent products of intact and plasmin degraded fibrin, fibrinogen and other proteins (Mosher, 1975). In vitro data suggests that fragment X derived from fibrin polymerises to form soluble macromolecular complexes. In addition, components of early plasmin-fibrin digest mixtures copolymerise with fibrinogen in the absence of thrombin to yield soluble complexes. These early fibrin digests also copolymerise with fibrin. Such copolymerisation also occurs in vivo (Chang and Bang, 1977).

3. Affinity chromatography methods

Stabilised insoluble fibrinogen derivatives of human fibrinogen were prepared by immobilisation of the protein onto CNBr-activated Sepharose 6B by Matthias and co-workers, 1977. When thrombin-treated plasma is passed through columns containing immobilised fibrinogen, the fibrin was absorbed

and could be eluted with buffers containing 1M NaCl or 2M Na Br. Plasma samples from a patient with DIC gave similar results (Matthias, Reinicke and Heene, 1977). However, in view of the studies by Chang and Bang (1977) on complex formation between early fibrin degradation products and fibrinogen, it is unlikely that the affinity method can distinguish between soluble fibrin and early plasmin-fibrin digestion product complexes in plasma samples.

Although the gel exclusion chromatography method is relatively specific in the detection of soluble fibrin-fibrinogen complexes when compared to the protamine sulphate and ethanol gelation tests it is rather time consuming as is the affinity chromatography of fibrinogen.

The gel exclusion and affinity chromatography methods cannot be specific either for thrombin or for plasmin action in vivo and may actually reflect the action of either or both of these enzymes.

4.3. Fibrinopeptide A

4.3.1. Introduction

Thrombin action on fibrinogen results in cleavage of arginyl-glycine bonds to release two molecules of fibrinopeptide A (FPA) from the NH_2 -terminal segment of the Aα chain and two molecules of fibrinopeptide B (FPB) from the NH_2 - terminal segment of the Bβ chain (see Chapter 1, 3.2.c). Thrombin susceptible bonds are not cleaved by plasmin (Blombäck, 1967), thus measurements of the fibrinopeptides should distinguish the action of thrombin from plasmin. Since thrombin cleaves the A peptide before the B peptide (Blombäck, 1967) efforts

have been focussed on the measurement of FPA as an index of thrombin generation.

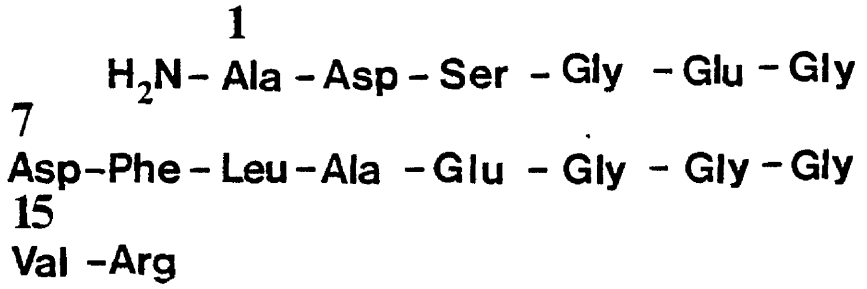
4.3.2. Isolation and characterisation of FPA

The release of fibrinopeptide A and B after thrombin cleavage of fibrinogen was first reported over 20 years ago (Bettelheim and Bailey, 1952) and subsequently confirmed by other workers (Blombäck and Vestermark, 1958; Blombäck, 1958; Nossel et al., 1974).

The most frequently used method for isolating the fibrinopeptide has been cation-exchange chromatography of fibrin clot supertnatant (Blombäck and Vestermark, 1958; Blombäck, Blombäck, Edman et al., 1966). Modifications of this method have been described (Murtaugh and Gladner, 1974).

Fibrinopeptide A is composed of 16 amino acids with a molecular weight of 1535 (see Figure 4.1.). Because of their small size solid-phase peptide synthesis has become an important alternative method for their preparation (Erickson and Merrifield, 1976). This method is of particular importance for immunologic and physiologic studies in which relatively large quantities of fibrinopeptides are required as tyrosyl analogs of fibrinopeptides are needed that may be conveniently radiolabelled. Synthesis of human fibrinopeptides A and B have been carried out by several workers (Blombäck, Blombäck, Olsson et al., 1969; Johnson and May, 1969; Budzynski and Marder, 1973; Wilner, Nossel, Canfield et al., 1976). Canine fibrinopeptide A, its amino tyrosyl analog (Wilner and Birken, 1975) and guinea pig fibrinopeptide A (Wilner, 1980) have also been

Fibrinopeptide A



Fibrinopeptide B

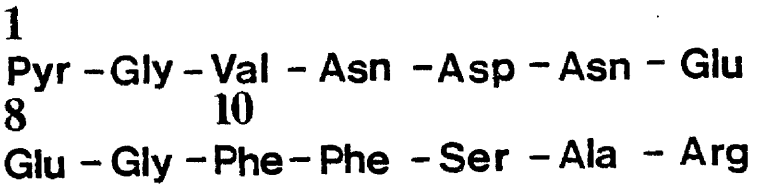


Figure 4.1

Amino acid sequences of fibrinopeptides A and B.

synthesised. A synthetic analog of fibrinopeptide A, N-benzoyl-L-phenyl-alanyl-L-valyl-L-arginine-P-nitro-anilide hydrochloride appears to be useful as a chromogenic substrate for quantitating the amidolytic activity of several enzymes (Svendsen, Blombäck, Blombäck et al., 1972).

Variants of fibrinopeptide structure are normally present within individuals of certain species. A minority of human A peptide molecules lack amino-terminal alanine (AY), and the serine residues in others are phosphorylated (AP)(Blombäck et al., 1966).

4.3.3. In vitro Release of FPA

In vitro release of FPA occurs after seven minutes in a syringe containing non-anticoagulated blood (Nossel et al., 1974; Prowse, Vigano, Borsey et al., 1980). Similar in vitro release was found in agitated blood at 37°C with agitation markedly accelerating the in vitro release (Kaplan, Drillings and Lesznik, 1981). As previously discussed, thrombin cleaves fibrinogen to release FPA and FPB sequentially. The generation of thrombin on the platelet surface has been suggested by several workers who demonstrated that FPA formation and α -granule protein release followed similar time courses (Prowse et al., 1980; Kaplan et al., 1981). Such results are in contrast to a previous report (Kaplan et al., 1978) which showed FPA release at thrombin concentrations at least one hundred fold lower than those which caused BTG and PF_4 release.

The earlier results of Prowse et al., and Kaplan et al., suggest that thrombin generated in the blood has equal access to plasma fibrinogen for fibrin formation and to platelets for induction of the release reaction in contrast to added thrombin which preferentially cleaves fibrinogen. Platelet release in the undisturbed system is entirely dependent on thrombin action since blocking thrombin action with hirudin completely prevents platelet release (Kaplan et al., 1981).

Collagen markedly increased in vitro release of FPA, with platelet release occurring earlier than FPA release (Kaplan et al., 1981). Such release by collagen may be as a result of collagen activation of the contact activation system (Wilner et al., 1968) and release of platelet factor V by collagen (Chesney, Pifer and Colman, 1978; Vicic, Lages and Weiss, 1980) with resulting thrombin formation.

ADP does not release significant amounts of FPA in vitro although initial rapid release of BTG and PF_4 does occur (Kaplan et al., 1981), although consistently small elevations of FPA release are present. This data supports the hypothesis that increased platelet release contributes to increased thrombin formation although the increment contributed by platelet release is clearly small.

FPA in vitro release does not occur in the presence of prostaglandin E_1 (PGE_1) and theophylline (Kaplan et al., 1981) suggesting that the relatively small amount of FPA present in control samples is unimportant in determining the role of thrombin generation.

Some snake venoms release FPA in vitro. Ancrod (Arvin), and Reptilase are partially purified fractions containing coagulant enzymes which can be isolated from the crude venoms of the Malayan (Agkistrodon rhodostoma) and South American (Bothrops atrox) pit vipers respectively (Esnouf and Tunnah, 1967 Klobusitzky and Konig, 1936). Ancrod contains both a thrombin-like coagulant enzyme, which cleaves only FPA from fibrinogen, and a less specific protease fraction, capable of degrading FPA as well as other proteins in vitro (Ewart, Hatton, Basford et al., 1979; Pizzo, Martin, Schwartz et al., 1972). Unlike thrombin ancrod does not activate factor XIII (Barlow, Holleman and Lorand, 1970). Reptilase also contains a thrombin-like coagulant enzyme which cleaves only FPA from fibrinogen in vitro. Unlike ancrod, reptilase does activate factor XIII (Pizzo, Martin, Schwartz et al., 1972).

4.3.4. In vivo release of FPA

Plasma FPA levels rise immediately in patients receiving intrauterine injection of hypertonic saline (Nossel, Wasser, Kaplan et al., 1979) to terminate pregnancy and has been shown to be associated with in vivo coagulation activation as reflected by reduction in fibrinogen, clotting factor levels, and platelet counts, and by elevated FDP levels (Stander, Fleossa, Glueck et al., 1971; Weiss, Easterling, Odomn et al., 1972; Schwartz, Greston and Kleiner, 1972; Van Royen, Treffers and ten Cate, 1974).

As discussed previously the FPA level rises gradually in blood collected in a plastic syringe and added at regular intervals to tubes that contain anticoagulant. Whereas FPA generation increases non-linearly when exogenous thrombin is mixed with normal blood in the syringe, generation is usually linear in clinical samples, and the rate can be calculated. Slow rates were found in normal blood and in blood from patients with venous thrombosis or aortic aneurysm and greatly elevated FPA levels. These results suggest that thrombin action might be predominantly localised in these conditions. Generation rates were four to 140 times faster than normal in blood from patients infused with prothrombin-complex concentrates and in patients with DIC, acute pulmonary embolism, active systemic lupus erythematosis or renal transplant rejection. These findings suggest that thrombin activity is greatly increased in the peripheral blood of patients with such diseases (Nossel, Ti, Kaplan et al., 1976).

Acute inflammation increases the absorption of FPA from extravascular sites, but the peak FPA levels achieved following intravenous injection are still 13 times greater than the peak levels obtained following injection of an equivalent dose of peptide into an acutely inflamed muscle. These findings suggest that intravascular thrombin action is far more effective in elevating plasma FPA levels than is extravascular thrombin.

4.3.5. Biologic Activity of FPA

The principal function of FPA appears to be to mask a specific polymerisation site within the E domain of the fibrinogen molecule (Blombäck, Hessel, Hogg et al., 1978). The exact location of such a polymerisation site has not been

completely resolved. The identification of an Aα 19 Arg→Ser amino acid exchange in the mutated fibrinogen Detroit (Blombäck, Blombäck, Mammen et al., 1968) associated with a polymerisation defect (see Chapter 4 .1 Blombäck, Hessel, Hogg et al., 1978) and the study of the kinetics of fibrinopeptide release from Aα chain fragments (Hogg and Blombäck, 1978) have both demonstrated the importance of specific sequences in the N-terminal section of the Aα chain for the interaction with thrombin and subsequent polymerisation.

The recent use of high performance liquid chromatography (HPLC) analysis of fibrinopeptide release with subsequent amino acid analysis of the fibrinopeptides has identified other amino acid exchanges in this region with resultant polymerisation defects (see Table 4.1).

Human FPA appears to cause potentiation of bradykinin-induced smooth contraction (Bayley, Clements and Osbahr, 1967) and vasoconstriction, presumably due to the direct stimulation of vascular smooth muscle cells (Osbahr, 1975).

Fibrinopeptides in relatively high concentrations are capable of competitively inhibiting the reaction of thrombin and fibrinogen as observed by the prolongation of the recalcification time of human plasma following addition of a mixture of FPA and FPB (Bettelheim, 1956). Studies with synthetic peptide analogues (Blombäck et al., 1969) demonstrated that the site on FPA recognised by thrombin involves the carboxy-terminal arginine (arg) and a phenylalanine (phe) residue located eight residues from the amino-terminal. The spacing between Phe and

TABLE 4.1

DYSFIBRINOGENAEMIA WITH DISORDERED FIBRINOPEPTIDE RELEASE

TYPE	DEFECTIVE RELEASE OF FIBRINOPEPTIDE	FIBRIN POLYMERISATION	IMMUNO-ELECTROPHORESIS	CLINICAL	MOLECULAR DATA
Detroit	B only	Slow	Anodal	Bleeding	A ($Arg^{19} \rightarrow Ser$)[1]
Manchester	A	Slow	Normal	-	A ($Arg^{15} \rightarrow His$)[2]
Petoskey	A	Normal	Normal	None	A ($Arg^{16} \rightarrow His$)[3]

References 1. Mammen, Prasad, Barnhardt et al., 1969

2. Lane, Van Ross, Kakkar et al., 1980: Lane, Southan, Ireland et al., 1982

3. Higgins, Penner and Shafer, 1981; Higgens and Shafer, 1981

Arg is apparently critical for thrombin inhibitory activity. This FPA site makes only a minor contribution to the specificity of thrombin action on fibrinogen. The critical thrombin recognition site on human fibrinogen Aα chains appears to be located in the region Aα 17-51.

4.3.6. Metabolism and clearance of FPA

A study on both the heparin induced reduction of plasma FPA in patients and the clearance of infused FPA from normal volunteers has determined a half-life for removal of FPA from the circulation of about three minutes (Nossel et al., 1974). Because only about 1% of plasma FPA is present in the urine (Tegger-Nilsson and Grondahl, 1974; Alkjaersig and Fletcher, 1982), it has been suggested that the kidney may play a direct role in the degradation of the peptide as it is cleared from the circulation (Nossel, 1976).

A recent study examined the clearance of ^{125}I desaminotyrosyl human FPA from the rat circulation. The results obtained in this study suggest that FPA clearance from the circulation can be described by a biphasic exponential curve. An initial rapid clearance had a half-life of less than 2 minutes and this was followed by a slower component with a half-life of approximately one hour when ^{125}I FPA was injected into control rats. Bilateral nephrectomy of the rats significantly elevated the plasma concentration and also prolonged the slow component half-life to 73 minutes. In rats with ureteral ligation identical clearance curves to the

nephrectomised rats were obtained, strongly suggesting that the labelled peptide was being directly filtered at the glomerulus and excreted by the kidney. The fast component was probably caused by equilibration of the peptide throughout intra and extravascular spaces and the slow component reflected the action of catabolic processes once equilibration was obtained. Blood samples withdrawn from control or nephrectomised rats at the completion of the experiments contained intact FPA and a major degradation fragment demonstrable by gel filtration on G10 Sephadex. A similar degradation could be elicited by in vitro incubation of ^{125}I-FPA with fresh citrated blood. Urine also degraded ^{125}I-FPA in vitro or in vivo to fragments, one of which was of similar molecular size to that produced in blood. This suggests that degradation of FPA occurs in plasma and urine rather than the kidney itself.

4.3.7. Immunochemistry of FPA

Fibrinogen and fragments of the fibrinogen molecule containing FPA react to variable degrees with anti-FPA antisera, and thus require processing procedures for removal of fibrinogen and FPA-containing fibrinogen fragments from plasma samples prior to assay (for a more detailed discussion see Chapter 5).

The immunoreactivity of 14 antisera to human FPA, prepared by immunising rabbits with this peptide conjugated to albumin carrier protein were studied (Canfield, Dean, Nossel et al., 1966; Nossel, Butler, Wilner et al., 1976). All the antisera tested showed comparable

reactivity to free human FPA in solution but disparate reactivities were observed when fibrinogen and fibrinogen fragments containing FPA were tested. The antisera could be divided into three groups: Group I could readily distinguish free fibrinopeptide A from fragments of fibrinogen containing the FPA sequence, whereas groups II and III could distinguish the free peptide from its parent molecule less well or not at all. Group I antiserum could distinguish free FPA (Aα 1-16) from the smallest plasmin derived fragment (Aα 1-23) that might be present by an order of one hundred fold difference in immunoreactivity (Canfield et al., 1966). This suggests that in the group I, but not in the groups II and III antisera, antigenic determinants were hidden in the larger FPA-containing fragments of the fibrinogen molecule, suggesting that the immunoreactive sites on the fibrinopeptide molecule detectable by these groups of antisera differ as well.

The reactivities of a Group I antiserum (R2) and a Group II antiserum (R33) were studied with synthetic COOH-terminal homologous of human FPA. The antigenic determinants of R2 antiserum specificity resided within the COOH-terminal ten residues of the FPA molecule, and the Phe 8, Asp 7 and Arg 16 contributed to R2 immunoreactivity. The antigenic determinants required for R33 specificity were clearly different and seemed to include residues in the more hydrophilic NH_2 terminal half of the FPA molecule as well. By the use of specific immunologic reagents and techniques, therefore, assays could be developed that are both sensitive and

specific and are clearly capable of distinguishing the products of thrombin proteolysis from those of plasmin proteolysis in clinical blood samples

4.3.8. Radioimmunoassay of FPA

The development of radioimmunossays for FPA has allowed more sensitive measurements of the peptide to be carried out in plasma (Nossel, Younger, Wilner et al., 1971; Nossel et al., 1974; Budzynski, Marder and Sherry, 1975; Gerrits, Flier and van der Meer, 1974; Kockum, 1976; Hofman and Straub, 1977). Iodinated tracers can be prepared by the radioiodination of desaminotyrosyl FPA with ^{125} Iodine. The tyrosyl analogue of the fibrinopeptide is used because all mammalian FPA and certain FPB do not contain tyrosine in their primary structure. Nossel et al., 1971, developed a method where they incubated antiserum and sample or standard overnight, radiolabelled tracer was added for one hour. Bound and free FPA were separated by the addition of charcoal, followed by centrifugation. The detection limit of the assay is 0.013 p moles. A commercial kit has now been developed according to this method. Normal values are around 1 pmole per ml.

4.3.9. FPA in clinical disorders

A. Deep venous thrombosis

Several studies have investigated fibrinopeptide A levels in patients with deep venous thrombosis (DVT). In one study FPA levels were measured in the plasma of 81 patients with deep venous thrombosis with and without

associated pulmonary embolism (Yudelman, Nossel, Kaplan et al., 1978). Of 47 patients with positive venography and/or lung scan, 42 had elevated FPA levels >1.3 pmol/ml (mean 7.4) and five had levels <1.3 pmol/ml. Of 34 patients with negative venography and/or lung scan, 29 had FPA levels of >1.3 pmol/ml and five had levels <1.3 pmol/ml.

In another study elevated levels of FPA were found in patients with DVT and in patients with pulmonary embolism (Peuscher, van Aken, Flier et al., 1980). The FPA level in patients with DVT confined to the calf veins was not elevated and was significantly lower in patients with proximal DVT.

A recent study assessed the predictive values of FPA and BTG in patients suspected of having acute DVT or pulmonary embolism (PE). Raised FPA and BTG levels were found in patients in whom DVT or PE was confirmed when compared to patients in whom these disorders were excluded. Raised FPA and BTG concentrations were also found in patients with inflammatory disorders and malignancy. Thus despite a specificity of 96% in symptomatic patients suspected of having a DVT or PE, raised levels of FPA and BTG are not specific for thrombosis.

The reduction in FPA concentrations in venous thromboembolism by the administration of heparin is evidence that elevated levels are caused by intravascular thrombin action (Nossel et al., 1976; Peuscher et al., 1980; van Hulsteijn, Briet, Koch et al., 1982; Yudelman and Greenberg, 1982). However FPA levels remained elevated during continuous

treatment with heparin in patients with pulmonary infarction; and/or pleural exudate; bronchopneumonia (Peuscher et al., 1980; van Hulsteijn, Fibbe, Bertina et al., 1982) or in patients with DVT or PE with concurrent sepsis or malignancy (Yudelman and Greenberg, 1982). This persistent elevation may be the result of extravascular formation of FPA which is not blocked by the action of heparin. Heparin has also been shown to reduce the elevation in FPA after surgery (Törngren, Norén and Savidge, 1979). Similar results were obtained after surgery in dogs (Medén-Britth and Radegran, 1980).

B. Myocardial infarction

As discussed in Chapter 1, Figure 1 (b), fibrin produced by the generation of thrombin, consolidates platelet thrombi and hence thrombin generation is suggested in the development of atherosclerosis. Plasma concentrations of FPA have, therefore, been measured in several studies in patients with myocardial infarction.

In one study, FPA levels were significantly increased in 29 patients admitted to hospital with myocardial infarction. The increase in FPA was more pronounced in patients with proven myocardial infarction and corresponded to the size of the infarction (Johnsson, Orinius and Paul, 1979).

In another study greatly elevated levels of plasma FPA were reported in patients with acute myocardial infarction as well as in spontaneous angina and effort angina. However, the reported mean values of FPA for each group had large standard deviations suggesting that the elevated levels may be the result of in vitro release in blood

sampling (Serneri et al., 1981).

In a recent study elevated levels of FPA were reported in patients admitted to hospital with a history and/or clinical symptoms of acute myocardial infarction. Within one week levels had returned to normal but this may be the result of inhibition of intravascular FPA formation by the use of anticoagulant therapy or the exhaustion of extra-vascular FPA generation in the infarcted tissue (van Hulsteijn, Kolff, Briët et al., 1982,

C. Coronary artery disease

In a recent study plasma FPA levels were measured in patients in whom myocardial infarction had occurred more than six months previously and in patients with abnormal coronary arteriograms without previous myocardial infarction. FPA levels were normal in each group. PF_4 and BTG were significantly elevated in the group with a previous myocardial infarction (Nichols, Owen, Kaplan et al., 1982). Similar results were reported in another study (van Hulsteijn et al., 1982c).

D. Malignancy

Elevated levels of plasma FPA were found pre-operatively in patients with malignancy (Törngren et al., 1979). In another study, 124 patients with various types of malignancy were studied. Elevated FPA levels were found in all patients with one third also having an accelerated$_{\Delta}$FPA Eight of the 45 patients with accelerated$_{\Delta}$FPA had signs of low grade DIC. While$_{\Delta}$FPA normalised in patients given heparin, FPA levels did not decrease. The results of this study

indicated that 30% of the selected patients with malignancy had evidence of intravascular thrombin activity and in 70% of these patients FPA is generated, at least in part, at a site accessible to thrombin. Elevated FPA levels appeared to be specific for active metastatic disease as patients without metastasis had normal FPA levels (Peuscher, Cleton, Armstrong et al., 1980). In another study in patients with disseminated neoplasia, an intravenous heparin bolus lowered the FPA level in 11/11 cases, while continuous heparin treatment led to suppression or normalisation of the FPA levels in 5/6 cases (Mombelli, Roux, Haeberli et al., 1982). The slow or incomplete decrease in FPA observed in some cases supports the findings of Peuscher et al., 1980. Elevated plasma levels of FPA have also been reported in another study on malignant disease (Yoda and Abe, 1981). Several studies have reported increased FPA levels either pre, during or after surgery (Torngren et al., 1979; Davies, Sobel and Salzman, 1980; Lane, Ireland, Wolff et al., 1982; Owen, Kvam, Nossel et al., 1983). An experimental study in dogs has suggested that this operative increase is caused by tissue damage during the operation and that FPA concentrations normalise within 24 hours (Medén-Britth and Radegran, 1980).

Increased plasma levels of FPA have also been reported in acute leukaemia (Myers, Pickles, Barb et al., 1981); liver cirrhosis (Coccheri, Mannucci, Palareti et al., 1982) and in major bacterial infections (van Hulsteijn et al., 1982).

Normal levels of FPA have been reported in type II hyperlipoproteinaemia (Nossel, Smith, Seplowitz et al., 1979; Killner and Lees, 1981) and in myeloproliferative disorders (Ireland et al., 1982).

4.4. B β 15-42 Peptide

4.4.1. Introduction

Following the initial release of FPA and fibrin monomer formation, further thrombin action releases fibrinopeptide B (FPB; B β 1 [Pyr] - 14 [Arg]) by cleaving B β 14 Arg-15 Gly (Blomback et al., 1978; Bilezikian, Nossel, Butler et al., 1975). Alternatively following the initial release of FPA if plasmin intervenes before further thrombin action the B β 1 [Pyr] - 42 [Arg] (B β1-42) fragment is released from fibrinogen and the B β15 [Gly] -42 [Arg] (B 15-42) fragment is released from fibrin, cleavage at B β42 [Arg] -43 [Ala] producing 'X-like' fragments (see Figure 2.1). These, it has been suggested can be readily removed from the circulation by the fibrinolytic system. Plasmin can be seen to act in a regulatory role, controlling the amount of pathological fibrin that is formed. Limited thrombin action can, therefore, take place in the circulation, increasing the concentration of FPA and soluble fibrin, without there being fibrin deposition.

Unfortunately the direct assay for FPB has been difficult in clinical situations. To date, two causes for the difficulty in specific FPB quantitation have been identified. First, larger FPB containing peptides

(eg Bβ 1-42) cross-react with anti FPB serum, thereby contributing to immunoreactive plasma FPB concentrations (Nossel et al., 1979). Second, it has been established that carboxypeptidase-B in human plasma cleaves the COOH-terminal arginine from FPB in vitro, the resulting Bβ1 [Pyr] - 13 [Ala] peptide, desarginyl-FPB, shows markedly decreased activity with some anti-FPB sera (La Gamma and Nossel, 1978), and hence, immunoreactive plasma FPB concentrations may be lowered by carboxy-peptidase-B in vivo and during the plasma processing procedcure. Recently a study which investigated the immunologic specificities of seven anti-FPB sera showed that certain antisera discriminate between the cross-reactivities of large peptide fragments containing the FPB sequence (eg fibrinogen, the NH_2 -terminal disulphide knot of fibrinogen (N-DSK) and Bβ1 [Pyr] -118 [Met]) and smaller fragments containing the FPB sequence (eg Bβ1-42 and desarginyl-FPB).

An assay specific for Bβ1-42 is not available however the slope of the inhibition curve for Bβ1-42 with the FPB antiserum is different from that of FPB and the immunoreactivity of Bβ1-42 increases ten-fold or more after treatment with thrombin, whereas that of FPB is unaltered. Hence Bβ1-42 can be distinguished from FPB by an increase in FPB immunoreactivity on treatment with thrombin (TIFPB). In vitro generation of Bβ1-42 occurs after treatment of plasma with streptokinase and is much more rapid in reptilase treated plasma in which fibrin I rather than fibrinogen is the substrate

(Nossel et al., 1979b). This increase in Bβ1-42 has also been demonstrated in vivo after streptokinase infusion in healthy volunteers (Prowse, Dawes, Lane et al., 1982). Increases in Bβ1-42 have also been observed to occur in vivo a few days after surgery (Lane et al., 1982a; Owen et al., 1983).

A problem associated with TIFPB immunoreactivity is that it probably includes the measurement of Bβ1-21 along with Bβ1-42 as Bβ1-21 also shows a marked increase in immunoreactivity after treatment with thrombin (Nossel et al., 1979b).

Radioimmunoassays have also been developed for the late plasmin cleavage products, fragment D and E (Gordon, Martin, Landon et al., 1975). Unfortunately, these methods have not yet been vigorously applied to the quantitation of derivatives in clinical plasma samples, largely because of the problems of production of sufficient quantities of highly specific antisera. Serum fragment E concentrations have been reported to be elevated in patients with DVT (Gordon, Cooke, Bowcock et al., 1977; Zielinsky, Hirsh, Straumanis et al., 1982) and in patients with acute partial stroke (De Boer et al., 1982). Butt et al., 1982).

Recently a new radioimmunoassay has been described for the detection of fragments containing the Bβ15-42 sequence (Kudryk, Robinson, Netre et al., 1982). Since this peptide sequence is part of a Bβ-chain fragment released during the earliest stage of plasmin proteolysis,

this assay should be of value in clinical investigations on all disease states associated with fibrino(geno) lysis.

4.4.2. Isolation and characterisation of Bβ15-42

Batroxobin digestion of N-DSK (Aα1-51, Bβ 1-118, γ1-78) derived from fibrinogen allows the partial release of the Bβ1-42 peptide. The latter, after partial purification, can be further digested with thrombin. This results in the cleavage of the Bβ14 Arg-15 Gly bond, and yields Bβ15-42 and free FPB. Separation of Bβ15-42 is achieved by fractionation on Sephadex G50 and G10. Bβ15-42 is composed of 28 amino acids with a molecular weight of 3040 (see figure 4.2). Since Bβ15-42 contains a tyrosine residue at position Bβ 41, it can be directly labelled with ^{125}Iodine.

4.4.3. Immunoreactivity of Bβ 15-42 antiserum

Pure fibrinogen cross-reacts with Bβ 15-42 antisera. a similar cross-reactivity is observed in plasma. FPB, fragments D and E do not show any cross-reactivity with Bβ 15-42 antiserum. Bβ 15-42 and Bβ 1-42 react with near identity in the assay system (Kudryk et al., 1982).

4.4.4. Metabolism and clearance of Bβ 15-42

The in vitro stability of Bβ 15-42 standard was measured in serum at 4°C and at 37°C. The half-life at 4°C was found to be 18-20 hours. At 37°C the half-life was 30 minutes. The addition of 1,10 phenanthroline prevented decay of Bβ 15-42 at both 4°C and 37°C (Kudryk et al., 1982).

Bβ15-42 Peptide

15 20
Gly - His - Arg - Pro - Leu - Asp - Lys - Lys - Arg - Glu

25 30
Gly - Ala - Pro - Ser - Leu - Arg - Pro - Ala - Pro - Pro

35 40
Pro - Ile - Ser - Gly - Gly - Gly - Tyr - Arg

Figure 4.2

Amino acid sequence of Bβ15-42 peptide.

The clearance of ^{125}I B β 15-42 has been examined in the rat circulation. The B β 15-42 clearance from the rat circulation is similar to that of FPA. An initial rapid clearance had a half-life of less than two minutes and this was followed by a slower component with a half-life of 228 minutes when ^{125}I B β 15-42 was injected into control rats. Bilateral nephrectomy prolonged the slow component to 420 seconds and significantly elevated the plasma concentration. In contrast to FPA, the clearance of ^{125}I B β 15-42 in rats with ureteral ligation was statistically indistinguishable from that of the control group. These results strongly suggest that ^{125}I B β 15-42 was being catabolised by the kidney (Lane, Marwick and Thomson, 1982).

4.4.5. Radioimmunoassay of B β 15-42

Recently a sensitive radioimmunoassay has been described for the measurement of fragments containing the B β15-42 sequence derived from fibrin(ogen) in clinical blood samples (Kudryk et al., 1982). Iodinated tracers were prepared by the radioiodination of B β 15-42 with ^{125}Iodine. In the assay, plasma samples were treated with ethanol in order to precipitate fibrinogen, which otherwise would cross-react with the antiserum. The supernatant or standard in appropriate dilutions was incubated with anti-B β 15-42 serum and a constant amount of ^{125}I B β 15-42 overnight. The antigen-antibody complex was then absorbed onto rabbit insolubilised anti-rabbit IgG. The latter complex was spun down and its

radioactivity determined,the detection limit for the assay is 0.008 p mole. Normal values were less than 3 p mole per ml. A radioimmunoassay kit has now been developed for this assay.

4.4.6. B β 15-42 in clinical conditions

Plasma levels of Bβ15-42 have been reported to be significantly higher in uraemic patients undergoing haemodialysis (Kudryk et al., 1982). This increase was suggested to occur as a result of decreased heparin concentrations in the blood. However, an alternative explanation of these results has been suggested: the elevated levels of Bβ15-42 are, at least in part, the result of impaired renal catabolism (Sherman, 1982) as shown in the rat circulation model (Lane et al., 1982b).

In conclusion, while it has been shown that increased levels of FPA are not specific for thrombosis, measurements of FPA and B β 15-42 are of great importance in determining the in vivo action of thrombin and plasmin on fibrin(ogen) in the pre-thrombotic, thrombotic and post-thrombotic situation, as well as in non-thrombotic clinical conditions. The measurement of plasma levels of FPA and B β 15-42 should, therefore, provide further insight into the molecular mechanisms involved in intravascular fibrin deposition.

CHAPTER V

MATERIALS and METHODS

5.1. Introduction

In this chapter the application of the technique of radioimmunoassay as a measurement of the platelet release reaction and thrombin and plasmin generation will be discussed. Radioimmunoassay will, therefore, be discussed first and then its application to the measurement of BTG, FPA and Bβ15-42.

5.2. Radioimmunoassay - Historical and Theoretical Aspects

The introduction of radioimmunoassay occurred two decades ago (Yalow and Berson, 1960). Radioimmunoassays have allowed a greater sensitivity, specificity, ease of performance and smaller sampling volumes than previously used biological assays.

The basic principle of a radioimmunoassay is illustrated in figure 5.1. If a given amount of antigen and antibody are allowed to react together, then at equilibrium they will form an antigen-antibody complex (the overlapping area B) together with a proportion of both the antibody and the antigen (F) which remain free. Figure 5.1. shows binding of antigen to antibody in the presence of different total masses of antigen. The

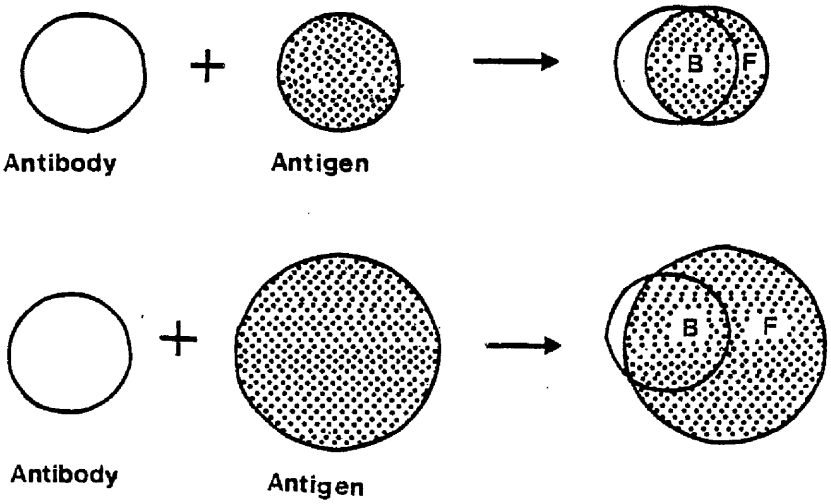


Figure 5.1

The basic principle of a binding assay, using immunoassay as an example. In a radioimmunoassay radiolabelled and unlabelled antigen compete for a fixed number of sites on the antibody, the higher the concentration of unlabelled antigen the lower the amount of radio-labelled antigen bound.

distribution of the antigen between the bound and free phases is directly related to the total amount of antigen present and thus provides a means for quantitating the latter.

The amount of antibody used in a radioimmunoassay is determined from the antibody dilution curve. This involves the incubation of a fixed amount of radiolabelled antigen (tracer) with different concentrations of the antiserum. After the appropriate incubation and separation of free and bound fractions, an antiserum dilution curve is obtained by plotting the percentage of tracer bound against dilution of the antiserum (see Figures 5.5 and 5.9). The concentration of antiserum chosen for use in a radioimmunoassay is usually that which is sufficient to bind approximately 50% of the tracer.

A standard curve is then obtained by the incubation of the predetermined amount of antiserum and radiolabelled tracer with different known concentrations of purified unlabelled antigen. Labelled and unlabelled antigen compete for a fixed number of binding sites on the antibody: the higher the concentration of unlabelled antigen the lower the percentage of tracer bound (see Figures 5.6 and 5.10). When a sample is substituted for a standard, the value obtained for the percentage bound is simply extrapolated by reading down from the curve to the the horizontal axis.

5.3. Radioimmunoassay of BTG

The radioimmunoassay method depends on competition between BTG and ^{125}I-labelled BTG for a limited number

of binding sites on a BTG specific antibody. The amount of ^{125}I-labelled BTG bound by the antibody will be inversely proportional to the concentration of unlabelled BTG present in platelet-poor plasma samples. The antibody-bound ^{125}I-labelled BTG is separated by a precipitation technique. After centrifugation, the precipitated radioactivity is measured in a gamma counter. By measuring the proportion of ^{125}I-labelled BTG bound in the presence of a series of BTG standards the concentration of BTG in unknown samples can be interpolated from a standard curve.

Method of Ludlam's Group

With the isolation of BTG and production of an antiserum in a rabbit (Moore, Pepper and Cash, 1975a), a radioimmunoassay was developed using these reagents (Ludlam, Moore, Bolton et al., 1975). BTG was radiolabelled with ^{125}I by the chloramine T method (Hunter and Greenwood, 1962). The antiserum was used at a final concentration (1:50,000) which bound approximately 60% of the tracer (final concentration of 1 ng ml). Antiserum, sample (or standard BTG) and tracer were incubated at 4^{o}C for three days before antibody bound BTG was separated from the free antigen by a double antibody method. The precipitate containing the bound antigen was counted for ^{125}I in a gamma counter. The working range of the radioimmunoassay as described was 2.5-150 ng/ml of plasma. However, by incubating the sample (or standard) for three days with antiserum at a final concentration of 1:300,000 prior to the addition of tracer (final concentration 100 pg/ml) and then

incubating for a further three days before separation of antibody bound protein, the detection limit could be decreased to 500 pg/ml of plasma.

5.3.1. Modifications by other workers

(A) Bolton, Ludlam, Moore et al., 1976

This group described three different radioimmunoassays for BTG:

Standard radioimmunoassay

This modified the method of Ludlam et al., 1975 from six days to two days. Antiserum, sample and tracer were incubated at 4 °C for 24 hours. Antibody bound was separated from free ^{125}I BTG as follows: after the addition of non-immune rabbit serum (to act as carrier for the precipitate of anti-BTG antiserum) and donkey anti-rabbit serum the tubes were incubated for 18 hours at 4°C and the precipitate containing the antibody bound BTG fraction collected by centrifugation at 1700 g for 45 minutes. The ^{125}I in the precipitate was measured in a gamma counter. The detection limit for this assay was 500 pg/ml.

Sensitive radioimmunoassay

To increase the sensitivity further, a tracer concentration of 200 pg/ml was used with a correspondingly greater dilution of antisera to bind about 50% of the tracer. Tracer was added after samples had been incubated with antiserum for 24 hours at 4°C as this has been shown to improve the sensitivity of the radioimmunoassay (Hales and

Randle, 1963). After a further 24 hours, antibody-bound ^{125}I BTG was separated from free ^{125}I BTG as before. The sensitivity of this assay was 40 pg/ml.

Rapid radioimmunoassay method

This modified method used antiserum covalently coupled to Sepharose-4B by the method of Cuatrecasas (1970). Plasma samples or standards were diluted in buffer and ^{125}I BTG was added. A suspension of solid-coupled antiserum, diluted to bind about 50% of the tracer was added. The tubes were mixed gently for one hour at room temperature, and the antibody bound separated from the free ^{125}I BTG by centrifugation (1700 g for ten minutes at room temperature) after the addition of diluent containing 15 Tween 80 as a wash. After discarding the supernatants, the residues were washed with further diluent and following centrifugation, the ^{125}I in the residue was counted. The sensitivity of this assay was 500 pg/ml.

(B) Kaplan, Nossel, Drillings et al., 1978

In this modified assay ^{125}I BTG tracer was used at a dilution which gives about 10,000 counts per minute (cpm) per tube (ie 19 pg BTG). Antiserum was used at a dilution of 1:160,000 to 1:320,000. These concentrations bound 0-50% of the tracer. After adding sample or standards, the assays were incubated overnight at 4°C, then antibody bound separated from the free BTG by the addition of 0.5 ml cold saturated $(NH_4)_2SO_4$ at 4°C. Tubes were centrifuged for 15 minutes at 3000g at 4°C. The ^{125}I

in the precipitate was counted in a gamma counter. The sensitivity of this assay was 14 pg ml

(C) Han, Butt, Turpie et al., 1980

(i) Routine assay

In this assay BTG antiserum was diluted with buffer (1:300,000) to bind 50% of tracer and incubated with sample or standard at 4°C for 24 hours. Antibody bound and free ^{125}I BTG was separated by the addition of goat anti-rabbit 1gG and polyethylene glycol (PEG). After mixing the tubes were left at room temperature for five minutes, centrifuged at 1500 g for 20 minutes. The ^{125}I in the precipitate was counted in a gamma counter. The sensitivity of this assay was 4 ng/ml.

(ii) One hour non-equilibrium assay

In this assay BTG antiserum was diluted (1: 50,000) to bind 50% of tracer. Incubation of antiserum, tracer and sample or standard was carried out at room temperature for one hour. Separation of antibody bound and free BTG was as described in the routine assay.

5.3.2. Method for radioimmunoassay of BTG used in this thesis

(A) Preparation of patient plasma

Blood samples were collected by a flawless clean venepuncture without trauma. Immediately after insertion of the needle, the tourniquet was released and 2.7 ml of blood was collected through a 21G Abbott butterfly in a sterile 3 ml plastic syringe and immediately transferred into a precooled polystyrene tube containing EDTA and theophylline as supplied in the BTG radioimmunoassay kit

supplied by the Radiochemical Centre (Amersham, England). The polystyrene tube was inverted three times and placed in an ice-water slurry at 4 °C for a minimum of 30 minutes and a maximum of 120 minutes before centrifugation at 3000 g for 30 minutes. 0.5 ml of the middle layer of platelet poor plasma was assayed either immediately or stored in plastic tubes at -70°C until assayed.

(B) Radioimmunoassay of BTG

This assay was carried out using a commercial kit supplied by the Radiochemical Centre. The kit contained:

1 vial containing up to 2 µCi ^{125}I BTG, freeze-dried.

5 reference standards of human BTG in buffer, nominally 10,20,50,100 and 225 ng/ml , freeze-dried.

1 vial ammonium sulphate solution.

24 sampling tubes containing EDTA and theophylline.

Method

1. Immediately before use, ^{125}I, anti-BTG serum and the reference standards were dissolved in distilled water.
2. 50 ul aliquots of the standards or platelet-poor plasma unknowns were pipetted into the assay tubes.
3. 200 ul of ^{125}I BTG was then added.
4. 200 ul of anti-BTG serum was aliquoted into the tubes.
5. All tubes were vortexed and incubated for one hour at room temperature.
6. 500 ul aliquots of the ammonium sulphate solution were pipetted into all tubes.
7. All tubes were vortexed and centrifuged immediately at 1500 g for ten minutes at room temperature.

8. The supernatant was decanted and the tubes were allowed to drain in racks and excess liquid removed from the rims of the tubes with paper tissues.
9. The ^{125}I in the precipitate was counted.

The tubes were then counted in a PRIAS programmable gamma counter, programmed to calculate the percentage of tracer bound in the tubes containing the set of standards. A standard curve was then printed out as shown in Figure 5.2. The percentage bound of tracer in the unknown samples was compared to this curve and the BTG values read off the horizontal axis.

5.3.3. Methodological variables in the BTG assay

(A) Reproducibility and accuracy of the BTG assay

The reproducibility of the BTG assay was tested as follows: in four different plasmas the BTG concentration was measured on four occasions (mean BTG levels ranging from 10-40 ng/ml); the mean inter assay coefficient of variation was 4.6%. The accuracy of the BTG assay was tested by examining four different plasmas four times within the same assay; the mean intra-assay coefficient of variance was 5.4%.

(B) Stability of BTG in frozen plasma

BTG levels determined in plasma samples immediately after venepuncture did not differ from BTG levels stored up to five months at -70°C (see Table 5.1).

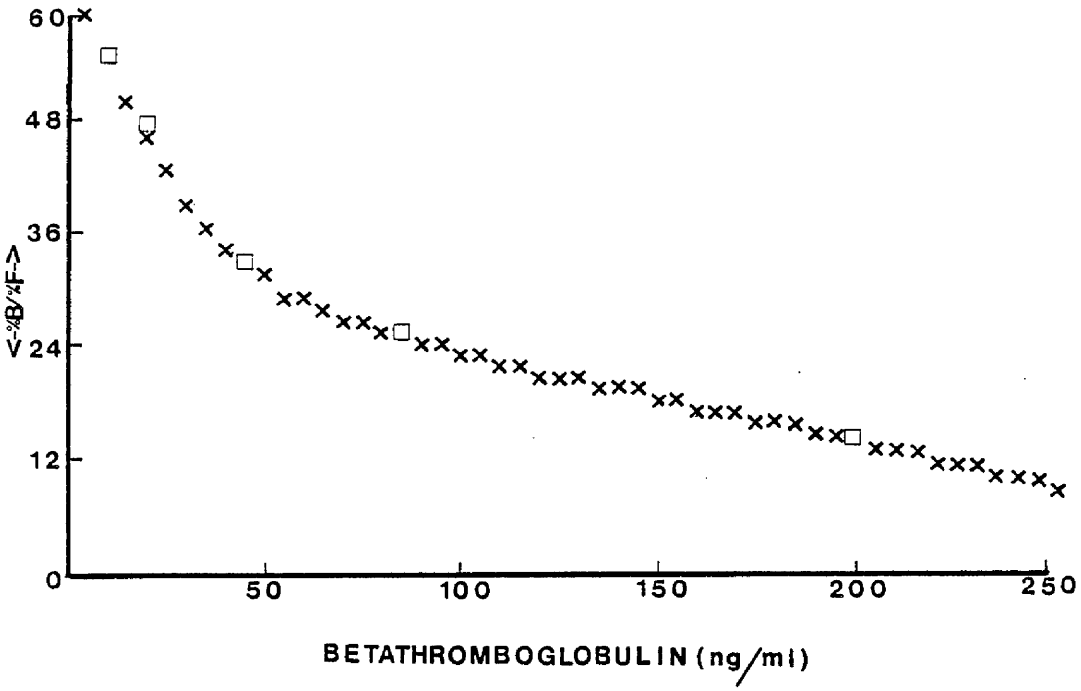


Figure 5.2

A programmed "print-out" of a standard curve for the radioimmunoassay of BTG, calculated from the percentage bound ^{125}I BTG. The percentage of the tracer bound (vertical axis) is progressively reduced with increasing concentrations of standard

TABLE 5.1

REPRODUCIBILITY AND ACCURACY OF BTG, FPA AND Bβ15-42 MEASUREMENTS

		INTRA ASSAY PRECISION	INTER ASSAY PRECISION
BTG	*CV%	5.4	4.6
FPA	CV%	4.4	3.2
Bβ15-42	CV%	9.7	10.2

*CV% = COEFFICIENT OF VARIATION

TABLE 5.2.

STABILITY OF BTG IN FROZEN PLASMA

PATIENT	PLASMA BTG (ng/ml) BEFORE STORAGE	PLASMA BTG (ng/ml) AFTER STORAGE	STORAGE TIME (MONTHS)
GB	37	39	1
CP	36	29	2
AT	53	47	3
AB	46	51	4
JS	39	44	5
MEAN	42.2	42	

(C) Effect of antiocoagulant mixtures on BTG concentrations

The tubes for blood sampling in the Amersham radio-immunoassay kit contain EDTA and theophylline in the anticoagulant. However, the original method (Ludlam et al., 1975) recommended the use of an anticoagulant mixture containing EDTA and two antiplatelet agents (theophylline and prostaglandin E_1, PGE_1)as this mixture has been shown to reduce in vitro release of BTG (Ludlam and Cash, 1976) during plasma preparation. To test this hypothesis, blood samples were obtained with a single venepuncture from 33 healthy male and female subjects (mean age 27.9 $\pm$ 1.3 (SEM) years, range 20-46 years) and equal volumes were immediately added to tubes containing either EDTA and theophylline or the "complete" mixture including PGE_1. Table 5.3 shows the results obtained after the assay of BTG in the samples. While the mean level obtained in samples collected without PGE_1 was higher than the mean level of samples collected with PGE_1, the difference was not statistically significant. In order to minise in vitro release of BTG during plasma processing 0.1 ml of 1 ug/ml PGE_1 was normally added to all BTG tubes unless otherwise stated.

TABLE 5.3

BTG CONCENTRATIONS (ng/ml) IN HEALTHY SUBJECTS WITH TWO DIFFERENT ANTICOAGULANT MIXTURES (n = 23)

ANTICOAGULANT MIXTURE	MEAN ± SEM	RANGE
With PGE_1	21.3 ± 2.3	10-51
Without PGE_1	24.1 ± 2.1	11-49
SIGNIFICANCE OF ANTICOAGULANT WITH AND WITHOUT PGE_1:	p	NS

5.3.4. Effect of physiological variables on the BTG assay

(A) Age and Sex

Blood samples were obtained from volunteers who comprised hospital staff, known to be healthy and on no drug treatment. These included two groups: a group of young volunteers, 47 females (mean age 23 ± 0.6 years, range 18-40 years) and 19 males (mean age 26.7 ± 0.7 years, range 18-40 years); a group of middle aged volunteers, 6 females (mean age 54.0 ± 2.2 years, range 41-60 years) and 4 males (mean age 50.8 ± 3.3 years, range 45-60 years). Blood samples were also obtained from a group of elderly controls who attended a Day Centre for the elderly in the city. An attempt was made to select only those who were apparently perfectly healthy and were not on drug treatment and did not have a past history of a vascular event. These included 20 females (mean age 74.6 ± 2.2 years, range 61-91 years) and 15 males (mean age 77.1 ± 1.9 years, range 62-89 years). The results obtained are shown in Table 5.4.

In the female group there is a statistically significant increase in BTG levels with age between the young age group compared to the middle aged and the elderly group. In the males, BTG shows an increase in the elderly group when compared to the young group but there appears to be a decrease in BTG in the middle aged group possibly as a result of the small sampling size. Plasma BTG in the males is higher than in the female groups, except in the middle aged group, a statistically significant increase being shown between males and females in the young age group. When all the groups were examined together, a positive correlation was found between age and BTG ($r = 0.39$, $p < 0.01$).

TABLE 5.4.

COMPARISON OF SEX AND AGE ON PLASMA BTG LEVELS (ng/ml) IN VOLUNTEERS (MEAN ± SEM)

*p < 0.05, **p < 0.001, NS (not significant)

	FEMALES		
	YOUNG(n=47)	MIDDLE AGED(n=6)	ELDERLY(n=20)
Age (years)	23.0 ± 0.6	54.0 ± 2.2	74.6 ± 2.2
BTG (ng/ml)	15.9 ± 1.2	20.5 ± 1.4*	27.2 ± 2.4**

	MALES		
	YOUNG(n=19)	MIDDLE AGED(n=4)	ELDERLY(n=15)
Age (years)	26.7 ± 0.7	50.8 ± 3.3	77.1 ± 1.9
BTG (ng/ml)	25.4 ± 2.8**	16.5 ± 4.3	28.3 ± 4.4

SIGNIFICANCE OF SEX DIFFERENCE:	p < 0.001	NS	NS

(B) Acute smoking

The effect of acute smoking on plasma BTG levels was studied on ten volunteers who included males and females (mean age 28.3 + 1.8 years, range 21-47) not taking any antiplatelet drugs, who were habitual smokers. They abstained from smoking nine hours prior to sampling. A blood sample was obtained from each volunteer prior to the smoking of three cigarettes, which were smoked within 20 minutes then ten minutes after the last cigarette had been smoked. The results obtained are shown in table 5.5. No significant difference was found in plasma BTG levels before and after acute smoking.

(C) Exercise

The effect of maximal exercise on plasma BTG levels was measured on nine male volunteers (mean age 27.6 + 2.1 years, range 23-32 years) not taking any anti-platelet drugs. A blood sample was obtained prior to exercise. The subjects then performed erect bicycle ergometry using a graded exercise protocol with an initial work-load of 300 k.p.m's (50 watts), increasing by 300 k.p.m. at 3 minute intervals until exhaustion. During the last 30 seconds of peak exercise a second venepuncture was performed and a blood sample for BTG estimation was removed. The results obtained are shown in Table 5.6. No significant difference was found in plasma BTG levels after maximal exercise.

TABLE 5.5

EFFECT OF ACUTE SMOKING ON PLASMA BTG LEVELS (ng/ml)
IN VOLUNTEERS (MEAN ± SEM) (n=10)

	PRE	POST
BTG (ng/ml)	27.4 ± 3.6	24.4 ± 3.7
SIGNIFICANCE OF ACUTE SMOKING	p	NS

TABLE 5.6.

EFFECT OF MAXIMAL EXERCISE ON PLASMA BTG LEVELS Ing/ml)
IN VOLUNTEERS (MEAN ± SEM (n=9)

	PRE	POST
BTG (ng/ml)	18.1 ± 1.4	20.1 ± 2.4
SIGNIFICANCE OF MAXIMAL EXERCISE	p	NS

5.4. Radioimmunoassay of FPA

The radioimmunoassay of FPA depends on the competition between unlabelled and ^{125}I labelled FPA for a limited number of binding sites on a FPA specific antibody. The amount of ^{125}I FPA bound to the antibody is inversely proportional to the concentration of unlabelled FPA in platelet poor plasma from which fibrinogen has been removed. After precipitation and centrifugation the antibody bound ^{125}I FPA is measured in a gamma counter. By measuring the amount of ^{125}I FPA bound in a series of standards the concentration of FPA in unknown samples is interpolated from a standard curve.

Method of Nossel's group

With the isolation of human FPA from plasma and production of an antiserum in a rabbit, a radioimmunoassay was developed using these reagents (Nossel et al., 1971). FPA lacks an amino acid which can be iodinated (see Figure 4.1), hence tyrosine was coupled to the amino terminal alanine of synthetic or native FPA by the use of the ONP (p-hydroxy-phenylpropionic acid p-nitrophenyl ester) ester method (Goodfriend and Ball, 1969). ^{125}I FPA was radiolabelled with ^{125}I by the chloramine T method (Hunter and Greenwood, 1962): 30 ul of 0.3M phosphate buffer (pH. 7.4) and 20 ul of carrier-free ^{125}I (6 mCi) were mixed, and 15 ul of tyrosylated FPA (5 ug) was added. 25 ul of chloramine T (100 ug) and, after 30 seconds, 50 ul of sodium metabisulphite (120 ug) were added, and after a further 30 seconds, 50 ul of ovalbumin (2 mg) was added as a carrier.

The mixture was passed over a Sephadex G-10 column (1.2 x 10 cm) equilibrated with 0.05M Tris, 0.1M saline (ph 7.5). 0.5 ml fractions were collected and radioactivity was counted. The early peak of radioactivity was used as the radiolabelled antigen, being diluted to give about 12,000 cpm per 50 ul. Radioimmunoassay was performed as follows: the assay tubes contained, in 0.5 ml final volume, 250 ul buffer, 50 ul ^{125}I FPA, 100 ul of the standard or sample to be assayed, and 100 ul of antiserum diluted 1:1000 (a dilution that binds about 30% of the total counts). Tubes were incubated for one hour at room temperature. Antibody bound ^{125}I FPA was separated from free ^{125}I FPA by precipitation of the bound peptide by the addition of 2 ml of dioxane-water 9:1 and immediate centrifugation ^{125}I in the precipitate was counted in a gamma counter. Tris-saline buffer (0.15M, pH 8.5) with 1 ng/ml ovalbumin, was used as a diluent for all reagents. The sensitivity of this assay was 0.1 ng FPA (2 ng FPA/ml plasma).

5.4.1. Modifications by other workers

(A) Nossel, Yudelman, Canfield et al., 1974

In this modified method the radiolabelling technique was improved: the following reagents were added sequentially into a conical tube - 10 ul solution containing 2-5 mCi ^{125}I; 30 ul 0.5M phosphate buffer pH 7.5; 10 ug FPA in 15 ul distilled water, and 15 ul chloramine T (50 ug) in

0.5 M phosphate buffer, pH 7.5. Thirty seconds later 20 ul sodium metabisulphite (96 ug) in 0.5M phosphate buffer, pH 7.5, 20 ul heparinised normal plasma and 200 ul 0.5M phosphate buffer, pH 7.5 were added. The iodinated peptide was separated on a Sephadex G10 column as described previously (Nossel et al., 1971).

The radioimmunoassay technique was modified by a preincubation and an increase in the amount of plasma tested: the assay tubes contained 100 ul of antiserum, 500 ul of test plasma or FPA standards made up to 500 ul with tris-ovalbumin buffer. After a 24 hour incubation at 4°C, 50 ul of tracer was added. A one hour incubation at 4°C took place before the separation of 500 ul of cold (4°C) charcoal suspension. After mixing the tubes were immediately centrifuged, the supernatant decanted and counted in a gamma counter. This modification resulted in an increase in specificity from 2 ng/ml in the original assay to 0.15 ng/ml.

Fibrinogen cross-reacts with the FPA antiserum and while extraction through an XM50 ultrafiltration membrane (a membrane that retains molecules of molecular weight 34,000 and above) gave reproducible results with buffer solutions, variable results were obtained when plasma samples were tested (Nossel et al., 1971). In this method the plasma was separated, 2.5 ml of ethanol was added to 2.5 plasma in a 12 ml conical centrifuge tube in an ice bath. After 30 minutes incubation, the tube was centrifuged at 1,500 g at 4°C for 20 minutes; 4 ml of the supernatant was pipetted into a fresh conical

centrifuge tube, which was recentrifuged at 1,500 g at 4°C for 20 minutes to eliminate any remaining precipitate, and the top 2 ml was pipetted into a segment of dialysis tubing and dialysed for at least 24 hours at 4°C. Manual inversion occurred once an hour when possible. Reproducible recovery of 75-100% of FPA added to plasma was obtained. The lowest FPA levels in plasma, thus reflecting the least in vitro generation of FPA were obtained with an anticoagulant mixture of heparin, 1000 U, and Trasylol, 1000 U, in 0.15M Na cl; 9 mls of blood being added to 1 ml anticoagulant.

(B) Harenberg, Hepp and Schmidt - Gayk, 1979

Three steps of the radioimmunoassay were modified:

Plasma dialysis was omitted. 200 ul of the supernatant of the ethanol precipitated plasma extract was added to microlitre cups. The extract was evaporated with nitrogen, then antiserum was added for 24 hours, then tracer for one hour. Bound and free ^{125}I FPA were separated by the addition of charcoal.

By the use of a second modification the bound ^{125}I FPA was separated by the use of a second antibody: normal rabbit serum, antiserum and sample or standard were incubated for one hour. Rabbit-immunoglobulin antiserum from goat and

tracer were added, and incubated overnight at 4 C. The microtitre plates were then centrifuged, the precipitate washed with buffer, recentrifuged and counted in a gamma counter. The sensitivity of this assay was 0.16 ng/ml.

(C) Kockum and Frebelius, 1980

In this modified method bentonite was used for the rapid removal of cross-reacting fibrinogen from plasma: bentonite was mixed with tris-saline buffer containing ovalbumin to a concentration of usually 100 ng/ml. The bentonite was mixed on a magnetic stirrer. While still stirring 0.5 ml of the bentonite suspension was pipetted into tubes followed by 1 ml of plasma. The tubes were capped and tilted for ten minutes on a reciprocal mixer at room temperature, then centrifuged (4^{o}C, 5000g for 10 minutes). The supernatant was pipetted off and assayed directly or frozen at -65^{o}C until analysis. Radioimmunoassay was performed as described by Nossel et al., 1974. The detection limit for this assay was 0.019 ng/ml.

5.4.2. Method for radioimmunoassay of FPA used in this thesis

(A) Preparation of patient plasma

Blood samples were collected by clean flawless venepuncture without trauma as described in 5.3.2. The first 2.7 ml of blood was removed and usually used for BTG estimation. Using a new syringe 9 ml of blood was removed into 1 ml of anticoagulant containing 1000 IU heparin (Heparin sodium, Weddel Pharmacueticals Ltd., West Smithfield, London); 1000 KIU trasylol (Trasylol, Bayer Pharmaceuticals Ltd. Haywards Heath, Sussex, England) in sodium chloride, 0.15M. After gentle inversion three times, the sample was

placed on ice or centrifuged immediately at 2000g for 20 minutes at 4°C. Plasma was assayed immediately or snap frozen and stored at -70°C until assayed.

(B) Radioimmunoassay of FPA

Iodination of desaminotyrosyl FPA

(i) Materials

Standard FPA, desaminotyrosyl FPA and rabbit anti-human FPA serum were purchased from IMCO Corporation Ltd (Stockholm, Sweden); Sodium ^{125}I (carrier free) was supplied by the Radiochemical Centre (Amersham, England). Ovalbumin, Grade V, and tris (Trizma (R)Base) were obtained from Sigma Chemicals Ltd., London. Sephadex G10 (Pharmacia (Great Britain) Ltd. Hounslow, Middlesex, England); sodium chloride (saline), sodium phosphate (phosphate); Chloramine T, sodium metabisulphite and sodium barbitone which were all "Analar" grade were purchased from BDH Chemicals Ltd., Poole, Dorset, England. Heparinised normal plasma was obtained by adding 9 ml of blood to 0.2 ml heparin, 5000 IU/ml and 0.8 ml saline 0.15M. After mixing the blood was centrifuged, plasma separated and stored frozen in aliquots at -70°C.

(ii) Radiolabelling of desaminotyrosyl FPA

1. To a conical reaction tube containing 10 ug desaminotyrosyl FPA in ammonium bicarbonate 0.1M, which was stored frozen at -70°C, add 30 ul phosphate buffer 0.5M, pH 7.5.

2. In rapid succession add 15 ul chloramine T (23.5 ug) and 10 ul Na ^{125}I (approximately 1 mCi). Mix for 30 seconds.
3. Add 20 ul sodium metabisulphite (48 ug), mix for 30 seconds.
4. Add 20 ul heparinised normal plasma
5. Add 200 ul phosphate buffer.
6. Apply reaction mixture to a 15 ml plastic pipette which contains 10 ml Sephadex G10 equilibrated with tris 0.05M - saline 0.1M, pH 7.5.
7. Collect 30 0.5 ml fractions.
8. Count each fraction in a Wilj single well gamma counter for 10 seconds.

The separation obtained on Sephadex G10 between ^{125}I FPA and free ^{125}I is illustrated in figure 5.3 (a). The fraction containing the highest ^{125}I FPA activity is diluted with 4.5 ml Tris-saline buffer containing 80 mg ovalbumin. Aliquots containing 100 ul ^{125}I FPA are stored frozen at - 70°C. ^{125}I FPA is usually stable for three weeks at -70°C after which the non specific binding is usually greater than 10% and thus the tracer should not be used after three weeks.

Specific activity of ^{125}I FPA

To obtain the specific activity of ^{125}I FPA 10 ul was removed from the iodination mixture before it was applied to Sephadex G10. 90 ul phosphate buffer was added, then 10 ul was applied to two presoaked 3MM chromatography strips, 3 cm wide (Whatman, UK, Maidstone, Kent). The strips were presoaked in 0.05M Barbitone

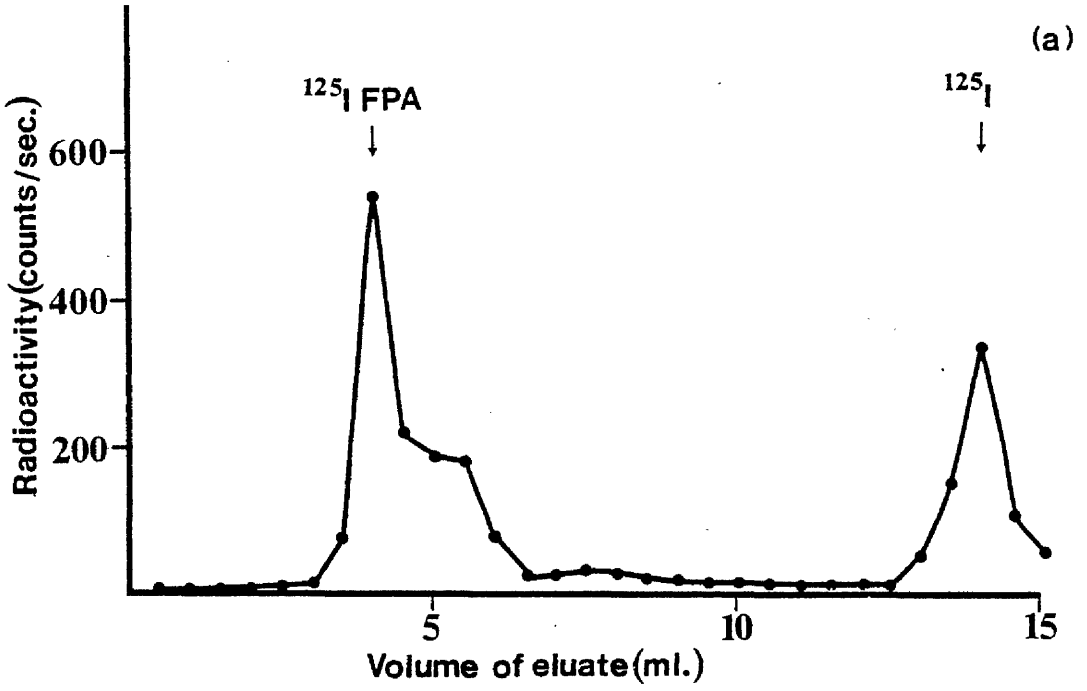


Figure 5.3 (a)

Separation of ^{125}I-labelled FPA from (^{125}I) iodide on Sephadex G-10. The separation was carried out by gel-filtration of an iodination reaction mixture containing ^{125}I FPA (10 ug) of specific radioactivity 61 uCi/ug, chloramine-T (23.5ug), sodium metabisulphite (48 ug) and phosphate buffer 230 ul and approximately 1 mCi of (^{125}) iodide in a total volume of 305 ul. The column (5g) of Sephadex G-10 was equilibrated with 0.05 M tris, 0.1 M NaCl buffer, pH 7.5. Unretarded iodinated protein was eluted first, followed by a smaller (^{125}I) iodide peak.

buffer pH 8.6. Electrophoresis was carried out using a current of 10 m amps, 500 volts in barbitone buffer. The strips were dried then divided into 0.5 cm and counted in a gamma counter. The separation of protein associated counts and free ^{125}I is shown in figure 5.3(b). The specific activity is consequently calculated:

Analysis of chromatography strips -

	Av of both strips
Total counts:	338350
Protein associated counts:	206294
% incorporation:	61

Specific activity:

10 ug = 1000 uCi ^{125}I

For 100% incorporation, Specific activity = 100 uCi/ug

For 6% incorporation, Specific activity = 61 uCi/ug

Preparation of samples from patients

This was carried out according to the IMCO protocol: frozen patient's plasma was thawed at 37°C. When just thawed, it was kept at 0°C. 2.5 ml of thawed or fresh plasma was put in a glass centrifuge tube at 0°C. 2.5 ml absolute ethanol (Burrough Ltd., London) (0°C) was added, the tube vortexed and kept at 0°C for 30 minutes. After centrifugation at 2000g for 20 minutes at 4°C, 4 ml of the supernatant was transferred to a new centrifuge tube and

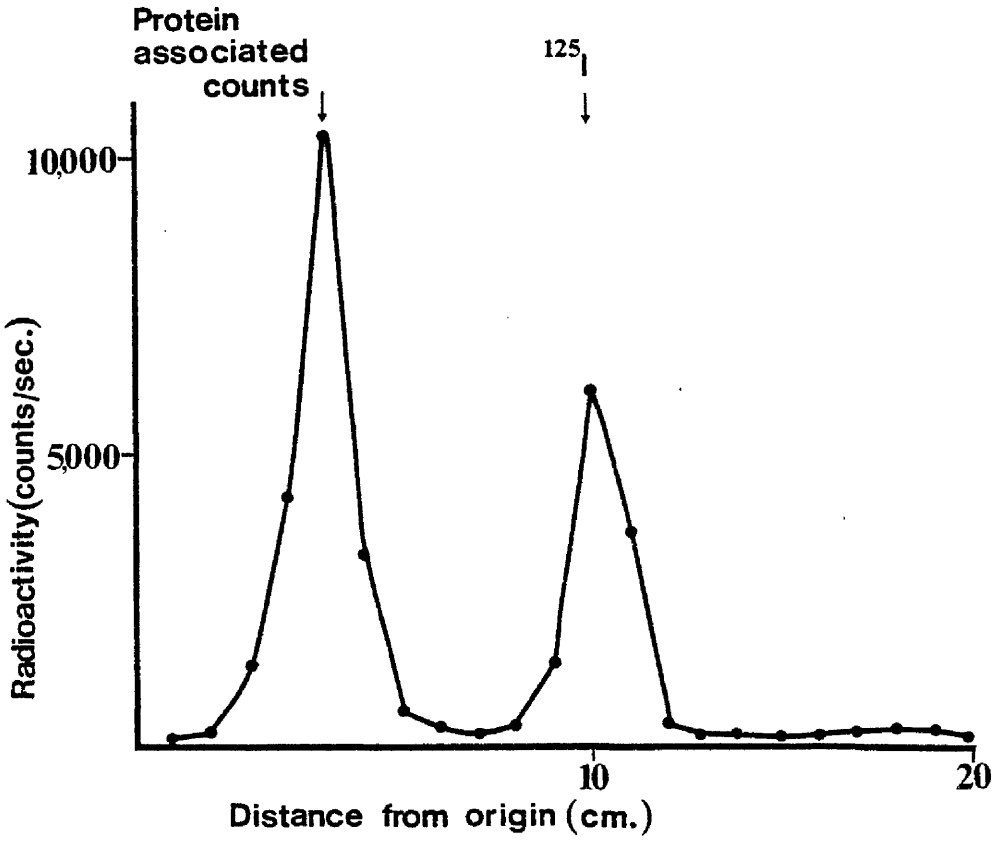


Figure 5.3 (b)

Electrophoresis separation of 10 ul (a 1:10 dilution of the iodination reaction mixture in 5.3 (a) on 3MM chromatography strips. Electrophoresis was carried out using a current of 10 m amps in 0.05M barbitone buffer, pH 8.6 for one hour.

kept at 0°C for 30 minutes. After centrifugation at 2000g for 20 minutes at 4°C, 2 ml of the supernatant was placed in a segment of 1-8 32" dialysis tubing (Medicell International Ltd., London) and dialysed against 8ml tris 0.05M, saline 0.1M, ovalbumin 0.1%, pH 8.5 on a rocking shaker at 4°C overnight. The dialysate was assayed immediately. Due to the cross-reaction of plasma fibrinogen with FPA antiserum (see Figure 5.4) it was necessary to remove the fibrinogen from plasma.

Assay procedure

This was set up according to the IMCO protocol:

1. Add ethanol and buffer according to Table 5.7. The buffer used was Tris 0.05M, saline 0.1M, ovalbumin 0.1%, pH 8.5.
2. Add 100 ul of FPA A(20 ng/ml) to tubes 7 and 8. Dilute 200 ul of FPA (20 ng/ml) with 200 ul buffer. Add 100 ul of this to tubes 9 and 10. Dilute the remaining 200 ul with 200 ul buffer and add 100 ul of this to tubes 11 and 12 etc.
3. Add 500 ul patient's dialysate to duplicate tubes.
4. Add 100 ul antiserum of adequate dilution to all tubes except 1-4.

In the assay protocol it is recommended that an antiserum concentration is used which results in 30-50% binding of the antiserum to the tracer. The protocol recommends a 1:1000 dilution of antiserum which, when an antiserum curve is set up, does result in a binding of 40-50% as shown in Figure 5.5.

5. Mix and cap tubes. incubate overnight at 4°C.

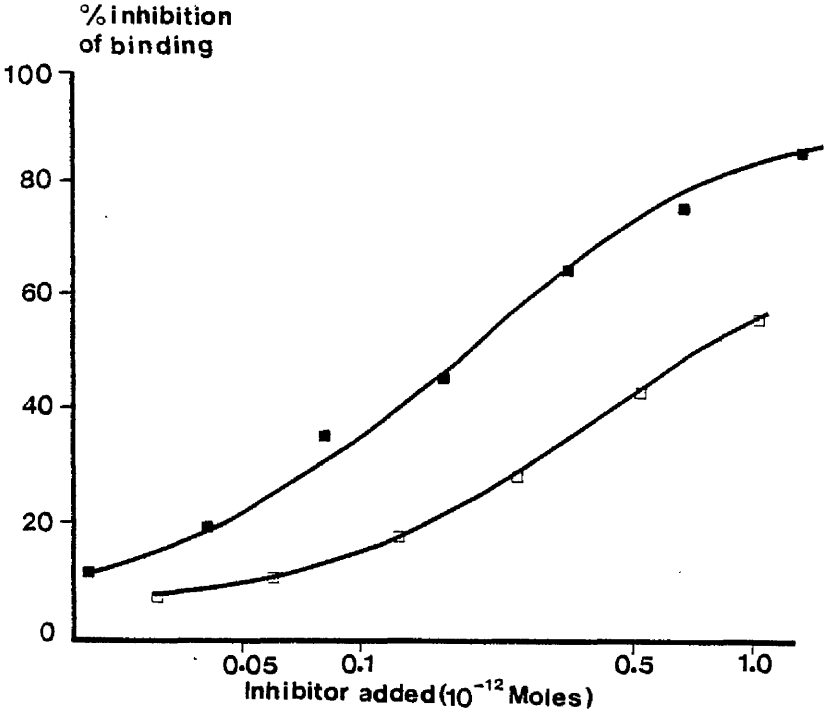


Figure 5.4

Inhibition of binding of FPA tracer by standard amounts of "cold" FPA (■—■) and pure fibrinogen (□—□). A 1:1000 dilution of FPA antiserum was used and this bound about 40% of tracer in absence of either competitor.

TABLE 5.7

FPA ASSAY PROTOCOL

Tube Number	Sample	Ethanol ul	Buffer ul	FPA/patients' samples. ul	Antiserum ul		Tracer ul		Charcoal ul
1- 2	buffer control	50	550	-	-		50		-
3- 4	charcoal control	50	550	-	-		50		500
5- 6	antiserum control	50	50	-	100		50		500
7- 8	FPA 1:1 = 2 ng/100 ul	50	350	100	100		50		500
9-10	FPA 1:2 = 1ng/100 ul	50	350	100	100		50		500
11-12	FPA 1:4 = 0.5 ng/100 ul	50	350	100	100		50		500
13-14	FPA 1:8 = 0.25 ng/100 ul	50	350	100	100		50		500
15-16	FPA 1:16= 0.125 ng/100 ul	50	350	100	100		50		500
17-18	FPA 1:32= 0.06 ng/100 ul	50	350	100	100		50		500
19-20	FPA 1:64= 0.03 ng/100 ul	50	350	100	100		50		500
21-22	Patient I	-	-	500	100		50		500
23-24	Patient II	-	-	500	100		50		500
25-26	Patient III	-	-	500	100		50		500
27-28	Patient IV	-	-	500	100		50		500
29-30	Patient V	-	-	500	100		50		500
31-32	Patient VI	-	-	500	100		50		500
33-44	Antiserum control	50	350	500	100		50		500

INCUBATE OVERNIGHT AT 4°C

INCUBATE ONE HOUR 4°C

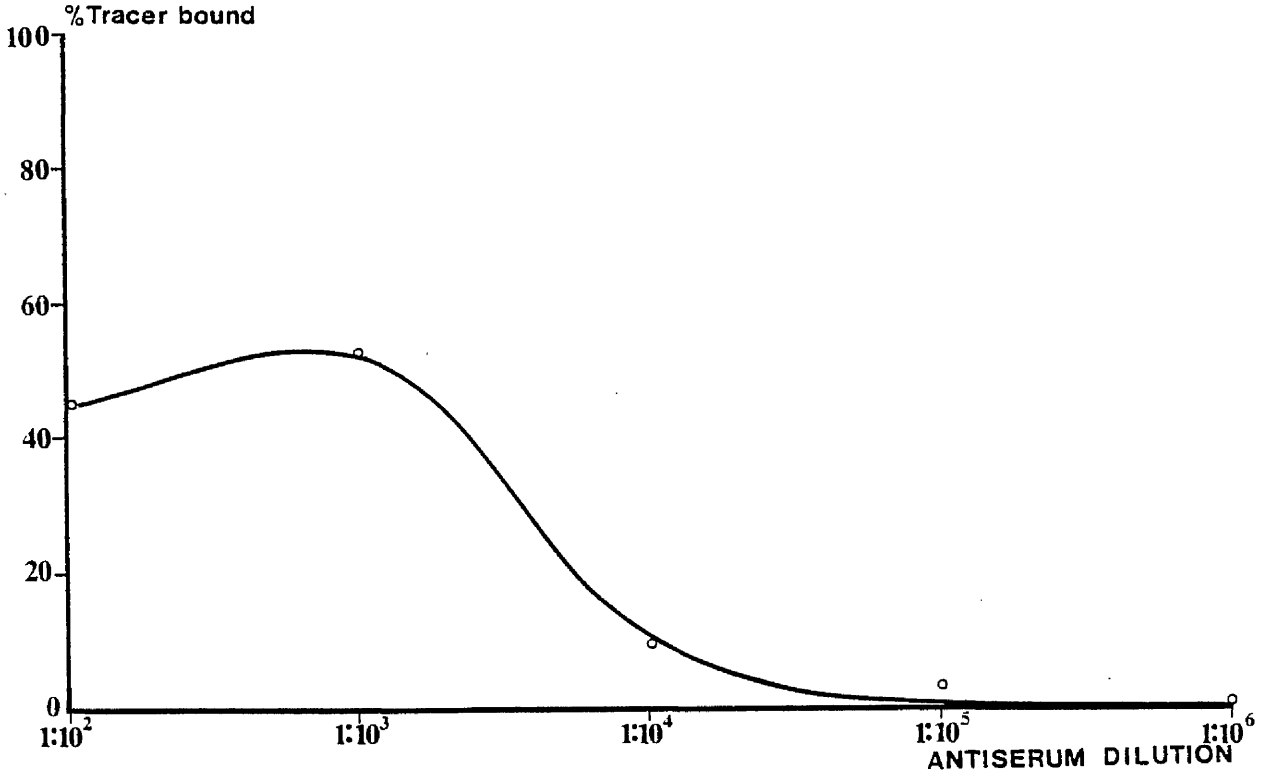


Figure 5.5

Antiserum dilution curve for FPA antiserum. 50% binding of the FPA tracer occurs at the 1:1000 dilution.

6. ^{125}I desaminotyrosyl FPA tracer is diluted with buffer to give about 10,000 cpm/50 ul. 50 ul is added to each tube.
7. Mix, cap and incubate for one hour at 4°C.
8. Add 500 ul charcoal suspension (200 ml buffer, 0.6 gram ovalbumin, 5 gram charcoal (activated untreated powder supplied by Sigma Chemicals Ltd., London) to all tubes except 1 and 2 and mix.
9. Centrifuge immediately (within 5 minutes after addition of charcoal) at 3000 g for 10 minutes at 4°C.
10. Decant the supernatant into new tubes, cap and count ^{125}I radioactivity in a PRIAS Auto-Gamma counter.

Determination of results

1. Calculate average of duplicates.
2. The charcoal control is the blank and is subtracted from all other figures.
3. $\frac{\text{Antiserum control}}{\text{buffer control}} \times 100 = \text{antiserum binding \%}$
4. $\frac{\text{Standard FPA or patient's sample}}{\text{antiserum control}} \times 100 = \text{binding \% of antiserum binding}$
5. Subtract % binding from 100% to obtain % inhibition of binding.
6. Draw the standard curve (see Figure 5.6) and calculate from the curve the amount of FPA in patients' samples.
7. Correct patients' FPA value to obtain ng/ml plasma (correction factor of 20).

Within the last year FPA concentrations in scientific journals has been expressed in picamoles per ml (pmol/ml). 1 pmole is equivalent to 1.535 ng/ml. Therefore the value of FPA obtained in patient's sample expressed in ng/ml is divided by 1.535.

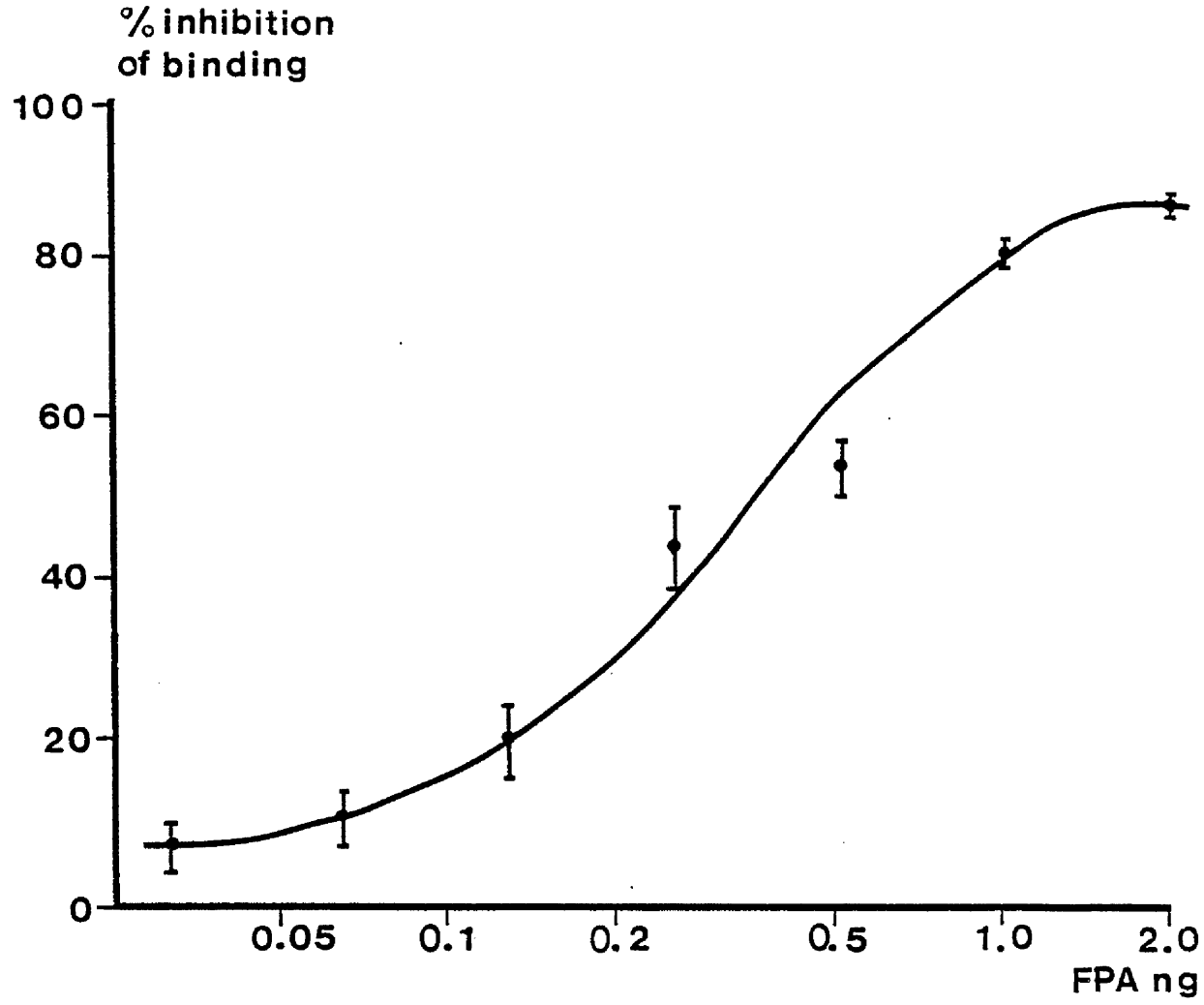


Figure 5.6

Standard curve for radioimmunoassay of human FPA. Values (average of doubles) represent the means of five experiments, standard deviation indicated by vertical bars. Separation of bound ^{125}I FPA by the double antibody technique.

5.4.3. Methodological variables in the FPA assay

(A) Reproducibility and accuracy of the FPA assay

The reproducibility of the FPA assay was tested as follows: in four different plasmas the FPA concentration was measured on four occasions (mean FPA levels ranging from 1.2-6.8 pmole/ml); the mean inter assay coefficient of variance was 3.2%. The accuracy of the FPA assay was tested by examining four different plasmas four times within the same assay; the mean intra-assay coefficient of variance was 4.4% (see Table 5.1).

(B) Stability of FPA in frozen plasma

FPA levels determined in plasma samples immediately after venepuncture did not differ from FPA levels stored up to five months at -70°C (see Table 5.8).

(C) Comparison of ethanol extraction and bentonite extraction on FPA recovery

Recovery was assessed by the addition to normal plasma of 1.5, 5 and 6.25 pmol/ml FPA before (a) ethanol precipitation and dialysis and (b) bentonite extraction. Ethanol precipitation was carried out as described in 5.4.2 (b). Bentonite extraction was carried out as follows: Bentonite (Sigma Chemicals Ltd. London) was mixed with TSO buffer to a concentration of usually 100 mg/ml.

The solution was stirred with a wooden applicator stick and then placedon a magnetic stirrer. While still stirring 0.5 ml of the bentonite suspension (50 mg of bentonite)was pipetted into tubes containing 1 ml fresh

TABLE 5.8

STABILITY OF FPA IN FROZEN PLASMA

	PLASMA FPA (pmol/ml)		
Patient	Before Storage	After Storage	Storage Time (Months)
JT	4.7	5.1	5
AMcK	6.8	6.8	3
JD	2.5	2.1	2
SM	1.2	1.2	1
Mean	3.8	3.8	

TABLE 5.9.

EFFECT OF ETHANOL OR BENTONITE EXTRACTION ON FPA RECOVERY

Amount of FPA added to plasma	Recovery after extraction	
	Ethanol then dialysis	Bentonite
2.5 pmol/ml	89	100
5 pmol/ml	106	112
6.25 pmol/ml	99	107

or thawed plasma. The tubes were capped, vortexed then tilted for 10 minutes on a reciprocal mixer at room temperature and centrifuged (4°C, 3000g for 10 minutes). 1 ml of plasma supernatant was removed into a clean tube and another 0.5 ml of bentonite suspension was added. After tilting on the mixer for another 10 minutes, centrifugation was again carried out. The supernatant was assayed immediately. The results obtained in Table 5.9 show that adequate recovery was obtained by both methods.

(D) Effectiveness of ethanol and bentonite in separating FPA from fibrinogen in plasma

To determine the actual amount of fibrinogen left in the ethanol and bentonite supernatants after extraction, fibrinogen concentrations were measured before and after extractions in the plasma of two normal controls. Fibrinogen was estimated according to the method of Clauss, 1957. The lowest detection limit of the fibrinogen assay was 0.04 mg/ml which is equivalent to 200 pmoles. From Table 5.10 it is evident that both methods are adequate in removing fibrinogen from plasma.

(E) A comparison of charcoal and solid phase double antibody in the separation of bound ^{125}I FPA

The use of powdered charcoal for the removal of free ^{125}I antigen, thus leaving the bound fraction in the supernatant, has been used for many years (Herbert, Leu, Gottlieb et al., 1965). Unfortunately charcoal also

TABLE 5.10

EFFECTIVENESS OF ETHANOL AND BENTONITE IN SEPARATING FPA FROM FIBRINOGEN IN PLASMA

	Fibrinogen in plasma (pmoles/ml)	Fibrinogen concentration after ethanol extraction (pmoles/ml)	Fibrinogen concentration after bentonite extraction (pmoles/ml)
1.	14,417	235	235
2.	8,529	235	235

TABLE 5.11

COMPARISON OF CHARCOAL AND SAC-CEL IN SEPARATING BOUND ^{125}I FPA

	Plasma FPA (pmol/ml ± SEM)	
	Charcoal Suspension	Sac-cel Suspension
n = 24	3.7 ± 0.8	3.6 ± 0.8
	(range 0.4-13 pmol/ml)	(range 0.4-13 pmol/ml)

"competes" with the antiserum and thus strips labelled antigen from the bound complex if strict adherence is not kept to immediate centrifugation once the charcoal suspension has been added. The use of a 'double' or 'second' antibody should circumvent any non specific binding to charcoal. The 'second' antibody is specific, the γ-globulin of the species in which the first antibody was raised - eg since FPA antiserum was raised in rabbits, the second antibody would be raised against a rabbit. More recently solid phase double antibodies in which the second antibody is coupled to an insoluble matrix such as cellulose have been developed (den Hollander and Schuurs, 1971). In 1982 a commercial solid phase double antibody - Sac-cel (Wellcome Diagnostics) became available. Sac-cel is a cellulose coupled anti-rabbit solid phase double antibody.

To determine the effectiveness of Sac-cel in separating the bound ^{125}I FPA, FPA estimations were carried out in 24 duplicate samples. In one sample the FPA estimation was carried out using the IMCO protocol with charcoal separation of bound and free ^{125}I FPA. In the second sample 0.1 ml of Sac-cel suspension was added to each tube after incubation with tracer. The tubes were vortexed then left for one hour at room temperature during which vortexing was repeated at 20 and 40 minutes. After one hour, 1 ml of distilled water was carefully layered into each tube, with minimum disturbance of the Sac-cel pellet, to reduce the non-specific binding to the sides of the tubes. The tubes were centrifuged at 2000 g for two minutes at room

temperature. The supernatant was removed by suction and the precipitate counted in a gamma counter. As shown in table 5.11 no difference was found in the values of FPA determined by the use of either charcoal or the double antibody body. In order to minimise any possible non-specific binding due to charcoal stripping, the double antibody method was used in the latter studies reported in this thesis.

5.4.4. Effect of physiological variables on the FPA assay

(A) Age and sex

Using the same protocol as described in 5.4.2 (A) blood samples were obtained from three groups; a group of young volunteers, 40 females (mean age 22.9 ± 0.7 years, range 18-40 years) and 19 males (mean age 26.7 ± 0.7, range 18-40 years); a group of middle aged volunteers; 4 females (mean age 53.5 ± 3.9 years, range 42-59 years) and 4 males (mean age 50.2 ± 3.7 years, range 42-59 years); an elderly volunteer group, 14 females (mean age 78.7 ± 2.3 years, range 64-91 years) and 12 males (mean age 76.8 ± 2.3 years, range 62-89 years). The results obtained are shown in Table 5.12.

In the female group there is a statistically significant increase in age from the young age group to the elderly group. Although the FPA level is increased in the middle aged group this difference is not significant due to the small sampling

TABLE 5.12

COMPARISON OF SEX AND AGE ON PLASMA FPA LEVELS (pmol/ml) IN VOLUNTEERS (MEAN ± SEM)

*p<0.05, ** p<0.02, *** p<0.001

FEMALES

	Young (n=40)	Middle aged (n=4)	Elderly (n=14)
Age (years)	22.9 ± 0.7	53.5 ± 3.9	78. 7 ± 2.3
FPA (pmol/ml)	0.76 ± 0./1	1.7 ± 0.4	1.75 ± 0.4*

MALES

	Young (n=19)	Middle aged (n=4)	Elderly (n=12)
Age (years)	26.7 ± 0.7	50.2 ± 3.7	76. 8 ± 2.3
FPA (pmol/ml)	0.4 ± 0	0.7 ± 0.3	2. 3 ± 0.5***
SIGNIFICANCE OF SEX DIFFERENCE	p<0.01	NS	NS

TABLE 5.13

EFFECT OF ACUTE SMOKING ON PLASMA FPA LEVELS (pmol/ml) IN VOLUNTEERS (MEAN ± SEM) (N=10)

	PRE	POST
FPA (pmol/ml)	0.4 ± 0	0.4 ± 0
SIGNIFICANCE OF ACUTE SMOKING	p	NS

TABLE 5.14

EFFECT OF MAXIMAL EXERCISE ON PLASMA FPA LEVELS (pmol/ml) IN VOLUNTEERS (MEAN ± SEM) (n=9)

	PRE	POST
FPA (pmol/ml)	0.4 ± 0	0.4 ± 0
SIGNIFICANCE OF MAXIMAL EXERCISE	p	NS

size. Similar results were obtained in the male groups with FPA being significantly increased in the older group. Plasma FPA appears to be increased in the female groups when compared to the male groups except for the older male group, a statistically significant increase being shown between males and females in the young age group. When all the groups were examined together, a correlation was found being age and FPA ($r=0.51$, $p< 0.001$).

(B) Acute smoking

The effect of acute smoking on plasma FPA levels was studied on the same ten volunteers as described in the protocol in 5.3.4 (B). The results obtained are shown in Table 5.13. No difference was found in plasma FPA levels before and after acute smoking.

(C) Exercise

The effect of maximal exercise on plasma FPA levels was studied on the same nine volunteers as described in the protocol in 5.3.4 (C). The results obtained are shown in Table 5.14. No difference was found in plasma FPA levels before and after maximal exercise.

5.5. Radioimmunoassay of Bβ15-42 Peptide

The radioimmunoassay of Bβ15-42 depends on the competition between unlabelled and ^{125}I labelled Bβ15-42 for a limited number of binding sites on a Bβ15-42 specific antibody. The amount of ^{125}I Bβ15-42 bound to the antibody is inversely proportional to the concentration

of unlabelled Bβ15-42 in platelet poor plasma from which fibrinogen has been removed. After precipitation and centrifugation the antibody bound ^{125}I Bβ15-42 is measured in a gamma counter. By measuring the amount of ^{125}I Bβ15-42 in a series of standards the concentration of Bβ15-42 in unknown samples is interpolated from a standard curve.

Method of Kudryk's Group

This group described the isolation of human Bβ15-42 from human plasma and production of an antiserum in a rabbit. A subsequent radioimmunoassay was developed using these reagents (Kudryk et al., 1982). The peptide contains a tyrosine residue at position Bβ41 (see Figure 4.2) and, therefore, can be labelled with ^{125}I. Bβ15-42 was radiolabelled with ^{125}I by the chloramine T method (Hunter and Greenwood, 1962): 25 ul; of sodium phosphate buffer, pH 7.5 containing 10 ug Bβ15-42 peptide was mixed with 5-10 ul of carrier free Na ^{125}I (1 mCi), 10 ul of chloramine T (50 ug) is added and after mixing (30 seconds) the reaction was terminated with 100 ul sodium metabisulphite (100 ug). The mixture was passed over a Sephadex G-10 column (0.38 x 30 cm) equilibrated with 0.05 M Tris - 0.1M Na Cl (pH 7.5). To minimise adsorption loss of the tracer, the reaction mixture was diluted with 1 mg/ml bovine serum albumen to act as carrier. The early peak of radioactivity was used as the radiolabelled antigen, being diluted to give 25,000 cpm per 50 ul. Radioimmunoassay was performed as follows: the assay tubes contained 0.25 ml final volume, 50 ul ^{125}IβB 15-42,

100 ul of the standard or sample to be assayed (this was diluted 1:2), and 100 ul of antiserum diluted 1:1500 (a dilution that binds about 30% of the total counts). Tubes were incubated for 18 hours at 4°C. Antibody bound ^{125}I Bβ15-42 was separated from free ^{125}I Bβ15-42 by the addition of an appropriate dilution of a second antibody. After end-over-end mixing for two hours at room temperature, then the tubes were centrifuged at 4000 g for 20 minutes at room temperature. The resulting pellet was washed three times and then the ^{125}I was counted in a gamma counter. The sensitivity of this assay was 0.16 pmol/ml. Fibrinogen was removed from plasma by extraction with ethanol. Recently a commercial kit has become available for the measurement of Bβ15-42.

5.5.1. Method for radioimmunoassay of Bβ15-42 used in this thesis

(A) Preparation of patient plasma

This was as described for the preparation of patient plasma in the FPA assay (see 5.4.2), plasma being assayed immediately or snap frozen and stored at -70°C until assayed.

(B) Radioimmunoassay of Bβ15-42

Iodination of Bβ15-42

(i) Materials

Standard Bβ15-42 and rabbit anti-human Bβ15-42 were purchased from IMCO Corporation Ltd (Stockholm, Sweden).

All other reagents were as described in the iodination of FPA.

(ii) Radiolabelling of Bβ15-42

1. To a conical reaction tube containing 10 ug Bβ15-42 peptide in 25 ul sodium phosphate (0.5M) pH 7.5, which is stored at -70°C, add in rapid succession 10 ul chloramine T (50 ug) and 10 ul Na ^{125}I (approximately 1 mCi). Mix for 30 seconds.
2. Add 100 ul sodium metabisulphite (100 ug). Mix for 30 seconds.
3. Apply the reaction mixture to a 15 ml plastic pipette which contains 10 ml sephadex G10 equilibrated with tris 0.05M-saline 0.1M, pH 7.5. Wash the reaction mixture with 100 ul tris-saline buffer containing 1 mg/ml bovine serum albumin (BSA).
4. Repeat step 3.
5. Collect thirty 0.5 ml fractions.
6. Count each fraction in a Wilj single well gamma counter for ten seconds.

The separation obtained on Sephadex G-10 between ^{125}I Bβ15-42 and free ^{125}I is illustrated in figure 5.7(a). The fractions containing the total ^{125}I Bβ15-42 activity are pooled then an equal volume of buffer containing BSA is added. Aliquots containing 50 ul ^{125}I Bβ15-42 are stored frozen at -70°C. The tracer is stable for two months at -70°C and is only used when the non specific binding is lower than 5%.

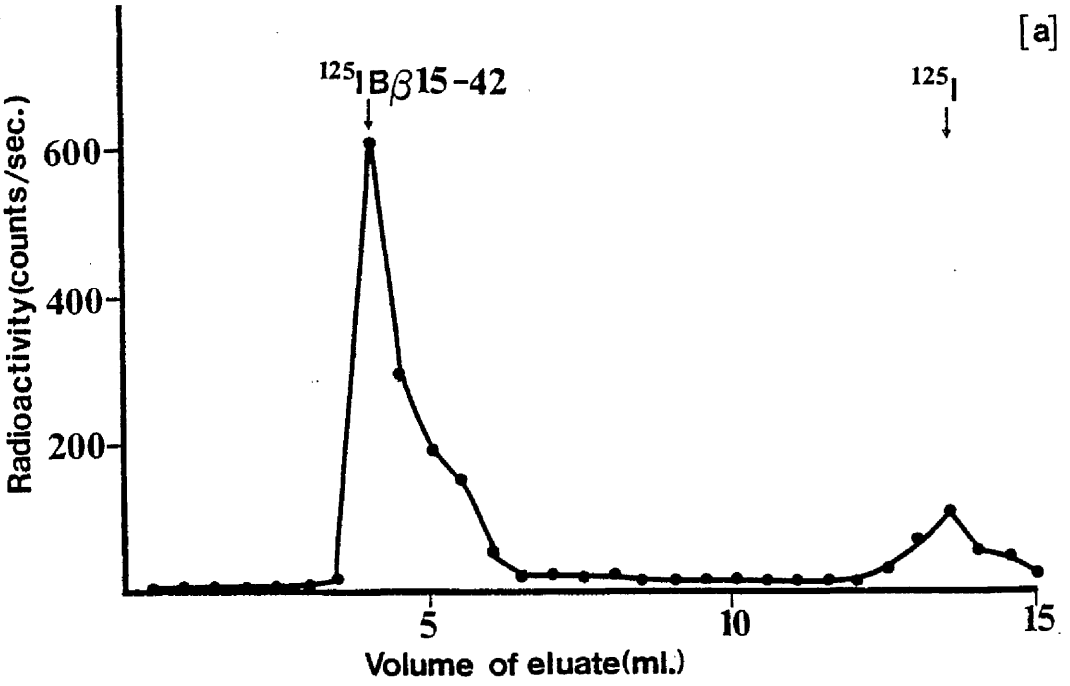


Figure 5.7(a)

Separation of ^{125}I-labelled Bβ15-42 from (^{125}I) iodide on Sephadex G-10. The separation was carried out by gel-filtration of an iodination mixture containing ^{125}I Bβ 15-42 (10 ug) of specific radioactivity 66 uCi/ug, chloramine-T (50 ug), sodium metabisulphite (100 ug), and phosphate buffer (25 ul) and approximately 1 mCi of (^{125}) iodide in total volume of 145 ul. The column (5g) of Sephadex G-10 was equilibrated with 0.05 M tris, 0.1 M NaCl buffer, pH 7.5. Unretarded iodinated protein was eluted first, followed by a smaller (^{125}I) iodide peak.

Specific activity of ^{125}I Bβ15-42

The specific activity of ^{125}I Bβ15-42 was measured as described for ^{125}I FPA (see 5.4.2 (B)). The separation of protein associated counts and free ^{125}I is shown in Figure 5.7 (b). The specific activity is consequently calculated:

Analysis of chromatography strips:

	Average of both strips
Total counts:	484621
Protein associated counts:	322046
% Incorporation:	66

Specific activity: 10 ug = uCi ^{125}I

For 100% incorporation, Specific activity = 100 uCi/ug

For 66% incorporation, Specific activity = 66 uCi/ug

Preparation of samples from patients

Due to the cross-reaction of plasma fibrinogen with Bβ15-42 antiserum (see Figure 5.8) it was necessary to remove the fibrinogen from plasma samples. This was carried out according to the method in the IMCO protocol: frozen plasma was thawed at 37°C. When just thawed, it was kept at 0°C. 1 ml of thawed or fresh plasma was put in a plastic centrifuge tube at 4°C. 1 ml of absolute ethanol was added, the tube vortexed and kept at 0°C for 30 minutes. After centrifugation at 2000g for 20 minutes at 4°C the supernatant was removed into a new centrifuge tube and kept at 4°C for a further 30 minutes. Centrifugation was repeated and an aliquot removed from the supernatant for immediate assay.

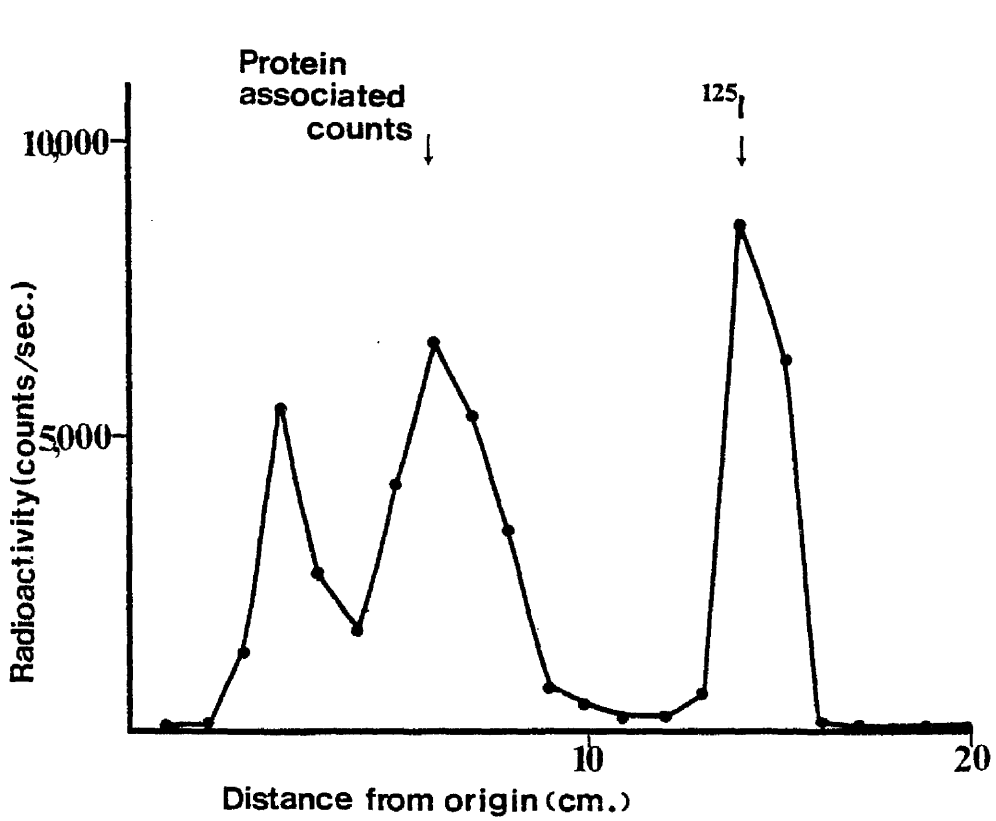


Figure 5.7(b)

Electrophoresis separation of 10 ul (a 1:10 dilution of the iodination mixture in 5.7 (a) on 3 MM chromatography strips. Electrophoresis was carried out using a current of 10 m amps in 0.05M barbitone buffer, pH 8.6 for one hour.

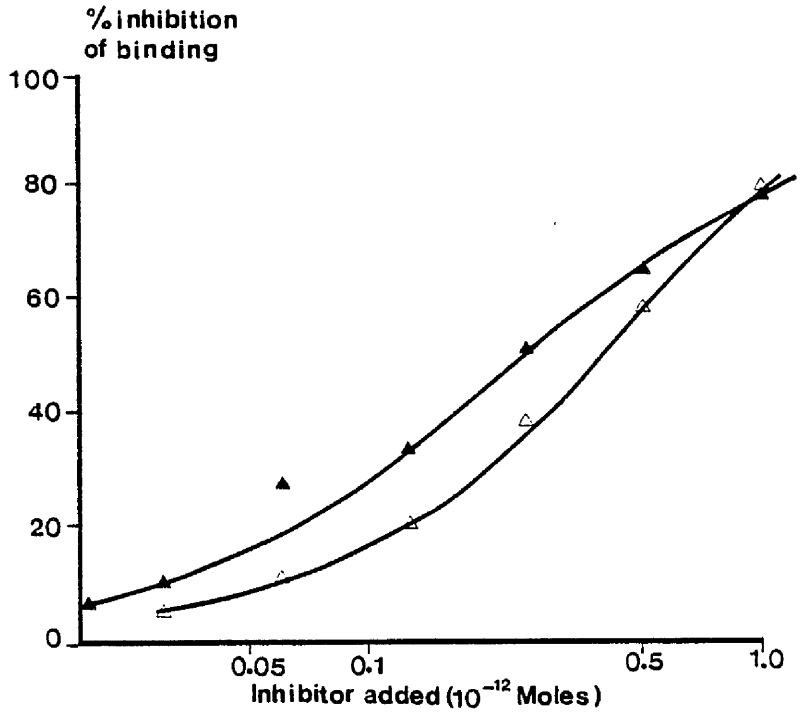


Figure 5.8

Inhibition of binding of B 15-42 tracer by standard amounts of "cold" Bβ 15-42 (▲—△) and pure fibrinogen (▲—▲). A 1:1500 dilution of Bβ 15-42 antiserum was used and this bound about 30% of the tracer in absence of either competitor.

Assay procedure

This was set up according to the IMCO protocol.

1. Add buffer according to Table 5.15. The buffer used was Tris 0.04M, Saline 0.11M, EDTA 0.01M, Na N_3 0.02%, Trasylol (20 KIU/ml), ovalbumin 1 ng/ml, pH 7.4.
2. Add 100 ul of Bβ15-42 standard (15 ng/ml) to tubes 7 and 8. Prepare doubling dilutions as shown in Table 5.15.
3. Dilute 0.5 ml of all patients' plasma extracts with 0.5 ml buffer. Aliquot 100 ul to the appropriate tubes.
4. ^{125}I Bβ15-42 is diluted with buffer to give about 25,000 cpm/50 ul. 50 ul is added to each tube.
5. Add 100 ul antiserum of adequate dilution to all tubes except 1-4.

In the assay protocol it is recommended that an antiserum concentration is used which results in 25-35% binding of the antiserum to the tracer. The protocol recommends a 1:1500 dilution of antiserum which, when an antiserum dilution curve is set up, does result in a binding of 25-35% as shown in Figure 5.9.

6. Mix and cap the tubes, incubate for 18 hours at 4°C.
7. Add 100 ul Sac-cel suspension. Mix, leave for one hour at room temperature, vortex at 20 and 40 minutes.
8. Add 1 ml of distilled water without disturbing pellet, centrifuge 2000g for two minutes. Aspirate supernatant.
9. Count ^{125}I radioactivity in a PRIAS Auto Gamma Counter.

TABLE 5.15

Bβ15-42 ASSAY PROTOCOL

Tube No	Sample	Buffer ul	Bβ15-42 Standard ul	Plasma Sample	Antiserum	Tracer			
1-2	Buffer control	200	-	-	-	50			
3-4	Sac-cel control	200	-	-	-	50			
5-6	Antiserum control	100	-	-	100	50			
7-8	Bβ15-42 1:1 (0.5)*	-	100	-	100	50			
9-10	1:2 (0.25)	-	100	-	100	50			
11-12	1:4 (0.125)	-	100	-	100	50			
13-14	1:8 (0.063)	-	100	-	100	50			
15-16	1:16(0.031)	-	100	-	100	50			
17-18	1:32(0.16)	-	100	-	100	50			
19-20	1:64(0.008)	-	100	-	100	50			
21-22	Patient 1	-		100	100	50			
23-24	Patient 2	-		100	100	50			
25-26	Patient 3	-		100	100	50			
	etc.						INCUBATE AT 4°C OVERNIGHT	ADD 100 ul Sac-cel	Leave one hour at room temperature. Centrifuge at 2000 G for two minutes. Count precipitate.

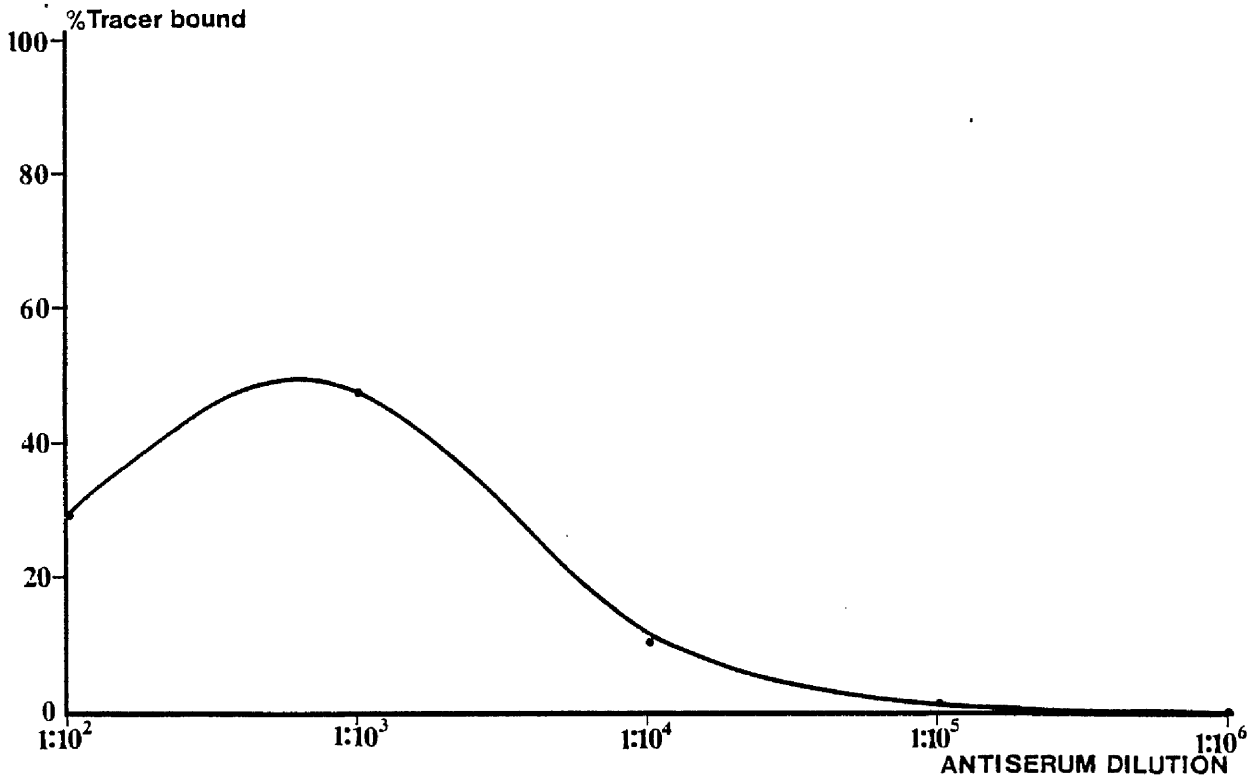


Figure 5.9

Antiserum dilution curve for Bβ15-42 antiserum. 50% binding of the Bβ 15-42 tracer occurs at the 1:1500 dilution.

Determination of results

1. Calculate average of cpm doubles. Subtract the value of the Sac-cel control (tubes 3 and 4) from all averages, except in the non specific binding estimation.
2. $\frac{\text{Sac-cel control}}{\text{buffer control}} \times 100$ = % non-specific binding of tracer (NSB). NSB is usually 2-4% of buffer control.
3. $\frac{\text{Antiserum control}}{\text{Buffer control}} \times 100$

   The value should be 25-30%.
4. Subtract % binding from 100% to obtain % inhibition of binding.
5. Construct the standard curve (see Figure 5.10) and calculate from the curve the amount of Bβ15-42 in patients' samples.
6. Correct patients Bβ15-42 value to obtain pmol/ml plasma (correction factor = 20).

5.5.2. Methodological variables in the Bβ15-42 assay

(A) Reproducibility and accuracy of the Bβ15-42 assay

The reproducibility of the Bβ15-42 assay was tested as follows: in four different plasmas the Bβ15-42 concentration was measured on four occasions (mean Bβ15-42 levels ranging 0.8-4.8 pmol/ml); the mean inter assay coefficient of variance was 10.2%. The accuracy of the Bβ15-42 assay was

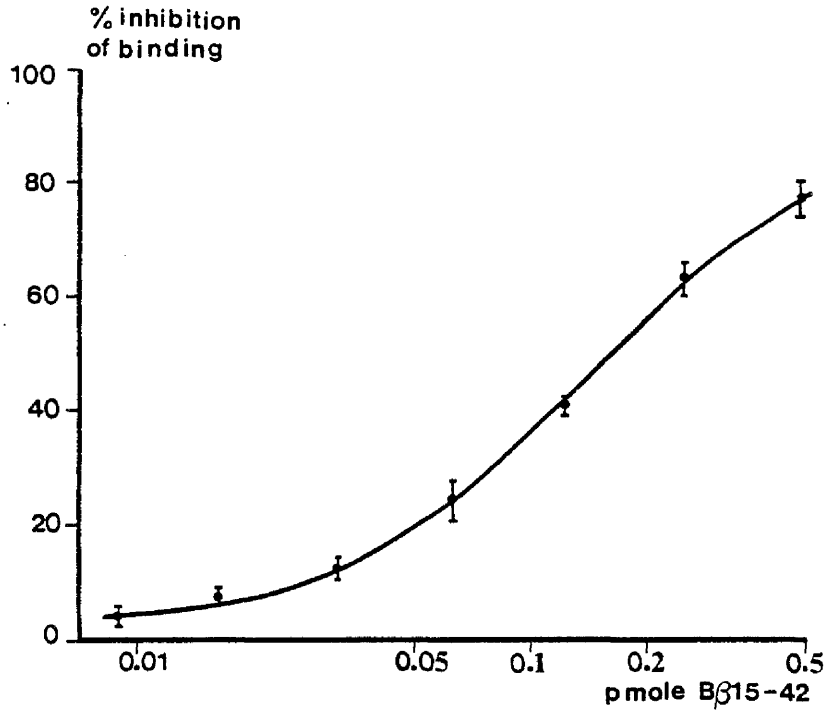


Figure 5.10

Standard curve for radioimmunoassay of human Bβ15-42. Values (average of doubles) represent the means of four experiments, the standard deviation indicated by vertical bars.

tested by examining four different plasmas four times within the same assay, the mean intra-assay coefficient of variation was 9.7% (see Table 5.1).

(B) Stability of Bβ15-42 in frozen plasma

Bβ15-42 levels determined in plasma samples immediately after venepuncture did not differ from Bβ15-42 levels in samples stored up to five months at -70°C (see Table 5.16).

5.5.3 Physiological variables in the Bβ15-42 assay

Effect of sex

Blood samples were obtained from a group of young volunteers, five females (mean age 25.2 ± 1.9 years, range 21-32 years) and twelve males (mean age 26.3 ± 1.2 years, range 18-33 years). The results obtained are shown in figure 5.17. Similar levels of plasma Bβ15-42 were found in males and females in the young age group.

TABLE 5.16

STABILITY OF Bβ15-42 IN FROZEN PLASMA

Patient	Before Storage	After Storage	Storage time (Months)
JT	10	10	5
AMcK	4	3.4	3
JD	0.8	0.7	2
SM	1	1.3	1
MEAN			

TABLE 5.17

EFFECT OF SEX ON PLASMA Bβ15-42 LEVELS (pmol/ml)

IN YOUNG VOLUNTEERS (mean ± SEM)

	AGE (YEARS)	Bβ15-42 (pmol/ml)
FEMALES (n=5)	25.2 ± 1.9	0.9 ± 0.1
MALES (n=12)	26.3 ± 1.2	0.8 ± 0.1

CHAPTER 6

Plasma Fibrinopeptide A and Betathromboglobulin in Pre-eclampsia and Pregnancy Hypertension

The aim of the studies undertaken in this chapter was to determine whether plasma levels of fibrinopeptide A (FPA) and betathromboglobulin (BTG) were abnormal in pregnant women with pre-eclampsia or essential hypertension when compared to pregnant women during normal pregnancy. Changes in platelet activity, blood coagulation and fibrinolysis in pre-eclampsia may be partly responsible for the increase in fibrin deposition seen in the placental bed in pre-eclampsia as well as the pathogenesis of this disease. A brief historical review on pre-eclampsia will be given in the first part of this chapter (6.1). In the second part (6.2) an initial study on pre-eclampsia and pregnancy hypertension will be described. In this study plasma samples from pregnant women with pre-eclampsia were compared to pregnant women with hypertension, healthy pregnant women in the third, second and first trimester of pregnancy, healthy non-pregnant women taking oral contraceptives and healthy non-pregnant women not taking oral contraceptives.

In the next part of this chapter (6.3) a second study on the administration of oral labetalol, a mixed α and β blocker, in the management of mild or moderate pre-eclampsia will be described. In this study plasma samples from pregnant women with mild or moderate pre-eclampsia taking oral labetalol are compared to the following not taking labetalol: pregnant women with mild or moderate pre-eclampsia, pregnant women with intrauterine growth retardation (IUGR) and normal healthy pregnant women. The conclusions from both studies will be discussed together in 6.4.

6.1. Introduction

In the fifth century B.C. Hippocrates noted that "drowsiness, fits and coma" were of serious prognostic importance in a pregnant woman, but the actual term "eclampsia" was first introduced by Verandeus in 1619. It is derived from the Greek word for a "flash" because many such patients complain of seeing "flashing" lights before the onset of a seizure. Lever in 1843 reported that proteinuria was present in patients with eclampsia and this led to the recognition of a "pre-eclamptic state".

It was believed for many years that the placenta manufactured a specific toxin, which was responsible for both pre-eclampsia and eclampsia (Holland, 1909) and this concept led to the use of the terms "toxaemia of pregnancy" or pre-eclamptic toxaemia (P.E.T). A toxin has never been discovered, so it is preferable to use the term "pre-eclampsia" to describe a clinical state which can potentially

progress to eclampsia. A number of hypotheses have been produced to explain the cause of pre-eclampsia, becoming so numerous, that as long ago as 1916 Zweifel termed it the "disease of theories" (Chesley, 1978).

A precise, generally acceptable definition of pre-eclampsia has not yet been reached and different clinical criteria have been used. The main features of this syndrome are systemic hypertension, proteinuria and oedema. The differences in definition lie in the degree of abnormality required to make a diagnosis. Oedema is difficult to quantitate and is, in any case, present in 40% of normal pregnant women (Thomson, Hytten and Billewicz, 1967). Proteinuria appears to indicate a much more severe form of the disease (MacGillvray, 1961). The diagnosis of pre-eclampsia is usually made, therefore, on the basis of sustained hypertension and proteinuria developing during the second half of pregnancy (Page, 1972). The use of the term "hypertensive disease of pregnancy" was suggested in the Lancet (1975) to avoid the problem of of whether a patient is "toxaemic" or not. Such a group, which would include patients with pre-existing hypertension and chronic renal disease, would be even more heterogeneous than that included under the term "pre-eclampsia".

The primary factor (or factors) responsible for the syndrome have not yet been identified despite many biochemical and other abnormalities being demonstrated along with pre-eclampsia.

Page (1972) suggested "an inner vicious circle" which tended to be self perpetuating in a cascade of events which is only broken by the termination of the pregnancy. Thus a reduction in uteroplacental circulation associated with vasoconstriction results in damage to the placenta, the release of trophoblast into the circulation precipitating DIC, fibrin deposition, glomerular endothelial lesions, reduced glomerular filtration rate, sodium retention, plasma volume with further reduction in placental perfusion. He also described an outer circle of predisposing factors such as excess sodium load, chronic renal and hypertensive diseases and posture. Recently pre-eclampsia has been likened to a disease of cascades (MacGillvray, 1981) such as the calcium transport system, the renin-angiotensin-aldosterone system, the kinin system, the prostaglandins, as well as the haemostatic system. Diet, genetic factors and immunologic factors are also involved. These factors may break into the chain at various points, thus precipitating a sequence of events which affects every system in the body.

Within the last few years, abnormalities in the prostaglandin system in pre-eclampsia, as suggested by a deficiency of prostacyclin have been reported. This deficiency may be the result of reduced prostacyclin synthetase (Lewis, 1981). This suggests an imbalance in the TXA_2/PGI_2 ration in pre-eclampsia similar to that suggested in Chapter 2 as a mechanism in thrombosis.

Changes in the haemostatic mechanism, suggestive of intravascular coagulation, have been observed in pre-eclampsia. These changes which are significantly greater than those occurring in normal pregnancy, include an increased rate of coagulation activation as suggested by an increased factor VIII related antigen/factor VIII clotting ratio (factor VIII ratio) (Boneu, Bierme, Fournie et al., 1977; Thornton and Bonnar, 1980; Fournie, Monrozies, Pontonnier et al., 1981) and increased soluble fibrin monomer complexes (McKillop et al., 1976).

Changes in the fibrinolytic system, which are different from those in normal pregnancy, have also been found in pre-eclampsia. In pre-eclampsia lower levels of plasma plasminogen are present than in normal pregnancy (Bonnar, McNicol and Douglas, 1971, Howie, Prentice and McNicol, 1971). There is also an increase in the level of inhibitor to urokinase in plasma from patients with pre-eclampsia (Bonnar et al., 1971, Howie et al., 1971). Serum FDP levels are higher in pre-eclamptic women than in normal pregnant controls (Bonnar et al., 1971; Howie et al., 1971; Condie 1976) although sequential studies suggest that consistently raised levels are not always present in pre-eclampsia (Gordon, Ratky, Sola et al., 1976). These results suggest that although there may be increased inhibition of fibrinolysis in pre-eclampsia, this is not complete and some lysis of insoluble fibrin is occurring. The results are, therefore, compatible with a degree of intravascular coagulation being present in pre-eclampsia.

Changes in platelet activity have also been found in pre-eclampsia. There is a reduction in platelet count (Bonnar et al., 1971) leading to thrombocytopenia (Pritchard, Weisman, Ratnoff et al., 1954; Howie et al., 1971). The lowered platelet count has been assumed to be due to the sequestration of platelets in the microcirculation of organs such as the placenta, kidney and liver as a result of increased platelet aggregation with a concurrent release of platlet granules. While lowered 5HT levels were found in severe pre-eclampsia when compared to normal pregnancy This may or may not be raised according to another study (Jelen, Fananapazier and Crawford, 1979). Another indication of increased platelet aggregation has been the report of increased BTG levels in pre-eclampsia (Redman, Allington, Bolton et al., 1977). Increased levels of BTG have also been reported in women taking oral contraceptives (Arandi, Saez, Abril et al., 1979; Duncan, 1979).

The estimation of plasma levels of BTG and FPA, markers of platelet release and thrombin generation respectively should provide more direct evidence of platelet and coagulation activation in pre-eclampsia.

6.2. Plasma BTG and FPA in Pre-eclampsia and Pregnancy Hypertension

2.1. Design of study

Six groups of patients were studied:

Group 1. Severe pre-eclampsia (n = 13): criteria

(1) Hypertension of 150/100mmHg or over before delivery

(2) Proteinuria ranging from 1.5-10g/day

(3) Generalised oedema

(4) Normal BP and no proteinuria at postnatal visit.

Group 2. Pregnancy hypertension (n = 11): criteria

(1) Persistent hypertension of more than 140/90mmHg in the first trimester of pregnancy

(2) No proteinuria or oedema

(3) Lowered BP at post natal visit.

Group 3. Normal pregnancy: criteria

(1) Uncomplicated pregnancy free from hypertension, oedema and proteinuria

(2) Should deliver infant of normal weight.

This group includes:

(A) 20 women in the 3rd trimester of pregnancy

(B) 15 women in the 2nd trimester of pregnancy

(C) 22 women in the 1st trimester of pregnancy

Group 4. This includes 14 healthy non-pregnancy women on on oral contraception with 30 ug oestrogen pills.

Group 5. This includes 20 healthy non-pregnant women not on oral contraception.

The pregnant groups were matched for age, parity and gestation. The non-pregnant women were matched for age with the pregnant groups, and sampled in mid-menstrual cycle.

2.2 Results

Betathromboglobulin

The plasma BTG levels (measured by the Amersham RIA kit) are shown in figure 6.1. No prostaglandin E_1 was added to the BTG blood sample tubes. No significant differences were found between the non-pregnant women not taking oral contraceptives and the women taking oral contraceptives; nor between non-pregnant women and healthy women in the first trimester of pregnancy. Elevated levels of BTG were found in the second trimester ($p < 0.01$) and third trimester ($p < 0.01$) of pregnancy, compared to the non-pregnant group and the first trimester of pregnancy Group.A further increase in BTG levels was found in pre-eclampsia ($p < 0.01$) and pregnancy hypertension ($p < 0.02$) when compared to healthy women in the third trimester of pregnancy. Despite higher levels being obtained in pre-eclampsia than in pregnancy hypertension, this difference was not statistically significant.

Fibrinopeptide A

The plasma FPA levels (measured by the IMCO kit) are shown in figure 6.2. No significant differences were found between the plasma FPA levels in the non-pregnant women not taking oral contraceptives and the women taking oral contraceptives; nor between non-pregnant women and any of the healthy pregnant groups. Significantly elevated FPA levels were found in pre-eclampsia ($p < 0.01$) and pregnancy hypertension ($p < 0.01$) compared to healthy women in the third trimester of pregnancy. Despite higher FPA levels being obtained in pre-eclampsia than in pregnancy hypertension, this difference was not statistically significant.

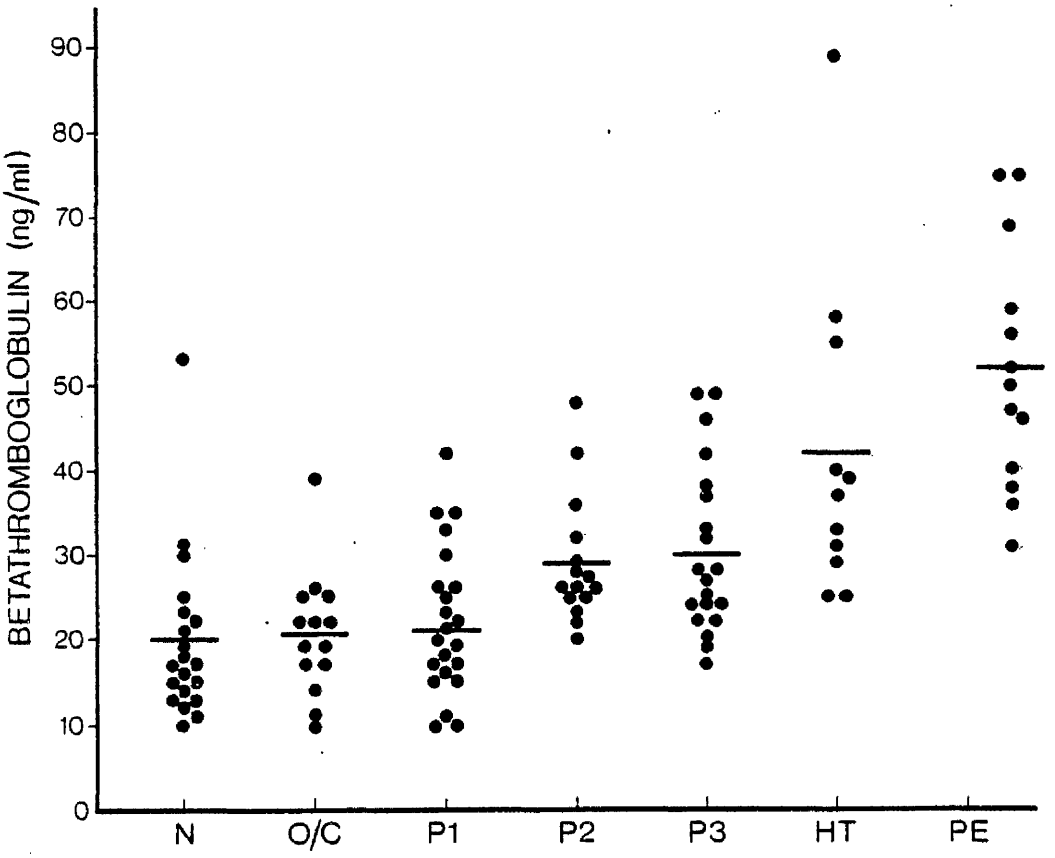


Figure 6.1

BTG levels in groups studied. N = normal non-pregnant. O/C = non-pregnant taking oral contraceptives. P1 = normal 1st trimester pregnant. P2 = normal 2nd trimester pregnant. P3 = normal 3rd trimester pregnant. HT = pregnancy hypertension. PE = pre-eclampsia.

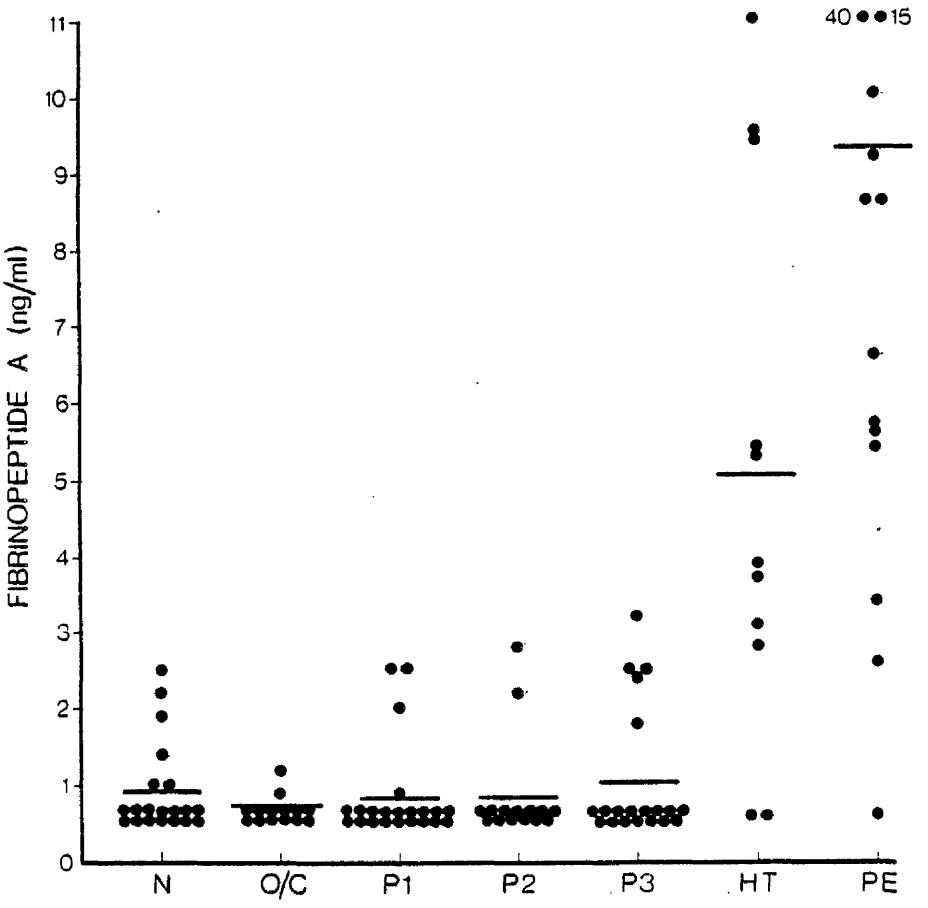


Figure 6.2.

FPA levels in groups studied.

6.3. Labetalol in the Management of Mild or Moderate Pre-eclampsia

6.3.1. Introduction

Labetalol is a unique antihypertensive agent which acts as a competitive inhibitor of alpha and beta adrenoreceptors, the beta blocking effects having been shown to be non-selective. α-adrenergic platelet stimulation is associated with the inhibition of adenyl-cyclase (Salzman and Levine, 1971), the decrease of cyclic AMP (Salzman, 1972a) and the induction of platelet aggregation. α-adrenergic blocking agents prevent this chain of reactions and specifically inhibit adrenaline-induced platelet aggregations (Praga and Pogliani, 1971). Oral labetalol undergoes considerable first pass metabolism in the liver, and is rapidly cleared via the bile and kidneys. Labetalol is less lipophilic than propranolol or oxprenolol and, therefore, may cross the placenta to a lesser extent.

The antihypertensive properties of labetalol in non-pregnant patients have been well documented. The acute and chronic haemodynamic effects of labetalol in hypertensive patients are quite different from those obtained with propranolol. Thus labetalol's antihypertensive action is largely due to a reduction in total peripheral resistance with only small changes in heart rate and cardiac output, whereas β-adrenoreceptor blocking drugs tend to cause a decrease in cardiac output with an initial rise in peripheral resistance. To date over 100 pregnant women with hypertension of varying aetiology have been studied (Michael, 1979; Lamming, Broughton and Symonds, 1980) and two possible advantages of labetalol over antihypertensive drugs used in the management of high blood pressure in pregnancy have been suggested. First it has been suggested that labetalol may encourage maturation of the

fetal lung (Michael, 1980; Nicholas, 1978) and secondly, labetalol may diminish the proteinuria of patients who have developed proteinuric pre-eclampsia before commencement of drug therapy (Michael, 1979). Recently work by Lunell (1982) suggests that labetalol has little, if any, effect on uterine blood flow.

If further studies confirm the efficacy, apparent safety and good tolerance of labetalol in pregnancy, it may well become the drug of choice for the control of hypertension in pregnancy.

The study undertaken assessed the use of oral labetalol in the management of mild to moderate pre-eclampsia. The aim was to reduce diastolic blood pressure to less than 90 mmHg and to determine the effect of a reduction in blood pressure on platelet release and thrombin generation by the measurement of plasma levels of BTG and FPA. Plasma levels of BTG and FPA were also determined in normal prenant women and pregnant women with intra uterine growth retardation. Recently it has been suggested that all hypertension in pregnancy and intra uterine growth retardation should be considered to have some "superimposition of pre-eclampsia" (Lewis, 1982).

6.3.2. Design of study

Four groups of patients were studied.

Group 1. Mild to moderate pre-eclampsia (n = 9) criteria:

(1) Hypertension consisting of a proteinuria <0.5g/day

Blood pressure >95 mmHg <110 mmHg in the third trimester of pregnancy

This group received drug administration according to the following protocol.

Labetalol tablets (Glaxo Group Research Ltd., Greenford, England) were administred at a starting dose of 100 mg b.d. Dose was increased daily according to the schedule below until a diastolic blood pressure <90 mmHg or the maximum daily dose of 1200 mg is reached.

Dose Schedule

Dose level 1 100 mg b.d.

Dose level 2 200 mg b.d.

Dose level 3 300 mg b.d.

Dose level 4 400 mg b.d.

Dose level 5 400 mg t.i.d.

Patients remained in hospital for the duration of the study.

Group 2

As for group 1 but no drug administration was given (n = 13).

Blood samples were obtained pre, 1 week, post last treatment and after delivery.

Group 3.

Intra uterine growth retardation (n = 8).

Group 4.

Normal pregnancy (n = 21): criteria

(1) Uncomplicated pregnancy free from hypertension, oedema and proteinurea in the third trimester of pregnancy.

Should deliver infant of normal weight.

All groups were matched for age, parity, and gestation.

6.3.3. Results

Blood pressure

The blood pressure values obtained are shown in Table 6.1. Blood pressures were taken from the left arm by the same operator on each occasion. Significantly higher differences were in pregnant women with pre-eclampsia when compared to normal pregnant women and pregnant women with I.U.G.R. Diastolic blood pressures were lowered with labetalol administration.

Betathromboglobulin

Plasma BTG levels are shown in Figure 6.3. No significant differences were found between the normal pregnant women with I.U.G.R. or pregnant women with pre-eclampsia either before or after drug treatment, or without drug treatment.

Fibrinopeptide A

Plasma FPA levels are shown in Figure 6.4. Elevated plasma levels of FPA were found in pregnant women with I.U.G.R. and pre-eclampsia when compared to normal pregnant women. This elevation was statistically significant in the pregnant women with I.U.G.R. In the group treated with labetalol, higher FPA levels were obtained after treatment and statistically elevated levels were obtained after delivery.

TABLE 6.1.
BLOOD PRESSURE VALUES (mmHg) IN GROUPS STUDIED
(MEAN VALUES)

Group Studied	Pre	Post (7 days treatment)	Post Delivery
	Systolic/ Diastolic	Systolic/ Diastolic	Systolic/ Diastolic
Pre-eclampsia Labetalol treatment	140/94**	133/89	128/83
Pre-eclampsia non-treatment	135/93*	133/91	130/86
I.U.G.R.	125/81		
Normal pregnancy	115/71		

** $p < 0.02$ * $p < 0.05$

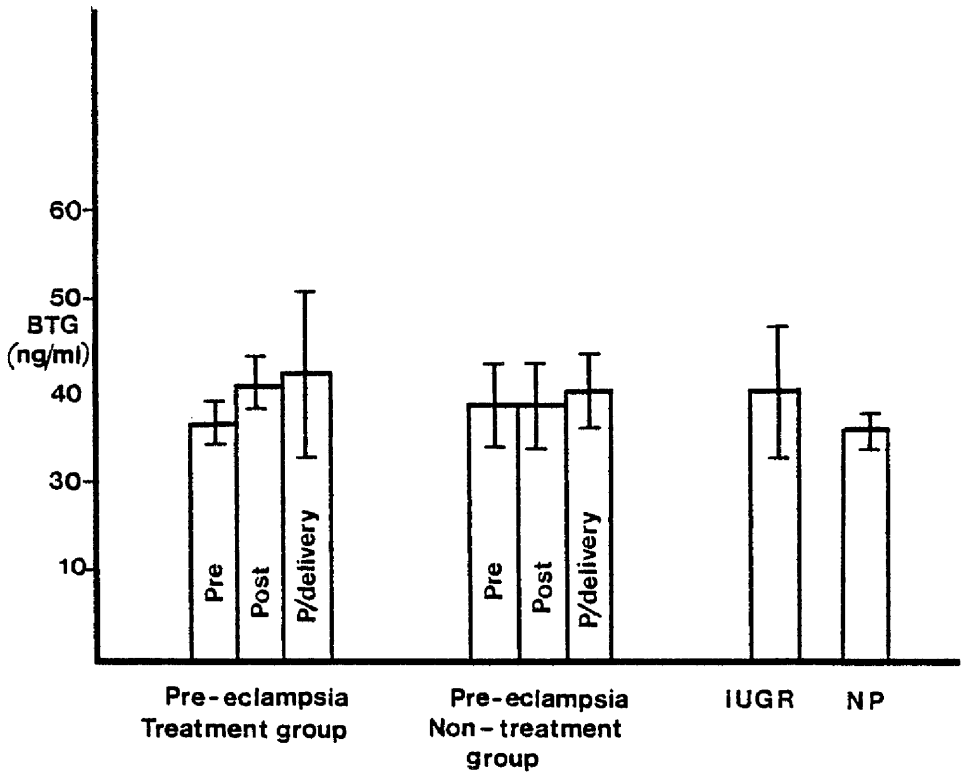


Figure 6.3

Betathromboglobulin levels in each of the four groups studied. P/delivery denotes post delivery, IUGR-intra uterine growth retardation, NP-normal pregnancy.

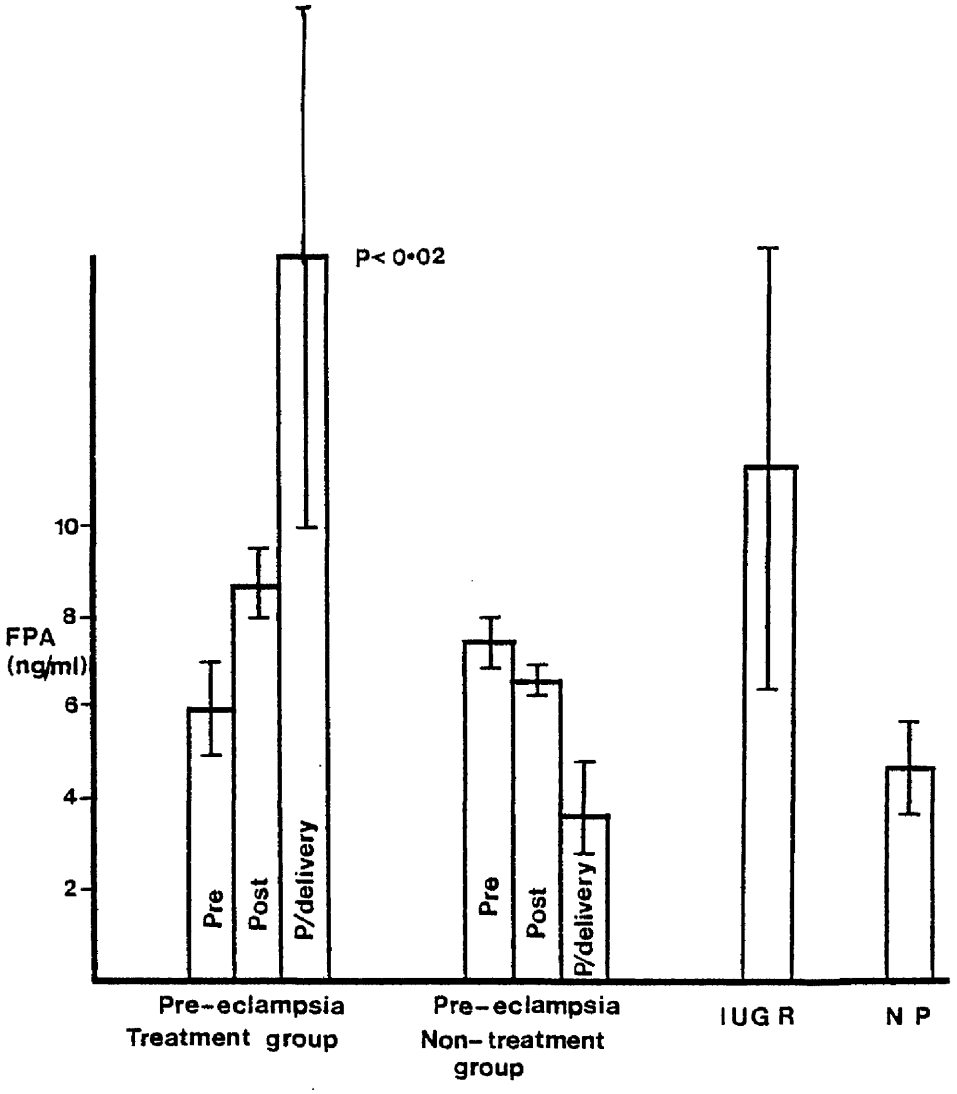


Figure 6.4.

Fibrinopeptide A levels in each of the four pregnancy groups studied.

6.4. Discussion

In the initial study no increase in plasma levels of BTG was found in women taking oral contraceptives, in contrast to two previous reports (Arandi et al., 1979; Duncan, 1979). Neither was there any increase in plasma levels of FPA. During normal pregnancy significant increases in BTG levels were observed in the second and third trimester of pregnancy. This suggests platelet activation is occurring in normal pregnancy which is in agreement with a report of a slight decrease in platelet survival in normal pregnancy (Rakoczi, Tallian, Bagdany et al., 1979). No increase in FPA levels was observed, suggesting no detectable increase in thrombin generation in normal pregnancy. This finding suggests that the increased soluble fibrin monomer complexes in normal pregnancy (McKillop et al., 1976) may be due either to increased thrombin generation which cannot be detected by the FPA assay, or to other mechanisms which may promote fibrin complex formation such as increased fibrinogen levels (Kanaide and Shainoff, 1975).

Pregnant women with pre-eclampsia had significant increases in plasma BTG, when compared to normal pregnant women, as previously reported (Redman et al., 1977). These findings are consistent with previously reported platelet activation (Whigham, Howie, Drummond et al., 1978) and decreased platelet survival in pre-eclampsia (Rakoczi et al., 1979).

Significant increases in plasma levels of FPA were shown, consistent with previous suggestions of increased thrombin generation in pre-eclampsia (Bonnar et al., 1971; McKillop et al., 1976). Similar but less marked changes were found in pregnant women with hypertension without proteinuria suggesting relationships between activation of coagulation and platelets and pregnancy hypertension.

In the second study no increase in plasma levels of BTG was found in pregnancy hypertension or mild pre-eclampsia, despite similar mean values being found for the normal pregnant women in the third trimester of pregnancy in both studies. Although similar elevated levels of plasma FPA were found in pregnant women with hypertension in both studies, in the labetalol study these differences were not significant as higher FPA levels were found in the normal pregnant women in the third trimester of pregnancy. This finding would be consistent with increased soluble fibrin complexes reported in normal pregnancy (McKillop et al., 1976). A statistically significant increase in FPA levels in women with intrauterine growth retardation when compared to normal pregnant women was found. This suggests an increased thrombin generation which may result in fibrin deposition in the placental bed with retarded growth of the fetus as a result.

In summary these results suggest that from the haemostatic aspect, elevated FPA levels in the I.U.G.R. pregnancy hypertension or mild pre-eclampsia and severe pre-eclampsia and elevated BTG in severe pre-eclampsia may reflect a continuous spectrum of disease rather than separate disorders, as has been suggested

recently (Lewis, 1982). The initial study suggested a relationship between hypercoagulability and hypertension, and that possibly increasing blood pressure per se may lead to secondary activation of the coagulation system. A recent study measured plasma BTG levels in mild to severe arterial hypertension and reported that disturbances of platelet function were correlated with the stage of hypertension (Petralito, Fiore, Mangiafico et al., 1982). As shown in the second study oral labetalol does lower diastolic blood pressure without any resultant decrease in platelet activation or thrombin generation. This suggests that once the suggested vicious circle of hypertension, intravascular coagulation and deteriorating renal function (Page, 1972; Gavras, Oliver, Aitchison et al., 1975) has been triggered, blockage of one event (hypertension) has no effect on the progress of other events. Until more is known about the underlying mechanisms in pre-eclampsia drug therapy must be closely monitored to measure as many factors with regard to all the systems concerned. In the second study labetalol administration also resulted in significantly higher levels of plasma FPA after delivery and further studies are suggested to clarify this situation.

CHAPTER 7

CHANGES IN PLASMA FIBRINOPEPTIDE A, BETATHROMBOGLOBULIN AND Bβ15-42 IN VENOUS THROMBOSIS AND IN PATIENTS WITH A HIGH RISK OF DEVELOPING DVT. WITH STUDIES ON ENHANCEMENT OF ENDOGENOUS FIBRINOLYSIS.

In this chapter studies were undertaken to determine whether plasma levels of fibrinopeptide A (FPA), betathromboglobulin (BTG) and Bβ15-42 were abnormal in a variety of medical conditions associated with a high risk of venous thrombosis as well as in patients with a high risk of post-operative deep vein thrombosis (DVT). Venous thrombi are composed primarily of fibrin in which are enmeshed red blood cells, white cells and platelets. Such intravascular fibrin deposition may theoretically be the result of decreased fibrinolysis as well as increased fibrin formation (Fearnley, 1965) and abnormal platelet release. In the first study described in this chapter (7.1) I have compared patients with longstanding retinal vein occlusion with control subjects. In the next study (7.2) plasma BTG levels were measured in patients with the nephrotic syndrome, who have a high risk of DVT, and compared to control subjects. The third study (7.3) analysed plasma Bβ15-42 levels in a group of volunteers who received the anabolic steroid stanozolol, an agent proven to alter many haemostatic parameters. The fourth study (7.4) evaluated the relationship of BTG, FPA and Bβ15-42 to post-operative DVT and examined the role of malignancy and stanozolol treatment. In the final study (7.5) plasma BTG

levels were measured in 'acute' illnesses such as malignancy and infection and compared to control patients, with the role of age and obesity also being considered. A summary of these results is given in 7.6.

7.1. Plasma FPA and BTG in Longstanding Retinal Vein Occlusion

7.1.1. Introduction

Retinal vein occlusion has several well recognised clinical associations, including hypertension, hyperlipidaemia, diabetes, polycythaemia and hypergammaglobulinaemia (McGrath, Wechsler, Hunyor et al., 1978). The latter two disorders cause increased blood viscosity, which slows retinal blood flow (Hume and Begg, 1969). Increased blood viscosity results in a higher haematocrit and resultant higher fibrinogen concentration which in association with reduced retinal blood flow might result in fibrin deposition and the formation of thrombi within the retinal area. Two previous studies reported increases in haematocrit, viscosity, fibrinogen and immunoglobulins in acute venous occlusion (Ring, Pearson, Sanders et al., 1976; McGrath, Wechsler, Hunyor et al., 1978). No studies have evaluated longstanding retinal vein occlusion. It is possible, therefore, that the increase in viscosity might be the result of an acute vascular event as the plasma fibrinogen level is known to increase in a variety of acute illnesses (Lowe, 1981). In addition, in both studies patients with retinal vein occlusion, half of whom had hypertension were compared with control subjects without hypertension. Since there is now good evidence that increased levels of blood viscosity, haematocrit and fibrinogen are found in subjects with hypertension (Letcher, Chien, Pickering et al., 1981), the

increased blood viscosity reported and hence the increased fibrinogen in both studies might be the result of the hypertension rather than the retinal vein occlusion. The increased fibrinogen concentrations in retinal vein occlusion suggests that abnormal coagulation, platelet and fibrinolytic activity might play a role in retinal vein occlusion or its ischaemic complications, especially if thrombosis contributes to vascular occlusion. The measurement of plasma BTG, FPA and fibrinogen should provide direct evidence of platelet activation and thrombin generation in retinal vein occlusion.

7.1.2. Design of study

Forty two unselected patients with retinal vein occlusion who were attending ophthalmology departments in Glasgow hospitals were studied after informed consent. All were in the chronic phase (three months to three years) since onset of symptoms. All underwent full ophthalmological and medical assessment, including fluorescein angiography and screening for hypertension and diabetes, which were treated appropriately. Thirty three control subjects were selected from patients attending hypertension clinics and patients admitted for elective surgery. This group was matched for age, sex, smoking habit, diabetes, hypertension, and treatment of hypertension with the retinal vein occlusion group. All subjects were ambulant and none had any acute illness. The patients were subdivided into those who had capillary non perfusion and/or neovascularisation at fluorescein angiography, Group 1 (20 patients) and those who did not, Group 2 (22 patients).

Methods

Serum immunoglobulins were measured by automated immunoprecipitation. Blood was collected into trisodium citrate, 0.129M, 9:1 V.V. for the measurement of fibrinogen as previously described (Lowe, McArdle, Stromberg et al., 1982). Plasma BTG was measured using the Amersham radioimmunoassay kit as described in Chapter 5.3.2. Plasma FPA was measured using the IMCO radioimmunoassay with ethanol precipitation to remove the cross-reacting fibrinogen and charcoal separation to remove free ^{125}I FPA as described in Chapter 5.4.2 (A) and 5.4.2 (B).

Statistical analysis

Statistical significance of difference in means was performed using Wilcoxon's rank sum test.

7.1.3. Results

Clinical Variables and Immunoglobulins

As shown in Table 7.1. 20 subjects with retinal vein occlusion had capillary non perfusion and/or neovascularisation at fluorescein angiography (Group 1) and 22 did not (Group 2). 52% of patients had hypertension and 52% had increased levels of at least one class of immunoglobulin in the following order of frequency: IgM, IgG, IgA. No patient with raised immunoglobulin had a definite haemoproliferative or connective tissue disorder. Table 7.1. shows that there were no significant differences in clinical factors or immunoglobulin abnormalities between Groups 1 and 2.

Plasma fibrinogen

A significantly higher fibrinogen concentration was found in group 1 (capillary non perfusion and/or new vessels) when compared to group 2 or the control group (see Figure 7.1).

TABLE 7.1
CLINICAL FEATURES AND IMMUNOGLOBULINS IN PATIENT GROUPS

	RETINAL VEIN OCCLUSION		CONTROLS
	GROUP 1 (Capillary non-perfusion and/or neovascularisation)	GROUP 2 (No non-perfusion or neovascularisation)	
Number	20	22	33
Age (years)	67.6 (SEM 2.1)	66.6 (2.4)	66.7 (2.0)
Males	13	8	17
Smokers	2	8	9
Hypertension	13	9	17
Diabetes	1	2	2
Site: Central	10	14	
Branch	8	8	
Both	2	0	
Abnormal Immunoglobulins	7/12	7/16	

SEM: Standard error of mean in parentheses

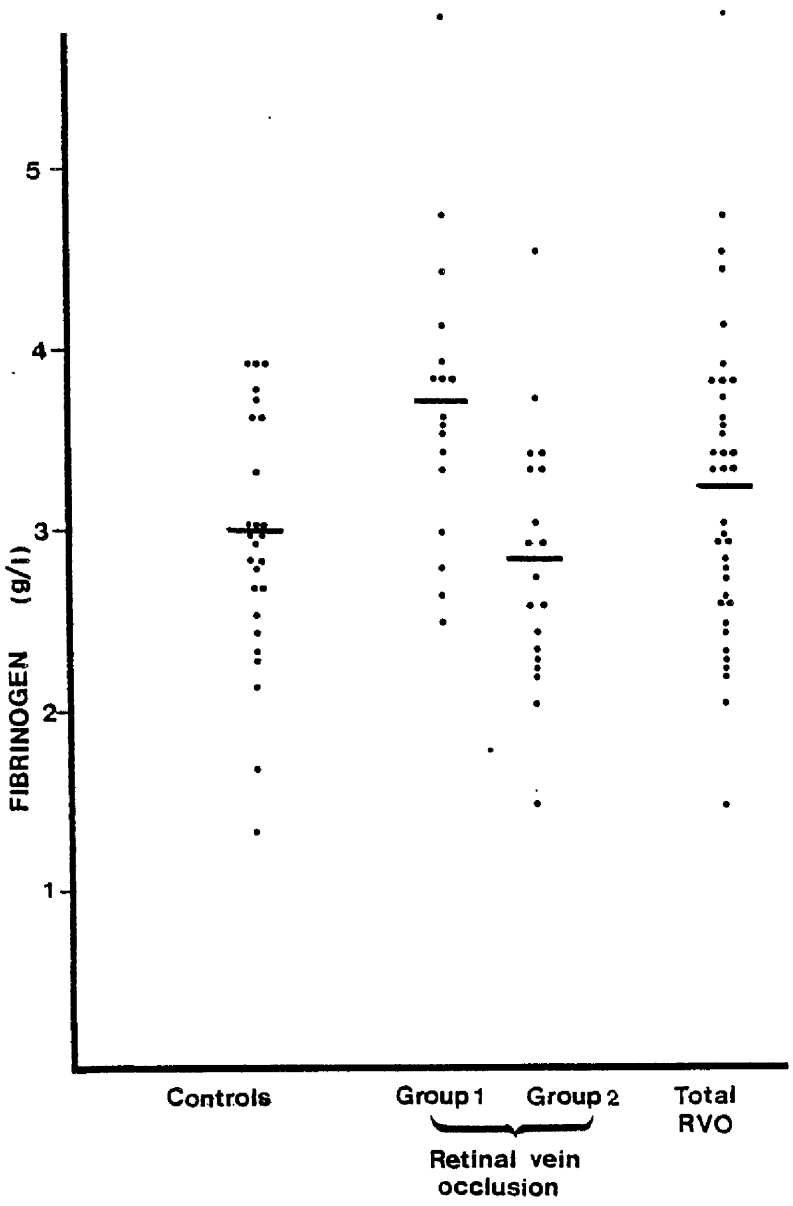


Figure 7.1

Plasma fibrinogen levels in each of the groups studied. Group 1 denotes patients with capillary non perfusion and/or new vessels. Group 2 denotes patients without capillary non perfusion. RVO denotes retinal vein occlusion.

Plasma betathromboglobulin

Subjects in group 1 had statistically higher levels of BTG 43 ± 6 ng/ml ($p < 0.05$) when compared to group 2, 32 ± 4 ng/ml, and the control group, 27 ± 2 ng/ml. BTG levels in the total retinal vein occlusion group, 37 ± 4 ng/ml ($p < 0.05$) were also significantly higher than group 1 and the control group (see Figure 7.2).

Plasma FPA

Plasma FPA levels were significantly higher in group 1, 7.2 ± 2.8 ng/ml ($p < 0.05$) when compared to group 2, 1.3 ± 0.2 ng/ml, the total retinal vein occlusion group, 4.1 ± 1.4 ng/ml and the control group, 1.0 ± 0.3 (see Figure 7.3).

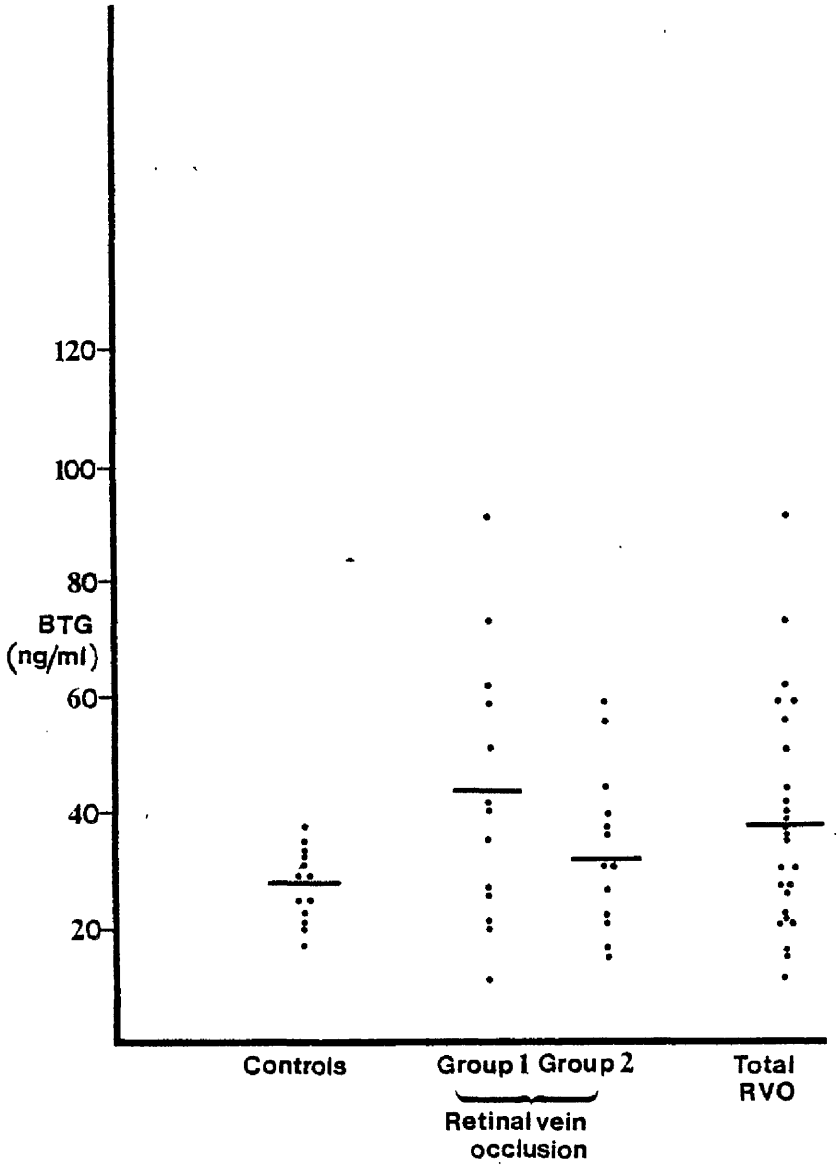


Figure 7.2

Plasma betathromboglobulin levels in each of the groups studied.

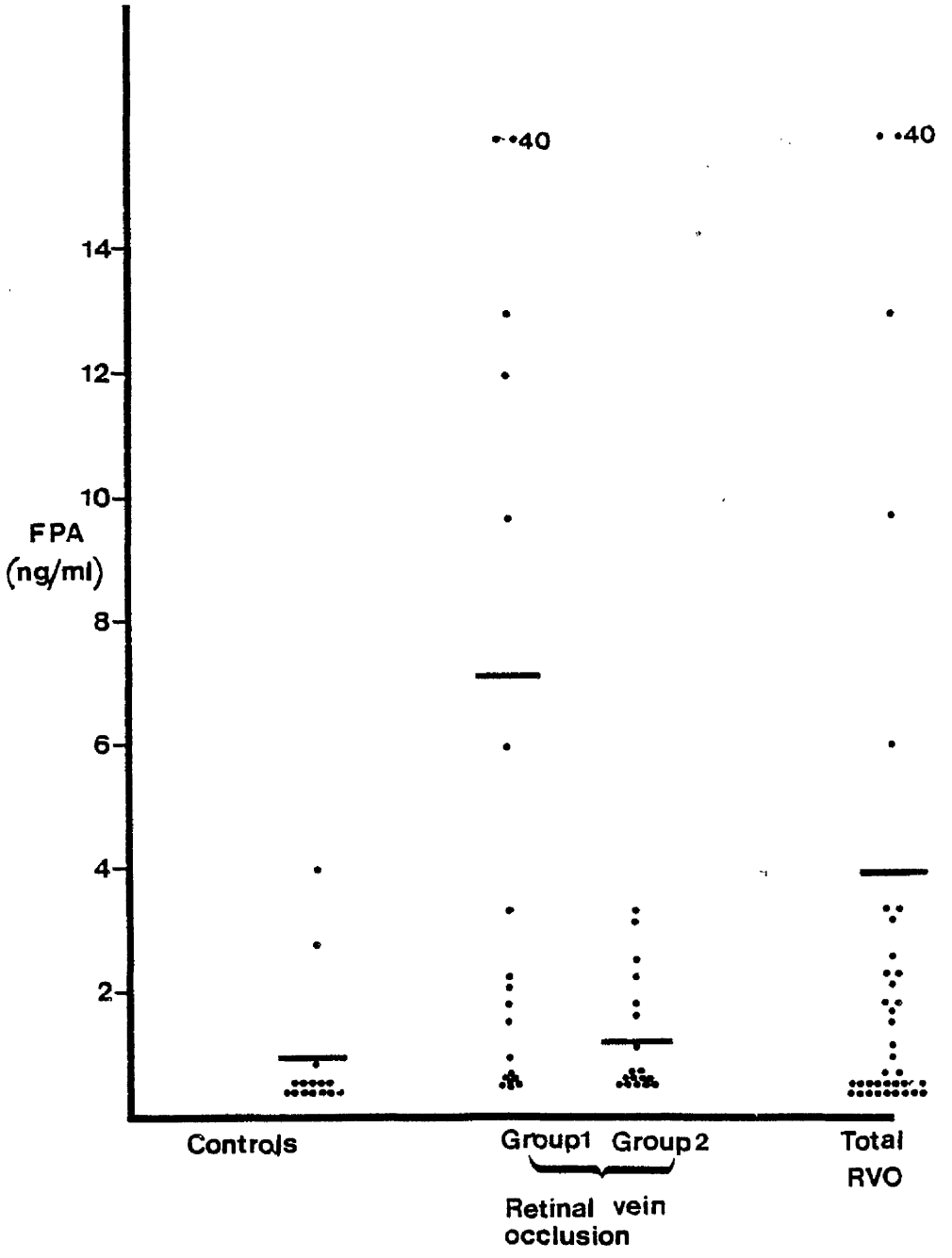


Figure 7.3

Plasma FPA levels in each of the groups studied.

7.1.4. Discussion

This study has shown that patients with longstanding retinal vein occlusion who have capillary non perfusion and/or new vessel formation (group 1) have increased fibrinogen levels, FPA and BTG levels. In patients with retinal vein occlusion but without ischaemic complication (group 2) no abnormalities were found in fibrinogen, FPA or BTG levels in comparison to those in the control group. The increase in fibrinogen and immunoglobulin levels in group 1 is similar to previous reports (Ring et al., 1976; McGrath et al., 1978). The present finding of a relationship of increased fibrinogen levels with capillary non perfusion differs from a previous report (Ring et al., 1976). It may be that since the previous study examined patients in the acute phase of retinal vein occlusion (Ring et al., 1976), acute phase increases may have obscured a relationship with capillary non perfusion. Uncertainty surrounds the question of whether or not increased fibrinogen levels favour the formation of a thrombus (Lowe, 1981) but patients in group 1 had increased levels of FPA suggesting that thrombin generation is present in some subjects with capillary non perfusion.

Platelet activation and consumption in diabetic retinopathy has been suggested by a lowered platelet count (Fuller, Keen, Jarret et al., 1979) and increased plasma BTG levels (Schernthaner, Sinzinger, Silberbaur et al., 1981). The observed increased plasma BTG levels in group 1 suggest that platelet activation is a feature of ischaemic/proliferative retinopathy after retinal vein occlusion and may be involved in retinal vein occlusion and its complications.

7.2. Plasma BTG Levels in the Nephrotic Syndrome

7.2.1. Introduction

Patients with the nephrotic syndrome (NS) present a group of patients with an increased risk of venous thrombosis, including renal and deep vein thrombosis (Chugh, Ma l ick, Uberoi et al., 1981). The possibility of increased arterial disease is somewhat controversial (Mallick and Shoirt, 1981; Wass and Cameron, 1981). Selective urinary loss of low molecular weight proteins results in plasma protein abnormalities characteristic of NS. A resultant increased hepatic synthesis of both low and high molecular weight proteins, include proteins that participate in coagulation and fibrinolysis (Thomson, Forbes, Prentice et al., 1974). Clotting factors, including plasma fibrinogen and factor VIII (Thomson et al., 1974; Kanfer, Kleinknecht, Broyer et al., 1971; Kendall, Lohmannand and Dosseter, 1971) and fibrinolytic factors such as plasminogen and α_2-macroglobulin (Thomson et al.,1974) are increased. Urinary loss may result in decreased concentrations of other low molecular weight inhibitors such as ATIII (Kauffmann, Veltkamp, von Tilberg et al., 1978) and α_2-antiplasmin (Taberner, Ralston and Ackrill, 1981). Some of these disturbances (increased fibrinogen and factor VIII; decreased ATIII) may be relevant to the increased risk of thrombosis in NS as may the rise in platelet count (Kanfer

et al., 1971; Walter, Deppermann, Andrassy et al., 1981), increased platelet adhesiveness and aggregation (Walter et al., 1981) and increased platelet release as shown by increased plasma levels of BTG (Adler, Lundin, Feinroth et al., 1980; Tomura, Ida, Kuriyama et al., 1982). Plasma BTG levels were measured in patients with NS and matched controls. Controls were matched for renal function (as measured by serum creatinine), since several haemostatic variables are abnormal in renal failure as previously discussed.

7.2.2. Design of study

Twenty one consecutive patients with the nephrotic syndrome were studied. All had proteinuria greater than 3 g/24 hours and serum albumin less than 35 g/litre at the time of study. Most were taking diuretic drugs. Twenty one controls were selected from hospital staff or non-nephrotic renal unit patients. They were matched for age, sex, smoking habit and serum creatinine with the nephrotic subjects. No subjects were taking anticoagulants or antiplatelet drugs at the time of study.

7.2.3. Method

Plasma BTG was measured using the radioimmunoassay kit (Amersham) as previously described. No PGE_1 was added to the blood sampling tubes. In nephrotic subjects, serum albumin, globulin and creatinine were measured by Technicon Auto-analyser with creatinine clearance and quantitative proteinuria being measured as previously described (Thomson et al., 1974).

Statistical analysis

Statistical significance of differences in means was performed using Wilcoxon's rank sum test, and correlations determined by the least squares method.

TABLE 7.2

Clinical and routine biochemical details (mean ± SEM) of nephrotic patients studied

Number	21	
Males	17	
Smokers	6	
Age	39.4 ± 3.6	
Serum albumin (g/l)	25 ± 1.1	(normal 35-55)
Serum globulin (g/l)	27.9 ± 0.9	(normal 22-33)
Serum cholesterol (mmol/l)	9.3 ± 0.8	(normal 4.1-7.4)
Serum creatinine (mol/l)	252 ± 41	(normal 40-130)
Urine protein (g/24 hr)	13.0 ± 1.6	(normal <0.2)
Creatinine clearance (ml/min)	56 ± 9	(normal 90-130)
Type of histological disease		
Membranous glomerulonephritis	7	
Minimal change glomerulonephritis	4	
Proliferative glomerulonephritis	4	
Focal glomerulonephritis	3	
SLE	1	
Amyloid	1	
Cryoglobulinaemia	1	

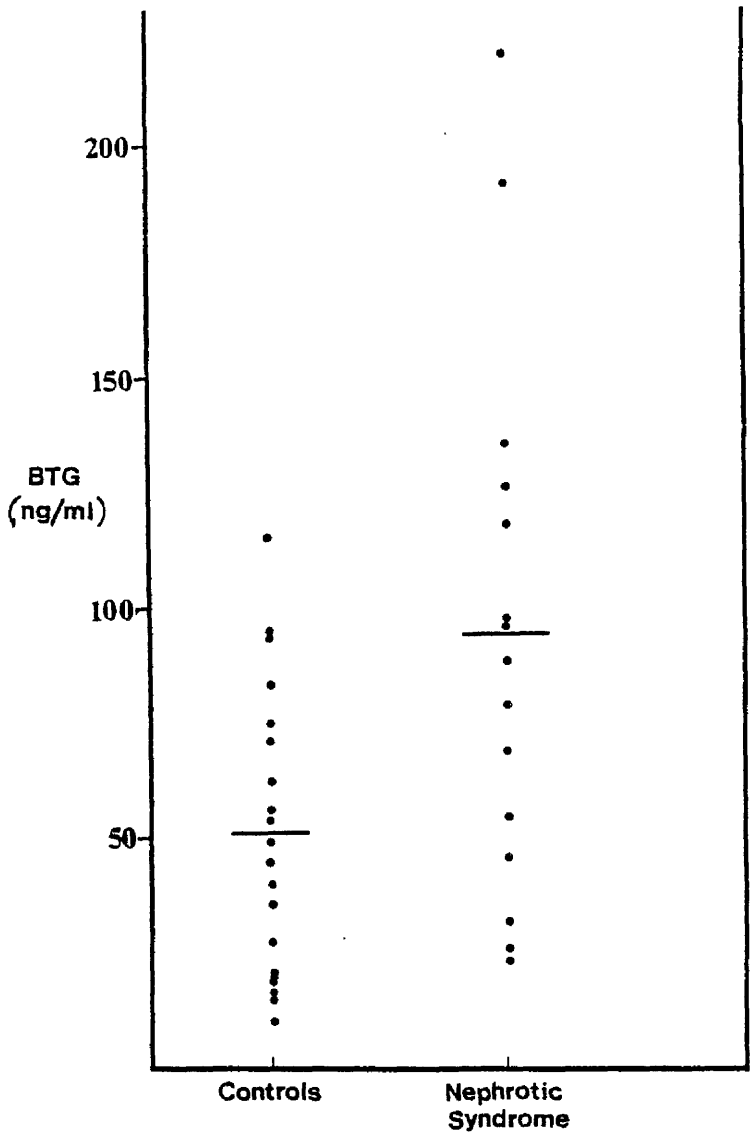


Figure 7.4.

Plasma betathromboglobulin levels in patients with the nephrotic syndrome and controls.

7.2.4. Results

Clinical details of the nephrotic patients are given in Table 7.2. No patient had active thrombosis at the time of study, but seven had a past history of venous or arterial thrombosis. A statistically significant increase in plasma BTG was found in the NS patients when compared to the control group as shown in Figure 7.4. In the NS patients there was no correlation between BTG and serum albumin, globulin, cholesterol, creatinine, urine protein or creatinine clearance.

No variable was significantly different in the patients with a history of thrombosis compared to those without such history.

7.2.5. Discussion

A significant increase in plasma BTG levels was shown in patients with nephrotic syndrome, confirming previous reports of increased plasma BTG levels in NS (Adler et al., 1980; Tomura, Ida, Kuriyama et al., 1982). These results may reflect platelet activation in vivo. In a fourth study, no significant increase in BTG was observed (Walter et al., 1981). All four studies used the Amersham BTG kit. Recently low albumin levels have been suggested to interfere with the BTG assay (Dr C Ludlam, personal communication). Plasma levels of BTG were not correlated with serum albumin levels in this present study.

7.3. Plasma Bβ15-42 in a Volunteer Study of Stanozolol Treatment

7.3.1. Introduction

Decreased fibrinolysis with inapproprite deposition of intravascular fibrin, may play a role in several vascular diseases (Almér, Pandolfi and Nilsson, 1975; Walker and Davidson 1978; Burnand, Whimster, Naiddo et al., 1982). Blood fibrinolytic activity has been shown to be increased after the oral administration of anabolic steroids, such as stanozolol (Stromba, Sterling Research Laboratories, England)to normal subjects (Davidson, Lockhead, McDonald et al., 1978; Preston, Burakowski, Porters et al., 1981) and in patients with vascular diseases (Burnand, Clemenson, Morland et al., 1980; Jarret, Morland and Browse, 1978). After a single intramuscular injection of 50 mg stanozolol in healthy volunteers we have shown a significant stimulated rise in plasma fibrinolytic activity (plasminogen activator) as well as plasma plasminogen levels, within 24-48 hours (Small, McArdle, Lowe et al., 1982). However this increased level of plasminogen activator indicates only a potential for stimulation of plasmin-mediated fibrinolysis and therefore a possibility for treatment of thrombotic disorders. Systemic fibrinolysis with raised FDP's does not occur with stanozolol but is observed after streptokinase and urokinase therapy.

With the recent introduction of the radioimmunoassay for fragment Bβ15-42 which is released during the earliest stage of plasmin proteolysis, (Kudryk, Robinson, Netre et al., 1982), a new marker may now be available for the measurement of fibrinolytic activity.

7.3.2. Design of Study

Plasma levels of Bβ15-42 and fibrinogen were measured in nine healthy male subjects, age 19-35 years, before, during and after a 14 day course of treatment with stanozolol (Stromba, Sterling Research Laboratories, England 10 mg orally/day).

7.3.3. Methods

Plasma Bβ15-42 levels were measured as described in Chapter 5.5.1. Plasma fibrinogen levels were measured on the Dade fibrometer. Statistical significance of difference in means was performed using Wilcoxon's rank sum test.

7.3.4. Results

Plasma Bβ15-42

The plasma Bβ15-42 levels obtained are shown in Table 7.3. Bβ15-42 levels increased from 0.84 ± 0.15 ($p < 0.01$) at 7 days, remained elevated at 1.18 ± 0.13 ($p < 0.05$) on day 14 and returned to baseline values of 0.9 ± 0.19, 14 days after cessation of therapy.

Plasma fibrinogen

The plasma fibrinogen levels observed are shown in Table 7.3. Plasma fibrinogen levels decreased from 2.32 ± 0.12 g/l (mean ± SEM) to 1.57 ± 0.1 ($p < 0.01$) at seven days, remained decreased at 1.71 ± 0.09 ($p < 0.01$) on day 14 and returned to baseline values 2.44 ± 0.20, 14 days after cessation of treatment.

TABLE 7.3.

PLASMA Bβ15-42 LEVELS IN VOLUNTEERS

(MEAN ± SEM, **p <0.01, *p <0.05)

DAYS	0	7	14	28
Bβ15-42 pmol/ml	0.84 ± 0.10	1.54** ± 0.15	1.18* ± 0.13	0.9 ± 0.19
Fibrinogen g/l	2.32 ± 0.12	1.57** ± 0.10	1.71** ± 0.09	2.44 ± 0.20

7.3.5. Discussion

The findings of this study are consistent with an increase in plasmin-mediated lysis of fibrin(ogen). A well recognised decrease in fibrinogen levels with stanozolol was also observed (Walker and Davidson, 1978; Preston et al., 1981). This decrease in fibrinogen levels may be due to altered synthesis (Preston et al., 1981) or may in part be explained by plasmin activation. Plasma levels of Bβ15-42 may therefore be used as a marker of plasmin-induced fibrinolysis. These volunteer obvservations were then extended to a study in high risk post-operative patients treated with stanozolol in an attempt to prevent DVT.

7.4. Relationship of BTG, FPA and Bβ15-42 to post-operative DVT, Malignancy and Stanozolol Treatment

7.4.1. Introduction

The occurrence of venous thrombosis remains an important cause of post-operative morbidity and mortality. Venous thrombi cannot be diagnosed on clinical grounds alone with sufficient precision for scientific studies. Venography enables venous thrombi above a certain size to be identified accurately in the deep veins of the leg, with venous thrombi (in the calf only) being identified by ^{125}I-labelled fibrinogen scanning. Pulmonary thromboemboli can be diagnosed safely but imprecisely using isotope lung scans, or precisely but with greater hazard (and therefore greater patient selection) using pulmonary arteriography.

Changes occurring in blood coagulation that initiate the development of post-operative deep vein thrombosis (DVT) may allow a more precise identification of venous thrombosis. However such

changes in blood coagulation remain the subject of speculation. A previous study reported that the prediction of DVT in post gynaecological surgery patients was improved by including laboratory variables, and found significant predictive value for pre-operative values of euglobulin-lysis time, fibrin-related antigen, fibrinogen, and factor VIII in patients in whom DVT developed (Clayton, Anderson and McNicol, 1976). An increase in pre-operative blood viscosity has also been associated with post-operative DVT (Dormandy 1975). In our recent study, which examined the incidence of deep vein thrombosis of the leg after major gastrointestinal surgery without prophylaxis, a comprehensive evaluation of pre-operative clinical and laboratory variables including BTG, showed no relation between DVT after surgery and haemostatic variables (Lowe, Osborne, McArdle et al., 1982). This finding is in agreement with most previous studies which observed no relationship between DVT after surgery and pre-operative haemostatic variables (Flute, Kakkar, Renney et al., 1972; Gallus, Hirsh and Gent, 1973; Mansfield 1972; Gordon-Smith, Hickman, Lequesne 1974; MacIntyre, Webber, Crispin et al., 1976) or pre-operative blood viscosity (Humphreys, Walker and Charlesworth, 1976; Lowe, Campbell, Meek et al., 1978). The study of Lowe et al., 1982a, observed significant elevations in plasma and blood viscosity, fibrinogen, factor VIII and fibrin-related antigen in patients with malignancy, infection or hyperbilirubinaemia before surgery. Since over half the patients had malignancy, infection or hyperbilirubinaemia before surgery, the failure of laboratory tests to predict DVT may be explained by the relation between the tests and the illness itself, whereas illness is not closely related to venous thrombosis.

Pre-operative illness may mask any relations between laboratory variables and DVT which may be more apparent in less sick patients, such as elective surgery in gynaecological patients (Clayton et al., 1976). The above study of Lowe et al., 1982, reported that a simple index of age and per cent overweight allowed prediction of a high-risk group (incidence of DVT >51%) and a low risk group (incidence of DVT <10%). The finding of no relation between DVT after surgery and pre-operative haemostatic variables may have been due in part to a lack of adequate methodology with which activation of coagulation in vivo can be monitored. With the advent of radioimmunoassays for the detection of peptide fragments of fibrin (ogen) released by the major proteases of the coagulation system and fibrinolytic systems ie. thrombin and plasmin new markers may now be available to assess the development of thrombotic disease in relation to thrombin and plasmin activity as well as with regard to platelet release.

It has been shown by many workers that following major elective surgery there is a decrease or 'shut-down' of blood fibrinolytic activity (plasminogen activator activity) which is maximum on the first day after surgery. Since the majority of postoperative DVT form at this time (Negus, Pinto, LeQuesne et al., 1968), it is possible that a decrease in plasmin activity at this point would result in an imbalance in the thrombin : plasmin ratio with thrombin action predominating and hence thrombosis as suggested by Nossel (1981) see Figure 2.1. Some studies have reported a greater degree of fibrinolytic shut down in patients who develop DVT (Mansfield 1972; Gordon-Smith et al., 1974) while others have not (Gallus et al., 1973; MacIntyre et al., 1976). Such

conflicting results may be due partly to the effect of other parameters on blood fibrinolytic activity, such as malignant disease (Browse, Gray and Morland, 1977) or methodology (Reilly, Burden and Fossand, 1980).

Blood fibrinolytic activity has been shown to be increased by drug treatment with anabolic steroids such as stanozolol (Stromba, Sterling Research Laboratories, England) when administered orally (Davidson et al., 1972; Preston et al., 1981). A recent study in healthy volunteers has shown that a single intramuscular injection of 50 mg stanozolol stimulated significant rises in plasma fibrinolytic activity (plasminogen activator) as well as plasma plasminogen levels within 24-28 hours (Small et al., 1982). A follow-up study as described in 7.3. has shown direct evidence of an increase in plasmin activity after stanozolol treatment by the measurement of plasma Bβ 15-42 fragment which was significantly elevated for at least seven days after treatment. The aim of the present study was to measure plasma levels of BTG, FPA and Bβ15-42, indices of platelet release, thrombin generation and plasmin activity respectively, in relation to postoperative DVT, malignancy and stanozolol treatment.

7.4.2. Design of study

Thirty two patients undergoing major elective surgery were studied. Seventeen patients underwent surgery for early malignancy of the stomach, duodenum, gall bladder, colon or rectum. Each patient was a high risk of postoperative DVT according to the age and obesity index of Lowe et al., 1982 (age in years plus 1.3 X per cent mean weight of a population of same age, sex and height, greater than 170). Informed consent was obtained from patients.

After overnight fasting, blood was taken between 8 and 10 am with minimal stasis as described in Chapter 5.4.2. All patients were sampled on the day prior to surgery, on the morning of surgery and on the first and seventh day after surgery (postoperative days -1, 0, 1 and 7 in Figures 7.5, 7.6 and 7.7). Seventeen control patients received no specific prophylactic drugs. The other 15 patients received an intramuscular injection of stanozolol, 50 mg in 1 ml, following the day -1 sample.

7.4.3. Methods

Plasma BTG levels were measured as described in Chapter 5.3.2. FPA levels were determined using the bentonite extraction method for the removal of cross-reacting fibrinogen and double antibody separation of bound and free ^{125}I FPA as described in Chapter 5.4.3. Plasma Bβ15-42 was measured as described in Chapter 5.5.1.

Statistical Analysis

Statistical significance of differences of means was performed using Wilcoxon's rank sum test.

7.4.4. Results

In order to determine the relationship of BTG, FPA and Bβ15-42 to postoperative DVT, malignancy and stanozolol treatment the overall patient population was studied under the three separate parameters. Group 1 divided the patient group into those patients who received no prophylactic treatment and those who received stanozolol. Group 2 divided the patient group into those patients who developed no DVT after surgery and those patients who did develop DVT postoperatively. Group 3 divided the patient group into those patients who did not undergo surgery for malignancy and those who did have surgery for early malignancy.

Clinical Details

Group 1

Seventeen patients received no prophylaxis. This group consisted of nine males and eight females. Nine patients developed a DVT postoperatively and eight had malignancy pre-operatively. Fifteen patients (eight males, seven females) received stanozolol treatment pre-operatively. Five patients developed a DVT and nine had malignancy.

Group 2

Eighteen patients (ten male, eight females) did not develop a DVT postoperatively. Ten patients received stanozolol treatment and eight had malignancy. Fourteen patients (seven males, seven females) developed a DVT postoperatively. Five received stanozolol treatment and nine had malignancy.

Group 3

Fifteen patients (six males, nine females) had no malignancy pre-operatively. Six patients had stanozolol treatment and five developed a DVT postoperatively. Seventeen patients (11 males, 6 females) had early malignancy. Nine received stanozolol treatment and nine developed DVT post-operatively.

Betathromboglobulin

Group 1 (Control:stanozolol)

Plasma BTG in the control group increased on day 1 (see Figure 7.5 (a)), with a significant increase being observed on day 7 ($p < 0.02$) when compared to day -1. In the stanozolol group significant increases were observed on day 1 ($p < 0.01$) and day 7 ($p < 0.002$) when compared to pre-operative day 0. No significant differences were obtained between the two groups.

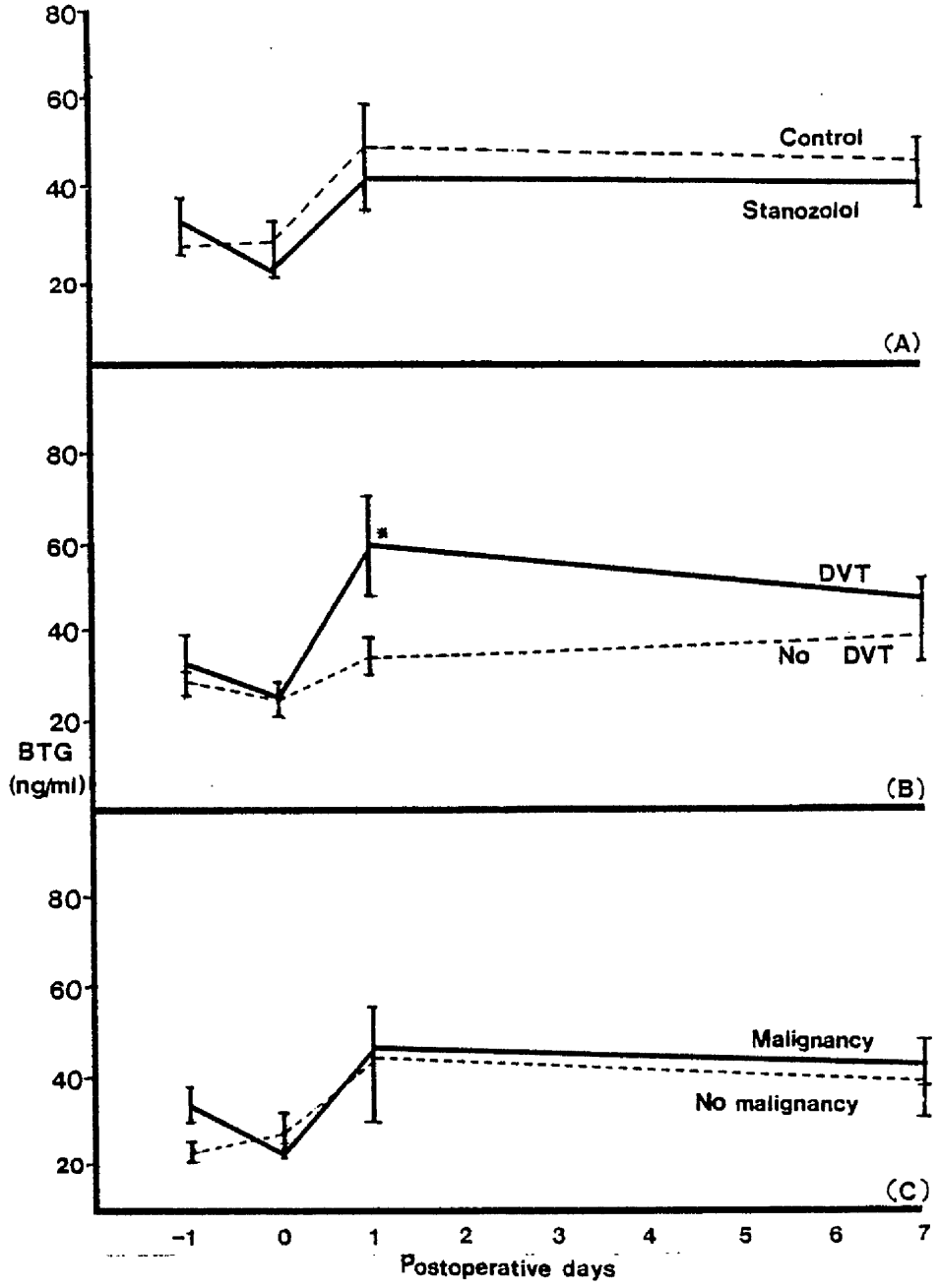


Figure 7.5

Plasma BTG levels in each of the groups studied.

Group 1; no prophylaxis: stanozolol.

Group 2; no DVT postoperatively: DVT postoperatively

Group 3; no malignancy preoperatively: malignancy preoperatively.

* $p < 0.02$

Group 2 (No DVT:DVT) - Figure 7.5(b)

Plasma BTG in the control group increased on day 1 with a significant increase being observed on day 7 ($p < 0.02$) when compared to day -1. In the group who developed a DVT post-operatively, plasma BTG levels were greatly elevated on day 1 ($p < 0.02$) and day 7 ($p < 0.02$) when compared to day -1. The difference between the two groups on day 1 was significant ($p < 0.05$).

Group 3 (No malignancy:malignancy) - Figure 7.5(c)

Plasma BTG in the control group increased on days 1 and 7 with the increase on day 7 being significant ($p < 0.05$) when compared to day -1. In the group with malignancy increased levels in day 1 were observed on days 1 and 7. The levels in day 1 were significant ($p < 0.05$) when compared to day -1. No significant differences were found on any of the days between each group.

Plasma Fibrinopeptide A

Group 1 - Figure 7.6 (a)

In the control group plasma FPA levels were significantly elevated on day 1 ($p < 0.005$) and day 7 ($p < 0.001$) when compared to day -1. In the group receiving stanozolol treatment significantly elevated FPA levels were found on day 1 ($p < 0.02$) when compared to day -1 with less elevated levels on day 7. There were no significant differences between the two groups.

Group 2 - Figure 7.6 (b)

In the control group plasma FPA levels, were significantly elevated on day 1 ($p < 0.005$) and day 7 ($p < 0.02$) when compared to day -1. In the group who developed a DVT, significantly elevated

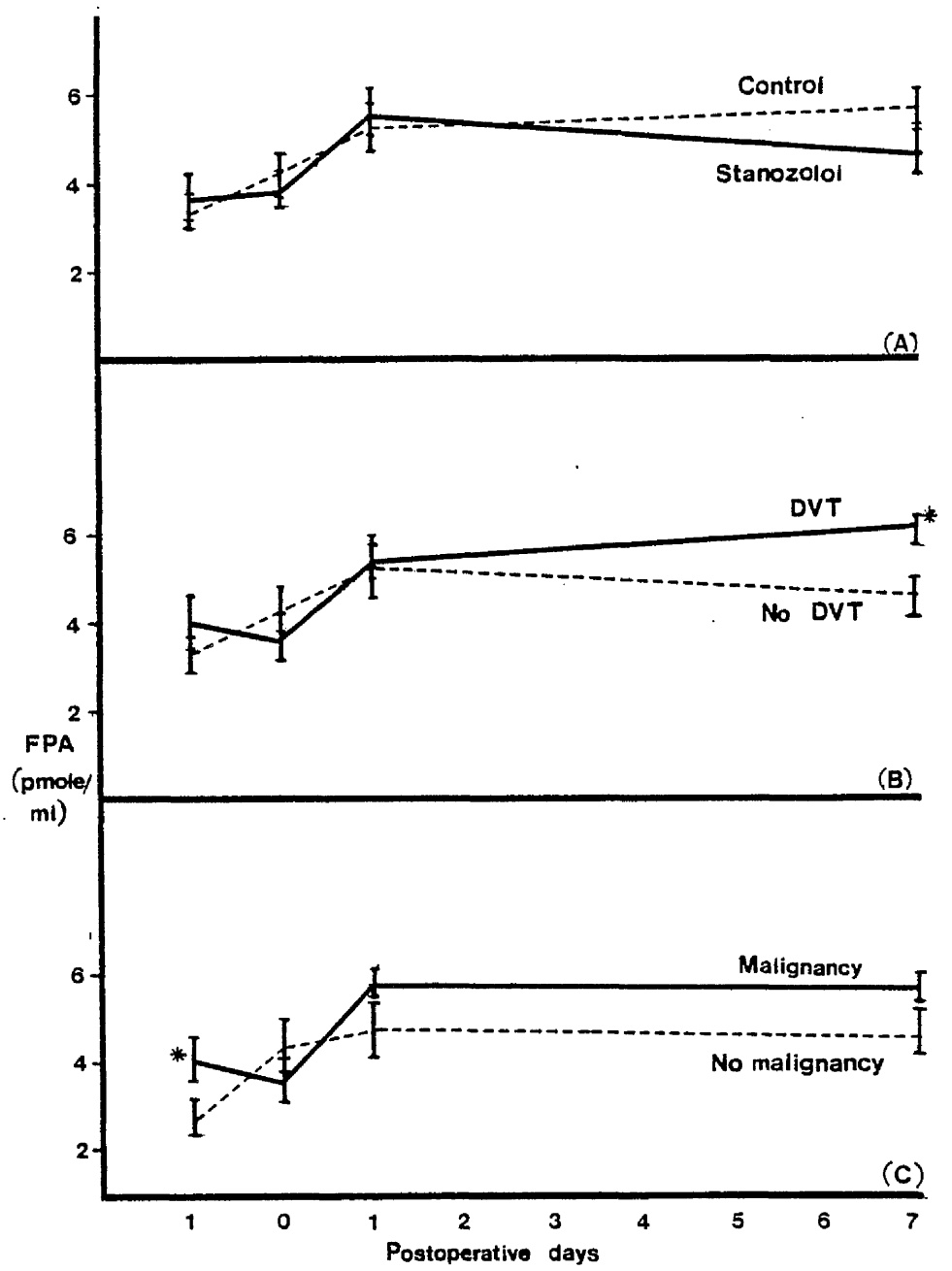


Figure 7.6

Plasma FPA levels in each of the groups studied.

* $p < 0.05$

values were found on day 1 ($p < 0.005$) and day 7 ($p < 0.02$) when compared to day -1. The FPA level on day 7 in the DVT group was statistically increased ($p < 0.05$) compared to the level in the control group on day 7.

Group 3 - Figure 7.6(c)

Significantly elevated FPA levels were found on day 0 ($p < 0.005$), day 1 ($p < 0.02$) and day 7 ($p < 0.005$) in the control group when compared to day -1. In the group with malignancy significantly elevated values were found on day 1 ($p < 0.01$) and day 7 ($p < 0.05$) when compared to day -1. A significant difference was observed in FPA levels in day -1 in the group with malignancy ($p < 0.05$) when compared to day -1 in the control group.

Bβ15-42

Group 1 - Figure 7.7 (a)

In the control group a significant difference in Bβ15-42 levels was found in day 7 ($p < 0.05$) when compared to day -1. In the Group receiving stanozolol treatment an elevated Bβ15-42 level was observed which was significant on day 7 ($p < 0.005$ when compared to day -1. A significant increase in Bβ15-42 levels was observed on day 7 between the stanozolol group ($p < 0.05$) and the control group.

Group 2 - Figure 7.7 (b)

A significant increase in Bβ15-42 levels was found in the control group on day 7 when compared to day -1. Although the value on day 7 in the DVT group was elevated, the difference when compared to day -1 was not elevated. However the Bβ15-42 level on day 0 in the DVT group was significantly higher ($p < 0.05$) than that of the control group.

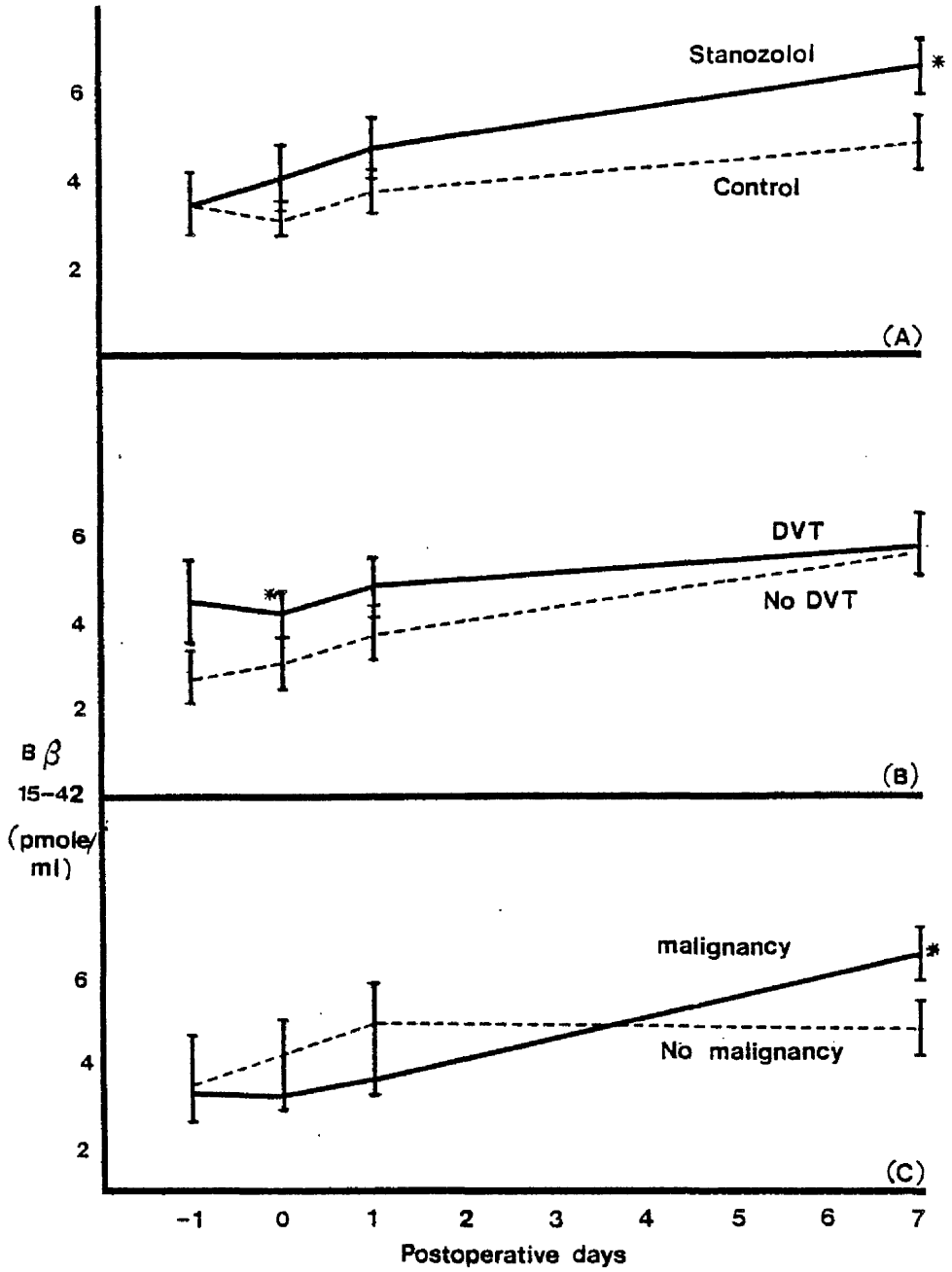


Figure 7.7

Plasma B β 15-42 levels in each of the groups studied.

* $p < 0.05$

Group 3 - Figure 7.7 (c)

In the control group there were no significant differences in the plasma Bβ15-42 levels on any of the days measured. In the group who had malignancy a significant difference was found on day 7 ($p < 0.001$) in Bβ15-42 levels when compared to day -1. significantly elevated Bβ15-42 levels were found in the malignancy group in day 7 ($p < 0.05$) when compared to the control group.

7.4.5. Discussion

1. The prevalence of malignancy and the incidence of DVT were similar in the two treatment groups. Thus stanozolol does not prevent the occurrence of DVT.
2. All groups of patients (see Figure 7.5) had a postoperative rise in plasma BTG levels on day 1 and day 7 as reported previously (de Boer et al., 1981; Lane et al., 1982a).
3. A statistically significant increase in plasma BTG levels was found on postoperative day 1 in the group who developed a DVT when compared to the group who did not. This finding has not been reported previously although an increase in BTG levels, which occurred after the postoperative day 1 increase, was reported just before the development of DVT after surgery (de Boer et al., 1981).
4. All groups of patients (see Figure 7.6) had a postoperative rise in plasma FPA levels on day 1 as reported previously (Törngren et al., 1979; Davies et al., 1980; Lane et al., 1982a; Owen et al., 1983).
5. A significant increase in plasma FPA levels was found on postoperative day 7 in the group who developed a DVT when

compared to the group who did not. This finding contrasts with a previous report which found no significant difference in the FPA levels in the groups with and without DVT on postoperative day 7 (Lane et al., 1982a). This difference may be due to differences in the occurrence of actual thrombosis.

6. In patients undergoing surgery for early malignancy, a pre-operative increase in FPA levels was observed when compared to patients without malignancy. This is in agreement with a previous finding (Törngren et al.,1979).
7. An increase in plasma Bβ15-42 levels on postoperative day 1 was observed in all groups (see Figure 7.7).
8. A significant increase in plasma levels of Bβ15-42 was observed on postoperative day 7in the group who received stanozolol when compared to the group who received no prophylaxis. This result is in agreement with the increase in levels of Bβ15-42 after administration of stanozolol in volunteers as reported in 7.3. The increase in Bβ15-42 after stanozolol treatment in major surgery is in agreement with the prevention of fibrinolytic 'shut-down' after major surgery, however this fibrinolytic 'shut-down' occurs on postoperative day 1, whereas an increase in Bβ15-42 was observed in all groups in this study. This suggests that rather than there being decreased fibrinolytic activity there is an enhanced expression of plasmin-like activity in the circulation. Although there may be decreased fibrinolytic activator it could be postulated that there is still sufficient present to produce an increased expression of fibrinolytic activity.

7.5.Betathromboglobulin and Platelet Counts - Effect of Malignancy, Infection, Age and Obesity

7.5.1. Introduction

In this chapter a study was undertaken to determine the effect of age, obesity and 'acute' illness such as malignancy and infection on plasma levels of BTG and platelet counts. One of the problems associated with the detection of markers for the detection of a thrombotic state is that such markers might be non-specific and might relate to non-thrombotic illnesses such as malignancy, infection and jaundice. Platelets, coagulation and fibrinolysis are sensitive to common stresses such as emotional stress, pain, injury and surgery, as well as many commonly used drugs. Few published studies report a 'sick' control group as well as a 'healthy' control group yet subjects with eg. respiratory infection or malignancy may well share the abnormality with the thrombotic group (Lowe et al., 1979d). Platelets may also be involved in inflammation or neoplasia and may be activated and deposited outside large blood vessels as well as inside. Hence the presence of increased circulating platelets or platelet release products may reflect participation in non-thrombotic pathology or extravascular events, rather than intravascular thrombosis. Furthermore, most 'healthy' control groups usually consist of hospital or laboratory staff in the 20-45 year age group whereas the majority of patient groups are in the 40-70 age group and hence older 'healthy' control groups should be considered.

Elevated plasma levels of BTG have been observed in patients with malignant disease (Bidet, Ferrier, Besse et al., 1980; Farrell, Duffy, Moriarty et al., 1980). Many patients in these studies have disseminated malignancy. The question arose as to

whether BTG levels would be elevated in early malignancy, in older subjects and in acute illness. The aims of the present study therefore were (a) to establish whether or not BTG levels were elevated in early operable cancer and (b) to establish whether or not BTG levels were elevated in a group of patients with acute non-vascular illness eg. infection.

7.5.2. Design of study

Four groups of subjects were studied. Group 1: comprised 40 healthy volunteers (mean age 27 ± 1.1 (SEM) years; this included 22 females not taking oral contraceptives, 18 males). Group 2: 29 patient controls (mean age 61.2 ± 2.1 years, 11 males, 18 females) admitted to hospital for major elective surgery for non-malignant disease, with no evidence of infection. Group 3: 19 patients (mean age 60.3 ± 2.2 years, 10 males, 9 females) admitted to hospital for proven operable gastrointestinal carcinoma. In all patients diagnosis was subsequently confirmed at operation - 10 patients had colorectal carcinoma, 7 had gastric adenocarcinoma and 2 had pancreatic carcinoma. Group 4: Nine patients (mean age 60 ± 3.5 years, three males, six females) admitted to hospital with acute bacterial infection but no malignant disease. Five had pneumonia and four had intra-abdominal sepsis (appendix abscess in one and cholecystitis in 3). No participant was taking any anti-platelet therapy.

7.5.3. Methods

Obesity was measured as per cent mean weight for height, age and sex, compared to adults in the Build and Blood Pressure Study (1962).

Plasma BTG was measured using the Amersham radioimmunoassay kit as described in chapter 5.3.2. No PGE_1 was added to the anticoagulant tubes.

Platelet counts were performed using a Coulter automatic platelet counter.

Serum creatinine levels were performed by Technicon autoanalyser.

Statistical analysis

All data are presented as the mean ± standard error of the mean. The significance of differences between groups was assessed by Wilcoxon's rank sum test. Correlations were determined by the method of least squares.

7.5.4. Results

Patient controls (Group 2) had significantly higher BTG levels ($p < 0.01$) and significantly lower platelet counts ($p < 0.01$) than normal controls (Group 1) (See Table 74). In the combined group of 69 control subjects there was a significant positive correlation between BTG and age ($r = 0.33$, $P < 0.01$) and a significant negative correlation between BTG and per cent overweight for age, sex and height ($r = -0.47$, $p < 0.01$). This correlation with obesity was found in Group 2 ($r = -0.58$, $p < 0.001$) but not in Group 1 - possibly as a result of the relatively small scatter of obesity in this group of young healthy subjects. A significant negative correlation was found between platelet count and age ($r = -0.55$, $p < 0.001$). There was no correlation between levels of BTG and sex or smoking habits. Patients with malignancy (Group 3) and infection (Group 4) were of similar mean age to patient controls (Group 2) and had similar levels of serum creatinine and obesity. Patients with malignancy (Group 3) had similar levels of BTG and platelet count compared to Group 2. Patients with infection (Group 4) had significantly higher levels of both BTG ($p < 0.05$ and platelet count ($p < 0.02$) compared to patient controls (Group 2)

TABLE 7.4

Age, BTG, platelet count, serum creatinine and obesity (mean ± SEM) in groups studied (* $p < 0.05$, ** $p < 0.02$ *** $p < 0.01$ compared to Group 2, patient controls)

	GROUP 1 Normal Control	GROUP 2 Patient Controls	GROUP 3 Malignancy	GROUP 4 Infection
No	40	29	19	9
Age (years)	27.0*** ± 1.1	61.2 ± 2.1	60.3 ± 2.2	60 ± 3.5
BTG (ng/ml)	26 *** ± 2	43 ± 4	4 ± 5	68 * ± 14
Platelet count $X10^9/L$	324 *** ± 16	221 ± 16	227 ± 19	294 * ± 19
Serum creatinine (umol/L)	79 ± 4	76 ± 3	76 ± 3	65 ± 10
Obesity	92 ± 2	90 ± 3	95 ± 5	97 ± 6

7.5.5. Discussion

The significant increase in plasma BTG levels found in the older patient controls (Group 2) when compared to the younger control group (Group 1) is not due to renal retention since all groups had similar serum creatinine levels. This increase is probably due to the significant rise in BTG with age which has been previously reported (Ludlam, 1979; Dewar, Marshall, Weightman et al., 1979; Zahavi, Jones, Leyton et al., 1980). The observed decrease in platelet count with age is supported by the results of a large epidemiological study (Meade and North, 1977). This may reflect a decrease in platelet survival with age (Abrahamsen, 1968). This increase in BTG levels with age may be a further reflection of platelet activation in vivo, possibly secondary to the increase in atherosclerosis with age.

In control subjects BTG was correlated to sex and smoking habit, but a highly significant negative association with obesity was observed and this has not been previously reported.

The finding of similar BTG concentrations in patients with early malignancy confirms a previous report (Farrell et al., 1980) which only observed significantly elevated BTG levels in the advanced stages of malignancy. This result suggests, therefore, that in the early stages of malignancy a small localised tumour does not result in measurable platelet release. In the advanced stages of malignant disease, the observed increased platelet release is presumably due to a greater vascular involvement.

Our finding of significantly increased BTG levels in patients with infection suggests that platelet activation occurs in acute "non-vascular illness". This is supported by increasing evidence

of platelet activation in acute "non-vascular" illnesses,such as infections and with associated "acute-phase" changes in plasma proteins such as fibrinogen levels (O'Brien, Etherington and Adams, 1980; Lowe et al., 1979d).

In conclusion, this study has shown that elevated BTG levels are not specific for "vascular" illnesses such as arterial occlusion or venous thrombosis. Studies of "vascular" illnesses should, therefore, contain a "control" group of patients with "non-vascular" illness, such as infection, and an "older" patient control group.

7.6. Summary

In the studies on venous thrombosis reported in this chapter several interesting findings have been observed.

1. Increased platelet activation and thrombin generation, as observed by increased BTG and FPA levels, were shown in patients with longstanding retinal vein occlusion who had capillary non perfusion and/or neovascular when compared to patients with retinal vein occlusion without ischaemic complications and controls.
2. An increased plasma level of BTG was found in patients with nephrotic syndrome suggesting ongoing in vivo activation is occurring in this syndrome.
3. A direct measurement of in vivo plasmin activity was observed in volunteers after the administration of stanozolol by the finding of increased levels of Bβ15-42 seven days after treatment.

4. The administration of the anabolic steroid, stanozolol, before major abdominal surgery does not prevent the occurrence of DVT postoperatively. Plasma levels of BTG appear to be an index of developing DVT, if measured on the first day after surgery, indicating ongoing in vivo platelet release before the development of the thrombosis. An increase in FPA levels in early malignancy suyggests that in vivo thrombin generation occurs in early malignancy but such in vivo thrombin generation does not predispose towards thrombosis. The observed increase in plasma FPA levels immediately after surgery suggests an increase in thrombin generation due to tissue damage and this cannot be regarded as predisposing factor towards thrombosis as this increase was observed in patients not developing a DVT. At postoperative day 7 increased levels of FPA appear to indicate an ongoing thrombotic process. The observed increase in plasma levels of Bβ15-42 immediately after surgery suggests ongoing in vivo plasmin activity in response to fibrin formation as a result of tissue damage, such in vivo plasmin activity does not prevent the development of thrombosis. In patients receiving stanozolol a greater increase in in vivo plasmin activity was observed on day 7 postoperatively.

CHAPTER 8

PLASMA FIBRINOPEPTIDE A, BETATHROMBOGLOBULIN AND Bβ15-42 IN ARTERIAL THROMBOSIS

8.1. Introduction

The clinical diagnosis of arterial thrombosis is usually made as a result of ischaemic damage to the tissue supplied by the vessel. This however does not give adequate precision for scientific studies. Indeed the diagnosis of arterial thrombosis is more difficult than venous thrombosis (Mitchell, 1978) as arteriographic 'occlusions' may be due to artefacts, arterial spasm, atherosclerotic plaques, thrombi, emboli or combinations of the phenomena. Acute occlusion of the limb arteries may be due to emboli from the heart or proximal vessels as well as thrombosis in situ: secondary thrombosis renders diagnosis difficult even at operation. It is now possible to differentiate cerebral infarction from cerebral haematoma by computerised axial tomography, but again CT scans cannot differentiate between thrombosis and embolism as the cause of the infarction. Myocardial infarction is almost always associated with a fresh occlusive thrombus. Most investigators believe that the thrombus antedates and causes the infarct (Chandler, Chapman, Erhardt et al., 1974; Fulton, 1978). The rediscovery that platelets play an initiating role in arterial thrombosis with activation of the coagulation system and deposition of fibrin due to a decreased fibrinolysis suggests the measurement of plasma BTG, FPA and Bβ15-42 as possible markers of platelet release, thrombin and plasmin generation in arterial thrombosis.

The first study described in this chapter (8.2) examines the levels of plasma FPA and BTG in patients with acute chest pain and normal subjects. The second study (8.3) measured plasma levels of BTG, FPA and Bβ15-42 in patients with coronary artery disease in order to determine if plasma levels of these proteins were related to the extent of coronary artery disease. The third study (8.4) examines plasma levels of BTG and FPA in patients with type II and IV hyperlipoproteinaemia and normal subjects to determine whether platelet release and fibrinogen activation were involved in the pathogenesis of the atherosclerosis in this condition. The fourth study (8.5) measured plasma levels of BTG and FPA in patients with diabetes mellitus and normal subjects in order to determine if there was a relationship between BTG and FPA levels and the occurrence of proliferative retinopathy in diabetes mellitus when compared to normal subjects. The fifth study (8.6) measured plasma levels of BTG and FPA in patients with transient cerebral ischaemic attacks and in both a group of 'elderly' and young controls to determine whether platelet release and fibrin formation were involved in the development of such attacks. The second part of this study assesses BTG and FPA levels as predictive indices of further vascular events. The last section of this chapter (8.7) summarises the results found in this chapter.

8.2. Plasma Fibrinopeptide A and Betathromboglobulin in Patients With Acute Chest Pain

8.2.1. Introduction

Activation of platelets and thrombin generation have been suggested as precursors of fresh occluding thrombus in the coronary arteries supplying an area of infarcted muscle (Turpie, de Boer and Genton, 1982). Further evidence in support of platelet activation

and thrombin generation is obtained from the findings of elevated levels of BTG (Rasi et al., 1980; Denham et al., 1977) and FPA (Johnsson et al., 1979). However these studies were obtained from a group of highly selected patients and were not representative of unselected patients with acute chest pain. The present study was therefore undertaken to determine whether increased plasma levels of BTG and FPA were specifically associated with myocardial infarction in an unselected group of patients admitted to hospital with acute chest pain.

8.2.2. Design of study

Forty eight consecutive patients (31 males, 17 females, age 41-82 years) were studied within 1-3 days after admission to hospital for acute chest pain in one general medical unit. Twenty one patients (14 males, 7 females mean age 65 ± 2 (SEM) years) had a definite or probable myocardial infarction (MI) as defined by Rowley and Hampton (1981) ie a convincing history of ischaemic type chest pain plus both ECG changes and a rise in serum levels of cardiac enzymes to more than twice the upper limit of normal (definite MI), or either ECG changes or enzyme rise (probable MI). Fifteen patients (10 males, 5 females, mean age 60.4 ± 3.4 years) had unstable angina, defined as a convincing history of ischaemic type chest pain without such ECG and enzyme evidence of infarction. Twelve patients (7 males, 5 females, mean age 59.7 ± 3.1 years) had chest pain due to non-cardiac causes (8 chest infection, 2 pneumothorax and 2 cause unknown). The control group consisted of 23 hospital patients (14 males, 9 females, mean age 55.8 ± 2.5 years) without evidence of heart disease, chest disease, inflammation or neoplasia.

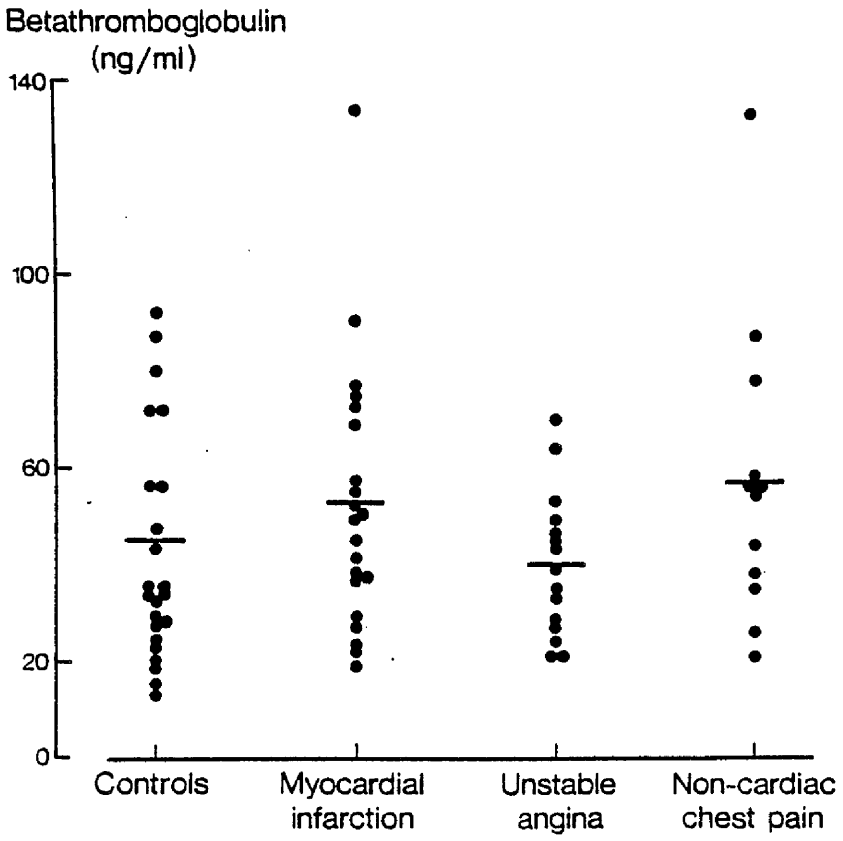


Figure 8.1.

Plasma betathromboglobulin levels in the groups studied.

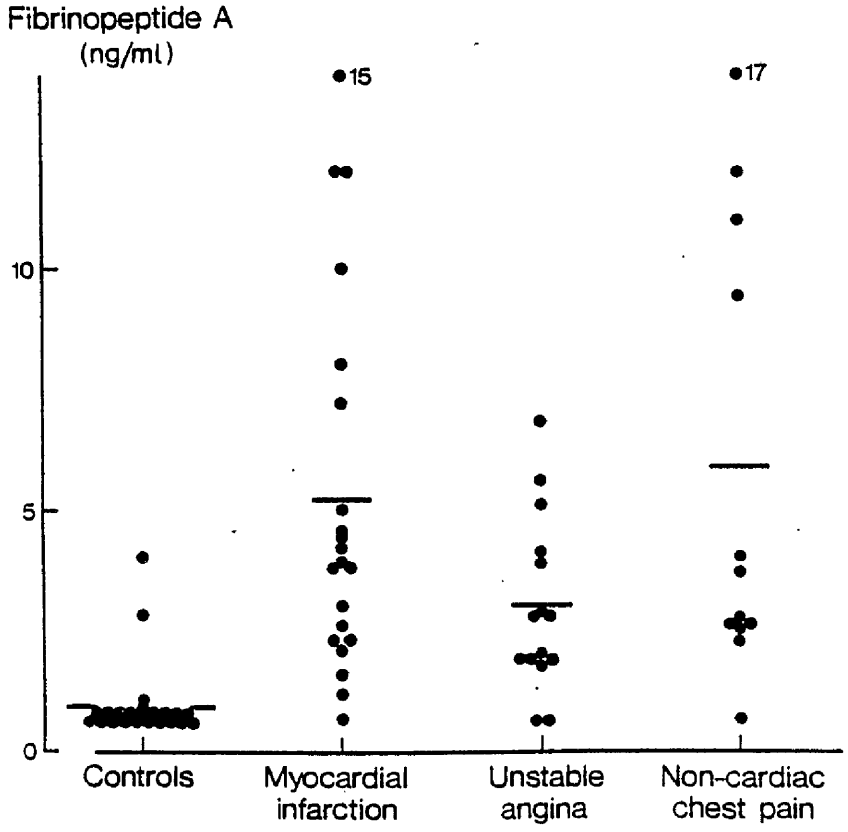


Figure 8.2.

Fibrinopeptide A levels in the groups studied.

8.2.3. Methods

Plasma BTG levels were measured as described in Chapter 5.3.2. No PGE_1 was added to the anticoagulant mixture. FPA levels were determined using the ethanol extraction method to remove cross-reacting fibrinogen as described in Chapter (5.4.2 (B) and the charcoal separation technique to separate bound and free ^{125}I as described in Chapter 5.4.2(B).

Statistical analysis of difference in means was performed using Wilcoxon's rank sum test.

8.2.4. Results

Betathromboglobulin

The levels obtained for plasma BTG in each group are shown in Figure 8.1. No significant difference was found between any of the four groups.

Fibrinopeptide A

The levels obtained for plasma FPA in each group are shown in Figure 8.2. Significantly elevated mean FPA levels were found in patients with myocardial infarction, unstable angina, and non-cardiac chest pain, when compared to normal subjects ($p < 0.01$). Despite higher levels being obtained in patients with myocardial infarction or non-cardiac chest pain compared to patients with unstable angina, these differences were not statistically significant. No significant correlation was found between BTG levels and FPA levels in any group of patients.

8.2.5. Discussion

1. Significant increases in plasma levels of FPA were shown in patients with myocardial infarction, in agreement with a previous report (Johnsson et al., 1979).

2. A significant increase was also shown in FPA levels in patients with unstable angina. The raised FPA levels therefore are not associated with myocardial necrosis.

3. No previous study compared patients with acute myocardial infarction or unstable angina to patients with other causes of acute chest pain. The present study, therefore, found similarly elevated levels of FPA in patients with non-cardiac chest pain (mostly chest infection). This result suggests that raised FPA levels are not specific for acute coronary insufficiency as suggested previously (Serneri et al., 1981).
4. No correlation was found between FPA levels and plasma fibrinogen levels (Dade fibrometer) in the present study, in agreement with a previous report (Harenberg, Haas and Zimmerman, 1981), hence the increase in FPA does not merely reflect the increased fibrinogen levels in patients with acute illness.
5. The mechanisms by which FPA levels rise in some patients with acute chest pain therefore, remain obscure.
6. No significant elevation in mean BTG levels was found in patients with myocardial infarction or unstable angina when compared to controls, although a few patients had elevated values. This result is in contrast to previous reports (Rasi et al., 1980; Denham et al., 1977; Hughes, Daunt, Vass et al. 1979). However, a non-significant elevation of PF_4 was reported in myocardial infarction when compared to controls (O'Brien, Etherington, Shuttlesworth et al., 1980). Conflicting evidence is available for increased platelet release in myocardial

infarction, either in vivo (raised BTG or PF_4) or in vitro (Heptinstall, Mulley, Taylor et al., 1980).

7. Measurements of BTG and FPA are therefore of no value in separating patients presenting with chest pain into those with and without myocardial infarction.

8.3. Plasma Fibrinopeptide A, Betathromboglobulin and Bβ15-42 in Coronary Artery Disease

8.3.1. Introduction

The possible involvement of haemostatic factors in atherosclerosis has been suggested in an editorial in the Lancet (1980). Recent clinical and experimental evidence suggests that platelet activation and release may play an important role in the pathogenesis of coronary atherosclerosis (Ross, Glomset, Kariya et al., 1974; Rutherford and Ross, 1976) and in the complications of ischaemic heart disease (Haerum 1972; de Wood, Spores, Notske et al., 1980). Fibrin formation has also been implicated in the development of coronary atheroma (Smith, Staples, Dietz et al., 1979; Meade, Chakrabarti, Stirling et al., 1980). However direct evidence of in vivo platelet activation and fibrin formation has been difficult to obtain in patients with coronary artery disease, since sensitive techniques of in vivo platelet activation and fibrin formation have not been available to date.

With the development of radioimmunoassays for BTG, FPA and Bβ15-42, in vivo markers of platelet release, fibrin formation and fibrinolysis respectively, it should now be possible to measure these parameters in vivo in coronary artery disease.

Elevated FPA levels have been previously reported in patients with coronary heart disease (Serneri et al., 1981). The present study was undertaken to determine whether platelet release, thrombin generation and fibrinolysis are related to the extent of coronary artery disease.

8.3.2. Design of study

One hundred men aged 33-65 years were studied after they had been admitted to Glasgow Royal Infirmary. They were all studied before they underwent selective coronary arteriography for assessment of chest pain: patients excluded were those with a history of myocardial infarction in the previous three months, those receiving treatment with diuretics (which causes haemoconcentration) or clofibrate (which lowers fibrinogen concentration and viscosity) and those with overt heart failure. The extent of angiographic coronary occlusion was graded according to the number of major vessels (right, left anterior descending, and left circumflex coronary arteries) in which the lumen was occluded by 50% or more; patients were thus classified as having one, two or three vessel disease (Lowe, Drummond, Lorimer et al., 1980). Twenty one patients had one vessel disease, 24 had two vessel and 52 had three vessel disease.

8.3.3. Methods

Plasma BTG levels were measured as described in Chapter 5.3.2. FPA levels were determined using the bentonite method to remove cross-reacting fibrinogen as described in Chapter 5.4.2. The double antibody technique was used to separate bound and free ^{125}I FPA. Plasma Bβ15-42 was measured as described in Chapter 5.5.1.

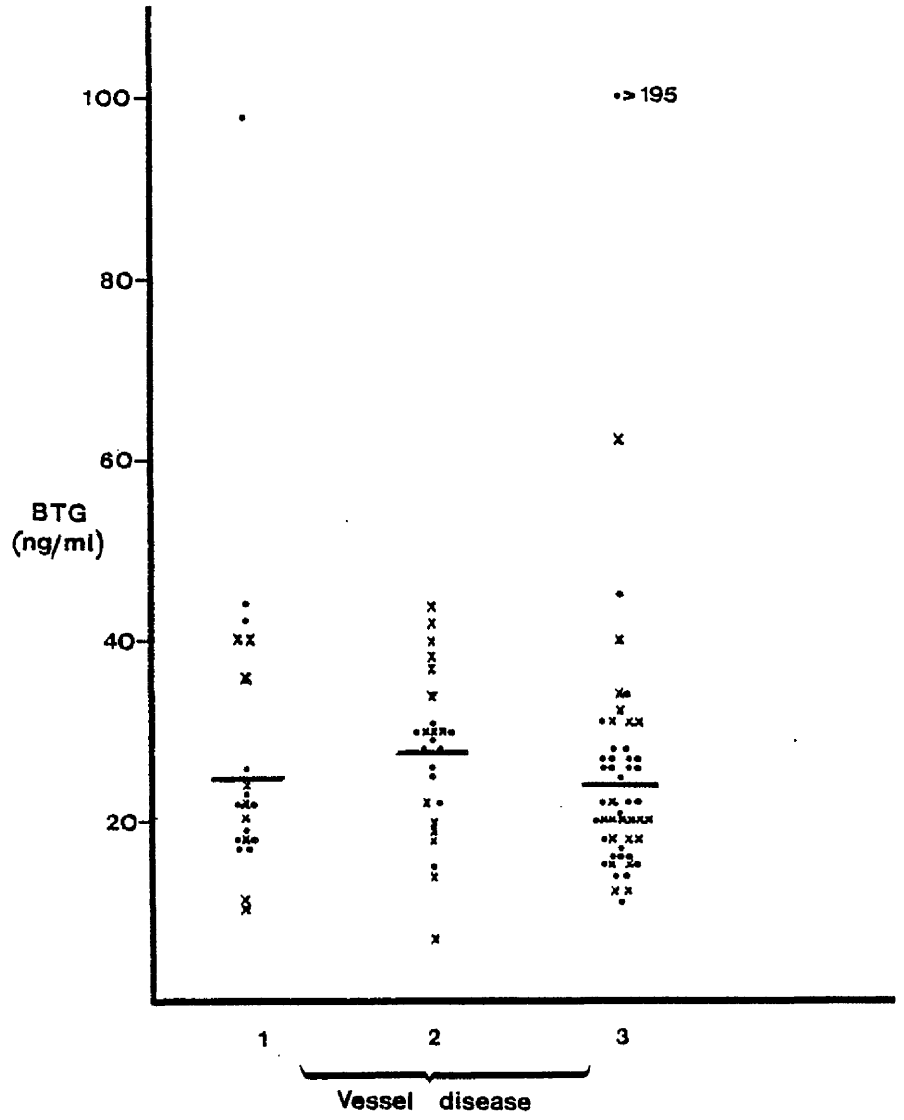


Figure 8.3

Plasma betathromboglobulin levels in patients with coronary artery disease. 1, 2 and 3 denotes patients having one, two or three vessel disease. x denotes those patients who had a previous myocardial infarction.

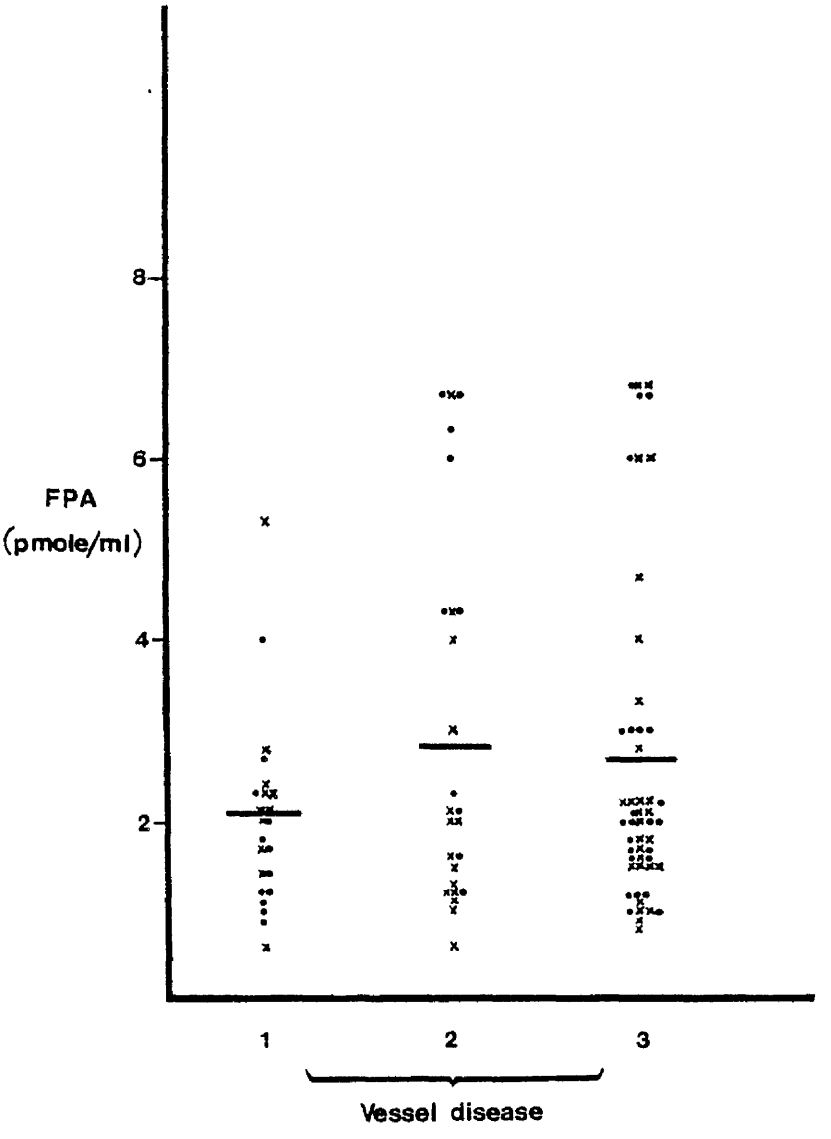


Figure 8.4

Plasma FPA levels in each of the groups with coronary artery disease, ie 1, 2 or 3 vessel disease. (x) denotes patients with a previous myocardial infarction.

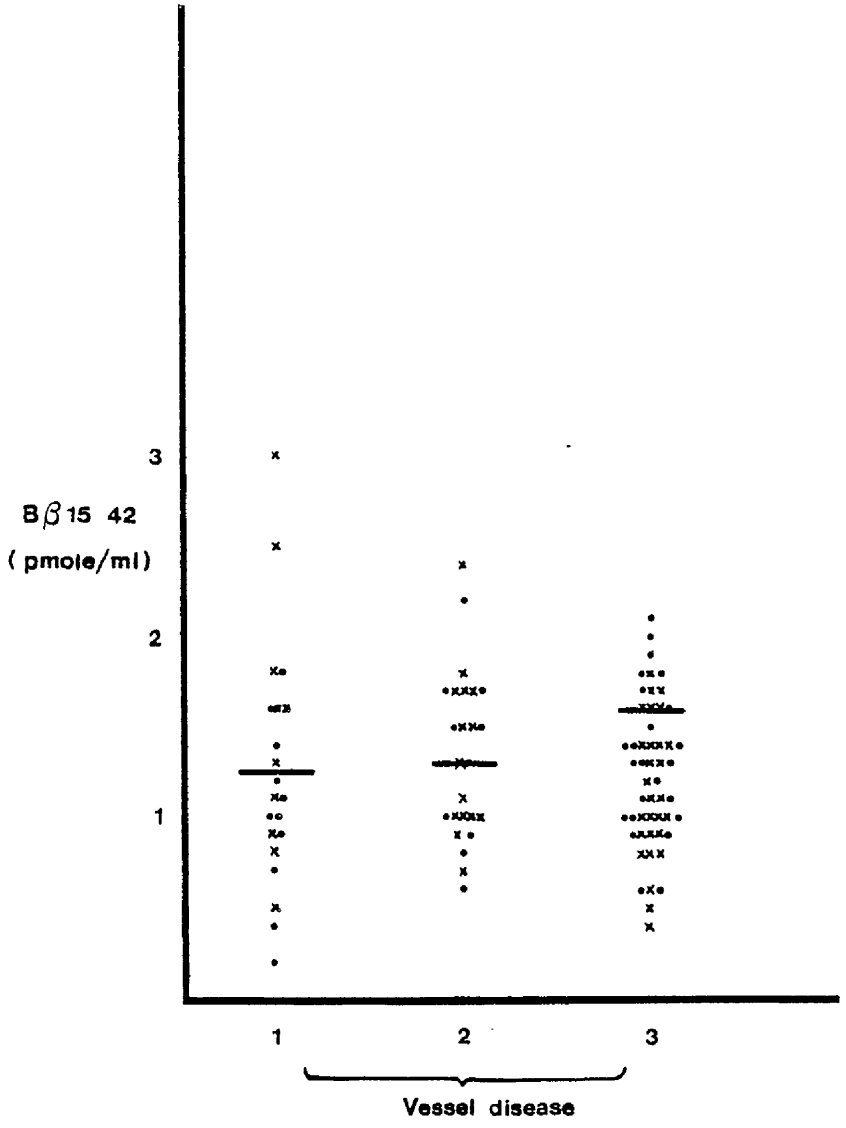


Figure 8.5

Plasma B β 15-42 levels in each of the groups with coronary artery disease, ie 1, 2 or 3 vessel disease. (x) denotes those patients with a previous myocardial infarction.

Statistical analysis

The significance of difference among groups was measured by the unpaired students' 't' test.

8.3.4. Results

Plasma levels of BTG, FPA Bβ15-42 obtained for each group are shown in figures 8.3, 8.4 and 8.5. The levels of BTG, FPA, and Bβ15-42 were not significantly different among the three patient groups.

8.3.5. Discussion

In the present study, plasma levels of BTG, FPA and Bβ15-42 were not elevated in patients with coronary artery disease. Furthermore, levels of BTG, FPA and Bβ15-42 were unrelated to the severity of the coronary artery disease assessed by the number of arteries with significant stenotic lesions or to the occurrence of a previous myocardial infarction. A previous study observed similar FPA results but observed elevated BTG and PF_4 levels in patients who had previously experienced myocardial infarction. This observed platelet release appeared to be unrelated to the extent of coronary artery disease but was suggested to be related to the presence of a previous myocardial infarction (Nicols et al., 1982). Such different findings in the two studies may be the result of different patient populations or different assay methods.

The observations in our study suggest that an ongoing state of increased platelet release and fibrin formation with abnormal fibrinolysis is not detectable in the peripheral blood of patients with coronary artery disease.

8.4. Plasma Fibrinopeptide A and Betathromboglobulin in Type II Hyperlipoproteinaemia

8.4.1. Introduction

Type II hyperlipoproteinaemia (HLP) is characterised by a primary increase in plasma low-density-lipoprotein (LDL) cholesterol. The increased risk of premature occlusive arterial disease in HLP is normally attributed to infiltration of the arterial wall by LDL, in proportion to its plasma concentration, and its concentration in developing arterial lesions. While evidence has been accumulating for the possible involvement of platelets in atherosclerosis less evidence exists for the involvement of the coagulation system in the pathogenesis of atherosclerosis. An increase in plasma fibrinogen in HLP (Lowe, Drummond, Third et al., 1979) and evidence suggesting the presence of increased intravascular coagulation in patients with hyperlipidaemia have been reported (Carvallo, Lees, Vaillancourt et al., 1979). This study was undertaken to determine plasma levels of FPA and BTG in patients with type II hyperlipoproteinaemia (a and b) patients with type IV hyperlipoproteinaemia and a group of normal subjects.

8.4.2. Design of study

Twenty one patients with primary type II HLP who were attending a lipid clinic were studied. Thirteen were male and eight were female: their mean age was 46 years (range 20-60). Fourteen were current cigarette smokers; none was diabetic; nine had a history of arterial disease (cardiac infarction or angina). Plasma cholesterol ranged from 6.5 to 15.3 mmol/l.

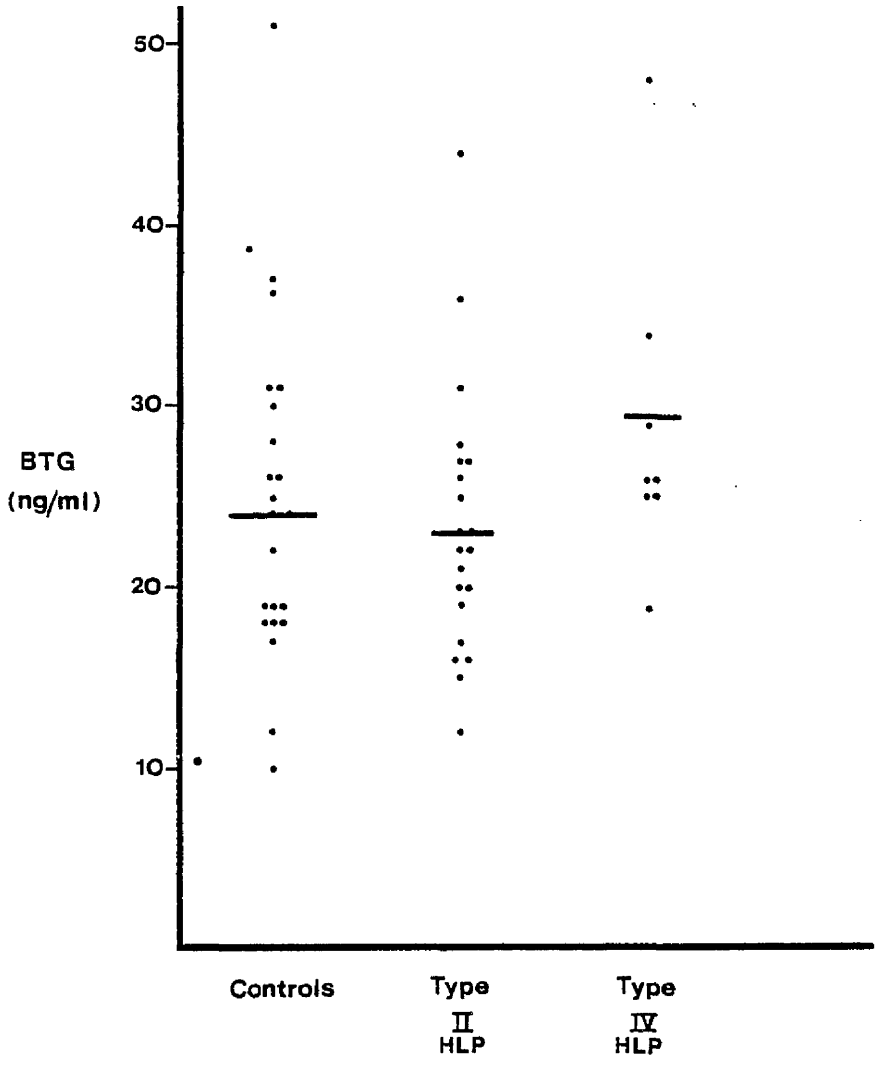


Figure 8.6

Plasma betathromboglobulin levels in patients with hyperlipoproteinaemia (HLP) and controls.

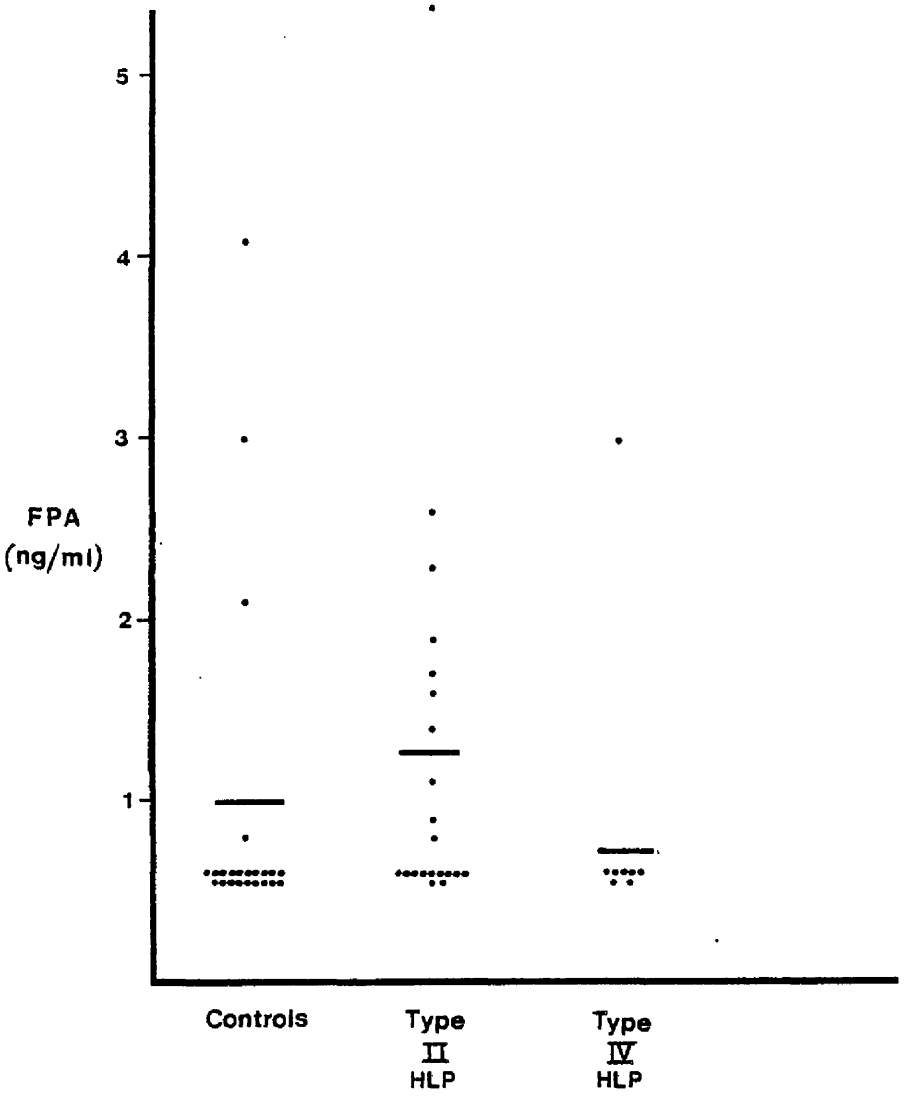


Figure 8.7

Plasma fibrinopeptide A levels in patients with hyperlipoproteinaemia and controls.

Eight patients with type IV HLP were also studied, six were male, two were female (age range 29-52 years), six were current smokers; none was diabetic. None had a history of arterial disease. Plasma cholesterol ranged from 5.6-16.5 mmol/l.

Twenty one controls, matched for sex, age and smoking habit, were selected from among hospital staff or patients admitted for elective minor surgery; all had normal total and LDL cholesterol concentrations.

8.4.3. Methods

Plasma BTG and FPA were measured as described in 8.2.3. Statistical analysis was performed by the use of the Wilcoxon rank sum test.

8.4.4. Results

The plasma levels of BTG and FPA obtained in this study are shown in Figures 8.6 and 8.7 respectively. The levels of BTG and FPA were not significantly different among the three patient groups.

8.4.5. Discussion

In this study plasma levels of BTG and FPA were not elevated in patients with type II or type IV HLP. Our findings are in agreement with a previous report which found no elevation of plasma FPA in patients with Type II HLP (Nossel et al., 1979). The results of both studies are inconsistent with the previous reported study Carvalho et al., 1979) which found evidence of intravascular coagulation in type II and IV hyperlipoproteinaemia. The patients in the former two studies were asymptomatic and may have had less extensive atherosclerosis than those studied by Carvalho et al., 1979.

Another explanation as suggested by Nossel et al., 1976, is that it is by no means certain that FPA levels and abnormal fibrinogen behaviour on agarose columns are equivalent measures of thrombin proteolysis of fibrinogen. Our finding of normal BTG levels in type II and IV HLP is inconsistent with the findings of a shortened platelet life-span in patients with arterial disease (Harker and Slichter, 1972) and platelet hyper-responsiveness in patients with HLP (Carvalho et al., 1974). This discrepancy may again be due to different populations or alternatively, since BTG is a marker of in vivo platelet activation it is a more sensitive method of platelet activation. The observations found in the study reported in this section suggests that ongoing activation of platelets and thrombin generation does not appear to be occurring in patients with type II and type IV hyperlipidaemia. This finding does not implicate the activation of platelets or thrombin generation in the pathogenesis of atherosclerosis.

8.5. Plasma Fibrinopeptide A and Betathromboglobulin in Diabetes Mellitus

8.5.1. Introduction

Patients with diabetes mellitus are known to have a high incidence of arterial disease. Haemostatic studies in diabetes have shown the presence of blood changes favouring thrombosis. A recent study reported that diabetics had higher platelet adhesiveness, fibrinogen, Factor VIIIc and ATIII, and a lower fibrinolytic activity and platelet count than control subjects (Fuller, Keen, Jarret et al., 1979). Furthermore in this study values were higher in diabetics with microvascular disease (defined as retinopathy or proteinuria) than in diabetics without disease. Activation of blood platelets in diabetes mellitus has been demonstrated by the finding of elevated plasma BTG levels in

diabetic patients, both with and without complications such as retinopathy (Burrows et al., 1978; Preston et al., 1978; Borsey, Dawes, Fraser et al., 1980). One study reported no significant difference between diabetic patients with complications and controls (Campbell et al., 1977). However, this latter study is subject to statistical criticism. A recent study found elevated FPA levels in diabetics although the patient population was small and it was not evident what complications were present in this group. Increased PF_{4} levels were also reported (Ek, Thunell and Blombäck, 1982).

The aim of the present study was to determine whether abnormal levels of BTG, an index of platelet release, and FPA, an index of thrombin generation, were abnormal in patients with diabetes mellitus and if there was a relationship between BTG and FPA levels and the presence of proliferative retinopathy when compared to control subjects.

8.5.2. Design of study

Seventeen patients with diabetes mellitus and proliferative retinopathy as diagnosed with fluorescein angiography were studied, ten were male and six female: their mean age was 54.6 ± 3.7 years (range 29-73) 16 diabetic patients without proliferative retinopathy were also studied. Ten were male and six were female; their mean age was 57.1 ± 3.0 years (range 18-76). Twenty one control subjects matched for sex, age and smoking habit were selected from among hospital staff or patients admitted for elective minor surgery.

8.5.3. Methods

Plasma BTG and FPA were measured as described in 8.2.3.

Statistical analysis

Analysis of the differences in mean values was performed by the use of the Wilcoxon rank sum test.

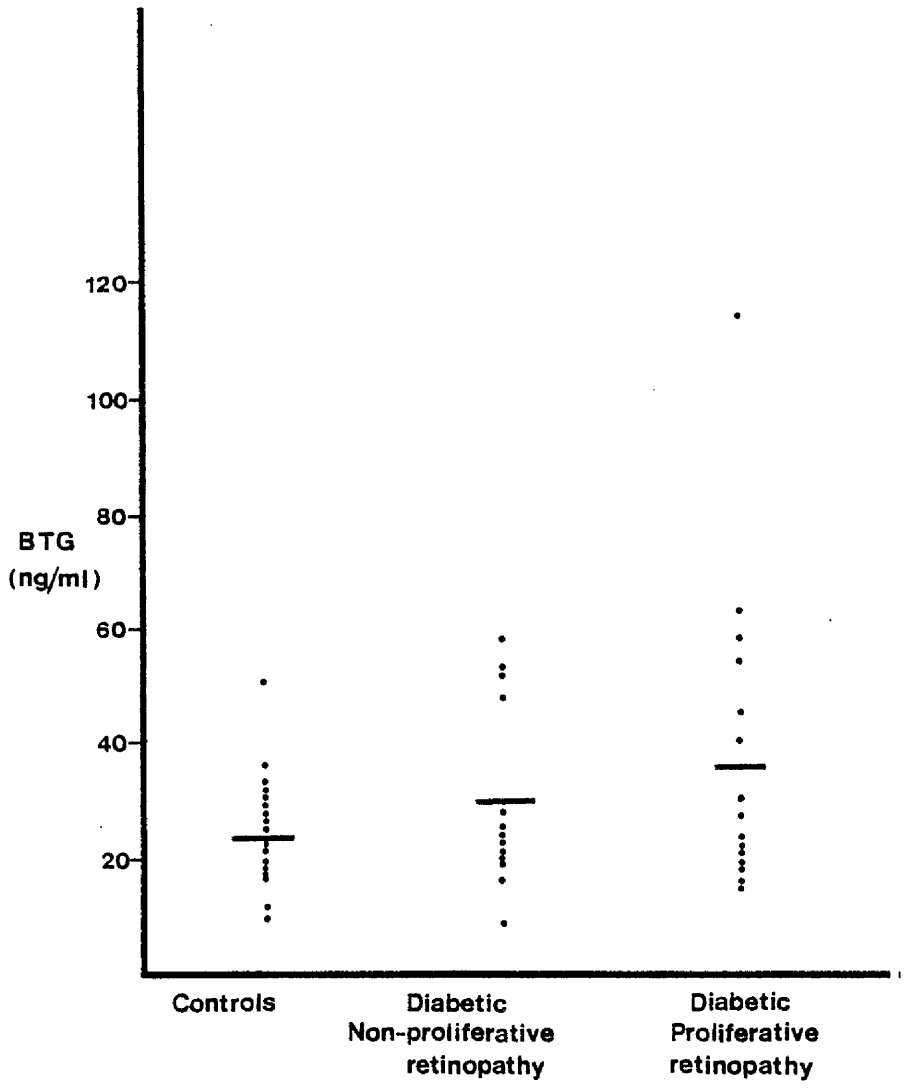


Figure 8.8

Plasma betathromboglobulin levels in patients with diabetes who had non-proliferative or proliferative retinopathy.

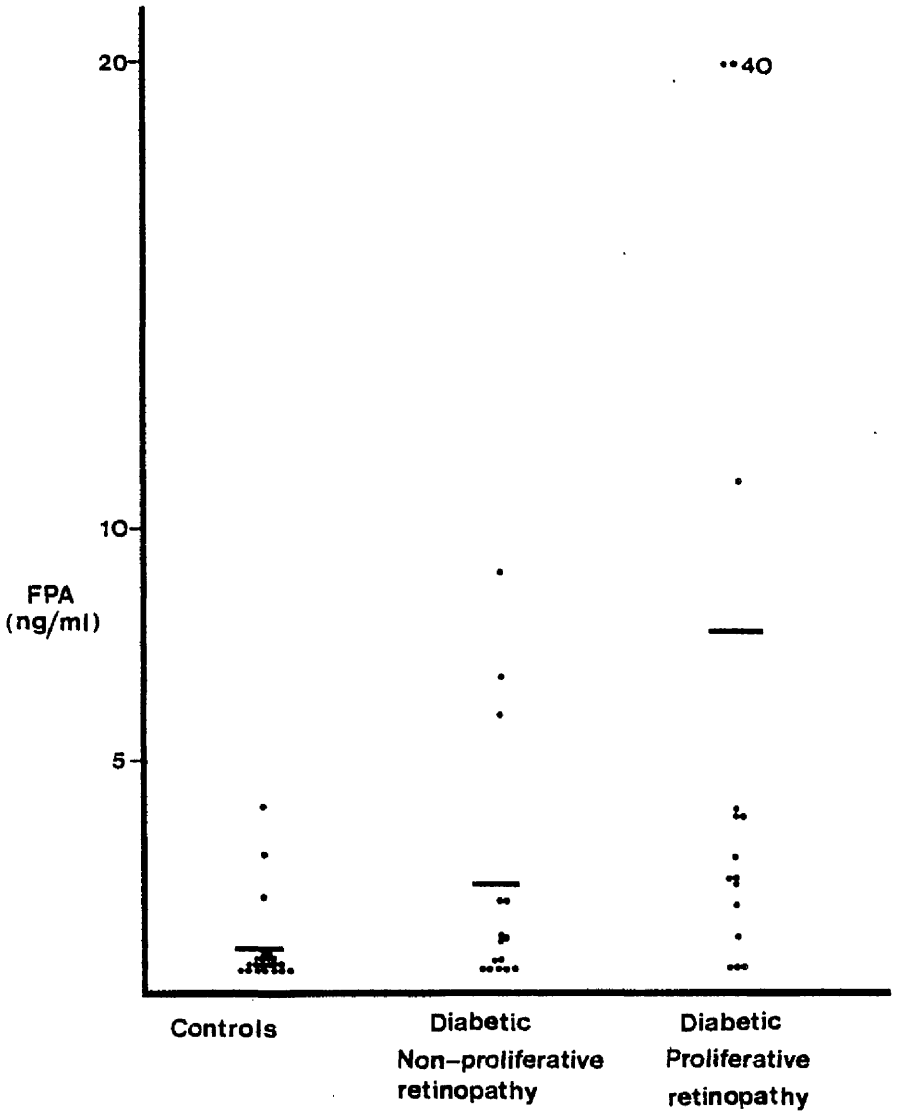


Figure 8.9

Plasma FPA levels in each of the groups studied.

8.5.4. Results

The plasma levels of BTG and FPA obtained in this study are shown in Figures 8.8 and 8.9 respectively. Elevated mean levels of BTG were found in diabetic patients with and without proliferative retinopathy when compared to controls. Only in the diabetic group with proliferative retinopathy was the difference significant. Elevated mean levels of plasma FPA were found in both diabetic groups when compared to control subjects. These differences were significant. Highly elevated mean levels of FPA were found in diabetic patients with proliferative retinopathy, the mean levels being significant when compared to the diabetic group without proliferative retinopathy and the control group.

8.5.5. Discussion

In this study elevated levels of BTG were observed in diabetic patients with retinopathy, the mean level in diabetes with proliferative retinopathy being significant when compared to the control group. This result is in agreement with previous reports (Burrows et al., 1978; Preston et al., 1978; Borsey et al., 1980). The elevated plasma BTG levels suggest in vivo activation of platelets is occurring in diabetes. Elevated mean FPA levels were found in both diabetic groups when compared to normal controls. Highly elevated mean FPA levels were observed in the diabetic group with proliferative retinopathy which were significant when compared to normal controls. A significant difference was found in mean FPA levels in the two diabetic groups. These results suggest that ongoing thrombin generation is taking place in diabetes and is more evident in the group with proliferative retinopathy.

8.6. Plasma Fibrinopeptide A and Betathromboglobulin in Transient Cerebral Ischaemic Attacks.

8.6.1. Introduction

Ischaemic stroke is a major cause of morbidity and mortality in arterial thrombosis. Transient ischaemic attacks (TIA) appear to be the result of emboli (platelet, fibrin or atheromatous) arising from atheromatous lesions in the major arteries to the brain (Fisher, 1959; Gunning, Pickering, Robb-Smith et al., 1964; Ehrenfeld, Hoyt and Wylie, 1966). TIA appear to be the most important warning symptoms of an impending stroke, with the greatest risk being in the first few months following the onset of TIA (Millikan and McDowell, 1978).

BTG and FPA have been reported to be increased in cerebrovascular disease (Matsuda et al., 1979; Hoogendijk et al., 1979; Lane, Gawel, Wolff et al., 1981). Since almost 74,000 people die annually in England and Wales from cerebrovascular disease (HMSO 1980), there is a need for predictive indices of major vascular events in TIA patients in order that trials of therapy can be directed at those most at risk.

The aim of the present study, therefore, was to compare levels of BTG, an index of platelet release, and FPA, an index of fibrin formation by thrombin, in patients with transient cerebral ischaemic attacks with levels in a group of 'elderly' and young controls and then to see if either BTG or FPA had a predictive value for vascular events in the following year.

8.6.2. Design of study

Plasma BTG and FPA levels were determined in 27 patients (20 female, 7 male), age range 49-70 years, who gave a recent history of TIA as defined by unequivocal focal neurological symptoms or signs lasting less than 24 hours. Patients with episodes of dizziness without focal signs, or with 'drop attacks' were not included. BTG and FPA levels were also determined in a group of 43 age-matched 'elderly controls' (27 females, 16 males) age range 42-91 years, who were attending an old people's luncheon club, residents of old people's home and from hospital staff. None had any acute illnesses, had no clinical evidence of cerebrovascular disease and were not taking anti-platelet therapy. Levels were also determined in a group of 32 healthy 'young controls' (28 females, 3 males) age range 18-28 years who were members of hospital staff.

8.6.3. Methods

Plasma BTG and FPA were measured as described in 8.3.3.

Statistical analysis

The statistical significance of difference in means was assessed by the Wilcoxon rank sum test.

8.6.4. Results

The values obtained for BTG are shown in Figure 8.10. Plasma BTG levels increased significantly with age ($p < 0.01$), as shown, but were higher in the TIA group compared to age-matched controls ($p < 0.01$). FPA levels obtained in each group are shown in Figure 8.11. FPA levels also increased significantly with age ($p < 0.05$), but no significant difference was found in FPA levels in the TIA patients when compared to age-matched controls. No sex difference in BTG or FPA levels was found.

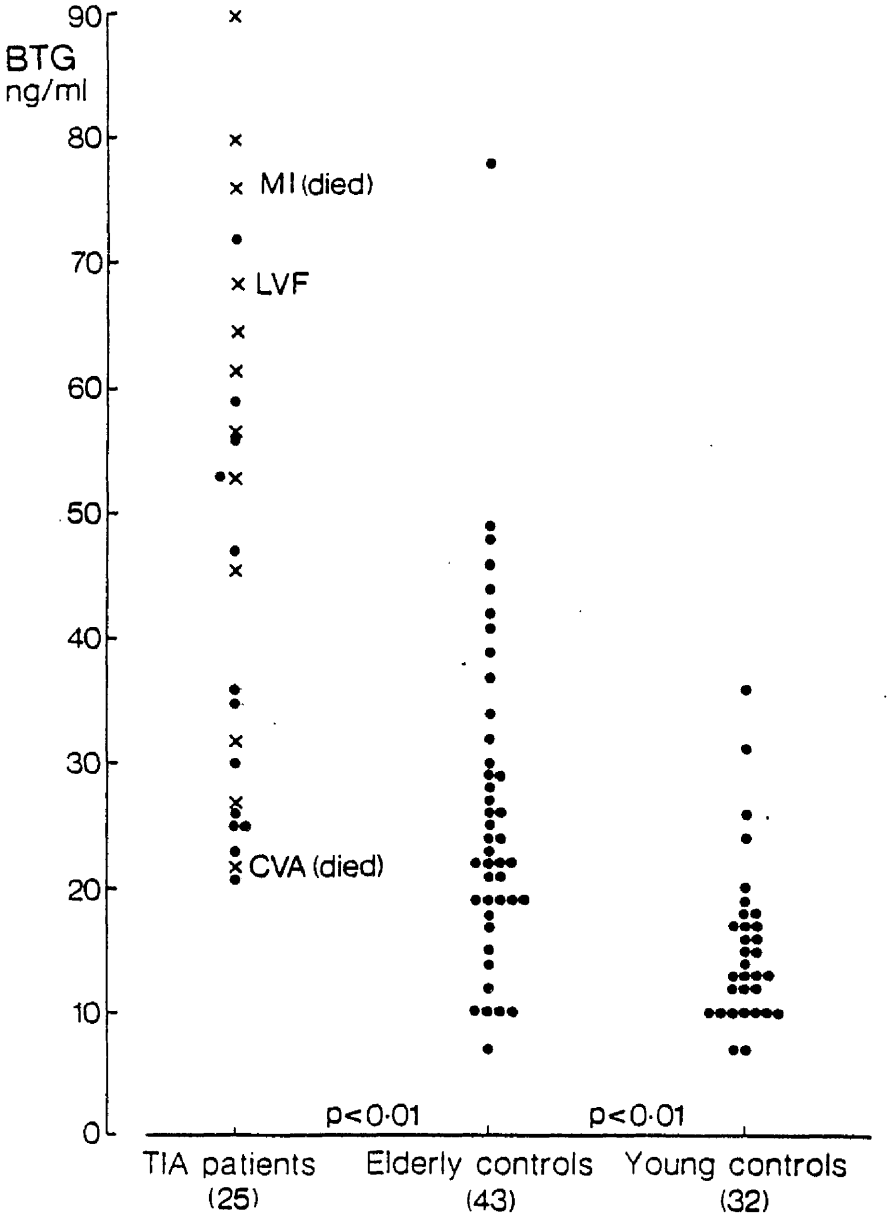


Figure 8.10

Betathromboglobulin levels in each of the groups studied.

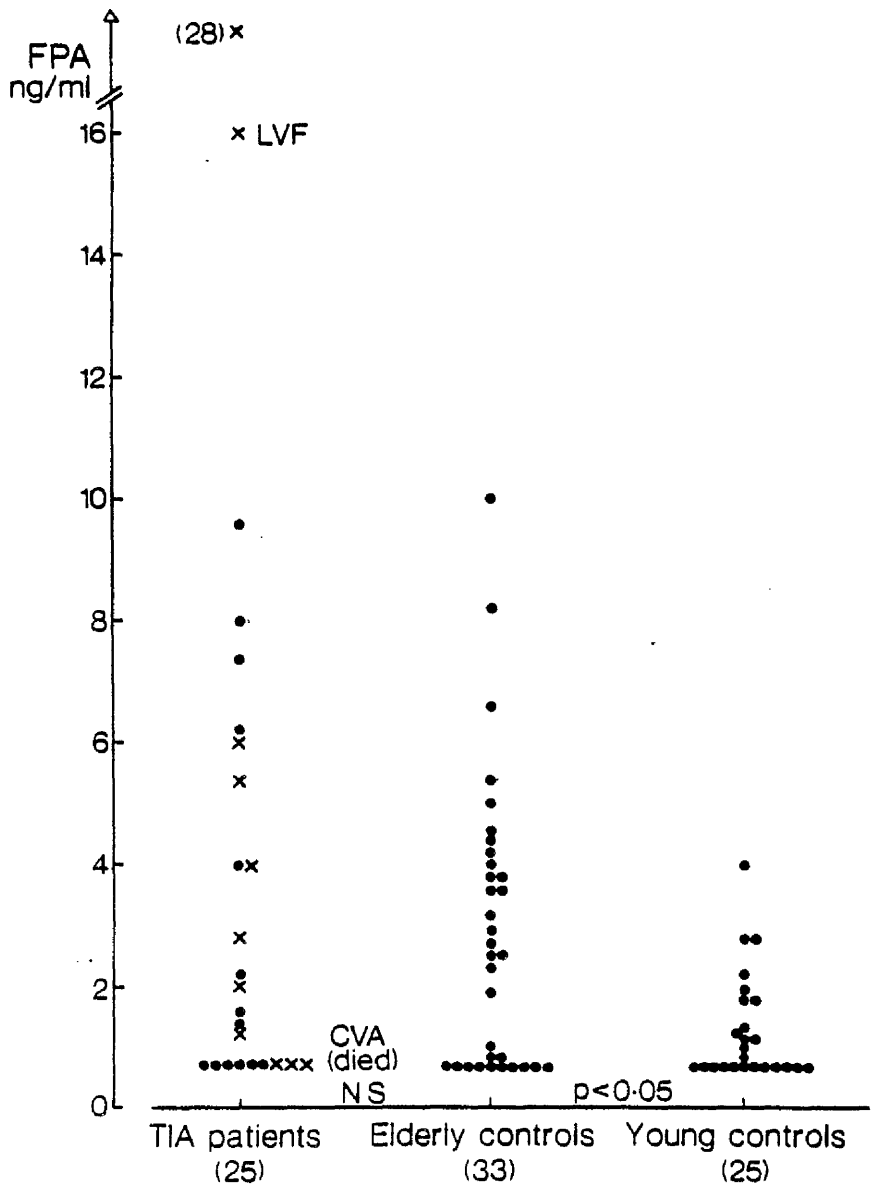


Figure 8.11.

Fibrinopeptide A levels in each of the groups studied.

In the TIA group, the time since the last TIA varied from two hours to 227 days (mean 45.5 days). In 14 of the patients the TIA was their first, in six multiple TIA had started in the previous year and in seven TIA had commenced in the previous 1-4 years. Nine of the patients were on aspirin therapy alone, three were on dipyridamole, and six were on both drugs. No correlation was found between the levels of BTG and FPA and the time since the last TIA, neither was there any correlation between BTG and FPA and aspirin or dipyridamole therapy in the TIA patients.

A predictive study was then carried out. All patients with TIA were followed for one year, and subsequent mortality or morbidity from vascular disease (completed stroke, myocardial infarction or continued TIA) was noted. At one year, repeat BTG samples were taken from as many patients as possible; 16 samples were obtained and two patients had died. Repeat samples were also obtained, at one year, from six of the elderly control patients with high BTG values, six with intermediate BTG values and six with low values. FPA estimations were not repeated since as shown in Figure 8.11 FPA was not of predictive value in TIA patients.

Twelve of the TIA patients suffered further vascular events in the subsequent year - nine had further TIA, one acute left ventricular failure (LVF), one fatal myocardial infarction (MI) and one fatal cerebrovascular accident (CVA). The mean BTG level of patients who had further vascular events, 56.7 ± 6.3 (SEM) ng/ml was significantly higher than the mean BTG level of those patients who did not have a further event, 39.1 ± 4.7 ($p < 0.05$). The group who had further vascular events had a similar mean age, 72.8 years, to that of the group who had no

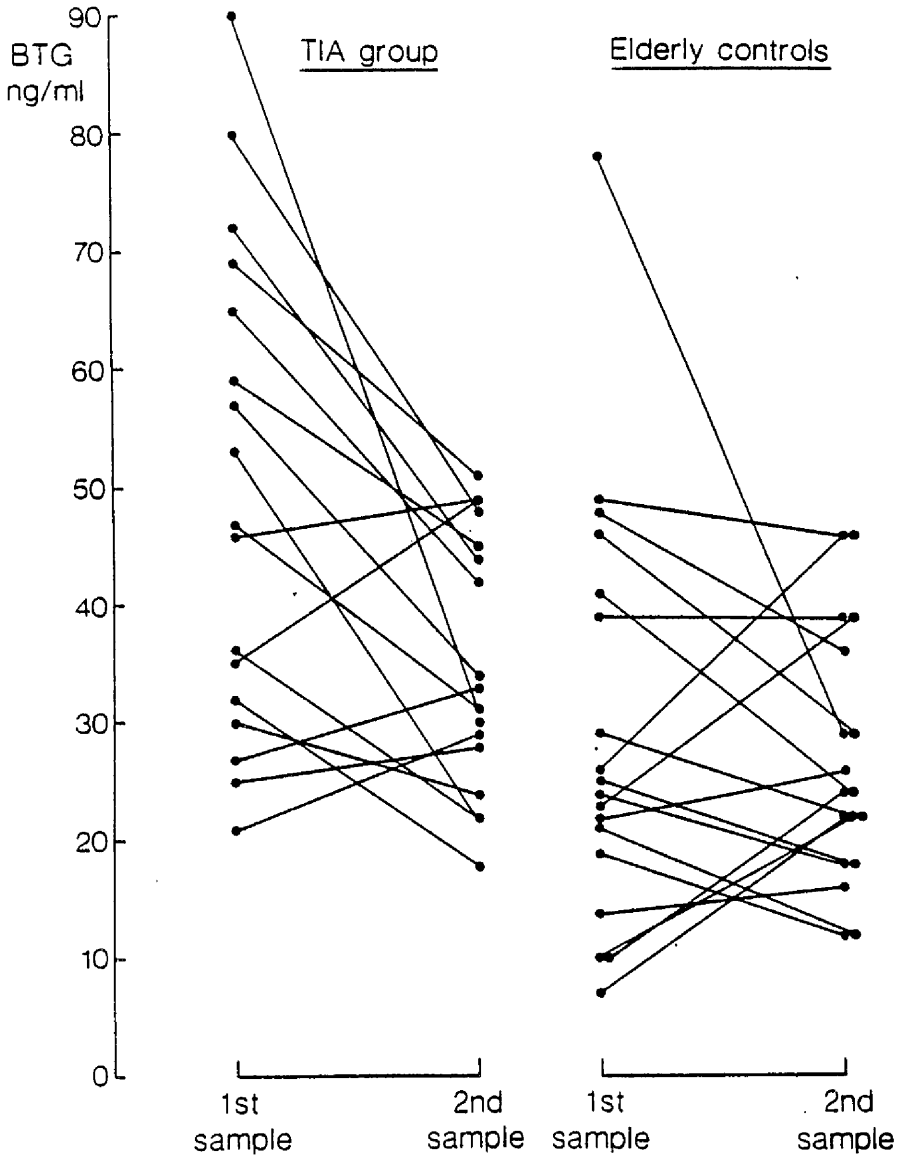


Figure 8.12

Betathromboglobulin levels in the TIA group and the elderly control group. The second sample was measured one year after the first sample.

further events, 73.9 years. There was no correlation between FPA levels and the occurrence of vascular events in the year subsequent to sampling, however in two patients who had a further TIA and fatal MI respectively, no FPA result was obtained.

Antiplatelet therapy was administered in all but two of the patients who suffered further vascular events.

8.6.5. Discussion

1. Significant increases in plasma levels of BTG were shown in TIA patients when compared to age-matched elderly controls, in agreement with previous reports (Matsuda et al., 1979; Hoogendijk et al.,1979). More importantly BTG was predictive of further vascular events.
2. The mean plasma BTG level, 21.8 ng/ml, in the 73 controls of all ages is consistent with previous reports (Zahavi, Cella, Dubiel et al., 1978; Cella et al., 1979).
3. A significant rise in BTG with age was shown as has been reported previously (Lane et al., 1981; Dewar et al., 1979; Zahavi et l., 1979; Ludlam, 1979).
4. The lack of sex difference in BTG levels is consistent with the report of Ludlam, 1979, but differs from other reports which found higher levels in old females compared to males (Zahavi, et al., 1979; Dewar et al., 1980) and lower levels in in females of all ages (Dewar et al., 1979).
5. Raised BTG levels in TIA have been reported by other workers (Matsudu et al., 1979; Hoogendijk et al., 1979). Elevated

levels have also been reported in acute cerebral infarction (Matsuda et al., 1979; chronic cerebral infarction (Lane et al., 1981) and in myocardial infarction (Matsuda et al., 1979; Denham et al., 1977). Increased BTG levels in the above diseases indicate enhanced platelet release and may indicate in vivo activation of the haemostatic mechanism, although it is not known if such activation is the result of abnormalities of platelet behaviour, of coagulation or of the vessel wall. The finding of no correlation between the time of the last TIA and BTG levels, suggests that raised levels are not simply acute changes associated with a TIA episode.

6. The mean FPA value in the young controls was 1.2 ng/ml which is consistent with previously reported values (Nossel et al., 1974, Hofmann and Straub, 1977).
7. A significant rise in FPA levels with age was shown, in agreement with a previous report (Lane et al., 1981).
8. FPA levels in the TIA patients were not significantly higher than the elderly control group.
9. Platelet activation rather than fibrin formation is characteristic of TIA.
10. No correlation was found between BTG and FPA levels and the taking of anti-platelet drugs, in agreement with previous reports of no effect with aspirin and dipyridamole on BTG levels in healthy volunteers (Hoogendijk, ten Cate, Ludlam et al., 1980; Lensing, Sturk and ten Cate, 1981), or in the case of aspirin, in patients with cerebrovascular accident. Sulphinpyrozone, another anti-platelet, does not reduce FPA levels in patients with stroke (Lane et al., 1981).

11. The predictive study suggests that raised BTG levels, but not FPA levels, appear to have a prognostic value in indicating TIA patients at risk of further vascular events. An arbitrary separation into two groups of TIA patients may be made, those with BTG levels above 50 ng/ml, a high risk group, and those with levels below 50 ng/ml, a low risk group.

12. The results shown in Figure 8.12 suggest that a state of increased platelet activation may be sustained for a considerable time in the high risk TIA patients, however the fall in BTG levels at the end of the year after initial sampling indicates that it is not maintained indefinitely. This might suggest a diminishing risk of further vascular events, for example, healing of ulcerated plaques in cranial arteries. However, the fact that BTG values in this group tended to remain in the upper part of the normal range may indicate ongoing platelet activation. Similar BTG levels in the low risk group over the year after initial sampling suggests no ongoing platelet activation is occurring in this group.

8.7. Summary

This chapter has described the measurement of plasma levels of FPA, BTG and Bβ15-42 as indices of platelet activation, thrombin generation and hence fibrin formation, and plasmin activity respectively in several studies related to arterial thrombosis. On the basis of the work reported in this chapter the following conclusions are made:

1. No evidence of platelet release was found in patients with acute chest pain as shown by similar plasma levels

of BTG in patients with myocardial infarction, unstable angina, non-cardiac chest pain and control patients. Similarly elevated levels of FPA were found in patients with myocardial infarction, unstable angina and non-cardiac chest pain when compared to control patients. Measurements of BTG and FPA are therefore of no value in separating patients presenting with chest pain into those with and without myocardial infarction.

2. Mean plasma levels of BTG, FPA and Bβ15-42 were not elevated in patients with coronary artery disease and were also unrelated to the extent of the coronary artery disease. However, a few patients did have elevated FPA levels and hence fibrin formation may occur in a small number of patients with coronary artery disease.

3. No evidence of platelet activation or thrombin generation was observed in patients with type II or type IV hyperlipoproteinaemia.

4. Ongoing platelet activation, as shown by elevated BTG levels, is suggested in diabetic patients with proliferative retinopathy. Increased thrombin generation and hence fibrin I formation is suggested in diabetic patients as a result of increased FPA levels but such thrombin generation appears to be greater in diabetic patients with proliferative retinopathy than in those without.

5. Platelet activation rather than fibrin formation appears to be characteristic of TIA patients as shown by increased plasma levels of BTG but not FPA in TIA patients having an increased risk of further vascular events.

CHAPTER 9

BETATHROMBOGLOBULIN IN IN VITRO STUDIES OF BLOOD-MATERIAL INTERACTIONS

9.1 Introduction

This chapter reports on a study undertaken to assess the suitability of BTG as a marker of *in vitro* blood compatibility. The term 'blood compatibility' as defined by Bruck (1980) denotes the absence of adverse effects of materials on whole blood, while the materials perform a given function alone or as parts of a device. Within the last decade the development of life support systems and devices to support failing organs has led to a demand for the production of synthetic materials that are blood compatible and may be left in the body for prolonged periods of time without stimulating a tissue reaction.

So far no ideal material has yet been produced that meets the demands of compatibility, strength and durability and few meet the criteria laid down by Salzman (1971). The main areas of clinical interest are in vascular grafts, dialysis membranes, cardiopulmonary oxygenators and vascular catheters. However despite such problems of thrombogenicity many such devices may serve their function by the use of anticoagulants in association with anti-platelet agents.

An invariable effect of exposure of foreign surfaces to native blood appears to be adhesion and aggregation of platelets as described previously (Mason, Mohammad, Chuang et al., 1976). A precondition to such adhesion and aggregation is the formation of a monolayer of proteins on the surface (vide infra) to which

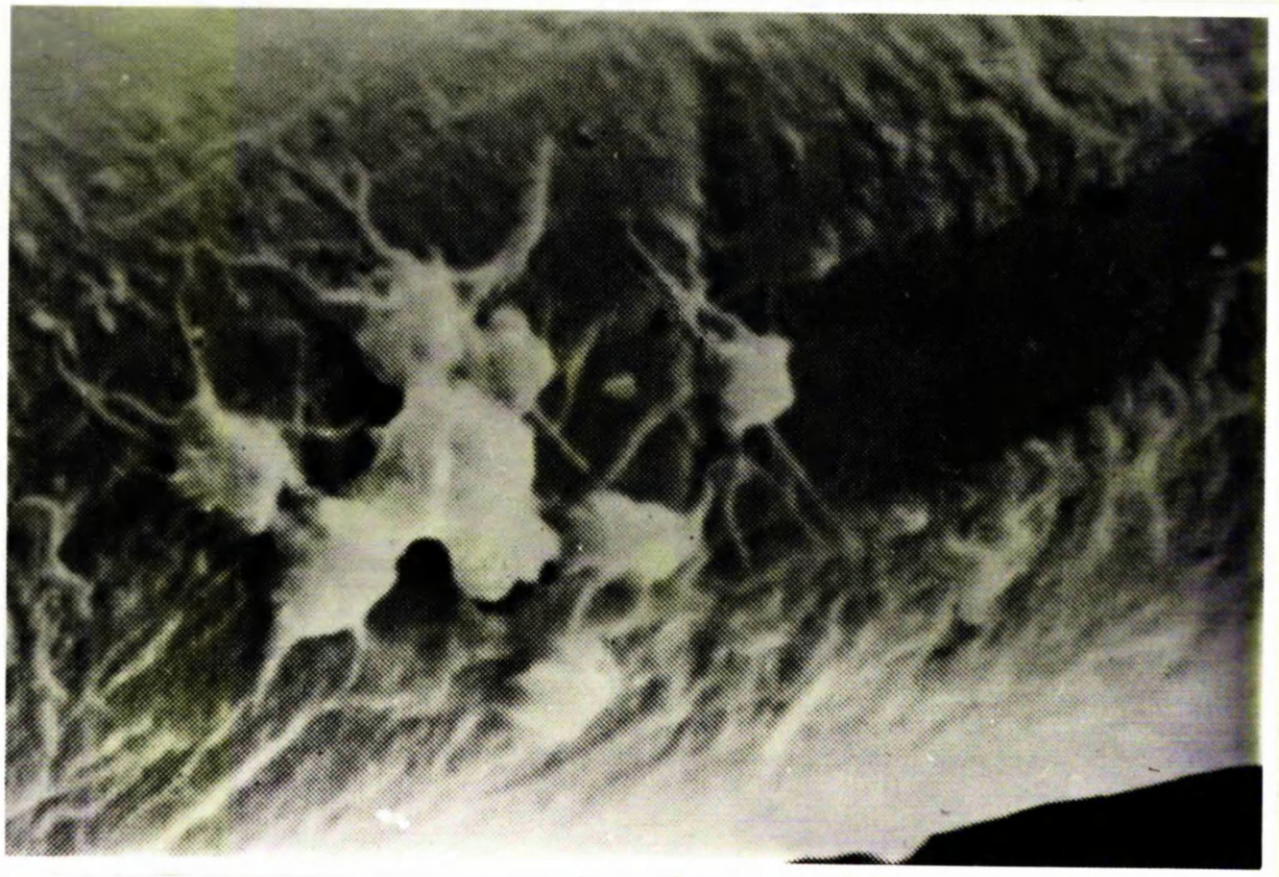


Figure 9.1

Scanning EM - magnified X3000 (courtesy of Dr R Wilkinson, Dept of Bioengineering, University of Strathclyde) of platelet adhesion to a foreign surface with pseudopodia thrown out in the direction of blood flow.

to which platelets then adhere. Such adherent platelets undergo viscous metamorphosis and this results in induction of platelet aggregation onto the surface (Baumgartner, Muggli, Tschopp et al., 1976). Analysis of the formation of cellular deposits show that previously deposited platelets throw out pseudopodia which orientate themselves in the direction of flow and these enhance further platelet deposition (see Figure 9.1). This in turn accelerates the formation of a network of fibrin which traps red and white cells (see Figure 9.2). Thrombus formation may then spread over the surface impairing function, or the adherent fibrin-platelet mixture may be washed off by the flowing blood and produce emboli in the patient. Studies of platelet deposition on artificial surfaces suggest that stagnation of flow accelerates platelet aggregation and it has been proposed that the preconditions responsible for this are the differences between arterial and venous blood, the varying blood flow patterns and the design and mechanical functioning of a medical device (Bruck, 1981). The results obtained in a recent study suggest that PGI_2 even at high concentrations does not prevent the formation of a monocellular platelet layer on collagen fibrils or artificial surfaces, but it does inhibit clumping of further platelets to this layer and prevents subsequent platelet thrombus formation (Fesus, Harsfalvi, Kovacs et al., 1981). In recent clinical trials conducted during cardioplumonary bypass surgery in animals (Longmore, Bennett, Gueirrara et al., 1979), and in human patients (Walker, Davidson, Faichney et al., 1981; Radegran, Aren and Teger-Nilsson, 1982)

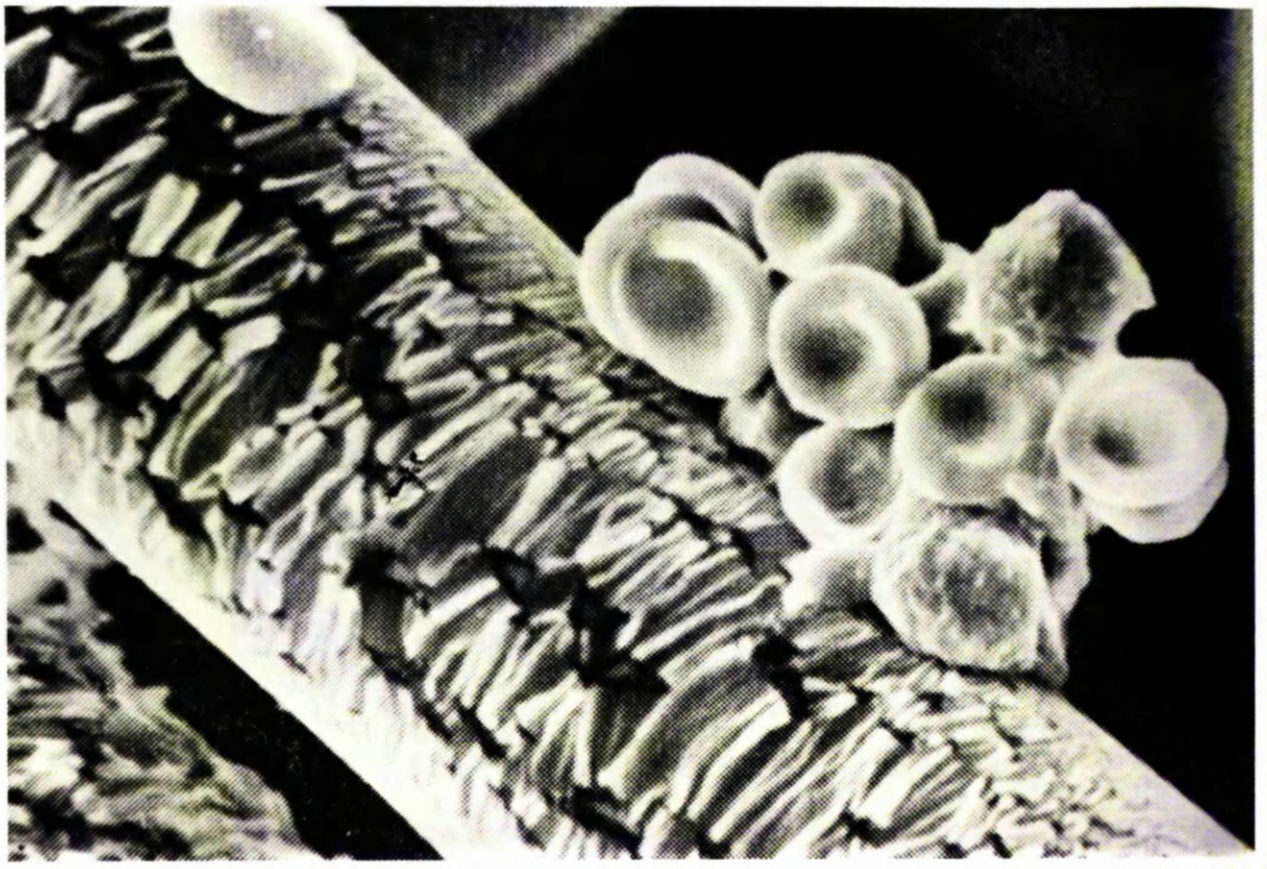


Figure 9.2

Scanning EM - magnified X3000 of red cell and white cell aggregates form on a protein monolayer of a foreign surface.

the administration of PGI_2 resulted in platelet protection, decreased formation of micro-aggregates and an improved haemostatic function after the appropriate procedure.

A wide spectrum of in vitro tests have been devised for the assessment of blood compatibility. Most depend on the measurement of activation of components of the coagulation system (see Figure 9.3). It is however, clear that the information gained from in vitro testing cannot be applied directly to the clinical situation and material assessment and development would therefore benefit from more reliable methods of evaluation. The available tests have been extensively reviewed (Salzman, 1972; Bruck, 1974; Forbes and Prentice, 1978) and a selection is set out in Table 9.1.

Since the release of granular material from within the platelets is accompanied by the formation of protrusions of the platelet plasma membrane and as morpholgical studies of adhesions to surfaces show that platelets extrude their granules into pseudopodia, it would seem that the adhesion of platelets to a foreign surface is accompanied by the release of platelet contents such as BTG. To date, no in vitro studies have been reported on the monitoring of changes in BTG levels when platelet adhesion and aggregation occur on synthetic materials. The aim of the present study reported in this chapter was to measure BTG levels under test conditions and with the use of different polymer materials.

9.2. Design of study - Methods

Values of BTG obtained in non-anticoagulated, citrated and heparinised blood were measured in normal individuals to standardise the method. Samples were obtained from young healthy members of hospital staff.

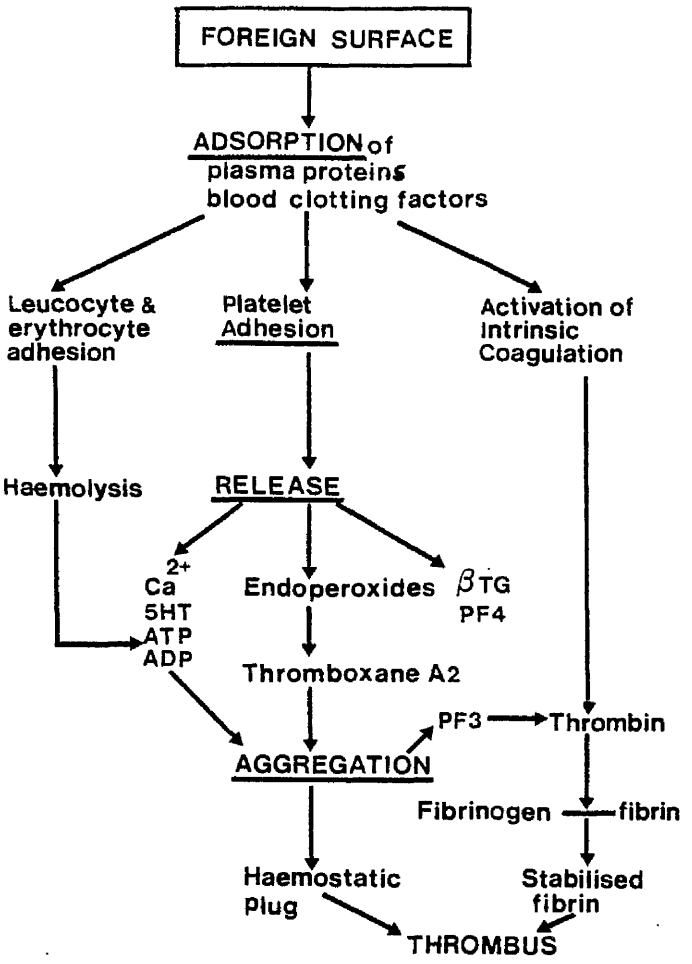


Figure 9.3

Blood-artificial surface interactions.

TABLE 9.1.

TESTS TO EVALUATE THROMBOGENICITY OF AN ARTIFICIAL SURFACE

PLATELET	BLOOD COAGULATION	GENERAL
Count	Partial thromboplastin time	Scanning of surfaces by electron microscopy
Release of constituents (BTG, Acid Phosphatase, 5HT, TXB_2, PF_4)	Fibrinogen and degradation products	Deterioration of function of prosthesis
Platelet survival	Assays of Factors XII, XI, and V	Tests cells constructed of materials under test
Adhesion to surface	Fibrinopeptide A (FPA)	Artificial circulations
Aggregation to ADP, 5HT, Collagen, Adrenaline	Radio-labelled fibrinogen turnover	Arterio-venous anastomose of materials to be tested
^{III}Indium labelled platelets	Immunofluorescent-staining for fibrinogen	Estimation of number of platelet-fibrin emboli downstream from implant

BTG release from blood in the syringe:

Venous blood was collected into a 30 ml syringe with citrate and heparin as anticoagulant and at various time intervals after the beginning of the venepuncture 2.7 ml aliquots were transferred into BTG sample tubes. The role of BTG in in vitro thrombus formation was studied by taking samples of non-anticoagulated blood at one minute intervals, and silastic tubes filled with blood, closed loops formed (Chandler, 1958) and the tubes rotated. Blood was removed at different time intervals for assay of BTG levels. The effect of oscillation on the platelet release reaction was measured by taking samples at specified times after oscillation had commenced.

Material assessment

Four materials were evaluated: siliconised glass tubes (Becton Dickinson Ltd),; polypropylene tubes (Sterilin Ltd.); plasticised polyvinyl chloride sheet (Avon Medical Ltd) and silicone rubber sheet (Dow Corning Inc).

Tubes

10 cm siliconised glass tubes, 10 cm X 1.5 cm, had a siliconised natural rubber stopper and the 10 cm polypropylene tubes, 10 cm X 1.5 cm had a polypropylene stopper. Ten ml of whole blood without anticoagulant was placed into each of the tubes and mixed on a roller mixer for six minutes. 2.7 ml of blood was transferred into two BTG sampling tubes and BTG assays carried out.

Flat sheets

A test cell with two sheets of polyvinyl chloride or silicone rubber mounted within it was attached to an oscillating rig, filled with 10 ml whole blood without anticoagulant and oscillated at 150 rev/min for six minutes. BTG assays were then carried out.

Measurement of BTG

Plasma BTG was measured as described in Chapter 5.32.

Measurement of FPA

Platelet aggregation and release of platelet contents can be induced by a number of agents including thrombin. Therefore, if BTG release is measured with any degree of certainty, some estimate of the levels of thrombin must be obtained. Therefore, FPA levels were measured in order to demonstrate that BTG release from platelets could be attributed to interaction of platelets with the polymer rather than the presence of thrombin.

9.3 Results

Normal values: Figure 9.4 shows the normal values of BTG in non-anticoagulated, citrated and heparinised blood. The mean values obtained were:

(1) non-anticoagulated blood, 36.9 ± 3.9 ng/ml (range 15-73)

(2) citrated blood, mean value, 69.0 ± 10.3 ng/ml (range 28-142)

(3) heparinised blood, mean value, 48.7 ± 8.2 ng/ml (range 26-76).

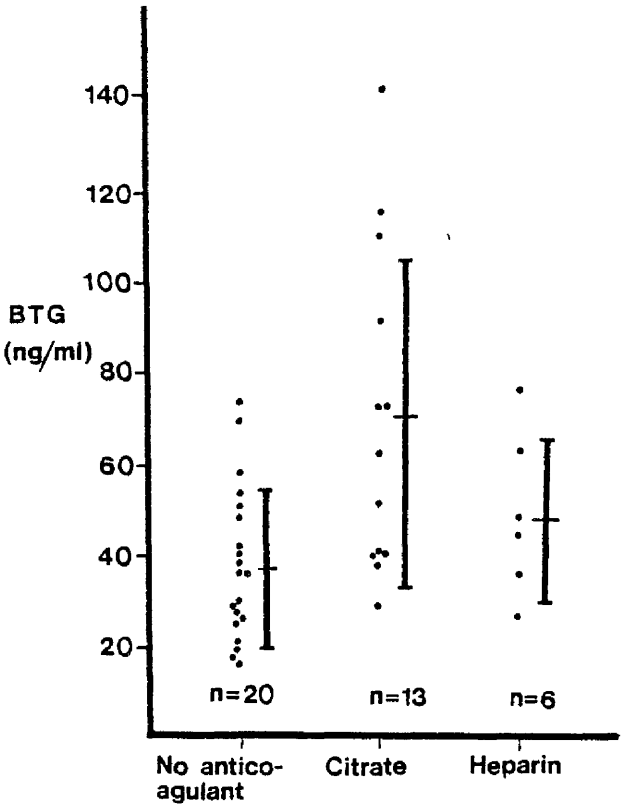


Figure 9.4

Concentrations of BTG in whole blood with and without anticoagulants. Individual values, and mean ± S.D.

BTG release from the syringe

As shown in Figure 9.5, in citrated blood there is a gradual increase in BTG levels till about eight minutes after which there is a steep rise in BTG levels. For comparison, the results of Ludlam and Cash (1976) who used no anticoagulant are also shown. They found a similar sharp increase in the levels of BTG after about nine minutes. With heparin, however, large rises were seen after 20 minutes. The results are the average of three experiments.

BTG:in vitro thrombus formation

Figure 9.6 shows the rapid increase of BTG levels, reaching the maximum measurable by the radioimmunoassay used, within 10-12 minutes in the three experiments that were carried out. The thrombus was visible in the tube about a minute before the maximum level of BTG is achieved.

BTG during oscillation of blood

The results obtained with the oscillation of blood are shown in Figure 9.7. No definite pattern of release was observed and there are different patterns of release in the three experiments.

Comparison of FPA level and BTG release

The release of FPA in a syringe with whole blood without anticoagulant is shown in Figure 9.8. Figure 9.9 shows the relationship between BTG release and FPA level in the same sample of blood. There is a gradual increase in the BTG level (as in the syringe, Figure 9.4) followed by a rapid increase. There is a corresponding increase in the FPA level after six minutes. Therefore, if BTG levels are measured for blood-polymer interactions, the blood-polymer contact time in the absence of an anticoagulant should be less than 6-8 minutes.

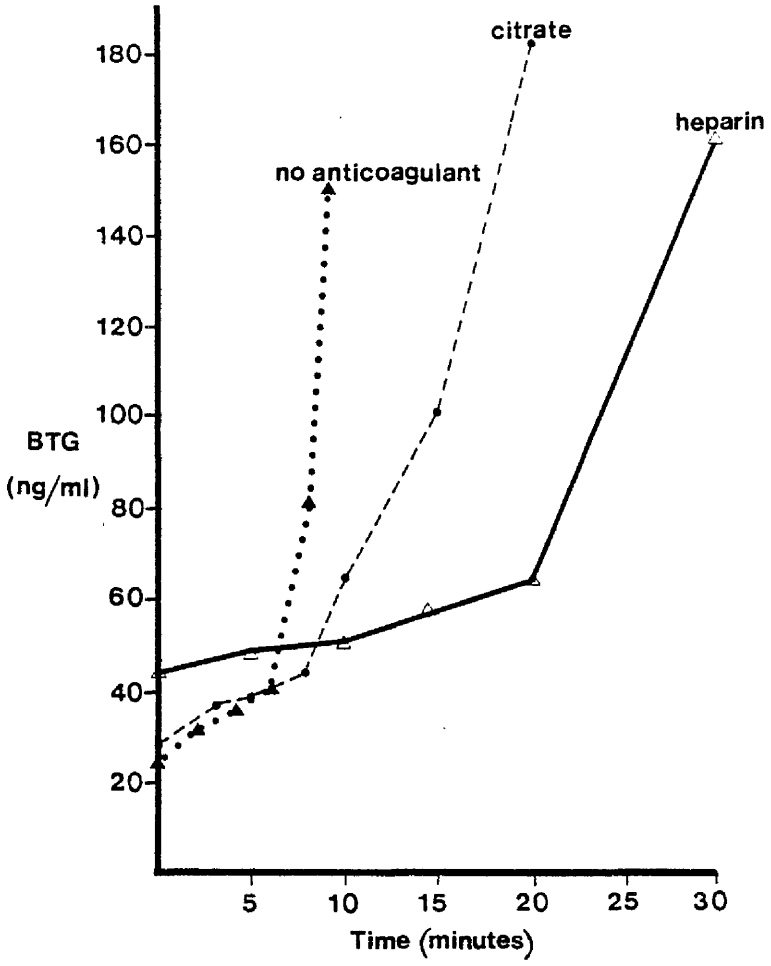


Figure 9.5

BTG release from blood in the syringe using citrate and heparin. Results are compared with BTG levels in whole blood without anticoagulant, obtained by Ludlam and Cash (1976).

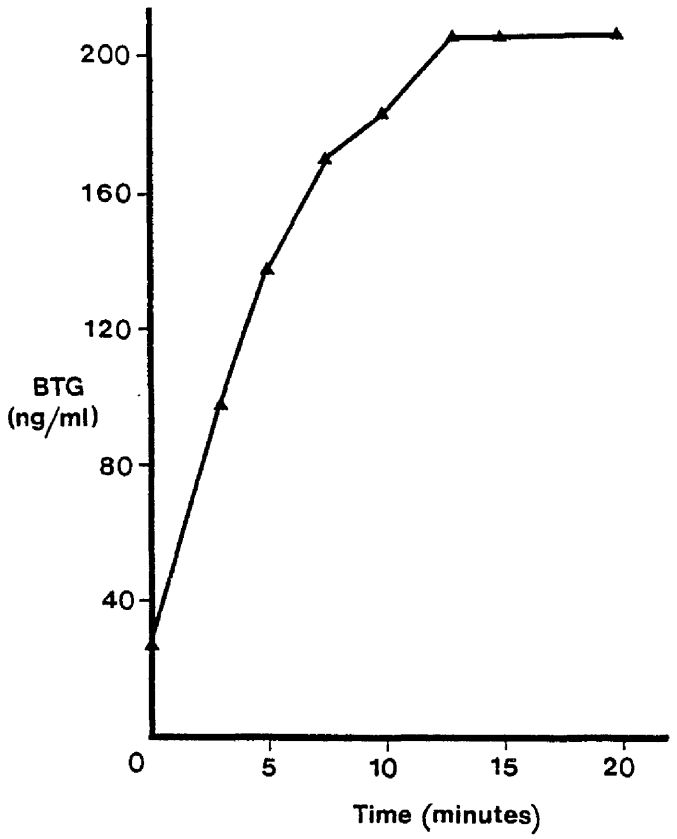


Figure 9.6

BTG release in in vitro thrombus formation;
a typical curve.

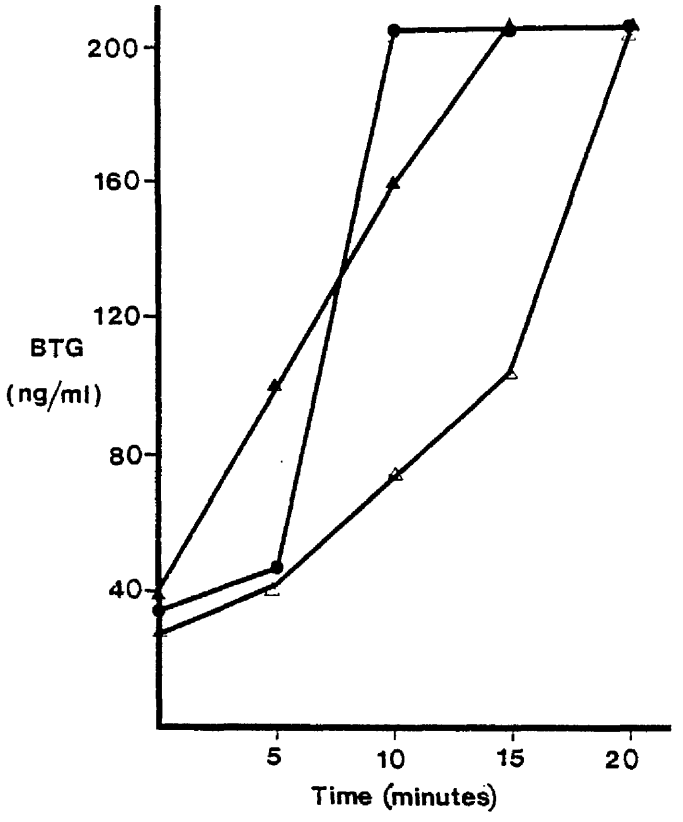


Figure 9.7

BTG release from citrated blood during oscillation; results of three test runs.

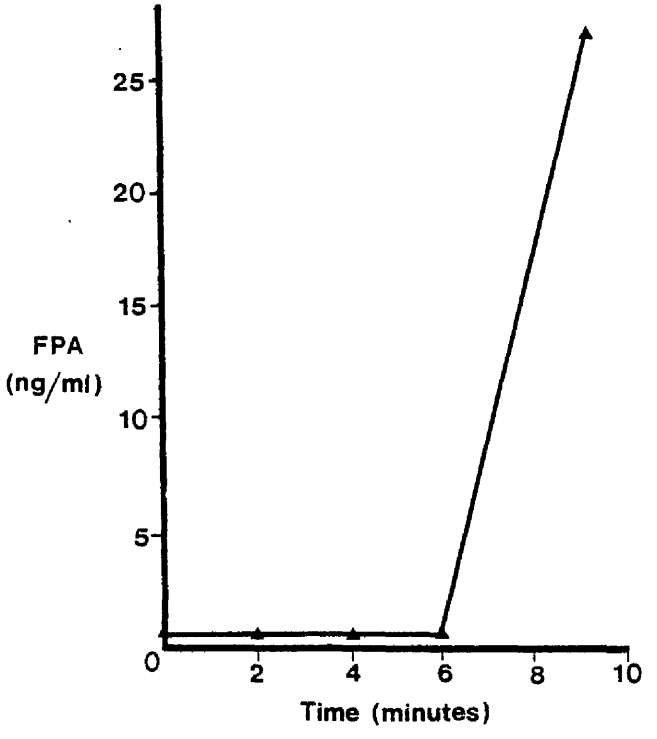


Figure 9.8

A typical curve of FPA levels in normal whole blood without anticoagulant.

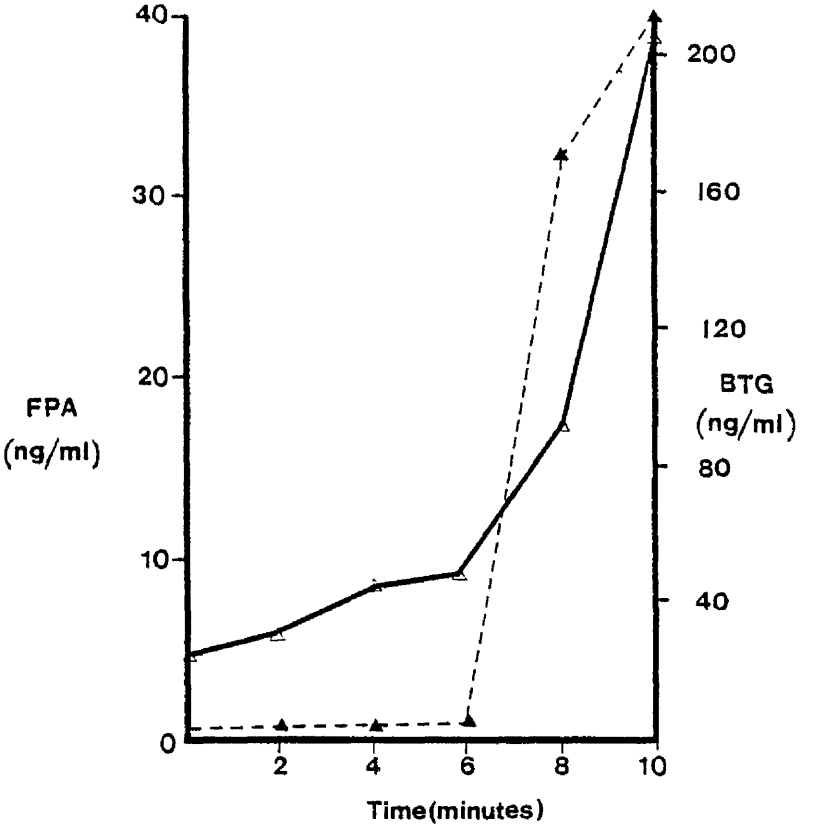


Figure 9.9

Relationship of BTG release (▵—▵) with FPA formation (▲···▲) in whole blood without anticoagulant, mixed in polypropylene tubes.

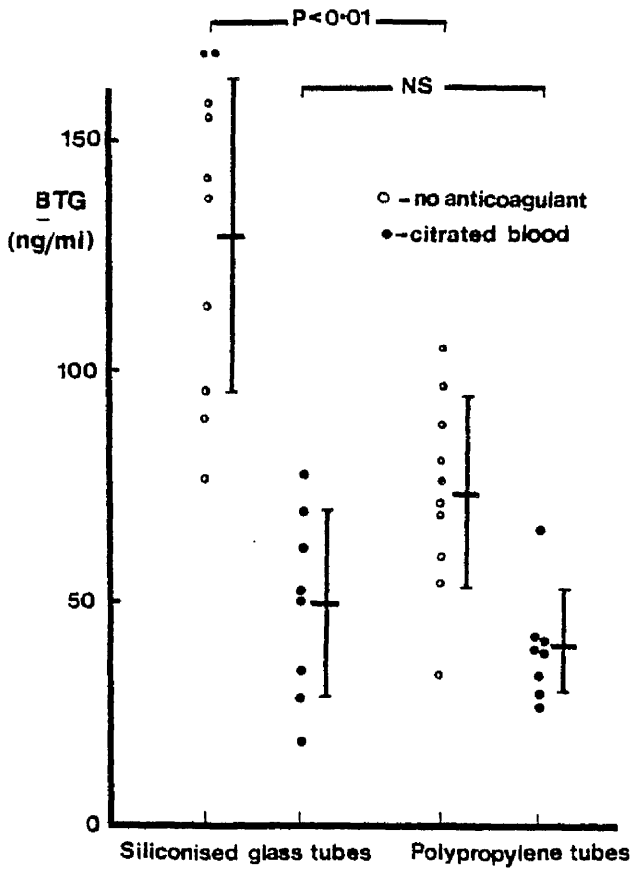


Figure 9.10

BTG release from whole blood in siliconised glass and polypropylene tubes.

Material assessment

Tubes

Siliconised glass tubes (Figure 9.10) caused greater release of BTG compared with the polypropylene tubes and, therefore, polypropylene initiates less thrombus formation than siliconised glass. However there was no significant difference between the tubes when the experiment was repeated with citrated blood.

Flat sheets

Polyvinyl chloride sheets gave mean BTG values of 120 ± 21 ng/ml over seven experiments. The mean BTG level for silicone rubber was 105 ± 18 ng/ml. The silicone rubbers appear, therefore to be less thrombogenic than polyvinyl chloride.

9.4 Discussion

Adler et al (1979) compared the effects of haemodialysis on plasma BTG levels with patients on either Cuprophan (Travenol) or regenerated cellulose (Cordis Dow) artificial kidneys. They found that there was an acute rise in the BTG levels in the first hour of dialysis which then remained constant throughout the remainder of the treatment when using the Travenol hollow fibre kidney; however, this effect was not observed with the Cordis Dow kidney, and the difference in the observations was related to the blood compatibility of the two types of hollow fibres. These experiments studied the overall performance of the device, and are not necessarily indicative of the blood compatibility of the materials of the kidneys as the effects of extracorporeal circulation, pumps etc contribute towards the measured BTG levels. By measuring BTG levels in patients with chronic renal failure on different

dialysers, Akizawa et al (1981) also concluded that BTG is an excellent marker for the blood compatibility of artificial kidneys. However, like the results of Adler et al (1980) this study did not differentiate between the blood compatibility of the material from the overall performance of the device and extracorporeal tubing.

The studies carried out in this chapter measured the potential use of BTG as a marker of blood compatibility. The acute rise in BTG from blood in the syringe is in agreement with the results of Adler et al (1980). The observation that oscillation of blood causes increased release of BTG, suggests that blood has to be mixed in different ways if BTG release is to be used as a marker for blood-material interactions. When the release of FPA was measured, abnormal values were obtained after 6-8 minutes indicating the presence of thrombin. Thus if BTG levels are to be measured for blood-material interctions in the absence of thrombin, the time blood-material contact occurs should be less than 6-8 minutes.

In vitro testing of different materials showed that polypropylene initiates less thrombus formation than siliconised glass. Silicone rubber was less thrombogenic than polyvinyl chloride.

9.5. Summary

This chapter has described the development of new haematological tests for blood compatibility assessment. BTG and FPA were regarded as fulfilling the necessary requirements of sensitivity and reproducibility. However, as the measurement of FPA required a large volume of blood, it was considered impracticable to include this test each time blood is exposed to materials. Siliconised glass tubes were more thrombogenic than polypropylene tubes. Polyvinyl chloride was more thrombogenic than silicone rubber.

In vitro blood compatibility assessment based on the release of BTG does provide relevant information on blood-polymer interaction. The studies in this chapter demonstrated significant differences in the release of BTG for different polymers and these findings suggest further assessment of other polymers is appropriate.

CHAPTER 10

CONCLUSIONS AND DISCUSSION

10.1. Introduction

In this thesis the role played by platelets, fibrin deposition and fibrinolysis in normal haemostasis has been discussed in particular detail (Chapter 1). Abnormalities of the above processes as possible mechanisms in thrombosis were consequently discussed in Chapter 2. To obtain a measurement of platelet activation, the radioimmunoassay of betathromboglobulin (BTG) was set up (as described in Chapter 3). The radioimmunoassays of fibrinopeptide A (FPA) and Bβ15-42 as indices of fibrin formation and plasmin activity were also set up (as described in Chapter 4). The methodology and measurement of these parameters and their assay in normal subjects was studied (Chapter 5).

Samples from normal pregnant women and pregnant women with hypertension of pregnancy or pre-eclampsia as well as the effect of an anti-hypertensive drug on patients with pre-eclampsia were studied (Chapter 6). Measurements of BTG, FPA and Bβ15-42 were studied in patients with deep venous thrombosis, chronic retinal vein occlusion, the nephrotic syndrome and compared to control subjects. A double blind trial of the administration of stanozolol, a fibrinolytic enhancing drug, was evaluated in the prevention of venous thrombosis after major gastrointesinal surgery. The administration of this drug to healthy volunteers was also studied. The effect of age, obesity and 'acute illness' on plasma BTG and platelet counts were also studied. These studies are described in Chapter 7.

Plasma levels of BTG, FPA and Bβ15-42 were also measured in clinical conditions associated with arterial thrombosis such as myocardial infarction and compared to patients with non-ischaemic chest pain and control patients. The extent of coronary artery disease on these measurements was also studied. BTG and FPA measurements were also studied in patients with type II and type IV hyperlipoproteinaemia, in patients with diabetes mellitus with or without proliferative retinopathy and compared to normal controls. Plasma BTG and FPA measurements were studied in patients with transient ischaemic attacks and compared to control groups. These patients were studied one year later. All these studies on arterial thrombosis are discussed in Chapter 8.

The measurement of BTG and FPA was evaluated in studies of different artificial surfaces and various parameters affecting the test conditions were evaluated (as described in Chapter 9).

10.2. An Assessment of Plasma BTG, FPA and Bβ15-42 as Markers for Thrombosis

The following questions have to be answered:

(1) What conclusions can be drawn from the various measurements obtained using the assays of plasma BTG, FPA and Bβ15-42? How do these measurements relate to each other?

(2) What practical problems are associated with these techniques and what modifications could be introduced to improve these techniques?

(3) Are these techniques likely to be useful as markers for thrombosis and the management of patients or do they solely have a role at the research level?

These questions will be discussed in turn beginning with a summary of results.

10.2.1. Summary of the results in this thesis

1. Normal non pregnant women (Group 5, Chapter 6.2)

Normal values of BTG, an index of platelet release and activity, and FPA, an index of fibrin formation, were observed in this group (see Figures 6.1. and 6.2). Hence there is no evidence of intravascular coagulation in this group.

2. Normal non pregnant women taking oral contraceptive therapy (Group 4, Chapter 6.2)

Normal values of BTG and FPA were obtained in this group (see Figures 6.1 and 6.2).

3. Normal pregnant women (Group 3, Chapter 6.2)

Increased BTG levels were observed in the 2nd and 3rd trimester of pregnancy suggesting increased platelet activation is occurring. However there is little evidence of fibrin deposition in this group as normal FPA levels were obtained (see Figures 6.1 and 6.2).

4. Essential hypertension in pregnancy (Group 2, Chapter 6.2)

Evidence of an increase in platelet activity as observed by an increase in BTG levels and fibrin formation, as suggested by increased FPA levels (see Figures 6.1 and 6.2) indicates intravascular coagulation is occurring in this group.

5. Intra uterine growth retardation (Group 3, 6.3)

Normal BTG levels were observed in this group (see Figure 6.3). An increase in fibrin formation as shown by an increase in FPA levels was observed (see Figure 6.4). This may reflect an increase in fibrin in the placental bed in I.U.G.R.

6. Mild to moderate pre-eclampsia (Group 1, Chapter 6.3)

No evidence of platelet release (see Figure 6.3) but an increase in fibrin formation as shown by an increase in FPA levels (see Figure 6.4) was observed in this group. Anti-platelet therapy, did not prevent this fibrin formation.

7. Severe pre-eclampsia (Group 1, Chapter 6.2)

Evidence of platelet release, an increase in BTG levels (see Figure 6.1) and an increase in fibrin formation, as observed by an increase in FPA levels (see Figure 6.2) suggests some degree of activated coagulation with fibrin deposition in the placental bed is occurring in these patients.

The measurements of both BTG and FPA were not able to distinguish the severe pre-eclamptic patients from the essential hypertension group.

8. Chronic retinal vein occlusion (Chapter 7.1)

An increase in plasma fibrinogen levels (see Figure 7.1) an increase in plasma BTG levels (see Figure 7.2) and an increase in FPA levels (see Figure 7.3) were observed in patients with capillary perfusion and/or neovascularisation. Measurements of fibrinogen, BTG and FPA did not distinguish between patients with and without RVO, or RVO patients with and without complications. It is possible that the observed activation of coagulation may be the result of activated coagulation in other vessels in the vasculature.

9. The nephrotic syndrome (Chapter 7.2)

Evidence of an increase in platelet release was observed in these patients when compared to control patients (see Figure 7.4). Unfortunately the FPA assay was not available when this study was carried out, thus evidence of fibrin formation is not available. Plasma BTG levels did not distinguish between either group.

10. Administration of stanozolol to healthy volunteers (Chapter 7.3)

An increase in plasma Bβ15-42 levels in normal volunteers after the administration of stanozolol, suggests direct evidence of in vivo plasmin activity is occurring for at least seven days after administration of the drug.

11. High risk DVT (Chapter 7.4)

Plasma BTG levels

An increase in BTG levels (see Figure 7.5) was observed one day post-operatively in each of the three sub groups of patients i.e. (a) those not receiving or those receiving stanozolol treatment, (b) those patients who did not and who did develop a post-operative DVT, (c) those patients with and without early operable malignancy. This observed increase in BTG is probably the result of tissue damage and trauma during surgery. However in patients who then developed a DVT this increase was significant suggesting that in these patients the surgical procedure triggered a greater platelet activation before the onset of thrombosis. However the increased values did not distinguish those with and without thrombosis.

Plasma FPA levels

An increase in FPA levels was observed post-operatively in all three sub groups. A significant rise in FPA levels was observed on post-operative day 7 in those patients who had developed a DVT, indicating an increased fibrin formation had occurred. However, these increased values did not distinguish those with and without DVT. A significant increase in FPA levels in patients with early operable cancer was observed pre-operatively suggesting ongoing fibrin formation is occurring in these patients. It is interesting to note that in each of the control groups of the three sub groups an increase in FPA levels was observed on the morning before surgery, presumably due to overnight stress.

Plasma Bβ15-42 levels

A rise in plasma Bβ15-42 levels, indicating an increase in plasmin activity, was observed in all three sub groups post-operatively. This would suggest that an increase in plasmin activity occurs after surgery despite previous reports of a fibrinolytic shut-down after surgery. A significant increase in plasma Bβ15-42 levels was observed on the seventh post-operative day in the group who received stanozolol, thus stanozolol has increased plasmin activity in vivo after surgery. A significant rise in Bβ15-42 levels was observed on the morning of surgery in those patients who then developed a DVT suggesting an increase in plasmin activity is already ongoing in the patients before surgery. While some of these patients did receive stanozolol this increase was also shown in patients who did not receive stanozolol. However post-operatively a greater increase, although not significant was observed between post-operative days 1-7 in patients who did not develop a DVT and those who did, suggesting a decrease in plasmin activity in patients developing a DVT. In patients with malignancy lower pre-operative values of Bβ15-42 were observed suggesting a reduced plasmin activity is present in these patients, however, post-operatively a significant increase in Bβ15-42 levels was observed in the seventh post-operative day. This may be the result of a steeper rise in FPA levels with resulting fibrin formation in this sub group on the first day post-operatively.

Overall the results obtained in this study suggest that BTG levels on the first day post-operatively are an index of developing DVT. As the prevalence of malignancy and DVT is similar in the two treatment groups, malignancy does not predispose towards thrombosis. Stanozolol despite increasing in vivo plasmin activity does not prevent a DVT. In early malignancy an increase in fibrin formation was observed.

12. Early malignancy and infection (Chapter 7.5)

As verified in the previous study no increase in BTG levels was observed in patients with early malignancy. An increase in plasma BTG levels was observed in patients with "non-vascular illness" chest infection when compared to control patients. This result suggests that elevated BTG levels are not specific to thrombosis and occur in non-thrombotic illnesses.

13. Acute chest pain (Chapter 8.2)

No increase in BTG levels was observed in patients with myocardial infarction, unstable angina and non-cardiac chest pain when compared to normal subjects (see Figure 8.1). Increased FPA levels, suggesting fibrin formation, were shown in patients with myocardial infarction, unstable angina and non-cardiac chest pain (see Figure 8.2). Hence raised FPA levels are not specific for acute coronary insufficiency. Neither are raised FPA levels specific for thrombosis. BTG and FPA levels are, therefore, of no value in separating patients with acute chest pain into those with and without myocardial infarction.

14. Coronary artery disease (Chapter 8.3)

Similar values of BTG, FPA and Bβ15-42 were found in patients with 1, 2 or 3 vessel disease of the coronary arteries. However some patients with 2 and 3 vessel disease had slightly higher BTG and FPA levels (see Figures 8.3 and 8.4), perhaps suggesting that slight activation of coagulation is occurring in these patients.

15. Type II and type IV hyperlipoproteinaemia (Chapter 8.4)

Plasma levels of BTG and FPA were not elevated in patients with type II and type IV hyperlipoproteinaemia. This result suggests that platelet release and fibrin formation are not implicated in the development of atherosclerosis.

16. Diabetes mellitus (Chapter 8.5)

No increase in BTG levels in diabetes mellitus was observed in patients with diabetes mellitus and retinopathy either proliferative or non-proliferative, suggesting no platelet activation is occurring in these patients. A significant increase in plasma FPA levels was found in diabetic patients with proliferative or non-proliferative retinopathy (see Figure 8.9). This suggests fibrin formation is ongoing in diabetes mellitus and appears to be higher in patients with proliferative retinopathy. This finding may reflect the severity of the disease and may be due to other abnormalities of this disease.

17. Transient ischaemic attacks (Chapter 8.6)

A significant increase in plasma BTG levels, ie platelet activation, was observed in patients with TIA when compared to elderly controls (see Figure 8.10). While an increase in FPA levels was observed in the TIA group, this was not significant when compared to elderly controls. Elevated plasma BTG levels were found to predict patients who developed further vascular events.

Artificial surfaces (Chapter 9)

Studies on different artificial surfaces showed that plasma BTG levels (see Figure 9.10) are an index of the thrombogenicity of these materials. Unfortunately FPA measurements were not applicable as the increases observed were not due to the material itself but to the thrombin release reaction (see Figure 9.9).

10.3. Problems Associated with the Radioimmunoassay Techniques

These problems have been discussed in previous chapters. The recommended protocols must be adhered to strictly. A perfect venepuncture must always be carried out. Preparation of plasma samples must be carried out immediately. This implies that the same person should be present or perform the venepunctures and this is not always practicable. With regard to the BTG assay, if these conditions are adhered to correct values should be obtained. One of the problems with the use of the Amersham radioimmunoassay kit is that the optimum value measured is around 210 ng/ml. While this would be adequate in most clinical samples, in assays carried out in the artificial surfaces studies, a greatly increased optimum value would be preferred.

The main problem associated with the FPA and Bβ15-42 assays is the removal of cross reacting fibrinogen from plasma samples before assay. While most of the studies in this thesis used the bentonite extraction method, this may not be adequate as the bentonite has a limited number of binding sites and a 75% extraction by ethanol has been recently recommended (personal communication H L Nossel). While this may suggest that some of the highly elevated clinical samples are erroneously high, a double bentonite extraction method was used and thus most assays should be the correct value. The use of the double antibody separation technique for the removal of bound antigen is preferred and should become standard practice.

10.4. Clinical Implications of the Measurement of Plasma BTG, FPA and Bβ15-42

While the assay of BTG can be performed within four hours, a time delay of 30 hours in obtaining the results of the FPA and Bβ15-42 assays limits their use in the diagnosis and management of thrombotic diseases.

The results in this thesis do suggest a role for plasma BTG in the development of venous thrombosis and possibly arterial thrombosis. An overall view of the results in this thesis suggest that it is not possible to use these assays as clinical markers for thrombosis. However, as a research tool they have a very important role as suggested at the beginning of this thesis. One of the main problems in developing tests for the detection of thrombosis is that the initial events in thrombosis are similar to those occurring in normal haemostasis and acute illnesses, as the results in this thesis have shown. Consequently patients with acute illnesses should be excluded from control groups. The results in this thesis also suggest the use of a "non-vascular illness" control group in all studies in order to be certain that abnormalities measured are specific to thrombosis.

With regard to the underlying mechanisms in thrombosis, the studies carried out in this thesis suggest that in venous thrombosis, platelet activation is occurring as an initial event, therefore BTG may be a marker of a pre-thrombotic state. Fibrin deposition occurs at a later event. Since no correlation was found in most studies between BTG and FPA, thrombin does not appear to be responsible for this platelet activation. It is interesting to note that in some

clinical conditions such as longstanding retinal vein occlusion, ongoing fibrin deposition and platelet release is still occurring months after the thrombotic episode occurs and may, therefore, indicate a continuous pre-thrombotic state in these patients. The results obtained in the DVT study support the earlier findings of McNicol and Douglas, 1976, that there is little evidence of a disturbance in the equilibrium between clotting and lysis in the development of DVT, however an examination of Figure 7.7 shows that in patients who developed a DVT after the first postoperative day the value of Bβ15-42 was not greater than the FPA value and perhaps some evidence does exist for the theory postulated by Nossel (1981) that it is the rate of proteolysis of the Bβ chain of fibrinogen which determines the development of thrombosis.

In arterial thrombosis, a greater increase in platelet activation would have been expected in clinical conditions associated with arterial thrombosis. The results obtained in this thesis do not support this hypothesis. The measurement of BTG was of no clinical value in the management of coronary artery disease. However in patients with cerebral arterial disease BTG measurements do have important implications in the management and detection of this disease. It is possible that differences observed in the BTG measurements obtained between the coronary artery and TIA study reflect differences in the different vascular compartments with obvious differences in blood flow and hence activation sites of thrombosis. Such differences may also apply to the diabetic retinopathy study, however in this study fibrin formation and not platelet activation appeared to be predominant.

The underlying mechanism in both arterial and venous thrombosis still appears unsolved and as suggested at the beginning of this thesis, the processes of platelet activation, clotting and fibrinolysis are closely interlinked in the thrombotic mechanism.

10.5. Final Comments and Aspects for Future Investigation

Thrombosis involves the complicated interactions of many factors. The complexity of these interactions suggests the need for new investigative tools in the search for an understanding of such a complicated event as thrombosis. Many of the studies in this thesis have validated previous research and other studies have shown new findings and consequently raised questions on the detection of thrombosis. Since the use of the B β 15-42 assay was only carried out in three of the studies reported, a reappraisal of the other studies with the inclusion of this assay is recommended to determine if a decrease in plasmin activity may be associated with thrombosis.

Recently new assays for FPB, des-Arg FPB, fragments D and E, have been available and hence their measurement is suggested in clinical conditions associated with thrombosis. With the development of new peptide fragments and epitopes of fibrinogen (see review by Plow and Edgington, 1982), other possible markers may soon be available. It may be that serial studies on the development of thrombosis will reveal more information of the DVT studies reported in this thesis. Daily blood samples might have revealed more information. The use of the BTG, FPA and B β15-42 assays may have potential in the development of in vitro and in vivo studies on the development of thrombosis especially in animal models.

The results in the artificial surfaces study have great potential with regard to artificial organ studies in animals and man, especially with the development of the artificial heart.

Sixty years ago Bordet wrote:

"Coagulation has been studied for years and years by many investigators, none of them can presume that the problem is yet solved, every one of them merely indulges in the hope of gathering more complementary data, a little more information".

I hope in this thesis I have gathered a little more information and complementary data.

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THROMBOSIS RESEARCH 25; 459-464, 1982
0049-3848/82/060459-06$03.00/0 Printed in the USA.

β-THROMBOGLOBULIN AND PLATELET COUNTS - EFFECT OF MALIGNANCY, INFECTION, AGE AND OBESITY

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(Received 32.11.1981; in revised form 11.1.1982.
Accepted by Editor D. Ogston)

ABSTRACT

Plasma levels of beta-thromboglobulin (BTG) and platelet count were studied in 69 control subjects, 19 patients with operable abdominal malignancy, and 9 patients with acute bacterial infection. In control subjects there was a significant rise in BTG and fall in platelet count with age, and a negative correlation of BTG with obesity. BTG and platelet count were normal in patients with operable malignancy, but significantly increased in patients with acute bacterial infection. These effects must be considered in studies of BTG and thrombosis.

INTRODUCTION

Increased levels of plasma β-thromboglobulin (BTG), a marker of platelet release, have been reported in vascular disorders such as deep vein thrombosis (1,2), pre-eclampsia (3), acute myocardial infarction (4-6), glomerulonephritis (7), diabetes mellitus (8) and in the nephrotic syndrome (9). Elevated levels of plasma BTG have also been reported in patients with malignant disease (10,11). In these studies many patients had disseminated malignancy. The aims of this study were (a) to establish whether or not BTG levels were elevated in early, operable cancer and (b) to establish whether or not BTG levels were elevat-

KEY WORDS: THROMBOGLOBULIN, PLATELET COUNTS, MALIGNANCY, INFECTION

ed in a group of patients with acute non-vascular illness, i.e., infection.

METHODS

We studied 4 groups of subjects. Group 1: comprised 40 healthy volunteers (mean age 27 ± 1.1 (S.E.M.) years; 22 females not taking oral contraceptives, 18 males). Group 2: 29 patient controls (mean age 61.2 ± 2.1 years, 11 males, 18 females) admitted to hospital for elective surgery for non-malignant disease, with no evidence of infection. Group 3: 19 patients (mean age 60.3 ± 2.2 years, 10 males, 9 females) admitted to hospital for elective surgery for proven operable gastrointestinal carcinoma. In all patients diagnosis was subsequently confirmed at operation - 10 patients had colorectal carcinoma, 7 had gastric adenocarcinoma and 2 had pancreatic carcinoma. Group 4: 9 patients (mean age 60 ± 3.5 years, 3 males, 6 females) admitted to hospital with acute bacterial infection but no malignant disease. Five had pneumonia and four had intra-abdominal sepsis (appendix abscess in one and cholecystitis in 3). Participants on drugs which may have had an influence on platelet function were excluded from this study. Obesity was measured as per cent mean weight for height, age and sex, compared to adults in the Build and Blood Pressure Study (12). Plasma β-thromboglobulin was measured by radioimmunoassay (13). 2.5 ml of venous blood was drawn without stasis through a 21G Abbott butterfly and immediately transferred into precooled polystyrene tube containing EDTA and theophylline. After 30 minutes in an ice-water slurry the blood was spun at 3,000 g at 4°C for 30 minutes and 0.5 ml of the middle layer of the platelet poor plasma was withdrawn for BTG determination. The concentration of BTG in platelet poor plasma was determined in duplicate by radioimmunoassay using a kit supplied by the Radiochemical Centre, Amersham. Whole Blood Platelet Counts were performed using a Coulter automatic platelet counter. Serum Creatinine Levels were performed by Technicon autoanalyser. Statistical Analysis: All data are presented as the mean ± standard error of the mean. The significance of differences between groups was assessed by Wilcoxon's rank sum test. Correlations were determined by the method of least squares.

RESULTS

Patient controls had significantly higher BTG levels ($p < 0.01$) and significantly lower platelet counts ($p < 0.01$) than normal controls. In the combined group of 69 control subjects there was a significant positive correlation between BTG and age ($r = 0.33$, $p < 0.01$) and a significant negative correlation between BTG and percent overweight for age, sex and height ($r = -0.47$, $p < 0.01$). This correlation with obesity was found in Group 2 ($r = -0.58$, $p < 0.001$), but not in Group 1 - possibly due to the relatively small scatter of obesity in this group of young healthy subjects.

A significant negative correlation was found between platelet count and age ($r = -0.55$, $p < 0.001$). There was no correlation between levels of BTG and sex or smoking habits.

TABLE 1.

Age, β-thromboglobulin, platelet count, serum creatinine and obesity (mean ± S.E.M.) in groups studied (*$p < 0.05$, **$p < 0.02$, ***$p < 0.01$ compared to Group 2, patient controls)

No.	GROUP 1 Normal Controls 40	GROUP 2 Patient Controls 29	GROUP 3 Malignancy 19	GROUP 4 Infection 9
Age (years)	27.0*** ± 1.1	61.2 ± 2.1	60.3 ± 2.2	60.0 ± 3.5
BTG (ng/ml)	26*** ± 2	43 ± 4	42 ± 5	68* ± 14
Platelet Count x 10^9/L	324*** ± 16	221 ± 16	227 ± 19	294** ± 19
Serum Creatinine (μmol/L)	79 ± 4	76 ± 3	76 ± 3	65 ± 10
Obesity (% mean weight)	92 ± 2	90 ± 3	95 ± 5	97 ± 6

Patients in group 3 (malignancy) and group 4 (infection) were of similar mean age to group 2 (patient controls) and had similar levels of serum creatinine and obesity. Patients with malignancy (group 3) had similar levels of BTG and platelet count compared to group 2. Patients with infection (group 4) had significantly higher levels of both BTG ($p < 0.05$) and platelet count ($p < 0.02$) compared to group 2.

DISCUSSION

Since all groups had similar mean serum creatinine levels differences in BTG levels cannot be ascribed to renal retention. We found the older patient controls (group 2) had higher levels of BTG than the younger normal controls (group 1) probably due to the significant rise in BTG with age which has been previously reported (14-16). We also found a decrease in platelet count with age, a finding supported by the results of a large epidemiological study (17). This may reflect a decrease in platelet survival with age (18). The increase in BTG levels with age may be a further reflection of platelet activation *in vivo*, possibly secondary to the increase in atherosclerosis with

age.

In control subjects BTG was unrelated to sex and smoking habit, but we found a highly significant negative association with obesity which has not been previously reported. The reasons for this are not known and this association requires further study.

Our finding of similar BTG concentrations in patients with early malignancy compared to control patients confirms the report of Farrell et al. (11) who only found significantly elevated BTG levels in the advanced stages of malignancy. It appears therefore that in the early stages of malignancy a small localised tumour does not cause measurable platelet release. In the advanced stages of malignant disease the demonstration of increased platelet release is presumably due to a greater vascular involvement.

There is increasing evidence that platelets are activated in acute"non-vascular" illnesses, such as infections,with associated "acute-phase" changes in plasma proteins such as elevated fibrinogen levels (19-20). Our finding of significantly increased BTG levels in patients with infection suggests that platelet activation occurs in acute "non-vascular" illness. It appears that, as with other tests of platelet behaviour, elevations of plasma BTG are not specific for "vascular" illnesses such as arterial occlusion or venous thrombosis.

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