COAGULATION DISTURBANCES AND FIBRONECTIN FRAGMENTATION IN PREECLAMPSIA

by

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This thesis is dedicated to Harry and my family

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ABSTRACT

COAGULATION DISTURBANCES AND FIBRONECTIN FRAGMENTATION IN PREECLAMPSIA

Preeclampsia or gestational proteinuric hypertension (GPH) is a major cause of maternal death worldwide. Patients may present with hypertension and proteinuria alone or may develop progressively more severe disease, ending in a fulminant syndrome with disturbed coagulation, vascular injury and end-organ damage. The reasons for this progression are unclear and can only be reversed by termination of pregnancy. There are currently no early, generally acceptable, laboratory markers of disease severity.

We have shown that patients with severe preeclampsia have significantly raised plasma levels of plasminogen activator-1, tissue plasminogen activator, fibronectin and fibrin(ogen) degradation product. The tissue plasminogen activator and fibrin(ogen) degradation product concentrations were found to progressively increase with progression of the syndrome. These findings support the hypothesis of the participation of disturbed coagulation and vascular injury processes in the aetiology of preeclampsia. The additional finding that the endothelium of umbilical cords from patients with preeclampsia was disrupted and characterised by increased deposits of fibronectin further supports of this proposal. Plasma fibronectin from patients with preeclampsia was found to be degraded, with fibronectin from plasma of patients with severe preeclampsia having larger amounts of low molecular weight fibronectin degradation products than the plasma from patients with mild peeclampsia. Comparison of the apparent molecular weights of these fibronectin degradation products, with those generated from pure fibronectin by plasmin, suggest that they may arise from the action of plasmin. These fibronectin degradation products were found to inhibit the opsonic activity of intact fibronectin and to cause an underestimation in the determination of plasma fibronectin concentration by enzyme-linked immunosorbent assay. Furthermore, plasma fibronectin degradation products and fibronectin degradation products, generated by plasmin cleavage of intact pure fibronectin, were able to disrupt endothelial monolayers in *in vitro* culture. This was not due to cytotoxic activity, but rather due to the generation of urokinase plasminogen activator from the endothelial cells,and subsequent urokinase plasminogen activator conversion of plasminogen to plasmin. The ability of a 29 kilodalton heparin-binding fragment to cause this disruption suggests that heparin-binding fragments may, in part, be responsible for the observed effect on endothelial cells in culture.

It is tenable from the results of this study that fibronectin degradation products, generated as a result of initial endothelial damage, perpetuates further damage to the endothelium. Escape of these products into the maternal circulation may be postulated to give rise to maternal endothelial cell damage and the generalised organ failure observed in preeclampsia.

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Table of Contents

INTRODUCTION

CHAPTER 1 - LITERATURE REVIEW
OVERVIEW OF THE PATHOLOGY OF PREECLAMPSIA **1**

1.1 INTRODUCTION 1

1.2 CLINICAL PRESENTATION 3

1.3 INCIDENCE 5

1.4 AETIOLOGY 5

1.5 PATHOLOGICAL AND PATHOPHYSIOLOGIC CHANGES IN PREECLAMPSIA 7

1.5.1 Placental changes 7

1.5.2 Renal changes 10

1.5.3 Cerebral Changes 11

1.5.4 Cardiovascular changes 11

1.5.5 Hepatic changes 12

CHAPTER 2 - LITERATURE REVIEW
VASCULAR ENDOTHELIAL DAMAGE IN PREECLAMPSIA. **13**

2.1 FUNCTION OF NORMAL ENDOTHELIUM 13

2.1.1 Maintenance of vascular tone 13

2.1.2 Vasoconstrictors secreted at the endothelial surface 16

2.1.3 Antithrombotic function of vascular endothelium 17

2.1.4 Role of vascular endothelium in fibrinolysis 17

2.2 ENDOTHELIAL DYSFUNCTION IN PREECLAMPSIA 18

2.2.1 Inflammatory mediators 18

2.2.2 Cytokine regulation of fibronectin (Fn), tissue factor (TF) and von Willebrand factor (vWF) expression by endothelial cells 19

2.2.3 Putative causative factors in endothelial injury 19

2.3.3 Mitogenic activity of endothelial-derived growth factors 22

2.3.4 Products of plasminolysis 24

2.4 EFFECTS OF ENDOTHELIAL INJURY 25

2.4.1 Evidence of vascular endothelial injury in preeclampsia 26

2.4.2 Biological responses to disruption of vascular integrity 27

2.5 CONCLUSION 35

CHAPTER 3 - LITERATURE REVIEW
HAEMOSTASIS IN PREECLAMPSIA **36**

3.1 NORMAL HAEMOSTASIS 36

3.2 FIBRINOGEN STRUCTURE 39

3.3 FIBRONECTIN (Fn) AND CROSSLINKING 41

3.4 FIBRINOLYSIS 43

3.5 BIOLOGICAL EFFECTS OF FDPs 49

3.6 HOMEOSTATIC CONTROL MECHANISMS 50

3.7 COAGULATION AND FIBRINOLYSIS IN NORMAL PREGNANCY 52

3.8 COAGULOPATHY IN PREECLAMPSIA 53

3.8.1 HELLP syndrome 54

3.8.2 Assessment of disseminated intravascular coagulopathy 54

3.8.3 The fibrinolytic system in preeclampsia 59

3.8.4 Treatment 60

3.9 CONCLUSIONS 61

CHAPTER 4 - LITERATURE REVIEW **62**
FIBRONECTIN - STRUCTURE AND FUNCTIONS **62**

4.1 INTRODUCTION 62

4.1.1 Gene transcription and molecular structure 63

4.1.2 Domain structure and functions 66

4.1.3 Fibronectin biosynthesis 71

4.1.4 Fibronectin receptors 73

4.2 PHYSIOLOGY OF FIBRONECTIN 77

4.2.1 Adhesive Functions 77

4.2.2 Immune-cell related functions 79

4.2.3 Mitogenic functions 81

4.2.4 Wound healing 81

4.3 EFFECTS OF FRAGMENTATION ON FIBRONECTIN FUNCTION 83

4.3.1 Chemotactic activity and immunosuppression 83

4.3.2 Phagocytic activity 84

4.3.3 Cell adhesion 86

4.3.4 DNA synthesis 86

4.3.5 Inhibition of endothelial cell growth 86

4.4 FIBRONECTIN IN PREGNANCY AND PREECLAMPSIA 87

4.4.1 Fibronectin distribution in the placenta 87

4.4.2 Plasma fibronectin levels in pregnant women 89

4.4.3 Neonatal plasma fibronectin levels 91

4.4.4 Amniotic fluid fibronectin 91

4.5 CONCLUSIONS REGARDING THE ROLE OF FIBRONECTIN IN PREECLAMPSIA 92

CHAPTER 5 - EXPERIMENTAL SECTION
FIBRONECTIN - BIOLOGICAL AND IMMUNOLOGICAL ROLE IN PREECLAMPSIA 93

5.1 HYPOTHESIS 93

5.2 AIMS OF THIS THESIS 94

5.3 STUDY DESIGN 95

CHAPTER 6 - EXPERIMENTAL SECTION
DEVELOPMENT OF ENZYME LINKED ABSORBENT ASSAYS (ELISA) FOR FIBRONECTIN AND FIBRIN(OGEN) DEGRADATION PRODUCTS 97

6.1 PURIFICATION OF FIBRONECTIN 97

6.1.1 Background 97

6.1.2 Methods and results 97

6.2 PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES TO FIBRONECTIN 101

6.2.1 Background 101

6.2.2 Methods and results 102

6.2.3 Results 103

6.3 POLYCLONAL ANTIFIBRONECTIN ANTIBODY (ANTISERUM) PRODUCTION AND CHARACTERISATION 107

6.3.1 Background 107

6.3.2 Methods and Results 108

6.4 DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF FIBRONECTIN 110

6.4.1 General principles of enzyme linked immunoassay (ELISA). 110

6.4.2 Method (After Ruoslahti et al, 1982) 112

6.4.3 Results 112

6.5 DISCUSSION 115

6.6 ENZYME-LINKED IMMUNOSORBENT ASSAY FOR FIBRIN- AND FIBRINOGEN-RELATED ANTIGENS (FRA/FDP) IN SERUM 117

6.6.1 Background 117

6.6.2 Methods 118

6.6.3 Results 118

6.6.4 Discussion 121

6.7 SUMMARY 121

CHAPTER 7 - EXPERIMENTAL SECTION
COAGULATION ASSOCIATED MARKERS IN PREECLAMPSIA 122

7.1 BACKGROUND 122

7.2 PATIENTS AND METHODS 123

7.2.1 General design of the study 123

7.2.2 Data Collection 123

7.2.3 Laboratory investigations 124

7.2.4 Statistical analysis 125

7.3 RESULTS 125

7.4 DISCUSSION 131

7.4.1 Fibrinogen degradation products (FDP) and tPA 131

7.4.2 Fibronectin (Fn) 132

7.4.3 Plasminogen activator inhibitor-1 (PAI-1) 132

7.5 CONCLUSION 133

CHAPTER 8 EXPERIMENTAL SECTION
FIBRONECTIN FRAGMENTATION IN SEVERE PREECLAMPSIA 134

8.1 BACKGROUND 134

8.2 PATIENTS AND METHODS 135

8.2.1 Patients 135

8.2.2 Laboratory Investigations 135

8.3 RESULTS 136

8.3.1 Integrity of fibronectin (Fn) by means of SDS-PAGE, Western blotting and Densitometric Scanning. 136

8.4 DISCUSSION 148

8.4.1 Fibronectin fragmentation 148

8.4.2 ELISA results 149

8.4.3 Opsonic Activity 150

8.5 CONCLUSION 151

CHAPTER 9 - EXPERIMENTAL SECTION 152
DEGRADATION OF FIBRONECTIN IN ASSOCIATION WITH VASCULAR ENDOTHELIAL DISRUPTION IN PREECLAMPSIA 152

9.1 INTRODUCTION 152

9.2 MATERIALS AND METHODS 154

9.2.1 Patient plasma studies 154

9.2.2 Umbilical cord studies 155

9.2.3 HUVEC studies 156

9.2.4 Data analysis 159

9.3 RESULTS 160

9.3.1 Fibronectin (Fn) and fibronectin degradation product (FnDP) detection by SDS-PAGE and Western blot 160

9.3.2 Umbilical cord studies 161

9.3.3 HUVEC studies 163

9.4 DISCUSSION 171

9.5 CONCLUSION 172

CHAPTER 10
CONCLUSIONS

APPENDIX I
ABBREVIATIONS

APPENDIX II
MATERIALS AND SUPPLIERS

REFERENCES

INTRODUCTION

Preeclampsia or Gestational Proteinuric Hypertension (GPH) is a major cause of maternal death worldwide (Romero *et al*, 1988). Patients may present with hypertension and proteinuria alone or may develop progressively more severe disease, ending in a fulminant syndrome with disturbed coagulation, vascular injury and end-organ damage (Roberts and Redman, 1993). The reasons for this progression are unclear and there are currently no early, generally acceptable, laboratory markers of severity.

Preeclampsia is a syndrome that affects virtually all maternal organs. The initial immunological mechanisms involved in the aetiology of preeclampsia are complex and not well defined, and seem to be related to poor placental perfusion occurring secondary to abnormal trophoblastic implantation (Rodgers *et al*, 1988b; Roberts and Redman, 1993). It is now accepted that widespread vascular endothelial damage, occurring as a result of an alteration of the haemostatic properties of the endothelium, is a major contributing factor to the pathogenesis of preeclampsia (Redman *et al*, 1978; de Boer *et al*, 1989; Weiner *et al*, 1982). The development of multi-organ vascular disease is believed to result from the ability of agents generated at the poorly perfused placenta to be transported to maternal organs through the maternal circulation. Morphological evidence of endothelial injury is provided by the observation of glomeruloendotheliosis (Spargo *et al*, 1976; Combs *et al*, 1993). Increased plasma levels of coagulation factors (de Boer *et al*, 1989; Taylor *et al*, 1990), and the cellular epitope of fibronectin (Taylor *et al*, 1991a), released from activated endothelial cells further supports endothelial damage in the preeclamptic syndrome.

Initiation of vascular endothelial cell damage occurs during the interaction of endothelial cells with blood-borne products, from the poorly perfused feto-placental unit and cytokines, released as a result of initial immune responses occurring during the development of the preeclamptic syndrome (Roberts and Redman, 1993). These interactions upregulate endothelial cell synthesis of coagulation factors (Stern *et al*, 1988), plasminogen activators (Loskutoff and Edgington, 1977) and plasminogen activator inhibitors (Colucci *et al*, 1983) to convert the normally anticoagulant endothelial surface to a procoagulant (Nawroth and

Stern, 1986; Bevilaqua *et al*, 1984; Colucci *et al*, 1983) and proadhesive (Springer, 1990; Stern *et al*, 1988; Scarpati and Sadler, 1989) surface. The acquirement of this procoagulant activity together with the induction of proadhesive properties on the endothelial surface appears to be related to the development of the disseminated intravascular coagulation and atherosclerosis observed in preeclampsia as well as deposition of fibrin and extracellular matrix proteins such as fibronectin.

This thesis involves the study of some of the markers of endothelial damage in an attempt to identify their role in the progression of preeclampsia to its fulminant form. In addition, it examines the possible role of fibronectin and its degradation products in endothelial damage. A review of preeclampsia, limited to those areas with which this thesis is concerned, is covered in chapter 1. Chapter 2 reviews the functions of normal endothelium and the alterations in these functions which result from vascular endothelial damage. Coagulation and lysis processes and the products generated during these processes are detailed in chapter 3. Chapter 4 includes a detailed description of the structure and function of fibronectin and its degradation products. Chapter 5 describes the hypothesis and the study design. Chapter 6 details the production of polyclonal and monoclonal antibodies against human fibronectin and the development of an enzyme linked immunoassay (ELISA) which was used to measure fibronectin. It also details the ELISA developed in our laboratory which was used to measure fibrin(ogen) degradation products. Chapter 7 describes the measurement of fibrin(ogen) degradation products, tissue plasminogen activator, and fibronectin in 21 patients with severe preeclampsia and 21 normotensive age and gestational age matched pregnant controls. The finding that plasma from patients with severe preeclampsia contains fibronectin degradation products is described in Chapter 8. This chapter also examines the effect of these fragments and fragments produced in vitro, by plasmic digestion of fibronectin, on the measurement of fibronectin concentration by ELISA and their effects on the opsonic activity of patient plasma fibronectin. Chapter 9 examines the effect of fibronectin degradation products from patients with mild and severe preeclampsia, and isolated fibronectin fragments, on the integrity of cultured human umbilical vein endothelial cells. Chapter 10 summarises the findings detailed in Chapters 7-9 and discusses the possible implications of these studies.

COAGULATION DISTURBANCES AND FIBRONECTIN FRAGMENTATION IN PREECLAMPSIA

SECTION I

LITERATURE REVIEW

CHAPTER 1 - LITERATURE REVIEW

OVERVIEW OF THE PATHOLOGY OF PREECLAMPSIA

1.1 INTRODUCTION

Hypertension is a complicating factor in many pregnancies, but is not a uniform condition. A subset of patients has an apparently distinct syndrome of acute hypertension with proteinuria occurring in the latter half of pregnancy which remits after delivery. This syndrome may progress in some patients to a fulminant state characterised by disturbed coagulation, vascular injury and multi-organ damage (Roberts *et al*, 1989). The disorder has been variably and sometimes interchangeably described as pregnancy induced hypertension, essential hypertension of pregnancy, preeclampsia, and more recently, gestational proteinuric hypertension (GPH). The pathogenesis of preeclampsia remains unclear and there are no definitive diagnostic laboratory markers of the disorder. Prediction of those patients who are likely to progress to a fulminant state appears to be largely dependent on the experience of the clinician.

The clinical nature of the classification systems in use reflects the lack of understanding of the underlying pathogenesis of preeclampsia. Although hypertension is an important feature in the diagnosis and management of preeclampsia, it appears to be more a consequence than a primary cause of the syndrome which is unique to human pregnancy. Absolute BP measurements provide the best guide to fetal and maternal prognosis and the development of proteinuria (Davey and MacGillivray, 1988). Where no previous record is available a BP of 140/90 is generally considered to be the cut off point for obstetric hypertension (Redman, 1983).

Incremental hypertension can also be used as a diagnostic criterion if a rise in systolic blood pressure (BP) of at least 30mmHg or in diastolic blood pressure of at least 15mmHg is measured. These changes must be manifest on at least two occasions 6h or more apart (Eastman *et al*, 1952). However, incremental measurements may confuse diagnosis as a rise of up to 30 or 40 mmHg may fall within the normal statistical range. Also the use of two observations increases the variability and may result in misdiagnosis if the blood pressure is abnormally low at any time in early pregnancy (Davey and MacGillivray, 1988).

There is also a variant of the disease called the HELLP (haemolysis, elevated liver enzyme activity, low platelets) syndrome which indicates the need for taking the whole pathophysiological picture into account for accurate diagnosis (Roberts and Redman, 1993).

The term "preeclampsia" was originally used to describe the syndrome which precedes eclampsia. Diagnosis of patients is difficult as the symptoms do not occur uniformly in all preeclamptic patients and some patients may be diagnosed only at or after delivery. Mild preeclampsia diagnosed clinically may erroneously include patients with latent renal disease or preexisting chronic hypertension, temporarily suppressed in midpregnancy because of vasodilation, and latent, essential hypertension (Chesley, 1978). The clinical diagnostic error for preeclampsia has been reported to be as high as 45% at the Chicago Lying-in Hospital, a reputable research centre (Chesley, 1985).

The clinical classification system of Davey and MacGillivray (1988) for the diagnosis of hypertensive disorders of pregnancy has been adopted for this thesis (Table 1.1). The terminology in this classification is specific for the diagnosis and distinguishes "true" preeclampsia, defined as gestational proteinuric hypertension (GPH)*, from gestational hypertension and gestational proteinuria as well as from other hypertensive diseases of pregnancy. All the preeclamptic patients studied for this thesis were diagnosed with proteinuria and hypertension during the latter half of pregnancy. GPH is defined as the occurrence of raised blood pressure (≥140/90 mmHg) with proteinuria (>0.3g per 24 hours). Fulminant disease is defined as acute, severe hypertension (a sustained blood pressure ≥160/110mmHg), proteinuria >0.3g per 24 hours and/or the occurrence of organ dysfunction (hepatic, neurological or renal) ascribed to GPH. For the purposes of this review the term "preeclampsia" is used to encompass studies using the slightly broader and more commonly used terminology, as defined in the NIH classification of hypertensive disorders of pregnancy (Roberts and Redman, 1993).

A **Gestational hypertension and/or proteinuria**

Hypertension and/or proteinuria developing during pregnancy, labour, or the puerperium in a previously normotensive nonproteinuric woman subdivided into:

1 Gestational hypertension (without proteinuria)
   a. Developing antepartum
   b. Developing intrapartum
   c. Developing postpartum
2 Gestational proteinuria (without hypertension)
   a. Developing antepartum
   b. Developing intrapartum
   c. Developing postpartum
3 Gestational proteinuric hypertension (preeclampsia)*
   a. Developing antepartum
   b. Developing intrapartum
   c. Developing postpartum

B **Chronic hypertension and chronic renal disease**

Hypertension and/or proteinuria in pregnancy in a woman with chronic hypertension or chronic renal disease diagnosed before, during, or after pregnancy subdivided into:

1 Chronic hypertension (without proteinuria)
2 Chronic renal disease (proteinuria with or without hypertension)
3 Chronic hypertension with superimposed preeclampsia (proteinuria developing during pregnancy in known chronic hypertension)

C **Unclassified hypertension and/or proteinuria**

Hypertension and/or proteinuria found either:

a. At first examination after 20 weeks (140 days) in a woman without known chronic hypertension or chronic renal disease or
b. during pregnancy, labour, or the puerperium where information is insufficient to permit classification and subdivided into
   1. Unclassified hypertension (without proteinuria)
   2. Unclassified proteinuria (without hypertension)
   3. Unclassified proteinuric hypertension

D **Eclampsia**

The occurrence of generalised convulsions during pregnancy, during labour, or within 7 days of delivery and not caused by epilepsy or other convulsive disorders.

Table 1.1 - Clinical classification of hypertensive disorders of pregnancy (Davey and MacGillivray, 1988)

1.2 CLINICAL PRESENTATION

The clinical presentation of preeclampsia includes both fetal and maternal syndromes. The fetal syndrome is classically that of intra-uterine growth retardation (IUGR) with intrauterine hypoxaemia, thought to be due to placental insufficiency. The maternal syndrome presents as a progressive disease which may culminate in severe, uncontrollable hypertension and organ dysfunction which appears to be due to poor perfusion and an associated coagulopathy (Table 1.2). In mild preeclampsia, the maternal condition is relatively stable due to compensatory mechanisms in the uteroplacental vasculature and is associated with hypertension and mild or no proteinuria. However, in severe preeclampsia, the damaged uteroplacental vasculature may not respond adequately to the pathologic processes taking place, with resulting imbalances in eicosanoid release. Although blood pressure rises, there is a decrease in uteroplacental blood flow, resulting in autoacceleration of maternal disease and worsening IUGR (Zeeman and Dekker, 1992).

Seizure activity is the diagnostic feature of eclampsia. Cerebral haemorrhage may occur as a complication of preeclampsia or eclampsia, and may be responsible for maternal death (Chesley, 1978).

With the GPH syndrome, management of pregnancy and timing of delivery are important for the health and viability of both the fetus and the mother. The only "cure" for preeclampsia is termination of pregnancy after which symptoms will regress. Treatment is merely supportive and is aimed at correcting the consequences of the disease such as hypertension, haemorrhage and pain. Thus, the need for accurate diagnosis and the ability to distinguish between patients with stable disease and those with progressive disease is vital.

Raised blood pressure	≥140/90mmHg (mild GPH) ≥160/110mmHg (severe GPH)
Proteinuria	≥0.3g per 24h
Oedema	Facial, digital or generalised
Rapid weight gain	>1.0 kg/wk
Placental changes	Placental ischaemia, infarction and insufficiency Placental abruption
Renal changes	Oliguria Hyperuricaemia Hypocalciuria Haemoglobinuria Haematuria Renal failure
Gastrointestinal symptoms and liver dysfunction	Nausea Vomiting Right upper quadrant pain Epigastric pain Mild jaundice Haematemesis
Visual changes	Retinal and posterior ciliary artery vasospasm Scotomata Blindness Blurred vision
Neurological symptoms	Dizziness, drowsiness Hyper-reflexia Headache and confusion Convulsions (eclampsia)

Table 1.2 - Clinical features of preeclampsia (based on Roberts, 1989a.)

1.3 INCIDENCE

Preeclampsia has an incidence of 7% (Roberts, 1989a), although Butler and Alberman, (1969) reported an incidence of 27.5% for "all grades of preeclampsia" in a study comprising 16981 singleton births. In 1972 a survey of 15 251 mothers delivered in the Cape Peninsula Maternity and Neonatal Service Region in South Africa, the region from which the patients

described in this thesis were drawn, showed the overall incidence of hypertension in pregnancy to be 33.8%. The incidence of antenatal hypertension was 15.7%, and that developing during labour was 18.1% (Knutzen and Davey, 1977). The highest incidence of preeclampsia is in primigravidae. However, as the disease recurs in up to 20% of subsequent pregnancies, it remains common in multiparae (Davey, 1988). In New Zealand, where eclampsia was previously a notifiable disease, the incidence dropped from 0.32% of all births in 1928-33 to 0.06% in 1959-61, while the case fatality rate fell from 18.9% to 0.8% over the same period (Medical Research Council of New Zealand, 1962).

The World Health Organisation (WHO) reports half a million maternal deaths annually from three major causes, namely, hypertensive disorders, sepsis and haemorrhage. Hypertensive disorders of pregnancy are presently the leading cause of maternal death in England and Wales (Loudon, 1991). Maternal deaths due to eclampsia are 100-200 times higher in developing countries than in Europe and North America and there is no indication that the risk of maternal death from hypertensive disorders of pregnancy is decreasing in Africa or Latin America (Duley, 1992).

1.4 AETIOLOGY

Although symptoms of disease only occur in the latter half of pregnancy, preeclampsia is thought to be initiated during placentation. Placentation is accomplished through invasion of uterine tissue by trophoblasts which infiltrate and disrupt the muscular wall of the decidual spiral arteries, converting them from potentially high resistance vessels into distended sinusoidal tubes that are insensitive to vasoconstrictive influences and able to maintain an adequate blood flow to the fetoplacental unit (King and Loke, 1991). The factors responsible for disturbed placentation have not yet been identified.

Certain factors appear to be associated with a higher risk of preeclampsia, but none of them is specifically diagnostic for the disease. These include genetic factors (Chesley and Cooper, 1986) and nulliparity (Chesley, 1978); conditions associated with a large placenta, namely, hydatiform mole (Page, 1939), fetal hydrops (Rote, 1985) and twin pregnancies (Roberts and Redman, 1993); diseases associated with pre-existing microvascular disease such as diabetes, collagen vascular disease and pre-existing hypertension, (Roberts and Redman, 1993), extremes of child-bearing age and malnourishment (Chesley, 1978).

Immunological factors may play an important role in the development of preeclampsia (Zeeman and Dekker, 1992). Immune mechanisms associated with the pathogenesis include immune complex disease or deposition (Redman, 1986; 1990; Rosenberg *et al*, 1971; Petruccho *et al*, 1974; Kitzmiller and Benirschke, 1973; Starzl *et al*, 1970) and non-adaptive immunity (King and Loke, 1991; Redman, 1990;1991;1992). Blood cells have been shown to be unduly activated in preeclampsia as shown by studies on markers of cell-mediated immunity such as raised concentrations of intracellular calcium ions in peripheral T lymphocytes (August *et al*, 1990); increased sensitivity to thromboplastin synthesis in monocytes from preeclamptic patients (Oian *et al*, 1983); raised concentrations of circulating tumour necrosis factor (TNF), TNF receptors and interleukin-6 (IL-6) (Vince *et al*, 1995) which are thought to be important in the pathogenesis of preeclampsia as disorders of spiral artery invasion and placental development may be associated with increased TNF which has been shown to inhibit trophoblast growth (Wegmann *et al*, 1993; Starke, 1993; Hunt, 1989; Heyborne *et al*, 1992); neutrophil activation indicated by raised plasma neutrophil elastase levels in preeclampsia (Butterworth *et al*, 1991) which may contribute to tissue and vascular damage (Greer *et al*, 1991) as elastase inactivates tissue factor pathway inhibitors. Neutrophils have been located both in fetal vessels as well as in areas of fibrin deposition in the placental bed. Other factors associated with the aetiology of preeclampsia, as mentioned above, are reviewed by Chesley (1978) and Davey (1985).

1.5 PATHOLOGICAL AND PATHOPHYSIOLOGIC CHANGES IN PREECLAMPSIA

1.5.1 Placental changes

(a) Spiral artery changes

The major blood supply of the decidua (or endometrium) is via the spiral arteries which penetrate from the myometrium (Figure 1.1). In normal pregnancy the spiral arteries increase greatly in diameter. The physiologic changes extend from the decidual course of these arteries into the myometrium and may even involve the terminal segments of the radial arteries (Brosens *et al*, 1967). Morphologically, the endothelium is replaced by trophoblasts, and the internal elastic lamina and smooth muscle of the media are replaced by both trophoblasts and an amorphous matrix containing extracellular matrix proteins including fibronectin (Fn) and fibrin (Brosens

et al, 1967). These vascular changes are effected in two stages. In the first trimester there is a conversion of the decidual segments of the spiral arteries by a wave of endovascular trophoblast migration. Conversion of the myometrial segments through a subsequent wave of trophoblast migration occurs in the second trimester at 16 to 20 weeks gestation (Khong *et al*, 1986). These changes result in increased uteroplacental blood flow and vascular perfusion of the placental site (Roberts *et al*, 1989).

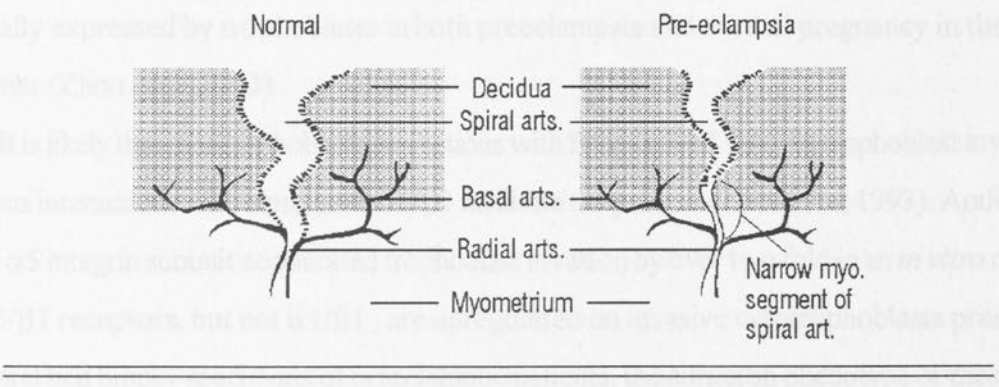


Figure 1.1 - Spiral arteries in normal and preeclamptic placentae.
The physiological changes in the spiral arteries do not extend into the myometrium in preeclampsia.

In preeclampsia changes may occur only in the decidual segments of the spiral arteries, while the myometrial segments retain the non-pregnant component of intima and smooth muscle (Figure 1.1). Since the diameter of these arteries is about 40% of the diameter of spiral arteries in normal pregnancy (Khong *et al*, 1986), the blood supply to the fetoplacental unit is impaired, resulting in placental ischaemia (Brosens *et al*, 1972). These changes may occur before clinical disease is noted (Redman, 1991).

In some preeclamptic and small-for-gestational-age (SGA) pregnancies complete absence of physiological changes throughout the entire length of some spiral arteries is seen (Khong *et al*, 1986). Up to 50% of all decidual segments may fail to undergo the physiological changes of pregnancy, but the myometrial segments of the spiral arteries are reportedly unchanged in all preeclamptic pregnancies. (Khong *et al*, 1986). Similar changes occur in some cases of pregnancy with chronic hypertension, first trimester abortion in primiparous women and with IUGR (Pijnenborg, 1991; Robert *et al*, 1989; Redman, 1991).

Fetal trophoblastic cells are present in the maternal circulation as early as the 18th week of gestation in healthy pregnant women (Redman, 1991a) and as early as the first trimester in

preeclampsia, and may still be present in the placental bed spiral arteries in the third trimester in preeclampsia and SGA pregnancies, a feature not seen beyond the second trimester in normal pregnancy (Khong *et al*, 1986; Pijnenborg *et al*, 1991).

Trophoblast invasion of the spiral arteries is thought to be modulated by the regulation of integrin receptor expression in the vascular tissue of the decidua and myometrium (Zhou *et al*, 1993). Abnormal regulation of expression of integrin receptors, specifically by cytotrophoblasts, is thought to occur in preeclampsia. Matrix proteins (fibronectin, laminin, collagen) are, however, normally expressed by trophoblasts in both preeclampsia and normal pregnancy in the third trimester (Zhou *et al*, 1993).

It is likely that cytotrophoblast interactions with Fn via $\alpha 5/\beta 1$ restrain trophoblast invasion, whereas interactions with laminin via $\alpha 1/\beta 1$ facilitate the process (Zhou *et al*, 1993). Antibodies to the $\alpha 5$ integrin subunit accelerated trophoblast invasion by over two fold in an *in vitro* model. As $\alpha 5/\beta 1$ receptors, but not $\alpha 1/\beta 1$, are upregulated on invasive cytotrophoblasts present in placental bed biopsy specimens of preeclamptic patients, the adhesion phenotype of these cells may not be optimal for invasion. This suggests that cytotrophoblasts fail to fully differentiate in preeclampsia. The differentiation process, though not yet well understood, may be under the control of cytokines. TNF-α has been suggested to play a role in limiting trophoblast invasion (Hunt *et al*, 1989).

b) Trophoblast migration

Trophoblast migration is known to occur in normal pregnancy (Pijnenborg *et al*, 1983). Increased trophoblast migration into uterine vein blood and the maternal circulation in women with eclampsia was first reported by Schmorl (1893) but also occurs in preeclampsia (Chua *et al*, 1991). After delivery trophoblasts may persist in the lungs and in the walls of the placental bed (Attwood and Park, 1961). There is no obvious correlation between gestational age or severity of preeclampsia and trophoblast concentration, although an acute maternal or fetal event, necessitating emergency delivery such as eclamptic convulsions, is associated with increased migration (Chua *et al*, 1991).

c) Acute atherosis

Atherosis is the development of aggregates of fibrin, platelets and lipid-laden macrophages which may partially or completely block the spiral arteries (Robertson *et al*, 1967; Pijnenborg *et al*, 1991). This pathology is related to endothelial cell injury as well as damage to underlying

muscle tissue (Robertson and Khong, 1987). Acute atherosis and associated thromboses cause placental infarction (Wallenburg, 1973) and are directly related to the severity of preeclampsia (Robertson *et al*, 1975). The features of atherosis are similar to those seen with allograft rejection (Kitzmiller *et al*, 1973).

d) Ischaemia

Abnormalities in the flow velocity wave forms of the uterine arcuate arteries have been detected by ultrasound Doppler techniques before preeclampsia is overt (18-24 weeks) (Campbell *et al*, 1986), but this is not present in all patients (Jacobson *et al*, 1990). It is supposed that these circulatory changes cause placental ischaemia, which in turn results in decidual haemorrhage (Brosens, 1964) and maternal systemic disease (Page, 1972). Hypoxia is thought to induce placental lesions such as the syncytial sprouts and knots (Tominga and Page, 1966) which are prominent in preeclampsia. Placental "toxins" associated with ischaemia are also suggested as causes of preeclampsia (Rodgers *et al*, 1988). A preeclampsia-like illness can be induced in animals by surgical restriction of the uteroplacental blood supply with resultant placental ischaemia (Horvath *et al*, 1990). Once preeclampsia is established uteroplacental and intervillous blood flow is reduced, intervillous blood becomes desaturated and there are functional changes ascribable to poor perfusion.

Other secondary placental abnormalities include infarcts, retroplacental haemorrhage, proliferation of the cytotrophoblast, and thickening of the trophoblastic basement membrane with fine fibrillar inclusions (Jones and Fox, 1980). There are areas where cytotrophoblast, which is resistant to hypoxia *in vitro*, is seen to degenerate in areas adjacent to normal syncytiotrophoblast, which is considered very susceptible to hypoxia. This suggests that hypoxia alone is not sufficient to cause the pathological changes. Either a factor, other than oxygen, that is required more by cytotrophoblast than by syncytiotrophoblast is absent, or factors specifically toxic to cytotrophoblast are present (Roberts *et al*, 1989).

In summary, abnormal trophoblast invasion appears to be pivotal to the development of preeclampsia, although symptoms of the syndrome only arise in the latter half of pregnancy.

1.5.2 Renal changes

Glomerular, tubular and arteriolar changes have been described as a consequence of preeclampsia. The endothelial cells lining the glomerular capillaries are greatly increased in size with electron-dense cytoplasmic inclusions and occlusions of the capillary lumen. This lesion,

termed capillary glomerular endotheliosis, is a characteristic feature of preeclampsia (Sheehan and Lynch, 1973). Thickened basement membrane, IgG, IgM, complement and fibrin deposition have been demonstrated in the glomeruli by Petruccho *et al* (1974). As a result of these changes, decreased glomerular filtration rate and proteinuria are common findings. (Roberts *et al*, 1989). Renal biopsy examination is considered to be the most reliable diagnostic test by some (Chesley, 1978), although similar changes have been seen in women with placental abruption with no signs of preeclampsia (Roberts *et al*, 1989). Lesions are reversible after delivery.

Characteristic changes in preeclampsia are present in 70% of primiparae, but only in 14% of multiparae. The glomerular changes described correlate more consistently with proteinuria than with hypertension, suggesting that proteins might simply be trapped in the glomerulus. These changes, which are not seen in other hypertensive conditions, suggest that preeclampsia is a unique disease of pregnancy associated with an endothelial pathology (Roberts and Redman, 1993).

Changes in sodium excretion

Excessive sodium retention has long been considered part of the pathophysiology of preeclampsia (Roberts *et al*, 1989). Eclamptic and severely preeclamptic women have low concentrations of chloride and sodium in their urine. After delivery chloride excretion increases dramatically. Angiotensin II sensitivity precedes obvious fluid retention by months. Oedema of hands and face is believed to be related to sodium retention, and may be massive in preeclampsia rendering the patient unrecognisable (Roberts *et al*, 1989).

Renal function changes in preeclampsia do not consistently antedate other sensitive indicators of preeclampsia such as changes in coagulation and plasma volume and are thus unlikely to be causal abnormalities.

1.5.3 Cerebral Changes

Normal cerebral blood flow, but depressed oxygen consumption, has been reported in preeclampsia (McCall, 1953). The maintenance of normal blood flow in the presence of hypertension indicates increased cerebral vascular resistance due to vasoconstriction (Chesley, 1978). Vasoconstriction of retinal vessels in preeclampsia may represent a global reduction in cerebral blood flow (Belfort, 1992). Cerebral oedema may occur in some patients. Convulsions are common in fulminant disease. Petechial haemorrhages are the most frequent lesions seen in the brain (Roberts and Redman, 1993) and may result in strokes which are the most common cause of death in eclampsia.

1.5.4 Cardiovascular changes

Blood pressure is directly related to cardiac output and total peripheral resistance (TPR) (Roberts *et al*, 1989). Cardiac output is increased by up to 50% in normal pregnancy, yet blood pressure (BP) does not usually increase. BP is lower during the first half of pregnancy than in the postpartum period, when cardiac output subsides toward non-pregnant levels. Thus, during normal pregnancy TPR decreases due possibly to increased vasodilator substances in the circulation or the establishment of new vascular beds (MacGillivray, 1989). Cardiac output in untreated preeclamptics may be similar to that in normal pregnant women or reduced (Wallenburg, 1988; Visser and Wallenburg, 1991). There have however been reports of increased cardiac output (Mabie *et al*, 1989; Easterling *et al*, 1990; Benedetti *et al*, 1980). Treatment such as fluid administration or use of vasodilators prior to measurement may change the cardiac output and account for some of the variability in the reports. The elevation in TPR may account for the increase in blood pressure in preeclamptics (Roberts *et al*, 1989).

There is evidence for arteriolar narrowing or vasoconstriction in preeclampsia (Gant *et al*, 1973). The increased TPR is unlikely to be determined by the autonomic nervous system (Assali and Prystowski, 1950), and must therefore be influenced by humoral factors (Roberts, 1989a). The most striking difference is seen in the increased sensitivity of the preeclamptic women to the pressor agent angiotensin II compared to normotensive pregnant and non-pregnant women (Talledo *et al*, 1968). Angiotensin II sensitivity increases as early as 14 weeks gestational age before the development of elevated blood pressure (Gant *et al*, 1973). Vasoconstriction and increased systemic vascular resistance may occur subsequent to this increased sensitivity.

The decreased sensitivity of normal pregnant women to angiotensin II and the lower total peripheral resistance in normal pregnancy suggest that arteriolar narrowing in preeclamptic women may be the result of a decrease in vasodilator substances such as prostacyclin (PGI_2) and NO, either circulating or local, rather than of increased circulating pressors. There is increasing evidence of a prostacyclin:thromboxane A_2 imbalance (Uotila *et al*, 1993).

Subendocardial haemorrhages, usually on the left side of the interventricular septum, are present in over one-half of women who die of preeclampsia. They are identical to haemorrhages found in patients dying of hypovolemic shock (Roberts, 1989). Focal degenerative changes often occur in the myocardium (Sheehan and Lynch, 1973).

1.5.5 Hepatic changes

Characteristic histopathological findings in preeclampsia and eclampsia include sinusoidal occlusions with fibrin clots and proximal haemorrhages into the space of Disse. This is followed by liver cell injury, degeneration and finally, necrosis. Fibrin deposition can also occur in portal venous vessels, and may sometimes occur as hepatic arterial thrombi or mural deposits (Alexander *et al*, 1987). Gross lesions are visible in a high percentage of women dying of eclampsia and a third of those without macroscopic lesions have histologic abnormalities. Initially, changes are consistent with haemorrhage into hepatic cellular columns. The haemorrhage is usually replaced by fibrin. This lesion may be due to sudden hypertension in the sinusoids, resulting from acute arteriolar dilatation. The second lesion is infarction, possibly due to intense vasospasm, which may be associated with focal or diffuse necrosis. Infarction and necrotic changes were shown post mortem in livers from 40% of the eclamptics and from 20% of the preeclamptics in a study by Sheehan and Lynch (1973).

CHAPTER 2 - LITERATURE REVIEW

VASCULAR ENDOTHELIAL DAMAGE IN PREECLAMPSIA.

Although not the initial event in the pathogenesis of preeclampsia, vascular endothelial damage appears to be an early event in its progression, and is associated with many of the clinical symptoms and biochemical markers of the disease. Background studies on endothelial function and causes of endothelial damage and dysfunction are therefore reviewed in this chapter.

2.1 FUNCTION OF NORMAL ENDOTHELIUM

Endothelium forms a monolayer at the interface between blood and tissues and is attached to the blood vessel wall by the extracellular matrix. It facilitates transport by maintaining the patency and tone of the vasculature by regulating smooth muscle contraction and dilation. It has a role in the permeability of the vascular tree to molecules and cells carried by the blood, and a regulatory role in relation to blood clotting and fibrinolysis (Nawroth and Stern, 1986). Endothelial tissue also has a physiological role in disease states, haemostasis, immune responses and inflammation (Scarpati and Sadler, 1989).

Products synthesised and secreted by endothelial cells include matrix proteins such as collagen, elastin, laminin, vitronectin and fibronectin (Fn). Vasoactive peptides (MacGillivray,1983) such as angiotensin and bradykinin are produced as regulators of permeability and migration. Plasminogen activators and inhibitors, factor VIII:von Willebrand factor (vWF), tissue factor and factor Va are synthesised by the endothelium as regulators of haemostasis (Jaffe, 1984; Nemerson, 1988). The outer layer of the endothelial cell is comprised of a number of glycosaminoglycans (GAGs) forming a glycocalyx which contributes to the anticoagulant function of the endothelium by combining with other endothelial cell products such as prostacyclin, antithrombin, and ADPases to establish a platelet repellant vascular layer (Nawroth *et al*, 1984; Stern *et al*, 1988).

2.1.1 Maintenance of vascular tone

Normal endothelial cells modify contractile responses of subjacent vascular smooth muscle by secreting factors which control smooth muscle cell responses. These include:

a) Endothelial derived relaxing factors

i) Endothelial derived relaxing factor (EDRF) or nitric oxide (NO), found to be identical to

EDRF by Ignarro *et al* (1987) and Palmer *et al* (1987), is a small gaseous molecule with one unpaired electron making it a free radical. NO is synthesised from L-arginine by nitric oxide synthase, an enzyme produced constitutively by endothelial cells (Moncada *et al*, 1991). NO diffuses into the vascular smooth muscle cells and platelets where it stimulates guanylate cyclase to increase the synthesis of cyclic guanosine monophosphate (cGMP) which causes vasodilation and inhibition of platelet aggregation (Durante *et al*, 1992). The fetal-placental circulation can generate NO intracellularly. There appears to be basal release of NO which helps to maintain the generalised vasodilation in these vessels (Myatt *et al*, 1991).

ii) Prostacyclin (prostaglandin I_2 - PGI_2), a derivative of cyclo-oxygenase conversion of arachidonic acid (Figure 2.1), is synthesised in response to mechanical or chemical perturbation (Levin *et al*, 1984) and is a potent vasodilator and inhibitor of platelet aggregation and adherence to the healthy vessel wall (Moncada and Vane, 1979). Endogenous stimulatory substances include bradykinin, thrombin, and platelet derived factors such as thromboxane A_2 (TxA_2), serotonin (5HT), ADP, IL-1 and platelet derived growth factor (PDGF). Endothelial cells and platelets express receptors for these substances.

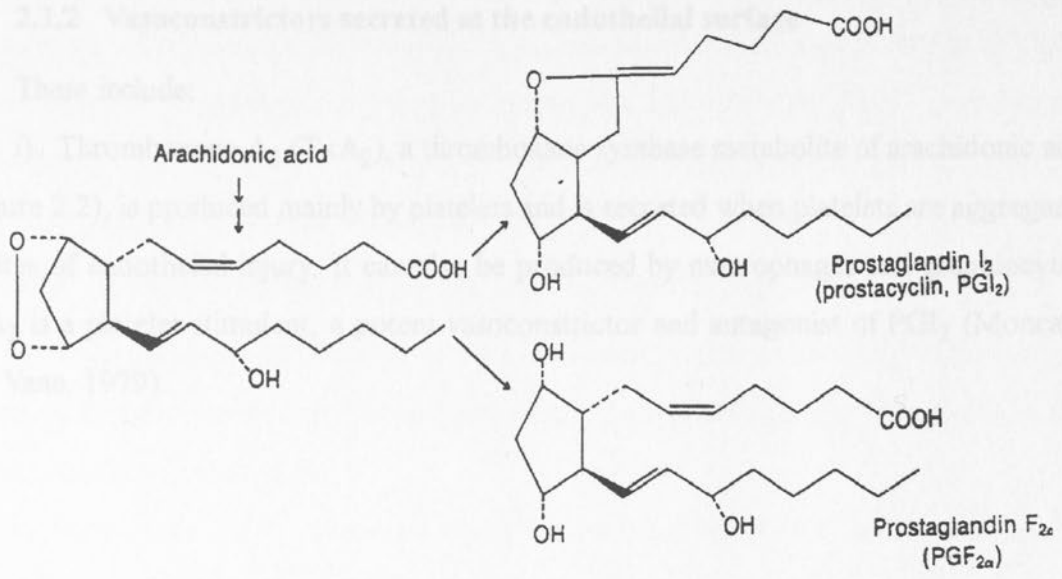


Figure 2.1 - Pathways of prostaglandin synthesis.
(Redrawn from Glew *et al*, 1992)

Increased intracellular calcium mediates the release of prostacyclin and NO. Prostacyclin acts locally by binding adenylate cyclase in response to an increase in cyclic AMP in the vascular smooth muscle cells and platelets (Vane and Botting, 1991; Ware, 1993). PGI_2 is readily hydrolysed to a stable, inactive form termed 6-keto-$PGF_{1\alpha}$, which is excreted in the urine.

b) Endothelial derived contracting factors

i) Endothelin-1 (ET-1) is a peptide apparently synthesised only by endothelial cells (Yanagisawa *et al*, 1988). Its release is usually slow but can be triggered by thrombin, epinephrine, shear stress and platelet aggregation. ET-1 has a rapid clearance and its action is that of a local hormone. ET-1 binds to specific membrane receptors to activate phospholipase C, with release of intracellular calcium resulting in activation of protein kinase C which modulates smooth muscle contraction (Maher *et al*, 1991). ET-1 has a prolonged pressor effect causing raised blood pressure, has a potency ten times that of angiotensin II (Vane and Botting, 1991) and may contribute to vasospasm. It can induce the release of PGI_2 and EDRF, and has mitogenic effects on vascular smooth muscle (Ware, 1993).

ii) Angiotensin converting enzyme (ACEII) is situated in the outer layer of endothelial cells. It converts angiotensin I to angiotensin II, and inactivates bradykinin (MAcGillivray, 1983).

2.1.2 Vasoconstrictors secreted at the endothelial surface

These include:

i) Thromboxane A_2 (TxA_2), a thromboxane synthase metabolite of arachidonic acid (Figure 2.2), is produced mainly by platelets and is secreted when platelets are aggregated at sites of endothelial injury. It can also be produced by macrophages and granulocytes. TxA_2 is a platelet stimulant, a potent vasoconstrictor and antagonist of PGI_2 (Moncada and Vane, 1979).

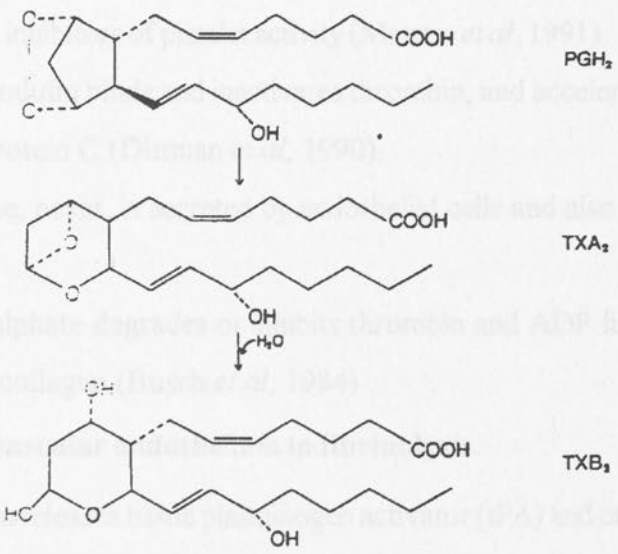


Figure 2.2 - Synthesis of thromboxanes, TXA_2 and TXB_2 from prostaglandin H_2. (Redrawn from Glew *et al*, 1992)

ii) Superoxide anion is formed when oxygen is reduced by a single electron to O2-, either during the cyclo-oxygenase pathway of arachidonic acid metabolism or under anoxic conditions from neutrophils and macrophages at the endothelial surface (Klebanoff, 1988; Uotila *et al*, 1990). Superoxide anion shifts the balance of PGI2:TxA2 in favour of TxA2, enhancing platelet aggregation and vasoconstriction, and inhibiting EDRF (Katusic and Vanhoutte,1989). Superoxide anion also oxidises membrane unsaturated fatty acids to form lipid peroxides which damage endothelial cells (Feher *et al*, 1987).

2.1.3 Antithrombotic function of vascular endothelium

Vascular endothelium actively prevents blood clot formation *in vivo* by means of cell surface and intracellular factors (Nawroth and stern, 1986). Endothelial-derived substances that prevent platelet aggregation are:

i) Nitric oxide (NO), release of which is stimulated by platelet ADP and serotonin released by aggregating platelets (Moncada *et al*, 1991).

ii) Prostacyclin (PGI_2), produced by endothelial cells in response to platelet ADP, serotonin and thrombin (Nolte *et al*, 1991). Synergistic interactions between PGI_2 and NO augment inhibition of platelet aggregation (Vanhoutte, 1989).

iii) Endothelial bound ADPase, which converts ADP from aggregating platelets to inactive AMP and adenosine, inhibitors of platelet activity (Marcus *et al*, 1991).

iv) Thrombomodulin binds and inactivates thrombin, and accelerates the activation of the anticoagulant, protein C (Dittman *et al*, 1990).

v) The protease, nexin, is secreted by endothelial cells and also inactivates thrombin (Jaffe, 1988).

vi) Heparan sulphate degrades or inhibits thrombin and ADP functions, and inhibits platelet adhesion to collagen (Busch *et al*, 1984)

2.1.4 Role of vascular endothelium in fibrinolysis.

The endothelium releases tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), which induce fibrinolysis by converting plasminogen into active plasmin. tPA is synthesised and released in response to endothelial cell damage, microthrombus formation, venous occlusion and pregnancy. Release of plasminogen activators is stimulated by thrombin, as is release of plasminogen activator inhibitor (PAI), from activated platelets (Ware, 1993). Other products released from platelet storage granules can induce the synthesis of PAI-1 by endothelial cells. Activated protein C stimulates fibrinolysis by complexing with, and inactivating, PAI (Coller, 1990).

tPA can have both aggregatory and inhibitory effects on platelets (Coller, 1990). Plasmin and tPA, in the concentrations found in blood after the administration of a thrombolytic agent, initially activate platelets and, if platelets are in contact with each other in a turbulent system, aggregation results (Penny, 1992). Thus, lysis of a clot induced by this mechanism may be followed immediately by re-occlusion because of the platelet-activating effects (Ware, 1993). *In vitro* studies have shown that tPA can degrade glycoprotein receptors on the platelet surface and reverse aggregation (Miles *et al*, 1986).

2.2 ENDOTHELIAL DYSFUNCTION IN PREECLAMPSIA

Consequences of endothelial cell activation due to inflammation or injury include thrombosis, increased production of vasoconstrictors and mitogens, increased membrane permeability, vasospasm and blood vessel remodelling (Piovella *et al*, 1992). While these are vital responses to disruption of vascular endothelial integrity, they can cause serious physiological disturbances when inappropriately activated (Roberts *et al*, 1989).

2.2.1 Inflammatory mediators

Lymphokines and cytokines released by lymphocytes and monocyte-macrophages in response to foreign antigen, injury, trauma and sepsis, are potent regulators of various functions of vascular cells, which include the synthesis and release of proteins involved in haemostatic reactions (Bevilaqua *et al*, 1984). Interleukin-1 (IL-1) and tumour necrosis factor (TNF) exert multiple effects on endothelial cells (Piovella *et al*, 1992). The balance of their action on endothelial metabolism induces the phenotype which is pro-inflammatory and pro-coagulant (Montesano *et al*, 1985). Tissue factor activity, PAI production and NO synthesis are enhanced by IL-1 and TNF, while surface thrombomodulin is decreased ((Culotta and Koshland, 1992; Cotran, 1987).

Levels of TNF-α, and soluble TNF receptor appear to be significantly higher in the plasma of women with severe preeclampsia compared to matched controls in the second and third trimester (Vince *et al*, 1995). It is possible that TNF and IL-1 function as "cytotoxic factors" when levels are increased above normal due to secretion by activated macrophages or lymphocytes. TNF has been described as directly toxic to endothelial cells (Heyborne *et al*, 1992). If these cytokines enter the maternal circulation from the preeclamptic placenta they could induce the procoagulant state in the vasculature, enhancing endothelial cell release of Fn, vWF and tissue factor. TNF added to endothelial cell cultures has been shown to alter the morphological appearance of the cells, so that they become elongated, overlap, rearrange their actin filaments and lose their stainable Fn (Hunt *et al*, 1989).

2.2.2 Cytokine regulation of fibronectin (Fn), tissue factor (TF) and von Willebrand factor (vWF) expression by endothelial cells

Stimulation of endothelial cells with IL-1 or TNF *in vitro* markedly increases tissue factor activity and the release of vWF (Piovella *et al*, 1991). Extracellular matrix (ECM) preparations treated with TNF stain more intensely for vWF than do control ECM preparations. Under immunofluorescent microscopic examination, endothelial cells show a decrease in cytoplasmic granular deposits of vWF. vWF is released more rapidly than Fn, and its levels in the medium quickly return to normal. Fn secretion increases slowly and remains elevated longer. Early rechallenge with these cytokines causes less intense release of vWF and does not affect Fn secretion, suggesting the presence of a mobilisable intracellular vWF pool in endothelial cells (Piovella *et al*, 1991).

2.2.3 Putative causative factors in endothelial injury

2.2.3.1 Cytotoxic serum factor

It has been hypothesised that placental damage with reduced perfusion results in the release of a circulating factor(s) injurious to vascular endothelium. Studies have shown that 10% predelivery serum from preeclamptic patients diluted in culture medium and added to normal human umbilical vein endothelial cells (HUVEC) causes increased chromate (^{51}Cr) release (a marker of cytotoxicity) as compared to that caused by normal pregnant predelivery serum (Roberts *et al*, 1991; Tsukimori *et al*, 1994).

Compared to predelivery serum, post-delivery serum from preeclamptics showed a decrease in ^{51}Cr release, while post-delivery serum from normal pregnant subjects showed increased release. Mixed preeclamptic serum with normal serum increased the chromate release. This suggested that preeclamptic sera contain an active cytotoxic component rather than being deficient in an endothelial stabiliser (Roberts *et al*, 1991).

Rapid reduction of cytotoxicity after delivery implies that the cytotoxic agent has a short half-life, and is therefore unlikely to be IgG, which has a half-life of several days to weeks (Roberts *et al*, 1991).

Other effects of serum from preeclamptic women on endothelial cells include increased accumulation of triglycerides (Endresen *et al* 1992), prostacyclin (Branch *et al*, 1992), cellular Fn (Taylor *et al*, 1991b) and decreased release of endothelin (Branch *et al*, 1991). These changes support the concept that changes in endothelial cell function are induced by circulating factors in preeclamptic patients.

Further studies reported no obvious morphological injury detectable by phase contrast microscopy of endothelial cells exposed to predelivery preeclamptic sera (Roberts et al, 1992). A sensitive assay for membrane integrity using propidium iodide exclusion and fluorescent activated cell sorter (FACS) analysis gave no evidence of altered membrane permeability.

Roberts et al (1992) suggest that preeclamptic serum is not actually cytotoxic but induces procoagulant activity in endothelial cells, secondary to changes distant from the site of endothelial disturbance, possibly the poorly perfused trophoblast.

2.2.3.2 Free radical activity

Lipid peroxides and oxygen free radicals have been suggested to be causative agents in vascular endothelial damage. A placental oxidant-antioxidant imbalance is thought to lead to an

increase in the release of placental lipid peroxidation products into the circulation (Hubel *et al*, 1989; Arbogast *et al*, 1994). Vascular contact with these products would promote peroxidative damage of endothelial cell membranes and selective inhibition of prostacyclin synthetase causing dysfunction of the vascular endothelium. A cycle of further release of lipid peroxides, with increased endothelial damage, could be established.

Free radicals are produced during normal physiological processes but their release is increased during ischaemia, hypoxia and in immune reactions, conditions described in the preeclamptic placenta (Hubel *et al*, 1989; Kloner *et al*, 1989). Increased peroxides in the circulation could promote haemolysis of red blood cells with possible doubling of serum iron concentrations which may occur in preeclampsia. Iron is a catalyst for lipid peroxidation and inactivation of NO in tissues. If lipid peroxide activity is increased relative to the counteractive capacity of blood antioxidants (ascorbic acid, uric acid, vitamin E), blood pressure would be elevated. After placental delivery and regression of hyperlipidaemia, the cycle would be broken (Hubel *et al*, 1989).

Lipid peroxidation products

The serum levels of lipid peroxidation products have been shown to be elevated in women with preeclampsia by the second trimester through analysis of conjugated dienes, malondialdehyde and fluorescent chromolipids, although their source is still uncertain (Uotila *et al*, 1993; Cester *et al*, 1994). Placentae from women with preeclampsia have a significantly increased triglyceride content compared to normal pregnancy placentae and a high degree of intrinsic lipoxygenase activity (Endresen *et al*, 1992). Syncytiotrophoblast plasma membranes have significantly increased lipoperoxide concentrations and an increased susceptibility to oxidative stress in preeclamptic patients compared with healthy pregnant subjects (Cester *et al*, 1994). Syncytiotrophoblast microvillus membrane preparations from normal and preeclamptic placentae have been shown to cause disruption of endothelial monolayers *in vitro*, whether grown on a matrix of Fn, laminin, gelatin or Matrigel (Smarason *et al*, 1993). Smarason *et al* suggest that the levels of membrane products entering the maternal circulation of preeclampsia patients may be higher than normal, thus causing significantly more endothelial disruption than that seen in the normal maternal vasculature. Aberrant lipoxygenase activities, leading to increased production of arachidonic acid peroxidation products may contribute to the complications of preeclampsia (Wang *et al*, 1992) and negatively affect the anti-oxidant state of the fetus (Uotila *et al*, 1993).

The cyclooxygenase pathway of arachidonic acid metabolism is another source of oxygen free radicals (Uotila *et al*, 1990).

Neutrophil activation

Neutrophils are activated in preeclampsia, possibly as a result of immune reactions or prolonged periods of ischaemia. The respiratory burst which follows activation is associated with a 20-fold rise in oxygen consumption and both superoxide anion and hydrogen peroxide production (Klebanoff, 1988). Neutrophils also metabolise arachidonic acid to leukotrienes and lipoxins which can degrade endothelial cell components (Klebanoff, 1988). The azurophilic granules of activated neutrophils release elastase, a protease which can destroy endothelial cells and their basement membranes, together with the subendothelial matrix (Greer *et al*, 1991).

Plasma neutrophil elastase levels are raised in preeclamptic patients (Butterworth *et al*, 1991). Fn fragments (120kDa) with cell attachment activity have been found to induce neutrophil elastase release from endothelial cells (Wachtfogel *et al*, 1988). A correlation exists between decidual elastase and plasma urate, which suggests that elastase release parallels disease severity as judged by plasma urate. Raised plasma urate was related to maternal vascular damage judged histologically on placental bed biopsies by McFadyen *et al* (1986).

Antioxidants

Little is known concerning antioxidant status in pregnancy. The antioxidant activity of serum is significantly higher in women with normal pregnancies relative to non-pregnant women (Uotila *et al*, 1993) and is correlated with ceruloplasmin, an antioxidant that may maintain iron in an oxidated state (Hubel, 1989). In mild preeclampsia the increase in levels of the anti-oxidant, vitamin E, may be even greater, but in severe forms of the disease the vitamin E concentrations may be deficient (Tsukatani, 1983; Mikhail *et al*, 1994). Raised levels of vitamin E have been measured in severe preeclampsia, as compared to normal pregnancy and mild preeclampsia (Uotila *et al*, 1993) while vitamin C is decreased in both mild and severe preeclampsia (Mikhail *et al*, 1994). High levels of activity of erythrocyte glutathione peroxidase, (another antioxidant), have been measured in severe preeclampsia (Uotila *et al*, 1990). The rise in antioxidants is probably a compensatory response to the increased lipid peroxide load in preeclampsia and may reflect the severity of the disease (Uotila *et al*, 1993).

Antioxidant therapy, perhaps in combination with dietary omega-3-fatty acids, might help to preserve the integrity of the vascular endothelium by shifting the TxA_2-PGI_2 balance towards a lesser thrombogenic state (Hubel et al, 1989).

2.3.3 Mitogenic activity of endothelial-derived growth factors

The endothelial cell function of secreting growth factors and chemoattractants may be important in processes such as vessel neogenesis and wound repair. Uncontrolled secretion of growth factors could be visualised as a trigger for development of atherotic lesions. Stimulatory agents may include thrombin-like factors or cytotoxic agents (Bizios *et al*, 1986; Zimmerman *et al*, 1985).

Release of growth factor mitogenic activity is commonly associated with endothelial damage, which lends support to the contention that endothelial cell injury or activation is an early pathogenetic feature of preeclampsia (Roberts et al, 1991). Growth promoting factors are released rapidly (5 min) from mechanically injured endothelial cells, but are not degraded for at least 24 hours thereafter. Release correlates poorly with cell death and is not due to release from the extracellular matrix. This suggests that mechanical disruption of the plasma membrane is the essential event leading to release of growth factors.

Serum or plasma from women with preeclampsia has been demonstrated to have increased mitogenic activity relative to normal pregnancy (even as early as the first trimester) as measured by ^{3}[H] thymidine incorporation into DNA of human fibroblasts (Musci *et al*, 1988; Roberts *et al*, 1991). Post-delivery incorporation was the same as in normal pregnancy. When reanalysed by trimester, the mitogenic index was significantly increased as early as the first trimester in women destined to become preeclamptic. However, this mitogen did not have characteristics anticipated for platelet-derived growth factor (PDGF), transforming growth factor - β (TGF-β) or fibroblast growth factor (FGF).

The increased mitogenic index (a marker of endothelial cell activation *in vivo*) precedes the clinical signs of preeclampsia by months, supporting the hypothesis that endothelial cell injury gives rise to the pathophysiological changes of the syndrome (Roberts *et al*, 1991). The marked variation in mitogenic activity among individuals indicates that the identification of the specific mitogenic factor is necessary to determine the potential of mitogenic activity as a predictive test for preeclampsia (Roberts *et al*, 1991).

2.3.3.1 Growth factors in preeclampsia

a) Platelet derived growth factor (PDGF)

PDGF concentration was increased in response to preeclamptic sera (Roberts at al, 1991). In hybridisation assays, predelivery serum from preeclamptic women induced double the PDGF-B chain mRNA of 48 hour postpartum serum. No difference was noted in normal pregnancy. PDGF in preeclampsia is a mitogen and a potent vasoconstrictor. PDGF may contribute to vascular proliferation in atherosis and hypertension and may cause release of endothelial derived relaxing factor (NO) from endothelium in response to denuding injury (Ware, 1993).

IL-1, IL-6, TNF and TGF-β have been shown to increase PDGF gene expression (Ristimaki *et al*, 1990). Interferon-γ (IFN-γ) has been shown to inhibit both basal and cytokine stimulated production of PDGF by HUVEC (Fox and Di Corleto, 1991). Omega-3 fatty acids also inhibit PDGF production and this inhibition can be blocked by antioxidants eg vitamin E (Hubel *et al*, 1989).

b) Transforming growth factor-β (TGF-β).

Endothelial cells secrete TGF-β in culture. TGF-β increases both PDGF-A-chain and B-chain gene expression (Ristimaki *et al*, 1990) and enhances the synthesis of endothelin-1, as does normal human serum *in vitro* (Ristimaki *et al*, 1991). TGF-β is an inhibitor of tPA and uPA, and is an attractant for macrophages (Ristimaki *et al*, 1990).

c) Fibroblast growth factor (FGF)

A basic FGF-like molecule appears to be partly responsible for endothelial growth promoting activity and wound healing. One possible route of release of β-FGF could be through mechanically induced membrane disruptions (lysis or leakage) of endothelial cells growing *in vivo* and *in vitro*. (McNeil, 1989).

Sato and Rifkin (1988) reported the finding that endogenous production of β-FGF is necessary for endothelial cell migration, as well as fibroblast migration to sites of injury. In addition, the basal level of synthesis of PA and the basal level of DNA synthesis in bovine aortic endothelial cells appear to be determined by the release of endogenous β-FGF, suggesting that β-FGF acts as an autocrine factor. TGF-β blocks the activity of β-FGF as well as the β-FGF induced synthesis of PA by endothelial cells.

2.3.4 Products of plasminolysis

In vitro experiments have revealed that the fibrinogen degradation product, fragment D, influences endothelial monolayer integrity and causes cell detachment from the substrate (Ge *et al*, 1992). This phenomenon, which involves remodelling of the ECM, has been demonstrated to involve plasminogen activators and plasminogen activator inhibitors and the plasmin system which play a role in the regulation of cell-cell and cell-ECM adhesion (Saksela *et al*, 1988). Endothelial cells incubated with fragment D showed increased concentration and activity of uPA and tPA in the culture medium in a concentration-dependent manner within 2 to 4 hours of incubation. Increased PA activity was followed by augmentation of cell-associated plasmin activity and subsequent increase in the degradation of ^{125}I-fibrinogen precoated in the subendothelial matrix (Ge *et al*, 1992).

An antibody directed against uPA and aprotinin (protease inhibitor) prevented approximately 60% of fragment D-induced endothelial cell detachment. Thus fragment D potentiates the proteolysis of the ECM and subsequent endothelial cell detachment. Fragment D may be a critical mediator linking activation of fibrinolysis to vascular injury in inflammatory disorders (Ge *et al*, 1992)

The effects of Fn fragments on endothelial cell integrity have not yet been studied. However, the effects of fibrinogen fragment D suggest the possibility that Fn fragments as products of plasminolysis, may have similar effects. This system is dealt with extensively in the experimental section.

2.4 EFFECTS OF ENDOTHELIAL INJURY

Response to injury hypothesis

Sites of atherosis may develop as a result of injury to the intimal vascular monolayer. Two types of endothelial injury are commonly described: a denuding injury, secondary to endothelial cell necrosis and desquamation, which triggers platelet/subendothelial interactions, and a non-denuding injury, which induces new endothelial properties and loss of intimal thromboresistance. Intravascular coagulation, mediated by inflammatory cytokines, may complicate atherosis at different stages (Piovella *et al*, 1992).

A sequence of responses, hypothesised as a "response to injury" model (Table 2.1) has been suggested to occur after endothelial injury.

1. Endothelial injury
2. Adhesion of blood platelets
3. Penetration of PDGF
4. Migration and proliferation of smooth muscle cells
5. Re-endothelialisation
6. Re-injury
7. Increased reaction (Repeat steps 2-5)
8. Infiltration of lipids and synthesis of connective tissue
9. Cell death

Table 2.1 The response to injury hypothesis
(from Ross and Glomset, 1976)

2.4.1 Evidence of vascular endothelial injury in preeclampsia

a) Morphological lesions

There is indirect evidence that generalised endothelial injury antedates the clinical syndrome of preeclampsia. Evidence of endothelial injury in preeclampsia is provided by the characteristic morphological lesions of the disease, namely acute atherotic lesions of the uterine spiral arteries (Shanklin and Sibai, 1989) and glomerular endotheliosis in the kidneys (Fisher *et al*, 1981; Sheehan and Lynch, 1973) which may result in localised occlusion of vascular capillaries.

Localised intralumenal endovascular trophoblast, attached to the arterial walls, can be demonstrated within the uteroplacental arteries in 80% of complicated pregnancies (Khong *et al*, 1992). This finding is commonly associated with localised disruption of the endothelium.

b) End-organ damage

The reduced perfusion of the placenta resulting from abnormal trophoblast invasion and undilated spiral arteries may explain the origin of the systemic manifestations of preeclampsia which include reduced organ perfusion (kidney, liver, brain) and may precipitate organ dysfunction. Factors produced by the poorly perfused fetoplacental unit may enter the maternal circulation and alter the functions of the vascular endothelium (Roberts and Redman, 1993).

c) Disturbance of tight junctions

Tight junctions may be disturbed with loss of regulated fluid and protein transport resulting in extravasation of fluid and proteins from the intravascular space (Roberts *et al*, 1989). This may cause oedema which is sometimes a symptom of preeclampsia (Ferris, 1991). The increased rate of disappearance of the albumin-bound dye, Evans blue, from the intravascular space in preeclamptic patients provides indirect evidence of loss of endothelial cell integrity (Roberts *et al*, 1989).

4.4.2 Biological responses to disruption of vascular integrity

Endothelial injury has a tendency to progress. Loss of the cytoprotective effect of normal endothelium may lead to increased vasospasm and intravascular coagulation, reducing perfusion distal to the site of injury. This is compatible with the accelerating clinical course in preeclampsia which only abates after delivery (Roberts *et al*, 1989).

a) Antibodies directed against vascular endothelial cells

Damaged endothelium may express antigens which induce the formation of anti-vascular endothelial cell antibodies. These antibodies have been detected in plasma of a significant number of patients with preeclampsia (Rappaport *et al*, 1990). It has been suggested that binding of these antibodies to the endothelial monolayer may have effects on PGI_2 secretion, increased platelet adherence, activation of the complement cascade and disruption of the monolayer (Zeeman and Dekker, 1992).

b) Activation of coagulation

Activation of the endothelial surface from an anticoagulant state to a procoagulant surface is indicated by increased plasma concentrations of vWF, cellular Fn (Lockwood and Peters, 1990), a disturbance of the tPA:PAI balance (de Boer *et al*, 1988) and increased platelet activation (Zemel *et al*, 1991).

i) Fibronectin (Fn)

Cellular Fn (cFn), released by activated or injured endothelial cells and endothelial cell matrix *in vivo*, is normally a minor component of circulating Fn, constituting <5% of the total plasma pool. Cellular Fn mediates platelet adherence and aggregation at sites of endothelial cell injury and is a sensitive indicator of activation of the coagulation cascade (Taylor *et al*,

1991a). To determine the extent of vascular injury during pregnancy, circulating cellular Fn has been quantified with a monoclonal antibody (MAb) specific for the ED1 region. Plasma cFn concentrations were significantly elevated as early as the second trimester, before the clinical manifestations of the syndrome, in women destined to have preeclampsia but not in women who have normal pregnancies or transient hypertension, suggesting that maternal vascular cell injury is an early and specific phenomenon in the pathogenesis of preeclampsia (Lockwood and Peters, 1990). The fact that elevated cFn levels were not found in the transient hypertension group indicates that elevated blood pressure per se is not responsible for the increase in plasma cFn levels in preeclampsia.

Although levels of total (ED1+ plus ED1-) Fn were also elevated in plasma samples from patients with preeclampsia, only the concentration of the ED1+ variant (cFn) correlated with severity of disease in two patients examined serially (Peters *et al*, 1989). Elevations in plasma content of ED1+ Fn, but not total Fn, were also noted in patients with acute vascular tissue injury associated with major trauma or sepsis syndrome. Western blot examination revealed the presence of intact dimeric ED1+ Fn in the circulation of all patients studied, although Fn fragments containing ED1+ were also detected (Peters *et al*, 1989).

ii) Tissue factor (TF) and von Willebrand factor (vWF)

Normal HUVEC exposed to sera were assayed for altered expression of cFn, tissue factor (TF) and vWF (Taylor *et al*, 1991b). TF is a potent endogenous, thrombogenic mediator. Serum from preeclamptic women caused increased cFn release from HUVEC *in vitro*, compared to normal pregnancy sera, but effects on TF and vWF were not observed. TF activity was measured in intact cells, and vWF was measured on fixed endothelial cells with a whole cell ELISA technique.

The results demonstrated that predelivery preeclamptic sera contain a factor(s) that stimulates the secretion or release of human endothelial cFn to a greater extent than sera from normal pregnancies. The elevated Fn concentrations in plasma of preeclamptic women might, in part, be due to the *in vivo* release of endothelial-derived cellular Fn (cFn) caused by this factor(s). However, in this model of endothelial cell activation neither tissue factor nor vWF expression appears to be increased preferentially by preeclamptic sera (Taylor *et al*, 1991a).

d) Alterations in vascular tone

i) PGI_2:TxA_2 imbalance

An early event in the development of preeclampsia is the reduction of refractoriness to vasoactive agents followed by vasoconstriction. A functional imbalance between vasodilator and vasoconstrictor eicosanoids may contribute to the early increase in sensitivity to angiotensin II (Myatt, 1986). The most important eicosanoid imbalance is that of PGI_2:TxA_2. Preeclampsia is associated with a relative deficiency in PGI_2 synthesis and an increase in TxA_2 production (Walsh *et al*, 1985) and TxA_2 synthase expression in the placenta (Woodworth *et al*, 1994) which may be associated with deficient glutathione peroxidase activity (Walsh and Wang, 1993). As these eicosanoids are antagonists in the regulation of interactions of platelets with the vessel wall, an imbalance, especially in the uteroplacental circulation and the kidney, may be an important factor in the development of the disease giving rise to some of its clinical manifestations.

Clinical effects of eicosanoid imbalance

Platelet destruction and the development of the HELLP syndrome, reduced uteroplacental blood flow with arterial thrombosis and placental infarction may all be initiated by the increased vasoconstriction due to this imbalance (Zeeman and Dekker, 1992). The absence of the normal stimulation of the renin-angiotensin-aldosterone system (which controls the plasma fluid volume), despite the occurrence of hypovolaemia, may be attributable to the PGI_2:TxA_2 imbalance (MacGillivray, 1983). Preeclampsia is associated with decreased intrarenal production of PGI_2 and PGE_2, both vasodilator prostaglandins, with decreased 6-keto-prostaglandin F1-α urinary excretion (Pedersen *et al*, 1984). This may explain the decrease in renal blood flow, glomerular filtration rate, urate clearance and the development of proteinuria (Ferris, 1988). Increased sensitivity to angiotensin II may lead to an impaired ability to excrete sodium, which may cause an increase in vascular tone and blood pressure (MacGillivray, 1983). However, findings suggest that PGI_2 deficiency is not a primary event in the pathogenesis of preeclampsia since prostaglandin vasodilators are not the major vasodepressor agents of pregnancy (Conrad and Colpoys, 1986; Ahokas *et al*, 1991) and pregnant women using high doses of prostaglandin-synthesis inhibitors do not have an increased incidence of preeclampsia (Everett et al, 1978).

ii) The nitric oxide (NO) system

It is hypothesised that the prostacyclin system is held in reserve to reinforce the NO system locally when endothelial damage occurs. Prostacyclin may be more important than NO in long

term prevention of atherosclerosis, whereas lack of NO may be causative in hypertension. PGI_2 production is therefore considered to function as a rescue mechanism, especially during periods of ischaemia and hypoxia (Spokas *et al*, 1983), and particularly in those pregnancies where uteroplacental perfusion and uteroplacental NO release is reduced and vasoconstriction, platelet activation and TxA_2 release is raised. TxA_2 may stimulate prostacyclin or NO formation by the 'endoperoxide shunt' (Ware, 1993). Vasodilatory prostaglandin release is evoked locally in order to re-establish normal function by regulating tissue perfusion and metabolism. According to this hypothesis, severe preeclampsia would only occur when uteroplacental PGI_2 synthesis, as the critical rescue mechanism, is deficient (Spokas et al, 1983).

Continuous basal release of NO keeps the smooth muscles of vessels dilated. Failure of NO release could lead to hypertension. Studies *in vitro* suggest that impairment of NO release is mediated by the activation of endothelial protein kinase C (Ware, 1993). In addition, leukocytes present in the diseased arterial wall may release superoxide anion ($O2^-$), which inactivates NO. Impairment of the EDRF mechanism in diseased arteries also hampers the ability of the endothelium to prevent platelet aggregation and release of vasoactive and mitogenic substances (Ware, 1993). Treatment with L-arginine has been reported to correct endothelial dysfunction in both an animal model of atherosclerosis (Girerd, 1990) and in patients (Drexler, 1991).

iii) Endothelin-1 (ET-1) release

Endothelin-1 (ET-1) may cause constriction of underlying smooth muscle in emergency or in defensive events such as haemostasis or wound repair. Excess ET-1 may lead to the genesis of hypertension (Ferris, 1991) and contribute to vasospasm (Faraci, 1993). ET is thought to exert its effects locally rather than systemically. ET-1 mRNA in placental villous tissue is significantly higher in preeclamptic women than in control subjects (McMahon *et al*, 1994).

e) Platelet activation

Platelets will interact and adhere to endothelium or subendothelial tissue if perturbed by injury or inflammation (Ware, 1993). Surface-mediated platelet activation may occur on the poorly endothelialised surfaces of the spiral arteries. Activated endothelial cells express selectins and immunoglobulin related ligands or cell adhesion molecules (ICAM, VCAM and PECAM) which bind to stimulated neutrophils, lymphocytes and platelets respectively by means of integrin and selectin receptors (Osborn, 1990; Albelda *et al*, 1991; Lawrence and Springer, 1991) (Figure 2.3a). Platelets also attach to neutrophils by means of a P-selectin-mediated mechanism, which facilitates transendothelial passage (Hamburger and McEver, 1990).

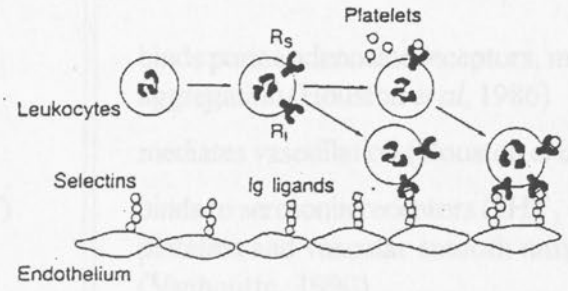


Figure 2.3a - Possible mechanisms of leucocyte-facilitated binding of platelets to the endothelium. Leukocytes interact with endothelium at inflammatory sites and attach with integrin (R_I) and selectin (R_S) receptors to selectins (circles) and immunoglobulin-related ligands (squares) on the endothelial surface. Platelets interact with neutrophils through a selectin-mediated mechanism, and may come into closer contact with the endothelium through these interactions.(Redrawn from Ware *et al*, 1993)

Activated platelets, even without adhering to the endothelium, affect endothelial function by release of contents of storage granules (Houston *et al*, 1986) (Figure 2.3b)

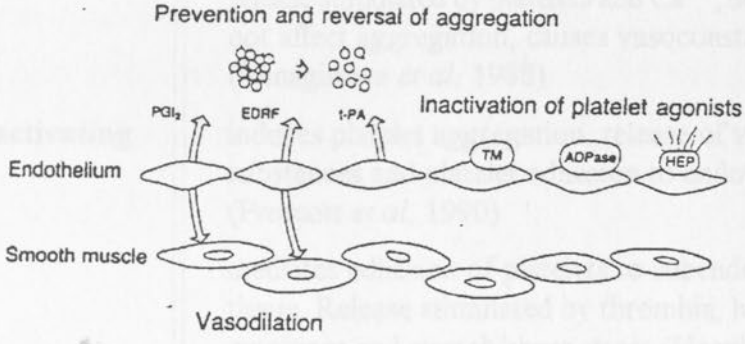


Figure 2.3b - Vasoactive and antiaggregatory substances released by normal endothelium. (Redrawn from Ware *et al*, 1993)

ADP	binds purine adenosine receptors, mediates platelet aggregation (Houston *et al*, 1986)
ATP	mediates vasodilation (Houston *et al*, 1986)
Serotonin (5HT)	binds to serotonin receptors ($5HT_1$ and $5HT_2$) on platelets and vascular smooth muscle cells (Vanhoutte, 1990)
TxA_2	causes vasoconstriction and platelet aggregation (Schafer *et al*, 1984)
Leukotrienes	from neutrophil precursors or arachidonic acid metabolism, cause vasoconstriction (Romano and Serhan, 1992)
Lipoxins	from neutrophil precursors causes vasodilation (Houston *et al*, 1986)

Table 2.2 - Factors released from storage granules of platelets.

ET-1	release stimulated by platelets and Ca^{++}, but does not affect aggregation, causes vasoconstriction (Yanagisawa *et al*, 1988)
Platelet activating factor	induces platelet aggregation, release of vasoactive substances and platelet adhesion to endothelium (Prescott *et al*, 1990)
vWF	mediates adhesion of platelets to subendothelial tissue. Release stimulated by thrombin, histamine, cytokines and stretch/shear stress (Hamilton *et al*, 1987; Penny *et al*, 1991)

Table 2.3 - Substances released by endothelial cells which affect platelet aggregation

i) Effects of serotonin on platelet aggregation

There is a 49% reduction in sensitivity to prostacyclin in preeclampsia which may contribute to an increase in coagulation and platelet aggregation at the endothelial surface

(Briel *et al*, 1984). This suggests that platelets of pregnant women may be less responsive to prostacyclin induced inhibition of platelet aggregation, and even more so in preeclampsia. The abnormality of prostaglandin metabolism may be associated with a decrease in placental capability to metabolise serotonin (Weiner *et al*, 1987). Plasma serotonin levels have been found to be significantly increased in preeclampsia (Weiner, 1990). Raised placental serotonin in preeclampsia, may be due to the decreased production of monoamine oxidase (which degrades serotonin) by the damaged placenta (Weiner, 1990).

The effect of serotonin released by aggregating platelets on vascular smooth muscle contraction depends on the state of the endothelial layer. The receptor for serotonin which mediates muscle relaxation is an S_1 or $5HT_1$ type, which is expressed by intact endothelium (Vanhoutte, 1990). Serotonin bound to the S_1 receptor will induce vasodilatory synthesis of PGI_2 and EDRF and inhibit further platelet aggregation and thrombus formation. However, serotonin may cause contraction of an injured vascular wall by binding to a different receptor, S_2, on the vascular smooth muscle cells beneath the damaged endothelial layer. Serotonin will then amplify platelet aggregation and exert a positive feedback on its own release. Serotonin may also amplify the vasoconstrictor action of catecholamines and angiotensin II. Serotonin interaction with these two receptors, dependent on the initial extent of uteroplacental vascular endothelial damage, may help to explain the progression to severe disease in some patients, while other patients remain stable (Vanhoutte, 1990) (Figure 2.3c).

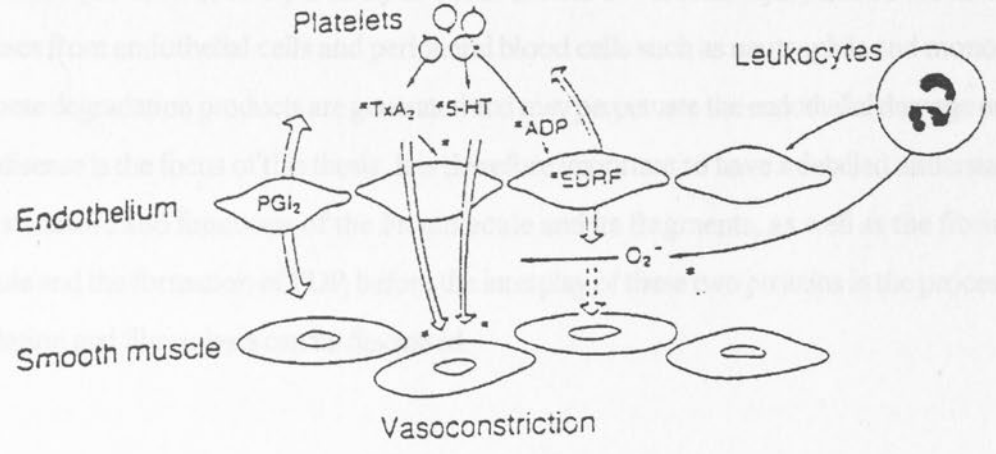


Figure 2.3c - Platelet interactions with diseased endothelium.
(Redrawn from Ware *et al*, 1989)

ii) Effects of platelet thromboxane (TxA_2)

Increased platelet thromboxane output can amplify platelet aggregation (Greer *et al*, 1989). This could account for the increased fibrin deposition in organs such as kidneys and for the atherosis developing in the placental blood vessels. Platelet aggregation and TxA_2 synthesis are inhibited by low-dose aspirin (60 mg/day) in non-pregnant, pregnant and preeclamptic women (Louden et al, 1991). Aspirin inhibits platelet aggregation by irreversibly inhibiting the action of cyclooxygenase. If used at a dosage determined to specifically reduce contractile prostanoid (TxA_2) synthesis much more than prostacyclin, the increased angiotensin II sensitivity of preeclamptic patients is reduced (Vane and Botting, 1991). The life of a platelet is 8-11 days, therefore aspirin treatment should lead to cumulative inhibition of TxA_2 formation while allowing endothelial cells to produce prostacyclin by new enzyme generation (Vane and Botting, 1991).

2.5 CONCLUSION

In summary, there are many indications that vascular endothelial damage is an early event in the development of the preeclamptic syndrome. As the endothelial layer has such a range of regulatory functions, any disturbance of this layer will undoubtedly have widespread effects. Endothelial lesions may be associated with activation of the coagulation system, resulting in an imbalance of circulating coagulation and fibrinolytic factors.

Abnormally increased fibrin and Fn deposition occurs and may not be restricted to the placenta, but may extend to the vasculature of a number of organs including the placenta, kidneys and liver. Degradation of these proteins to fibrin degradation products (FDP) and fibronectin degradation products (FnDP) is likely to occur at sites of vascular injury due to the release of proteases from endothelial cells and peripheral blood cells such as neutrophils and monocytes. That these degradation products are generated and may perpetuate the endothelial damage process of the disease is the focus of this thesis. It is therefore important to have a detailed understanding of the structure and functions of the Fn molecule and its fragments, as well as the fibrinogen molecule and the formation of FDP, before the interplay of these two proteins in the processes of coagulation and fibrinolysis can be discussed.

CHAPTER 3 - LITERATURE REVIEW

HAEMOSTASIS IN PREECLAMPSIA

This chapter deals with the process of blood coagulation in healthy subjects, in normal pregnancy and in preeclampsia. Blood coagulation involves the conversion of fibrinogen to fibrin and is essential for normal platelet function and wound healing. It is important to have a basic understanding of the clotting factors and of the coagulation pathways in order to appreciate the changes and abnormalities that occur in coagulation in pregnancy and preeclampsia.

3.1 NORMAL HAEMOSTASIS

Blood normally circulates through the vasculature without appreciable coagulation or haemorrhage. Localised or diffuse vascular injury will result in platelet adherence to the damaged endothelium or exposed subendothelium (Weiss, 1975). Subsequently, activation of the coagulation cascade occurs (MacFarlane, 1964; Davie and Ratnoff, 1964) either due to contact activation (intrinsic pathway) (Saito, 1987) or through expression of tissue factor at the site of injury (extrinsic pathway) (Nemerson, 1988). Vascular injury or activation of factor XII also results in stimulation of fibrinolytic pathways (Mullertz, 1984) (Figure 3.1). DURING the course of the coagulation cascade, coagulation factors which normally exist in the plasma as inert precursors are amplified and converted into active enzymes (Doolittle, 1973). Many of these enzymes are made more efficient and effective by accelerators, although thrombin, the most active of all these enzymes, has no accelerator (Fenton *et al*, 1977).

Clotting factors in the coagulation cascade

The liver is the major site of synthesis of the coagulation proteins (except von Willebrand factor (vWF)), the fibrinolytic proteins and protease inhibitors such as protein C, antithrombin III (ATIII) and heparin cofactor II (Kelly and Summerfield, 1987). The clotting factors can be divided into three groups. The contact system includes factors XI and XII which are involved in the initial phase of intrinsic activation *in vitro* and are not consumed (Figure 3.1). The prothrombin group includes factor II (prothrombin), VII, IX and X. These are dependent on vitamin K for the N-terminal γ-carboxylation of specific glutamic acid residues

which provide calcium binding sites. They are stable and well preserved in plasma, except for prothrombin. The third group includes Factor I (fibrinogen), V, VIII and XIII which lose their activity during the clotting process. These substances behave as acute phase reactants in that their concentration is increased during inflammatory illnesses and in pregnancy (Jacobs and Bird, 1983). Factor VIII circulates as a complex of two proteins, the Factor VIII coagulant protein (VIIIC) and the Factor VIII-related protein or antigen (VIIIR Ag) which contains vWF. Factor VIIIC is an element of the coagulation cascade, while FVIII:vWF is a cofactor for platelet adhesion to subendothelium. Approximately 85% of FVIIIC in normal plasma is bound to vWF as a FVIII:vWF complex (Moake *et al*, 1983).

The intrinsic pathway is initiated by activation of factor XII to a hydrophobic state (Fair *et al*, 1977) by contact with foci of negative charges (active surfaces) (Hubbard *et al*, 1960) on damaged endothelium, bacterial particles or other foreign material (Figure 3.1). High molecular weight (HMW) kininogen may act as a cofactor for factor XIIa in activating factor XI, which is accelerated by platelet factor 3 (PF-3). The pathway continues with activation of factor IX (a serine protease) and factor VIII (cleaved by thrombin, IXa or plasmin) to accelerate factor Xa formation in the presence of divalent calcium ions and PF-3 or phospholipid (Rick, 1982; Schiffman *et al*, 1973). Prekallikrein, with an active-site serine, also accelerates coagulation. The platelet membrane may participate in contact activation as an assembly site for coagulation factors (Walsh and Griffin, 1981).

The extrinsic pathway is initiated by tissue thromboplastin or tissue factor (TF) and the activation of the proenzyme factor VII. A TF:factor VII complex is formed, which in the presence of calcium, also activates factor X which then enters the common pathway illustrated in Figure 3.1. TF contains specific calcium-binding sites (Wijngaards *et al*, 1977) and may act as a site for calcium bridging in the same way as factor V and VIII (Davie and Fujikawa, 1975). Kallikrein and factor XIIf are apparently the major activators of factor VII under physiologic circumstances (Zur *et al*, 1982). Several other enzymes may also activate factor VII, for example, factor Xa, plasmin and factor IXa with inactivation of complement inhibitor, C-1 (Gordon *et al*, 1980).

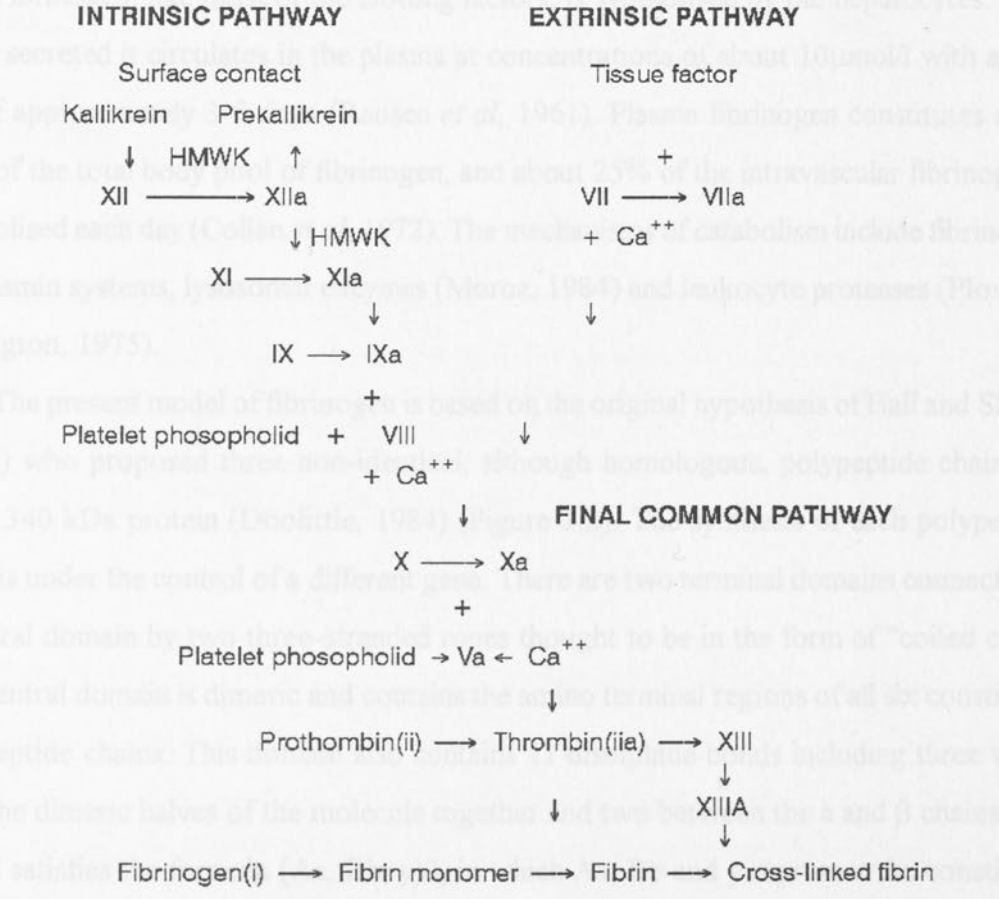


Figure 3.1 - Coagulation cascade

The common pathway involves a series of reactions between activated factor X, factor V, platelet phospholipid, prothrombin and fibrinogen, resulting in the formation of fibrin. Activated factor X in the presence of factor V, calcium and platelet-derived phospholipid, forms a prothrombinase (a protein-membrane-phospholipid complex with factor Xa as the active enzyme centre) which converts prothrombin to thrombin (Krishnaswamy *et al*, 1988).

Thrombin is a serine protease which has a number of activities. It modifies factors V and VIII, accelerating activity of the common pathway, it induces platelet aggregation and brings about proteolytic conversion of fibrinogen into fibrin monomers by cleaving four arginyl-glycine bonds, releasing fibrinopeptides A and B (Ryan *et al*, 1976).

3.2 FIBRINOGEN STRUCTURE

Fibrinogen, like most of the clotting factors, is synthesised by the hepatocytes. After being secreted it circulates in the plasma at concentrations of about 10μmol/l with a half-life of approximately 3-5 days (Rausen *et al*, 1961). Plasma fibrinogen constitutes about 75% of the total body pool of fibrinogen, and about 25% of the intravascular fibrinogen is catabolised each day (Collen *et al*, 1972). The mechanisms of catabolism include fibrinolysis by plasmin systems, lysosomal enzymes (Moroz, 1984) and leukocyte proteases (Plow and Edgington, 1975).

The present model of fibrinogen is based on the original hypothesis of Hall and Slayter (1959) who proposed three non-identical, although homologous, polypeptide chains for every 340 kDa protein (Doolittle, 1984) (Figure 3.2). The synthesis of each polypeptide chain is under the control of a different gene. There are two terminal domains connected to a central domain by two three-stranded ropes thought to be in the form of "coiled coils". The central domain is dimeric and contains the amino terminal regions of all six constituent polypeptide chains. This domain also contains 11 disulphide bonds including three which hold the dimeric halves of the molecule together and two between the a and β chains. The model satisfies the formula (Aa, Bb, y)2, in which Aa, Bb and y represent the constituent chains of the protein having approximate molecular weights of 73, 60 and 50 kDa respectively (McKee *et al*, 1970).

The Aα-chain has two RGD sequences, one near the N-terminal (α95-97) and one near the C-terminal (α572-574). These sequences are the same as the sequence in the fibronectin (Fn) molecule which is essential for cell attachment. Two crosslinking glutamine residues occur at α328 and α366 for crosslinking of Fn to fibrin by activated factor XIII in the formation of extended α-chain multimers (McKee *et al*, 1970).

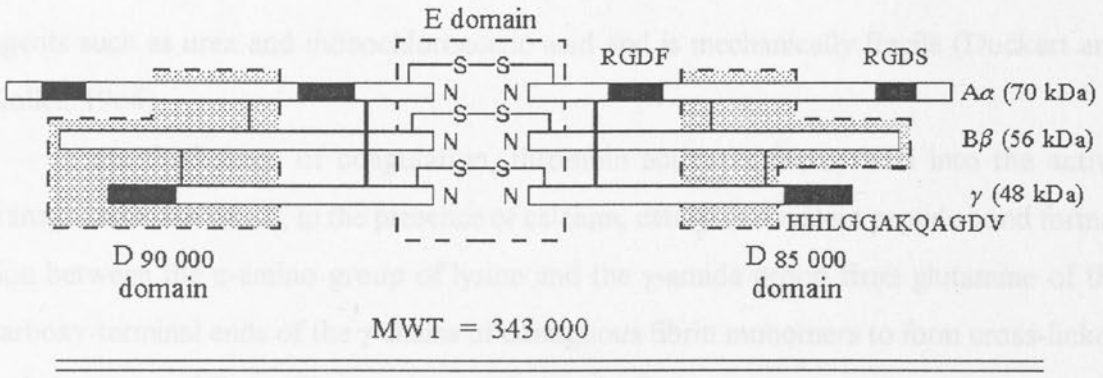


Figure 3.2. Scheme of the structure of the human fibrinogen molecule. Black stretches on Aα and γ chains indicate the localisation of RGD and HHLGGAKQAGDV sequences recognsed by integrins gpIIb-IIIa and the vitronectin receptor. Boxes outlined with dashed lines define the E, D90 000 and D85 000 domains obtained after plasmin digestion. (Redrawn from Levesque *et al*, 1991)

Carbohydrate sidechains are attached to the Bβ and the γ chains at asparagine residues in the characteristic asparagine-X-threonine sequence (Pepper *et al*, 1974). These moieties consist of sialic acid, galactose, mannose and N-acetylglucosamine. There are also several calcium-binding sites on the molecule of either high or low affinity (Marguerie *et al*, 1977).

The Aα chain contains the N-terminal 16 amino acid sequence of fibrinopeptide A (FPA). The Bβ chain has an N-terminal 14 amino acid sequence which represents fibrinopeptide B (FPB). Both fibrinopeptides are released by the hydrolytic action of the serine protease thrombin on the arginyl-glycine bonds at the initiation of fibrin formation. This exposes the Glycine-Proline-Arginine sequence which is apparently essential for polymerisation (Doolittle *et al*, 1979).

Complementary hydrogen bond donors and acceptors are exposed as the result of the loss of fibrinopeptides. The fibrin monomers thus undergo spontaneous polymerisation by hydrogen bonding between sites in the central E-domain of one molecule (N-terminal α-chain) and the lateral D-domain of another molecule (C-terminal γ-chain) to form a half-overlapped dimer. Two lateral domains then interact in an end-to-end manner. Additional interactions produce a long two-stranded fibrin protofibril (Fowler *et al*, 1981). Progressive end-to-end and side-to-side associations of these protofibrils form a thick, loose, gelatinous fibrin network (Erikson and Fowler, 1983). At this stage the fibrin is soluble in

agents such as urea and monochloroacetic acid and is mechanically fragile (Duckert and Koller, 1966).

In the final stage of coagulation, thrombin activates factor XIII into the active transglutaminase which, in the presence of calcium, catalyses covalent peptide bond formation between the ε-amino group of lysine and the γ-amide group from glutamine of the carboxy-terminal ends of the γ chains of contiguous fibrin monomers to form cross-linked γ-chain dimers of fibrin. Fibronectin may bind to the fibrinogen α-chain via transglutaminase activity during crosslinking to form α-chain polymers (Hermans and McDonagh, 1982), aiding in the stabilisation of the fibrin clot which is more resistant to plasmin digestion than the loose clot. Thus, there are three major forms of fibrin which may exist *in vivo*: non cross-linked fibrin (fibrin monomer), partially cross-linked fibrin and totally cross-linked fibrin (Gaffney, 1981).

The role of platelets in clot formation

Stimulated platelets play a role in clot formation, by adhering to exposed subendothelial collagen and Fn at sites of endothelial disruption. Fn enhances platelet spreading and aggregation on collagen (Grinnell *et al*, 1979; Arneson *et al*, 1980). This process requires the presence of vWF which binds to specific platelet receptors on the platelet membrane. The platelet receptor complex, GPIIb/IIIa, binds to the RGD sequences of Fn, vWf and fibrinogen via the GPIIIa residue. The receptor complex may also bind to the γ chain dodecapeptide HHLGGAKQAGDV of fibrinogen via the GPIIb residue (Perutelli and Mori, 1992). Platelet-bound fibrinogen mediates platelet aggregation by bridging the GPIIb/IIIa complexes on two adjacent platelets. Binding of fibrinogen alters the receptor conformation and results in the expression of neoantigens that may participate in signal transduction pathways (Perutelli and Mori, 1992). Platelet aggregation is amplified as stimulated platelets secrete dense bodies and α-granules containing adenosine diphosphate (ADP), Fn, fibrinogen, calcium, serotonin, thromboxane and β-thromboglobulin together with other coagulation and platelet factors (Jacobs and Bird, 1983).

3.3 FIBRONECTIN (Fn) AND CROSSLINKING

Fn is one of four proteins in plasma which serves as a substrate for transglutaminases during clotting and can be cross-linked to itself, *Staphylococcus aureus (S. aureus)*, fibrin,

and collagen (Mosher *et al*, 1980) by both the 30kDa amino-terminal domain (Mosher *et al*, 1980) and the 29kDa carboxy-terminal plasmic digest fragment (Iwanaga *et al*, 1978) of Fn. Incorporation of Fn into the clot is due to factor XIIIa-catalysed covalent cross-linking between Fn and the Aα-chain of fibrin (Mosher, 1975). Fn in the ECM of cultured fibroblasts is also a substrate and can be cross-linked extensively by exogenous plasma transglutaminase (Keski-Oja *et al*, 1976). Other factor XIIIa substrates in clotting plasma are fibrin, α2-macroglobulin (Mosher, 1976a) and α2-plasmin inhibitor (Sakata and Aoki, 1980).

The concentration of Fn in serum is 20-50% less than that of plasma (Mosesson & Umfleet, 1970) and the difference is even greater if clotting is carried out at 0-4°C (Mosher, 1976b), as incorporation of Fn into the clot at lower temperatures is due to noncovalent binding as well as covalent crosslinking (Mosher 1976b). Fn will comprise 4.4% of the clot assuming that 35% of the plasma Fn is incorporated.

Fn which has been cross-linked to fibrin alters the properties of the clot. Fine clots (consisting of fine fibrils with branch points, formed at high ionic strength and pH), to which Fn is cross-linked at 22°C, have half the elastic modulus of cross-linked fine clots which lack Fn, whereas "coarse clots" (consisting of coarse fibrils with many branch points, formed at low pH and ionic strength), to which Fn is cross-linked at 22°C, have twice the elastic modulus of crosslinked coarse clots which lack Fn (Kamykowski *et al*, 1981). The thrombin clotting time of fibrinogen is inhibited by Fn, especially when the ratio of Fn to fibrinogen approaches 1:1 (Niewiarowski and Cierniewski, 1982). *In vitro*, cross-linking of Fn to fibrin enhances the attachment and spreading of invading fibroblasts, endothelial cells and macrophages on a fibrin-coated substrate (Grinell *et al*, 1980).

It is possible that Fn has a role in directing whether the activation of fibrinogen results in the formation of soluble fibrin monomers, crosslinked fibrin gel, or some other intermediate product. Fn may also function to regulate fibrin degradation. Under some conditions Fn may serve to maintain soluble fibrin monomers *in vivo*, both as a consequence of its effect on the physical properties of fibrin and through mediation of the rate of plg activation. Fn further functions as an opsonin (Saba *et al*, 1978) by enhancing the binding of fibrin microaggregates to activated monocytes for ingestion (Bevilaqua *et al*, 1981). Thus, once fibrinogen is activated *in vivo*, potential mechanisms exist for Fn to influence each subsequent event involving fibrin. Fibrin and Fn also have important interactions in the cellular

reactions involved in wound healing, and possibly in clot retraction. (Grinnell *et al*, 1980). Thrombin and serum are mitogenic and stimulate synthesis of Fn by fibroblasts (Mosher and Vaheri, 1978). Fn and its thrombin-cleaved fragments are chemotactic for fibroblasts (Postlethwaite *et al*, 1981).

The interaction of soluble Fn with substrate attached C-reactive protein (CRP) (Salonen *et al*, 1983) may link Fn in injured tissue. CRP is an acute phase reactant, synthesised by hepatocytes, which increases in plasma from trace levels up to 1000-fold in concentration during inflammatory reactions and tissue injury and is deposited in the lesions (Du Clos *et al*, 1981). Fn bound to CRP retains its ability to promote fibroblast anchorage and may act as a scaffold for ingrowing connective tissue cells during early stages in the repair process (Vaheri *et al*, 1983).

3.4 FIBRINOLYSIS

This is a physiological process closely linked to activation of the coagulation factors, in which insoluble fibrin deposits are removed by enzymatic cleavage into soluble fragments. The fibrinolytic system is activated by the local release of tissue plasminogen activator (tPA), or urokinase plasminogen activator (uPA) or streptokinase plasminogen activator, which converts the inert proenzyme, plasminogen, to plasmin. Plasmin is a protease which cleaves factor VIII, fibrinogen and fibrin to produce fibrinogen/fibrin degradation products (FDP). These products impair platelet aggregation.

Components of the fibrinolytic enzyme system include:

a) Plasminogen (plg)

Human plasminogen (plg) is a single chain glycoprotein with a molecular weight (MWT) of about 92 000 daltons and contains approximately 2% carbohydrate. Its concentration in plasma is approximately 1.5-2μM. The molecule consists of 790 amino acids with 24 disulphide bridges and five homologous triple loop structures or 'kringles' (Wiman, 1978). Native plg has NH_2-terminal glutamic acid (Glu-plg) and can be converted to modified forms such as Lys-plg by limited plasmic digestion.

Lys-plg is converted to plasmin by cleavage of a single Arg-Val peptide bond (Robbins *et al*, 1967) by both tPA and uPA. The two-chain plasmin molecule is composed of a heavy

chain or A-chain, originating from the N-terminal region of plg, and a light chain or B-chain constituting the C-terminal region (Groskopf *et al*, 1969). The active site of plasmin comprises histidine 602, asparagine 645, and serine 740 (Sottrup-Jensen *et al*, 1978). Activation of Glu-plg to plasmin by urokinase in purified systems is much slower than that of Lys-plg and occurs in the presence of inhibitors by cleavage of the Arg 560-Val 561 bond.

Plasminogen (plg) can specifically bind to fibrin through its lysine-binding sites (Wiman and Collen, 1978). These sites also mediate the interaction of plasmin or plg with α_2-antiplasmin.

b) Plasminogen activators (PA)

Endogenous PA can be immunochemically subdivided into two types: urokinase-type plasminogen activators (uPA), and tissue-type plasminogen activators (tPA).

Tissue-type plasminogen activator (tPA)

Tissue plasminogen activator (tPA) is synthesised mainly by vascular endothelial cells (Todd, 1984), where its concentration is greatest in small veins and in the renal vasculature (McConnell *et al*, 1966). tPA is also synthesised by smooth muscle cells and fibroblasts and is present in urine, milk, tears, saliva, bile and semen where it may play a physiologic role in maintaining the patency of excretory ducts (McNicol *et al*, 1961). tPA is normally present in the plasma in trace amounts, estimated at 5 to 10 μg/L (Collen and Lijnen, 1975) and is rapidly cleared from the circulation by the liver (Korninger *et al*, 1982). The half-life of human tPA has been estimated to be 2-15 min (Kluft, 1981).

tPA occurs either as a single-chain glycoprotein of 527 amino acids (MWT 70 000) (Pennica *et al*, 1983), or the molecule can be cleaved by plasmin at the centre to form a disulphide-bonded two-chain molecule, the chains being designated A and B (Rijken and Collen, 1981). Both forms are active serine proteases with similar enzymatic properties. The B-chain originates from the the C-terminal side of the molecule and is homologous to other serine proteases such as trypsin, prothrombin, elastase, plg and uPA (Banyai *et al*, 1983). In the A-chain, two regions with a high degree of sequence homology with the 'kringle' regions of plg, uPA and prothrombin are found, suggesting that tPA contains two kringles. A 43-residue long amino-terminal region, which has no counterpart in uPA, is homologous with the so-called finger domains of Fn which are responsible for the affinity

of Fn for fibrin (Banyai *et al*, 1983). Limited proteolysis of the N-terminal region of tPA leads to a loss of the fibrin affinity of tPA (Banyai *et al*, 1983). Although tPA is a glycoprotein, no evidence is available that the sugar moieties are essential for its enzymatic properties (Rijken, 1983).

Urokinase-type plasminogen activator (uPA)

One or more urokinase-type plasminogen activators (or pro-activators) is present in plasma. A discrepancy appears to exist between the plasma levels of uPA as determined immunologically, and as found functionally. Urokinase does not bind avidly to fibrin, presumably because it lacks the finger domain of tPA. It selectively activates free plg in the plasma by a mechanism that is independent of fibrin binding (Pannell and Gurewich, 1986) and appears to be responsible for fibrinogenolysis (Loskutoff and Levin, 1984).

Human urinary uPA occurs in three molecular forms: a single-chain proenzyme form (Wun *et al*, 1982), a high molecular weight, two-chain form (HMW-uPA) of 54 000 daltons, and a low molecular weight, two-chain form (LMW-uPA) of 33 000 daltons (Barlow *et al*, 1981). Both two-chain forms are linked by one disulphide bond (Barlow *et al*, 1981). The heavy (B) chain contains the active site (Pannell and Gurewich, 1986).

The B-chain of uPA shows sequence homology with the B-chain of tPA and of plasmin and with other trypsin-like proteases (Banyai *et al*, 1983). A domain in the A-chain of HMW-uPA shows homology with the kringle domains of plg and tPA. The N-terminal part of the A-chain shows homology with the N-terminal part of coagulation factor X and with epidermal growth factor (Gunzler *et al*, 1983).

c) Antiplasmins

At least five plasma proteins neutralise free plasmin. Two of these act very rapidly, namely, α2-macroglobulin and α2-antiplasmin. α2-Antiplasmin complexes with plasmin thereby diminishing plasmin binding to fibrin and is crosslinked to fibrin by factor XIIIa after thrombin formation during the common pathway , which contributes to its inhibitory effects (Tamaki and Aoki, 1982). α2-Antiplasmin also inactivates urokinase and inhibits fibrinolysis associated with transformed cells (Harpel, 1987). Studies have shown that plasmin does not react with α2-macroglobulin or α2-antitrypsin until the available α2-antiplasmin

is saturated (Sherman, 1977). This implies that α2-antiplasmin may be the most important component of the plasma antiplasmin system (Kaplan, 1978). In plasma the total antiplasmin activity normally exceeds the available plasmin by at least ten-fold (Harpel, 1987).

d) Antithrombin III

Antithrombin III (ATIII), an antiserine protease with a half-life of 48 hours, is a major inhibitor of coagulation. It forms complexes with thrombin by attachment of an arginine residue in ATIII to the active-site serine of thrombin (Matheson and Travis, 1976) to form a bimolecular complex which is rapidly cleared from the circulation by the hepatic cells (Shifman and Pizzo, 1982). The anticoagulant activity of ATIII is enhanced by heparin which may expose enzyme-binding sites on ATIII by binding to an ε-lysine residue (Rosenberg and Damus, 1973). ATIII inactivates factor Xa (Marciniak, 1973), factor IXa (Rosenberg, 1975), kallikrein (Vennerod *et al*, 1976) and several other enzymes with serine active sites (factor VII, XI, XII), in addition to thrombin (Rosenberg *et al*, 1973). The action of ATIII is regulated by platelet factor 2 and by a complement inhibitor, S-protein, which inhibits its activity (Jenne *et al*, 1985).

e) Plasminogen activator inhibitors (PAI)

The discovery of an inhibitor for tPA was made when addition of tPA to plasma samples was seen to result in the rapid disappearance of part of the tPA activity and in the formation of HMW complexes of tPA and inhibitor detectable by the SDS-PAGE/fibrin indicator gel method (Kruithof *et al*, 1983). Similar results were obtained with uPA. In both cases the molecular weights of the formed complexes differed from those of free activator by 40 000 - 50 000 daltons. PAI-1 has been isolated from many tissues including platelets (Erikson *et al*, 1985) and endothelium (van Mourik *et al*, 1984). In platelets, PAI-1 is stored and secreted from α-granules, a phenomenon that may stabilise haemostatic plugs (Sprenger and Kluft, 1987). The primary structure of PAI-1 suggests that it is a serine protease. Cytokines interleukin-1β (IL-1β)and tumour necrosis factor α (TNFα) diminish the secretion of tPA and increase the production of PAI-1 from endothelial cell cultures (Schleef *et al*, 1988). The neutralisation of PAI-1 by factor XIa may be an important mechanism by which contact activation triggers fibrinolysis (Berrettini *et al*, 1989). PAI-1 is sig-

nificantly less active when bound to fibrin. A different form of PA has been isolated from placenta as well as from monocytes, namely, PAI-2 (Sprenger and Kluft, 1987).

f) Mechanism of plasminogen activation

Fibrin deposition or some other closely related factor stimulates the vascular endothelial cells to secrete stored PA. The enzyme binds to the Aα chain of fibrin at a site, Aα-(148-160), that is exposed following thrombin cleavage of FPA (Lloyd *et al*, 1981). Small quantities of plg also bind specifically to the Aα chain of fibrin via the regulatory kringle $region_{1-3}$ of plg (Sottrup-Jensen *et al*, 1978). Thus, plg will be in close contact with PA, which very rapidly activates the bound plg to plasmin and initiates the process of fibrin degradation (Cederholm-Williams and Fennell, 1981). This interaction with fibrin greatly accelerates the rate of plg activation. Fibrin-bound plasmin is protected from inactivation by α_2-antiplasmin, by blocking of the antiplasmin binding site. Once plasmin is dissociated from fibrin it is immediately inhibited, protecting other plasma proteins from plasminolysis (Wiman *et al*, 1979).

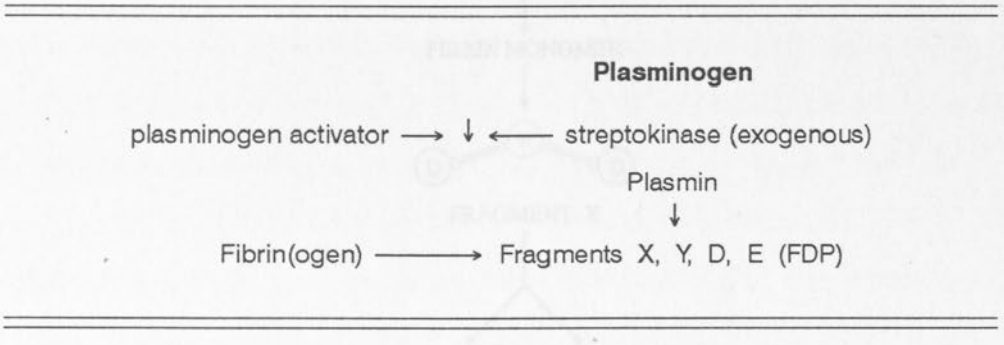


Figure 3.3 - Diagrammatic representation of fibrinolysis

Plasmin cleaves fibrinogen and fibrin at specific peptide bonds. The Aα chain of fibrinogen has 11 plasmin cleavage sites, four in the α helical section at the amino-terminus which also contains the thrombin cleavage site, four in the interzonal stretch which has four proline residues, and a net positive charge which may be a factor for its sensitivity to plasmin, and three at the carboxy-terminal region (Doolittle *et al*, 1979). The first cut, which forms fragment X, is relatively major, and consists of the removal of the Aα-chain tails that are not part of the D-domains (Marder *et al*, 1969). Fragment X (Figure 3.3) is able to clot, but more slowly than native fibrinogen.

The next cleavage is the scission of all three polypeptide chains, Aα, Bβ and γ, in one connector region (coiled-coil), where several lysine-arginine bonds (Nussenzweig *et al*, 1961) are broken to produce fragments D and Y (Marder *et al*, 1969) (Figure 3.4). The former corresponds approximately to one D-domain, the latter to one E- and one D-domain held together by the remaining connector. The last major cut produces fragments D and E from fragment Y. Additional cuts trim these fragments to smaller sizes that depend on solvent conditions. Approximate molecular weights of the fragments produced by plasmin digestion in the presence of calcium are: Fragment X 250 000, Y 150 000, D 90 000 to 100 000, E 52 000 (Mihalyi, 1980) (Figure 3.4). Prolonged lysis of crosslinked fibrin produces one additional major fragment, γ-crosslinked D-dimer (Kopec *et al*, 1973). Fragment E binds to D-dimer with high affinity to form a D-dimer-E complex (Gaffney *et al*, 1975).

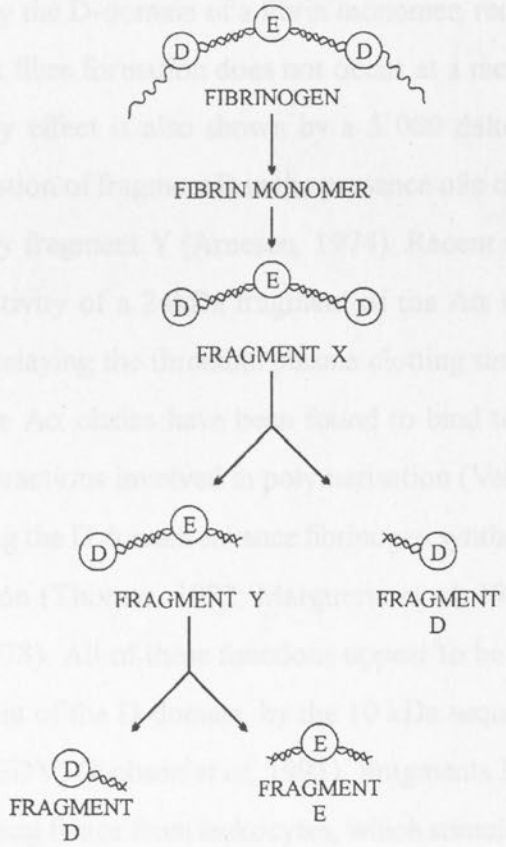


Figure 3.4 - Proposed pathways of the plasminolysis of human fibrinogen. (Redrawn from Marder *et al*, 1969).

3.5 BIOLOGICAL EFFECTS OF FIBRINOGEN DEGRADATION PRODUCTS

Fibrin(ogen) degradation products (FDP) and their complexes impair the haemostatic process and are thought to contribute to haemorrhage in intravascular coagulation and fibrinogenolysis. They also function as antithrombins, and form complexes with fibrin monomer or fibrinogen. Addition of early plasmin digests to a fibrinogen solution leads to inhibition of clotting, with both the rate of clotting and clot rigidity being reduced. No clotting is observed if the ratio of fragments to intact fibrinogen is sufficiently large. More extensive digests are also effective (Marder and Shulman, 1969). Of the two major fragments in the terminal digest, fragment D, was apparently found to carry the major anticoagulant potential (Larrieu *et al*, 1972). The mechanism of inhibition of fibrin formation by fragment D occurs at the second step, that is, the polymerisation of monomeric fibrin to protofibrils (Hermans and McDonagh, 1982). Fragment D blocks the polymerisation site that could be occupied by the D-domain of a fibrin monomer, reducing the average length of the protofibrils so that fibre formation does not occur at a measurable rate (Fletcher *et al*, 1966). This inhibitory effect is also shown by a 5 000 dalton γ-chain peptide (D1), obtained by plasmin digestion of fragment D in the presence of a chelating agent (Olexa and Budzynski, 1981), and by fragment Y (Arnesen, 1974). Recent studies have revealed the specific anticoagulant activity of a 24kDa fragment of the Aα chain which affects fibrin monomer formation by delaying the thrombin plasma clotting time (Lau, 1993). Carboxy-terminal fragments of the Aα chains have been found to bind to, and compete with, the normal AαC domain interactions involved in polymerisation (Veklich *et al*, 1993).

Fragments containing the D domain enhance fibrinogen synthesis (Kalvaria *et al*, 1986), inhibit platelet aggregation (Thomas, 1972; Marguerie *et al*, 1982) and mediate calcium binding (Purves *et al*, 1978). All of these functions appear to be mediated by an intact C-terminal end of the γ-chain of the D-domain, by the 10 kDa sequence containing Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) (Robson *et al*, 1993). Fragments D and E effect the release of an hepatocyte-stimulating factor from leukocytes, which stimulates the synthesis of acute phase proteins, including fibrinogen and modulate leukocyte microbicidal activity (Doolittle, 1984). Fragment DD may inhibit lymphocyte activation by binding to CD11b/CD18 on adherent monocytes and thereby modulate inflammatory processes associated with disseminated intravascular coagulation (DIC) (Robson *et al*, 1993). Purified fragment E is a

potent inhibitor of thrombin (Arnesen, 1974). Fibrin monomers may accelerate coagulation (Hansen *et al*, 1975).

Small dialysable FDPs may act as vasoconstrictors (Osbahr and Custodio, 1975), have chemotactic properties (Senior *et al*, 1986), may increase vascular permeability (Sueishi *et al*, 1981) and may inhibit contractility of uterine muscle (Kumar Basu, 1969).

3.6 HOMEOSTATIC CONTROL MECHANISMS

Control mechanisms are necessary to limit the size of the fibrin clot and to neutralise active procoagulants that may enter the general circulation. These mechanisms operate at three different levels: locally in the haemostatic plug, in the circulation, and in various cellular clearance mechanisms residing in the reticulo-endothelial system (RES), the liver and the lung.

Local processes	Washout and dilution Restriction of coagulation to platelet membrane "Interiorisation" by fibrin Antithrombin
Humoral processes	Self-damping processes 1. Consumption of coagulation factors 2. Factor XII fragmentation 3. Cleavage of HMWK by factor Xa 4. Formation of two-chain factor VII 5. Polymerisation of factor V subunits
Physiological inhibitors of coagulation	1. Antiproteases a. Antithrombin III b. Heparin cofactor II c. α2-Macroglobulin d. α1-Proteinase inhibitor e. C'-1 inhibitor f. Plasminogen activator inhibitors g. α2-Antiplasmin h. Activated protein C inhibitor 2. Miscellaneous coagulation inhibitors
Cellular Processes	Reticuloendothelial clearance (of prothrombinase complexes, Xa, tissue factor, FDP) Hepatocellular clearance (of factors IXa, Xa and others) Liver cell-associated inhibitors (of thrombin, and factor Xa) Pulmonary vascular bed (retention of particulate fibrin, FDP, tissue factor) Phagocytosis or specific binding by leukocytes and macrophages (of fibrin, FDP, other particulate debris) Cell associated proteases Fibrinolytic enzyme system.

Table 3.1 - Homeostatic mechanisms that regulate blood coagulation

3.7 COAGULATION AND FIBRINOLYSIS IN NORMAL PREGNANCY

The structural modification of the spiral arteries during pregnancy increases blood flow to the feto-placental unit (Stirling, 1984). The subsequent development of a low level of intravascular coagulation in the placental area is compatible with the finding of fibrin in normal placental vessels. A physiological role for the increase in coagulation and the decrease in systemic fibrinolysis during pregnancy may be in dealing with bleeding during abortion or following delivery. Raised levels of clotting factors are associated with an increased risk of arterial and venous thrombosis and may explain the increased incidence of thrombo-embolism in pregnancy and the puerperium (Stirling, 1984).

The effect of pregnancy on the coagulation system has similarities to a low level of compensated DIC confined to the placental area (Bonnar *et al*, 1990; Bern, 1990; Stirling, 1984). Circulating coagulation factors which occur in increased levels are listed in Table 3.2. The increases can be partly explained by hormonal effects. Increased activation of the clotting system may occur as a response to the fetus. The placenta may be a source of TF, with consequent thrombin generation via the extrinsic pathway. There is evidence that thrombin activates factor V and factor VIIIC which are then inactivated by proteolytic degradation. This could explain the increasing factor VIIIR:Ag/VIIIC ratio. Activation of factor VII by placental phospholipid and/or complex formation (tissue factor-factor VII-factor Xa) could account for the elevation of both factor VII and factor X (Kasper *et al*, 1964).

Inhibitors of fibrinolysis may also be important. While α1 antiproteinase, an acute phase reactant, is increased during pregnancy the other inhibitors, such as α2 macroglobulin (which act as a defense mechanism by inhibiting factor Xa, thrombin and activated factors) may remain largely unchanged. Levels of antithrombin III and C'-1 inhibitor may decrease during normal pregnancy (Gjonnaess and Fagerhol, 1975).

Low levels of intravascular coagulation confined to the placental area can be detected as early as 11-15 weeks (Bonnar *et al*, 1990). The appearance of high molecular weight fibrinogen/fibrin complexes by the second month of pregnancy indicates the presence of uteroplacental fibrin formation. Fibrinolytic activity declines steadily during pregnancy, with a slight increase in FDPs from about 21 - 25 weeks, and an increase after delivery. It is reasonable to assume that the increased levels of fibrinogen, thrombin generation and inhi-

bition of fibrinolysis are part of a mechanism to ensure the integrity of vessels, for example, in placental areas (Stirling, 1984). Levels of plg closely parallel those of fibrinogen (Bonnar *et al*, 1990). The sudden increase in fibrinolysis on delivery supports the idea that inhibition is mediated by the placenta. The further increase of FVIIIRag:FVIIIC, fibrinogen and α1 antitrypsin after delivery is probably due to an acute phase reaction similar to that seen post-operatively (Stirling, 1984).

Increased levels	**Decreased levels**
fibrinogen x2 (20 wks)	factor XI 70% normal
factor VII x4	factor XIII 70% normal
factor VIIIC (peak-3rd trimester)	factor II
factor VIIIR:ag "	AT III activity (sl)
factor X	
FDPs (slight)	
fibrinfibrinopeptide A (slight)	
β-thromboglobulin (slight 3rd trimester)	
platelet factor 4 (slight 3rd trimester)	

Table 3.2 - Coagulation factors in normal pregnancy. (Corash, 1986)

3.7.1 Effects of labor and delivery on haemostatic variables.

There may be a low-grade DIC associated with normal delivery with slight decreases in fibrinogen, Factor VIII and Factor XII levels and slight increases in fibrinogen related antigen. Platelet count may drop substantially (Yoshimura et al, 1992). Fibrinopeptide A (FPA) and thrombin-antithrombin (TAT) III complexes show no changes during labour, but after delivery levels may increase significantly. Alpha$_2$-antiplasmin levels may drop due to neutralisation of plasmin, although alpha$_2$-plasmin inhibitor-plasmin complexes may show significant increases after delivery (Yoshimura et al, 1992).

Activation of the maternal fibrinolytic system during labour is indicated by increased tPA antigen (Bremer et al, 1994; Yoshimura et al, 1992). After delivery of the placenta a marked further increase in fibrinolytic potential has been demonstrated with a significant rise in tPA antigen and activity levels accompanied by a fall in PAI-1 antigen and activity levels (Bremer et al, 1994).Findings suggest that activation of the fibrinolytic system begins during labour before pla-

cental separation to compensate for the hypercoagulable state which develops at the time of delivery when activation of the thrombotic system occurs (Yoshimura et al, 1992).

Separation of the placenta results in a return to normal of the haemostatic system by four weeks post partum with the loss of the placental source of PAI-2 (Wiman et al, 1984); contact system activation and platelet consumption occur immediately after delivery, then an increase in fibrinogen, Factor VIIIC and platelet count shortly afterwards (Bonnar et al, 1970; Hellgren and Blomback, 1981).

3.8 COAGULOPATHY IN PREECLAMPSIA

Disseminated intravascular coagulopathy (DIC) is a pathological process in which a generalised activation of the haemostatic system occurs, causing widespread fibrin deposition and subsequent lysis within the vascular system, leading to microthrombosis and consumption of platelets and clotting factors, including fibrinogen. This may result in organ dysfunction (esp. kidneys, lungs and liver), haemorrhage and a haemolytic anaemia due to RBC fragmentation in the microvasculature (Hathaway, 1989)

DIC in preeclampsia and eclampsia is associated with endothelial injury, platelet activation and activation of the blood clotting and fibrinolytic systems. A hypercoagulable state is reflected by the presence of fibrin deposits in the microvasculature and platelet/fibrin thrombi in the liver, spleen, brain, adrenal glands, lungs, heart and kidney (Page, 1972). This may lead to obstruction of the vessels and distal tissue necrosis. Thrombus formation may be followed by a haemorrhagic phase in which there is consumption of clotting factors and widespread conversion of plg to plasmin by tPA or uPA, with digestion of fibrinogen and fibrin and the liberation of FDP into the circulation (Jacobs and Bird, 1983).

Thrombocytopaenia with prolonged bleeding and thrombin times, haemolysis and platelet activation are all associated with preeclampsia (Perry and Martin, 1992). The intrinsic pathway may be activated in preeclampsia, with changes in activities of a number of circulating clotting factors. Endothelial injury could facilitate the activation of factor XII due to exposure of the extracellular matrix (Perry and Martin, 1992).

3.8.1 HELLP syndrome

Haemolysis, elevated liver enzymes and low platelet counts, abbreviated as HELLP syndrome, occurs in severe forms of preeclampsia. DIC is found in all these patients as well as in

some preeclamptic patients without the HELLP syndrome (De Boer *et al*, 1991). Thrombocytopenia occurs in approximately 15% of preeclamptic or eclamptic patients with or without coagulopathy. The mechanism responsible is unknown, but possibilities include increased platelet aggregation or agglutination, immune mechanisms, or thrombin activity (Perry and Martin, 1992). In one study where 50% of preeclamptic patients had low platelet counts, platelet dysfunction was reflected by increased bleeding times and a decrease in thromboxane A_2 (TxA_2) synthesis.

3.8.2 Assessment of disseminated intravascular coagulopathy

The presence of DIC is assessed by specific tests including reduced platelet counts, alteration in prothrombin time, activated partial thromboplastin time, and/or bleeding time and measurement of fibrin monomer and D-dimer which will be present at the same time due to simultaneous coagulation and fibrinolysis occurring with DIC (Kruskal *et al*, 1987).

These conventional tests for DIC are observed in less than 20% of patients with preeclampsia or eclampsia (Perry and Martin, 1992). However, the presence of low grade DIC can now be assessed by tests for prothrombin complex, fibrinogen, factorV, FVIII, vWf, FXII, plg, antiplasmin, antithrombin III, kallikrein-inhibiting activity and spontaneous proteolytic activity (Brodin *et al*, 1985), FPA and FPB, D-dimer and other FDPs which reflect activation of the coagulation system as well as factors released by the α-granules of activated platelets. Immunological techniques using specific monoclonal antibodies are sensitive enough to measure nanomolar amounts of D-dimer in serum (Kruskal *et al*, 1987), while D-dimer detected by latex agglutination assay with *S. Aureus* gives only a qualitative or semi-quantitative result, and is acceptable only as a screening technique. Latex agglutination assays for FDPs show a decrease in sensitivity during progressive digestion of fibrinogen by plasmin (Marder *et al*, 1971). An assay has now been developed which is capable of determining the proportions of crosslinked and non-crosslinked fibrin and fibrinogen derivatives in clinical samples (Kruskal *et al*, 1987).

Increased levels	Decreased levels
Intrinsic pathway activity	Fibrinogen
Fibrin degradation products	Prothrombin
D-dimer positive	Antithrombin III
Fibrinopeptide A	Factor XII
Fibronectin	Platelets
Factor VIII Ag:activity	FVIIIC:FVIIIRag
Thrombin:antithrombin III complexes	
β-thromboglobulin	
Platelet aggregation	

Table 3.3 - Changes that imply hypercoagulation in preeclampsia
(from Perry and Martin, 1992)

a) Fibrinogen and related peptides

FPA and FPB, released by thrombin activity in the formation of fibrin monomers during coagulation, fibrin or fibrinogen degradation products of plasminolysis, namely fragments X, Y, D and E, and D-dimer from crosslinked fibrin, can be measured in patient plasma (Kruskal *et al*, 1987). An elevation in any of these products is considered to reflect thrombin generation and the conversion of fibrinogen into fibrin (Perry and Martin, 1992). The simultaneous occurrence of FPA and FPB with products of lysis is indicative of DIC. Products of fibrin degradation as measured by conventional techniques represent the sum of all the products of fibrin and fibrinogen breakdown, at least 15 of which have been identified (Graeff and Hafter, 1982; Francis and Marder, 1982).

Fibrinogen levels are significantly reduced in severe preeclampsia as compared to normal pregnancy levels (Thorburn *et al*, 1982) where fibrinogen levels may be increased as part of an acute phase response, especially at delivery. FDPs, especially the cross-linked X oligomer (Gaffney *et al*, 1988) and FPA levels have been shown to be raised in preeclampsia, especially in severe cases, where raised levels persisted for 3-7 days postpartum (Borok *et al*, 1984; Weiner *et al*, 1983). FPA has a half-life of 5 minutes and is therefore a very sensitive marker of thrombin generation *in vivo*. As fibrinogen has a half-life of 3-6 days, this suggests abrupt cessation of fibrinogen production at delivery, with a gradual resolution of DIC as the preeclamptic condition abates.

b) D-dimer

D-dimer levels indicate the extent of cross-linked fibrin breakdown and may be a useful screening test for coagulopathy in preeclamptic patients (Perry and Martin, 1992). The presence of D-dimer (half-life = 8 hours, Franks *et al*, 1984) detected with a monoclonal antibody was found to correlate with elevated FDPs and low platelets (<100 000/μl) in preeclamptic patients (Trofatter *et al*, 1989). Two thirds of patients without D-dimer had lower FDPs (<10mg/dl) and 80% had platelets >100 000/μl. D-dimer positive patients had higher blood pressure, greater proteinuria and more abnormal liver function tests than D-dimer negative patients. Raised D-dimer levels in preeclamptic patients have also been reported by Ballegeer *et al* (1988). It is possible that FDPs may not originate at localised sites of DIC, but from sites of fibrin deposition (Kruskal *et al*, 1987), which must be taken into account when assessing the presence of active clot formation.

c) Factor VIII Ag:Activity Ratio

Endothelial damage in preeclampsia may result in an increased factor VIII ratio due to increased release of factor VIII antigen, especially in severe preeclampsia (Thorton, 1977). FVIIIAg refers to the antigenic expression of FVIIIC which is measured in immunoassays with homologous antibodies. This may reflect increased thrombin generation. The increased factor VIIIAg:activity ratio precedes the decrease in antithrombin III levels in patients destined to have preeclampsia (Pekonen *et al*, 1986).

d) Antithrombin III

A decrease in circulating antithrombin III indicates binding or consumption secondary to thrombin production. Antithrombin III levels are low in preeclampsia and eclampsia (Weiner *et al*, 1982; Weenink *et al*, 1983) and may be associated with severity of disease and maternal morbidity (Saleh *et al*, 1987). An increase in circulating thrombin:antithrombin III complexes is indicative of increased thrombin production. Complexes occur in normal pregnancy (de Boer *et al*, 1989), preeclampsia (Kobayashi *et al*, 1987) and pregnancy complicated with hypertension (Reinthaller *et al*, 1990), but levels are significantly higher in preeclampsia (Kobayashi *et al*, 1987), possibly due to the intravascular coagulation associated with preeclampsia.

Significant correlations have been found between antithrombin III activity and platelet count, and FPA (Kobayashi *et al*, 1987; Weiner *et al*, 1988), but not between platelets and FPA. It is possible that antithrombin III consumption by fibrin generation is initiated by platelet activation, and is one cause of low antithrombin III activation in preeclampsia (Weiner *et al*, 1988). However, antithrombin III and α2-antiplasmin correlate less with platelet activation in preeclampsia than with plasma Fn levels (Saleh *et al*, 1992).

Women with renal histological evidence of gestational proteinuric hypertension had lower antithrombin III activation than those without histological evidence. This suggests a high frequency of underlying renal disease in women with early onset gestational proteinuric hypertension (delivery before 34 weeks gestational age) (Weiner, 1990).

e) Platelet-associated IgG and IgM

An increase in platelet-associated IgG that correlates inversely with severity of disease has been shown, suggesting underlying immune mechanisms, or possibly damaged platelets (Burrows *et al*, 1987). FPA levels were normal in nulliparous patients, therefore thrombin activity is not responsible.

In a similar study platelet-bound and circulating platelet-bindable immune globulin (IgG) and complement (C3) were assayed. There was a high frequency of platelet IgG and C3 in women with preeclampsia and their neonates. The patients with abnormal platelet antiglobulin levels had normal levels 6 weeks postpartum (Samuels *et al*, 1987). These may be autoreactive antibodies, externalised IgG from platelet α-granules or immune complexes formed with antigen released from the placenta (Perry and Martin, 1992).

Neonates of preeclamptic mothers may have thrombocytopenia (Pritchard *et al*, 1987), neutropenia, oedema, proteinuria (Mirro *et al*, 1982) and some abnormal clotting parameters associated with abnormal liver function (Lox *et al*, 1985). The thrombocytopenia does not correlate with the severity of thrombocytopenia in the mother. These findings indicate that either a causal factor in plasma of preeclamptic mothers crosses a normal placenta and initiates an immune response, or that preeclampsia damages the placenta causing a breakdown in selective protein transfer. There were increased levels of IgG in platelets of these neonates which also bound increased amounts of anti-IgM. Damage to placental vessels could allow transplacental passage of maternal IgM or IgG which may initiate IgM production by the fetus.

These results show that the placental surface is altered in women with preeclampsia and their neonates, even in the absence of bleeding (Samuels, 1987). The haemostatic abnormalities in the neonate have little clinical significance as offspring of preeclamptic mothers do not suffer from bleeding disorders other than those associated with prematurity (Perry *et al*, 1990).

f) Platelet activation

Activation of platelets has been proposed as a major contributor to the development of DIC in preeclampsia (Roberts *et al*, 1989). Markers of activation noted include decreased platelet counts, increased platelet aggregation, increased release of β-thromboglobulin (Lieberman *et al*, 1988), platelet factor 4 and a shorter platelet half-life (Inglis *et al*, 1982; Saleh *et al*, 1992). The younger platelet population is indicated by the presence of megathrombocytes and a trend to increased mean platelet volume in those who progress to severe disease (Samuels *et al*, 1987). The activation may be localised to the placental bed (Perry and Martin, 1992).

One conflicting study showed low platelet counts, increased platelet aggregation and circulating aggregates in pregnant women as compared to non-pregnant women, but there were no observed differences in these tests between normal pregnant women and patients with preeclampsia or chronic hypertension, suggesting that platelet dysfunction may not be increased in preeclampsia (O'Brien *et al*, 1986).

In a study of third trimester patients and controls platelet β-thromboglobulin levels were raised in preeclampsia and in chronic hypertension with preeclampsia, most likely due to platelet response rather than consumption, as platelet aggregation ratios were normal when assessed by the amount of collagen required for half-maximal aggregation (Socol *et al*, 1985). β-thromboglobulin levels in the preeclampsia group alone correlated inversely with 24 hour urinary protein, serum creatinine and creatinine clearance, but not with blood pressure (Socol *et al*, 1985). This suggests that altered renal function contributes to the rise in β-thromboglobulin in preeclampsia. Aggregates of degranulated platelets are associated with glomerular endotheliosis in the kidney. The levels of platelet factor 4 and β-thromboglobulin released during platelet aggregation show a correlation, although β-thromboglobulin has a half-life of 100 minutes, while platelet factor 4 is cleared very rapidly by the renal glomeruli, possibly by adsorption to endothelial cells (Socol *et al*, 1985).

tion to endothelial cells (Socol *et al*, 1985).

Platelet activating factor (PAF) was measured by a receptor-mediated platelet aggregation mechanism with release of platelet contents (Benedetto *et al*, 1989). There was a decrease in the inhibition of PAF activity in the serum of preeclamptic patients. This defect in serum inhibitory potential may contribute to platelet activation in these patients.

g) Platelet-associated fibronectin (Fn)

Microvascular occlusion is associated with Fn release and subsequent adhesion of platelets to sites of endothelial injury. Platelets must be activated before expressing receptors for Fn which may accelerate the interaction of platelets with vessel wall components (Samuels *et al*, 1991).

I^{125}-FnAb binding to platelets was significantly higher in patients with preeclampsia than in normal pregnant women or idiopathic thrombocytic puerpura (ITP) patients. Plasma from the preeclamptic patients caused significant binding of Fn to platelets as well. (Samuels *et al*, 1991).

3.8.3 The fibrinolytic system in preeclampsia

Fibrin deposition is a common feature in preeclampsia, which suggests that activation of the fibrinolytic system may occur to dispose of the fibrin. However, there is conflicting data on markers of fibrinolysis. Gow *et al* (1984) showed no difference in mean fibrinolytic activity or inhibition of uPA between normal pregnant and preeclamptic women. Most reports indicate that plg levels are raised in pregnancy, but the balance of plg activators and inhibitors may be altered in preeclampsia (Bonnar *et al*, 1990).

tPA may be released secondary to endothelial damage in preeclampsia, although placentae from preeclampsia patients have reduced tPA activity compared to normal (Yoshimura *et al*, 1985). In normal pregnancy PAI-1 and PAI-2 are increased, while in severe preeclampsia PAI-1 levels are raised but PAI-2 (a placental PA inhibitor localised in the trophoblastic epithelium) is decreased (Estelles *et al*, 1989). Increased PAI-1 levels are associated with decreased fibrinolytic activity (de Boer *et al*, 1988).

Deposition of fibrin and formation of thrombi in the placenta in preeclampsia may be due to defects in the fibrinolytic system. PAI-1 antigen levels and activity are elevated in

plasma, serum and platelet lysate, and PAI-1 levels correlate with the presence of placental infarcts and may be associated with severity of placental damage (Estelles *et al*, 1989). The reduced levels of PAI-2 may be a marker of this impaired placental function in preeclampsia. Cord blood tPA and PAI levels are also higher from neonates of mothers with preeclampsia than from normal pregnant women (Estelles *et al*, 1989). Platelets, as well as placental endothelium contribute to the increased PAI-1 levels (Gilabert *et al*, 1990), although the source of the total high PAI activity in preeclampsia is uncertain (Estelles *et al*, 1989). Active and latent PAI-1 from platelets (Sprengers *et al*, 1986) may explain the higher ratios between PAI-1 Ag and net PAI activity in severe preeclampsia.

In preeclampsia poor fetal outcome on APGAR scores correlates positively with low PAI-2. Low PAI-2 correlates directly with low birth weight and placental weight, but PAI-2 does not correlate with ATIII. There is a negative correlation between birth weight as well as placental weight to total PAI activity and PAI-1 Ag (Estelles *et al*, 1989).

3.8.4 Treatment

Efforts to arrest the progressive coagulation changes in preeclampsia have had limited success. A trial of antithrombin III treatment was performed with 27 patients treated for 7 days. DIC was normalised and there was good fetal outcome (Buller *et al*, 1980).

Plasma exchange with fresh frozen plasma was used 10 days postpartum in patients with HELLP syndrome in whom coagulation abnormalities persisted, possibly due to residual placenta in the uterus or trophoblasts in the lungs. Three exchanges of 3 litres each were required to produce a sustained increase in platelet count for 72 hours, and the treatment is recommended for selected patients with this unusual problem (Martin *et al*, 1990).

Attempts have been made to alleviate the haemostatic abnormalities in patients with preeclampsia with antiplatelet agents such as aspirin. The incidence of preeclampsia was thought to decrease with low-dose aspirin treatment of nulliparous women, primarily through its effect in those who have initially elevated systolic blood pressure (Sibai et al, 1993). However, these effects were overestimated as shown by the CLASP study of 9364 pregnant women. No beneficial effect of aspirin treatment in lowering the incidence of preeclampsia was shown (CLASP, 1994).

3.9 CONCLUSIONS

A degree of activation of coagulation with thrombin-mediated fibrin generation and possibly increased compensatory fibrinolysis appears to be a physiologic feature of pregnancy, although some changes are very subtle. The concentration of coagulation factors may be influenced by the severity of the plasma volume contraction which occurs in preeclamptic patients.

The commonest haemostatic abnormalities in preeclampsia are a rise in FVIIIR Antigen and low antithrombin III (ATIII) concentration. In severe cases thrombin action may be markedly increased. However, the mechanism designed to limit the spread of haemostatic reactions, namely ATIII, protein C and the fibrinolytic system, appear to be rarely overwhelmed and DIC is an exception in preeclampsia, and even in eclampsia (Wallenburg, 1989).

Episodes of platelet activation and consumption are rather common in patients with preeclampsia; activation is not thrombin-, but rather surface-mediated. Anticoagulant therapy has not been shown to be beneficial, and the side effects in pregnancy can be dangerous. (Wallenburg, 1989).

There have been many attempts to identify laboratory testable markers that would reliably predict the clinical appearance of preeclampsia and eclampsia later in gestation. In a study of 400 primigravidae at 28 weeks gestation tests for haematological parameters including partial thromboplastin time, fibrin(ogen) degration products (FDP), platelet factor 4, β-thromboglobulin and fibrinopeptide A (FPA) were performed. However, none of these factors were predictive (Thornton *et al*, 1989). From this review it appears that uric acid levels (Redman *et al*, 1976), platelet calcium (Zemel, 1990) and fibronectin (Fn) levels (Lockwood *et al*, 1990; Eriksen *et al*, 1987) are possible, early predictors of preeclampsia/eclampsia, with raised platelet calcium flux and cellular fibronectin (cFn) levels occurring in the first trimester.

The question of predictive tests and their associated problems is addressed in the second chapter of the experimental section of this thesis. A number of factors were assessed including fibrin(ogen) degradation products (FDP), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and fibronectin (Fn). Emphasis was placed on the use of these tests to distinguish between those patients with stable disease and those with progressive disease.

CHAPTER 4 - LITERATURE REVIEW

FIBRONECTIN - STRUCTURE AND FUNCTIONS

4.1 INTRODUCTION

The name fibronectin derives from the Latin *fibra* (fibre) and *nectere* (to bind or connect) and reflects the roles of this protein (Ruoslahti *et al*, 1982). Fibronectin (Fn) was initially described as cold insoluble globulin (CIg) (Morrison, 1948) due to the observation that it precipitates (with or without heparin) at 4°C (but not at room temperature) with fibrinogen and fibrin to form a component of cryoprecipitate. CIg appears to act as a nucleus with multiple binding sites for fibrin-fibrinogen complexes (Stathakis *et al*, 1978).

Fn binds tightly but not covalently to cell membrane receptors and requires denaturing solvents with high urea concentrations for extraction. It is released from the cell by several proteases including trypsin, chymotrypsin, plasmin, papain, thermolysin, subtilisin, cathepsin D or G and elastase (Chen *et al*, 1977). Multiple molecular forms of the molecule are present in plasma.

Fn molecules can assemble into fibrils, bind to cells and link cells to other kinds of fibrils in the extracellular matrix (ECM) (Mosher, 1984). Fn is also involved in the internal organisation of cells through interactions with actin. Fn deposition regulates highly organised processes such as cell migration during embryonic development, wound healing, tissue regeneration and mitogenesis, as well as anchoring of cells to basement membranes. The adhesive characteristics of Fn make it a crucial component of blood coagulative processes (Hynes, 1986). Fn added to cultures of tumour cells produces a marked improvement in cell adhesion and cytoskeletal organisation, and stimulates migration of the cells (Yamada *et al*, 1976; Ali *et al*, 1977). Fn binds specifically to collagen, heparin, fibrin and *Staphylococcus aureus (S. aureus)* and has opsonic properties (D'Ardenne and McGee, 1984).

These discoveries of the multifunctional nature of Fn have led to extensive investigations into the structure of the molecule and in defining the functions of its different domains. Many of the biological roles of intact Fn are apparently shared by its proteolytic fragments, which also seem to have effects of their own (Vartio and Vaheri, 1983).

4.1.1 Gene transcription and molecular structure

Fn occurs in a soluble form in plasma (in high concentrations of approximately 300ug/ml) (Mosher, 1984) and other body fluids such as amniotic fluid, as well as in an insoluble structural form in the pericellular connective tissue matrix in the subendothelial tissues of blood vessels and smooth muscle cells, in stroma of lymphatic tissue, in basement membranes and at epithelial and endothelial cell surfaces such as perisinusoidal tissue of the liver and glomerular mesangium (Vartio and Vaheri, 1983; Mosher, 1984).

The structure of Fn was originally deduced from the plasma form which is easily purified (Vartio and Vaheri, 1983). It has been characterised as a large 440-450kDa, dimeric glycoprotein which has two large polypeptide chains of approximately 220-240kDa linked by disulphide bonds at the carboxy-terminus. Peptide mapping initially revealed no differences between the two chains as separated by SDS-PAGE gels (Ruoslahti *et al*, 1982). The DNA sequences of bovine, rat and human Fn are similar and have been used to deduce the amino acid sequence. When analysed by electron microscopy the molecule looks like a "V". Each polypeptide chain is 60nm in length, and although the two chains usually come together at an angle of approximately 70°, they are flexible and can fold back on themselves to form a fairly compact molecule (Mosher, 1984) (Figure 4.1).

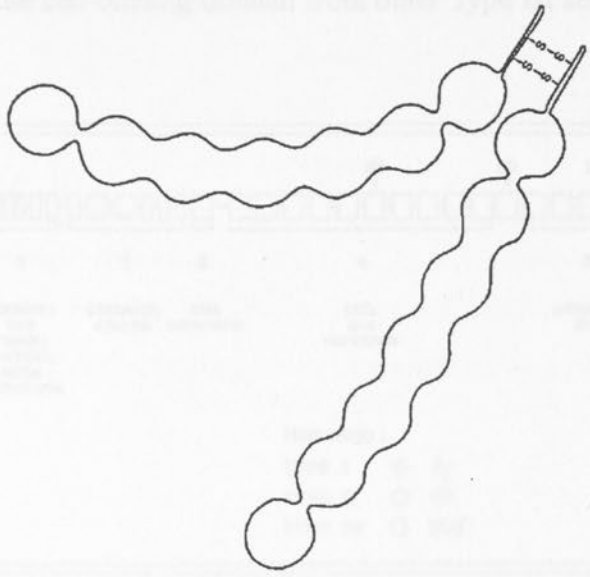


Figure 4.1 - A cartoon depicting the two subunits of the fibronectin molecule joined by a pair of disulphide bonds (S-S). (Redrawn from Hynes, 1985)

Proteolytic degradation of Fn demonstrates that each subunit consists of a number of stable domains, each capable of different binding activities. The Fn subunits are approximately 2000 amino acid residues long and associate to form a modular protein made up of short amino acid sequences repeated many times. The sequences or homologous repeats are of three general types (first described by Petersen, Skorstengaard and colleagues, 1983), Type I,II and III, and each of the structural and functional domains of Fn contain one or more of the specific repeats (Hynes, 1985). Each functional module is encoded by a single exon. The Fn gene, localised on chromosome 2, is close to 50 kilobases long and comprises 48 exons of constant size, each coding for 45-50 amino acids. The repeating structure confers multivalency and potential for high and low affinity binding (Hynes, 1985).

There are 12 Type I homologies, 25-50% identical and all with the same pattern of cysteine residues, except that the twelfth repeat contains two extra cysteines (Hynes, 1985). It is assumed that all Type I homologies show the same disulphide-bonding pattern, between cys1 and cys 3, and cys 2 and cys 4 (Petersen *et al*, 1983). The 2 Type II repeats also have disulphide bonds between cys 1 and 3, and cys 2 and 4, but the cysteine residues are evenly spaced. The Type III homologies are about 90 amino acids long, each encoded by two exons, and have no disulphide bonds (Hynes, 1985). The sequences of Types I-III can vary somewhat, eg. the amino acid sequence Arg-Gly-Asp-Ser (RGDS) distinguishes the type III sequence of the cell-binding domain from other Type III sequences (Figure 4.2).

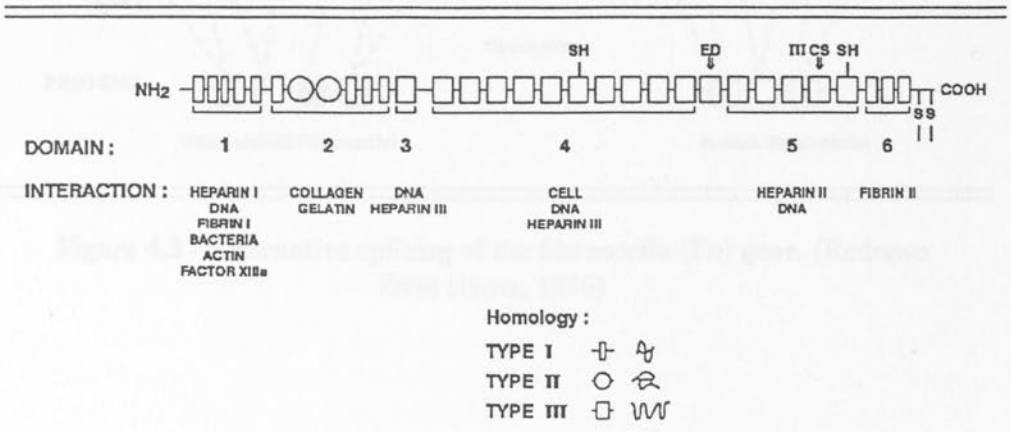


Figure 4.2 - Schematic representation of the domain structure of fibronectin. ED and IIICS represent two known regions of variability due to alternative splicing of mRNA precursors.
(Redrawn from Carnemolla *et al*, 1987).

Although the exons making up the Fn gene correspond directly to functional units in the protein, not all of them are expressed in every Fn molecule. The gene itself does not vary (only one hybridisation band was found by Southern blotting when a cDNA probe was reacted with human genomic DNA digested with several restriction enzymes) (Mosher, 1984). Differences observed among subunits of Fn arise from mRNA transcription or post-transcriptional events. At least 10 versions of the protein have been identified (Hynes, 1986). Different cell types synthesise different sets of variants (Paul and Hynes, 1984).

With RNA splicing most Fn exons are retained, but a few of them can be cut out along with the introns (Figure 4.3). This may have functional consequences, for example, soluble plasma Fn made in the liver always lacks a Type III repeat. This repeat is included in the Fn synthesised by fibroblasts and endothelial cells - a form of the protein that readily polymerises into insoluble fibrillar matrices. The regulation of alternative splicing is not yet understood (Hynes, 1986).

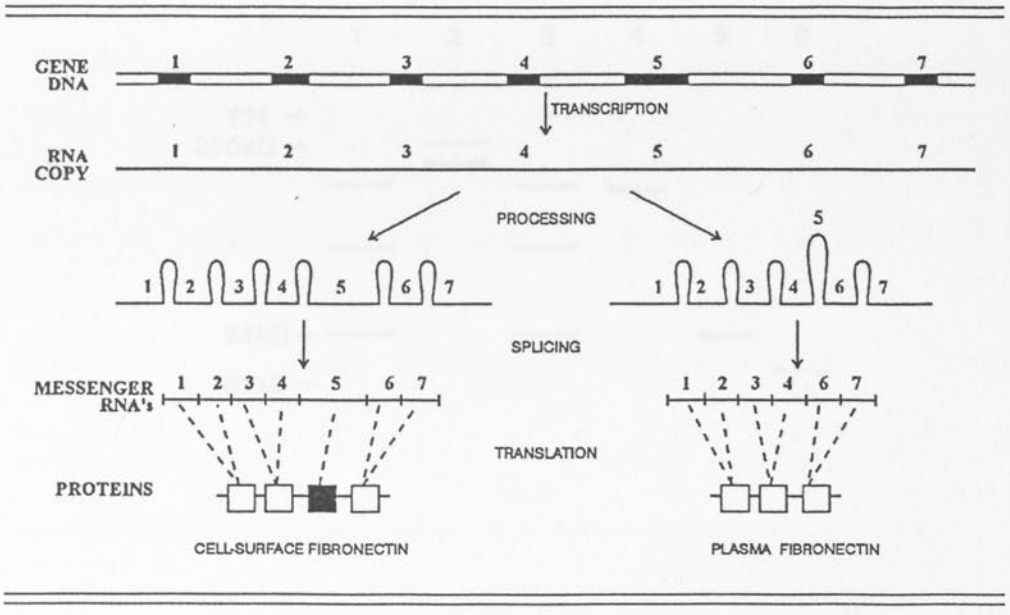


Figure 4.3 - Alternative splicing of the fibronectin (Fn) gene. (Redrawn from Hynes, 1986)

The carbohydrate content of Fn varies between 5 and 10%, depending on the source, eg. Fn from amniotic fluid has a carbohydrate content of 9.5%, whereas Fn from plasma and fibroblasts have approximately 5% carbohydrate (Mosesson *et al*, 1975; Yamada *et al*, 1977). Amniotic fluid Fn and cellular fibronectin (cFn) are larger than the plasma form and have slighty less mobility on SDS gels than plasma Fn (Crouch *et al*, 1980). However, intact Fn from plasma and cells are both recognised by conventional antisera (Ruoslahti *et al*, 1978).

4.1.2 Domain structure and functions

Several binding functions have been mapped onto the Fn molecule. A model of the domain structure of Fn has been deduced using cryoprecipitates rich in Fn. Proteolytic fragments have been separated by SDS-PAGE (Ruoslahti *et al*, 1981) (Figure 4.4a and b).

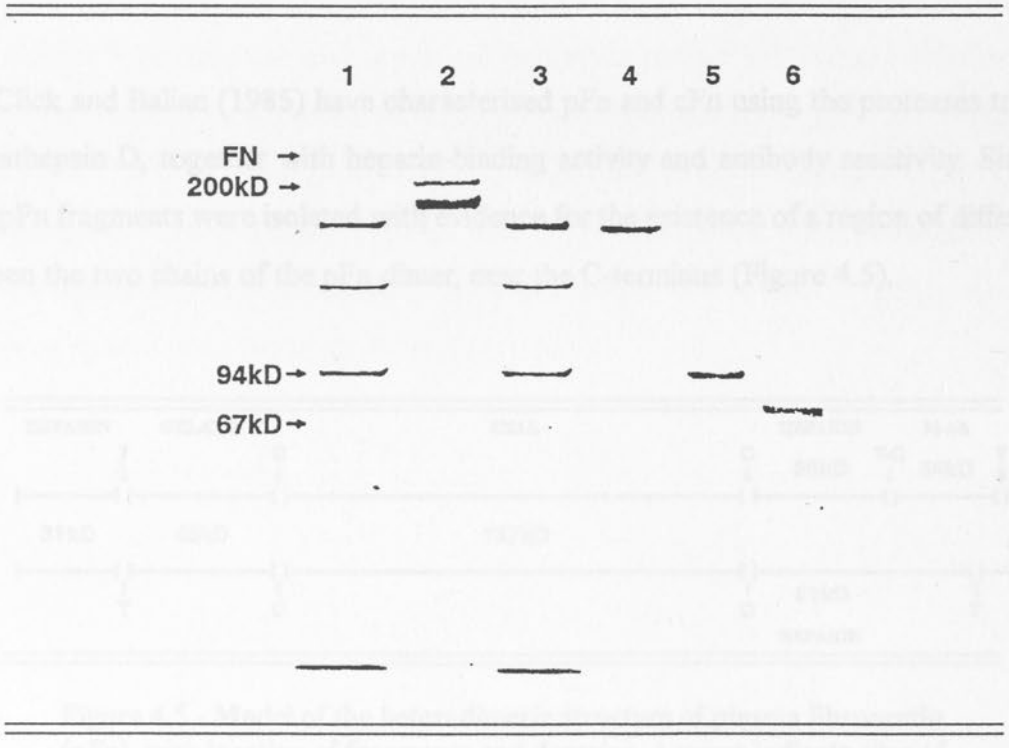


Figure 4.4a - SDS-PAGE gel of fibronectin (Fn). Lane 1, gelatin-Sepharose bound fraction of human plasma cryoprecipitate, lane 2, heparin-Sepharose-bound fraction (200kD fragment), lane 3, heparin-Sepharose non-bound fraction of material in lane1, lane 4, 170kD fragment, lane 5, 100kD fragment, lane 6, 80kD fragment. Molecular weights are shown on the left. (Redrawn from Ruoslahti *et al*, 1981)

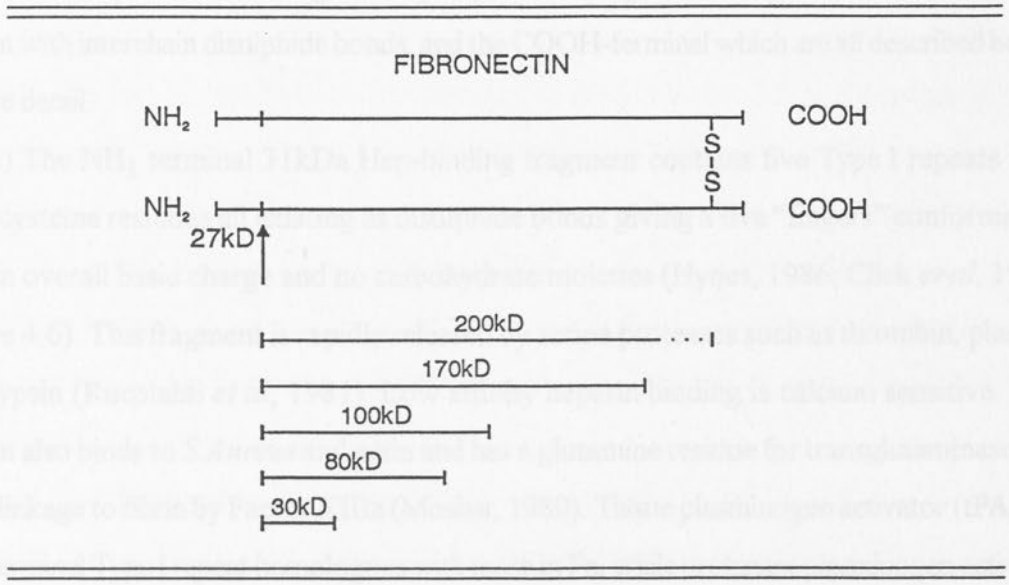


Figure 4.4b - Schematic representation of the arrangement of fibronectin fragments found in cryoprecipitate. (Redrawn from Ruoslahti *et al*, 1981)

Click and Balian (1985) have characterised pFn and cFn using the proteases trypsin and cathepsin D, together with heparin-binding activity and antibody reactivity. Six discrete pFn fragments were isolated with evidence for the existence of a region of difference between the two chains of the pFn dimer, near the C-terminus (Figure 4.5).

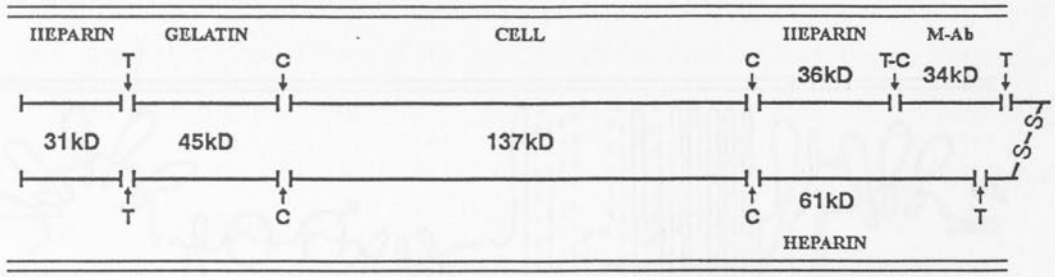


Figure 4.5 - Model of the heterodimeric structure of plasma fibronectin (pFn) with location of fragments and domains. Arrows indicate sites of cleavage with trypsin (T) and cathepsin D (C).
(Redrawn from Click and Balian, 1985)

The model of the Fn domains comprises of: a) a 28-31kDa amino-terminal low affinity heparin-binding domain, b) a 45kDa gelatin-binding domain, c) a 137kDa cell attach-

ment domain, d) a 35-38kDa high affinity heparin-binding domain, e) a 34kDa fibrin-binding domain with interchain disulphide bonds, and the COOH-terminal which are all described below in more detail.

a) The NH_2 terminal 31kDa Hep-binding fragment contains five Type I repeats with many cysteine residues all existing as disulphide bonds giving a five "fingers" conformation with an overall basic charge and no carbohydrate moieties (Hynes, 1986; Click *et al*, 1985) (Figure 4.6). This fragment is rapidly released by serine proteases such as thrombin, plasmin and trypsin (Ruoslahti *et al*, 1981). Low affinity heparin binding is calcium sensitive. This domain also binds to *S.Aureus* and actin and has a glutamine residue for transglutaminase and cross-linkage to fibrin by Factor XIIIa (Mosher, 1980). Tissue plasminogen activator (tPA) has an N-terminal Type I repeat homologous with those in Fn, while urokinase plasminogen activator (uPA) lacks this homology (Banyai *et al*, 1983). Whereas tPA binds to fibrin, uPA does not. This confirms the idea that Type I homology confers affinity for fibrin (Hynes, 1986).

b) The 45 kDa gelatin-binding fragment is adjacent to the NH_2 terminal domain. It has four Type I and two Type II repeats (Figure 4.6), with numerous disulphide bonds and carbohydrate moieties which may confer its strong protease resistance (Bernard *et al*, 1982). The disulphide bonds form two different types of loops (Petersen *et al*, 1983) which give a conformation essential for gelatin and collagen binding (Balian *et al*, 1979). Reduction of the disulphide bonds results in a lack of reactivity with gelatin. This fragment also binds to intact Fn polyamines and transformation enhancing factor (de Petro *et al*, 1981).

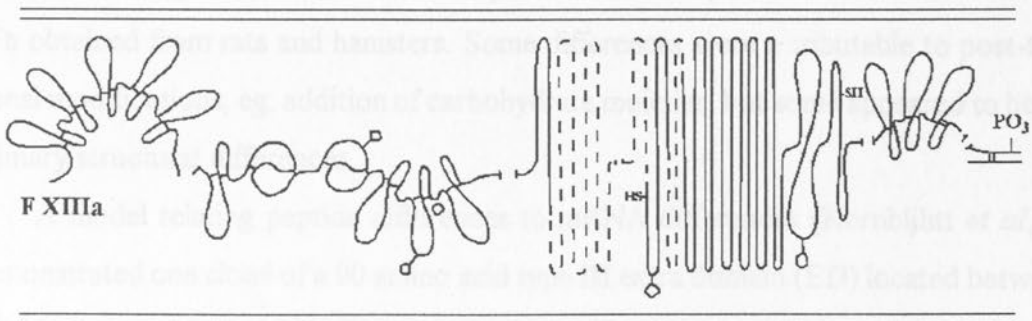


Figure 4.6 - Diagram representing the loop structure of fibronectin with type I, II and III homologies. (Redrawn from Homandberg *et al*, 1986)

c) A 137 kDa protease sensitive segment chemotactic for fibroblasts, which has a specific site for cell attachment. Ruoslahti *et al* (1981) showed that the essential site for attachment is contained in an 11.5 kDa fragment which has a RGD sequence and is unglycosylated. Amino acid analysis shows little or no cysteine as this region consists almost exclusively of Type III homologies (Click and Balian, 1985). This domain may bind weakly to heparin at low ionic strength as there is probably an affinity for heparin near the N-terminus in a 29K fragment (isolated by Gold *et al*, 1983) equivalent to the DNA binding site mapped by Pande and Shively (1982).

d) C-terminal heparin-binding region - two fragments of 36 kDa and 61 kDa were isolated and shown to have homology by peptide mapping at their N-terminal ends, thus are derived from the A and B chains respectively (Click and Balian, 1985). The fragments have high affinity for heparin, with little or no cysteine, but are insensitive to divalent cation concentration (Hayashi and Yamada, 1982).

e) The COOH terminus is adjacent to the heparin-binding domain, found in both subunits but released as a discrete 34 kDa trypsin digest-fragment only from the A chain. The B chain has a 200 kDa fragment with heparin binding and contains a fibrin II binding domain (Click and Balian, 1985). The fragment has numerous disulphide bonds in three loops of the Type I repeats and one free sulphydryl group (Petersen *et al*, 1983).

Fibronectin variants

Paul and Hynes (1984) showed the presence of multiple Fn subunits for both cFn and pFn obtained from rats and hamsters. Some differences were attributable to post-translational modifications, eg. addition of carbohydrate moieties, but some appeared to be due to primary structural differences.

A model relating peptide differences to mRNA differences (Kornblihtt *et al*, 1984) demonstrated one clone of a 90 amino acid type III extra domain (ED) located between the cell-binding and heparin-binding domains which is not seen in amino acid sequences of human pFn (Gold *et al*, 1983) or cDNA sequences of rat liver clones (Schwarzbauer *et al*, 1983). The liver synthesises mainly plasma Fn, therefore these results are consistent with the presence of the ED in cFn only (Figure 4.2). This represents part of the difference in apparent MWT between the cFn 50 kDa and pFn 36 kDa heparin-binding fragments of the

A chain (Click and Balian, 1985) (Figure 4.5). pFn and cFn can be distinguished by means of monoclonal antibodies (MAb) specific for the extra domain (ED1, or ED-A and -B) as described (Borsi *et al*, 1987; Lockwood *et al*, 1990; Carnemolla *et al*, 1987; Peters *et al*,1989).

The second region of difference is the connecting segment region (IIICS) of proteolytic susceptibility between the heparin binding and fibrin-binding domains which gives rise to five different transcripts (CS1-V) due to alternative splicing (Figure 4.2). Four transcripts have been identified in cDNA clones (Humphries *et al*, 1987) (Figure 4.6). There are several arginine and lysine residues that could be susceptible to cleavage by trypsin if folding of the native conformation placed one of these residues in an exposed position. The non-Type III difference segment contains an active sequence, RGDV, which could possibly confer a second cell-binding site on some Fn subunits (Hynes, 1985).

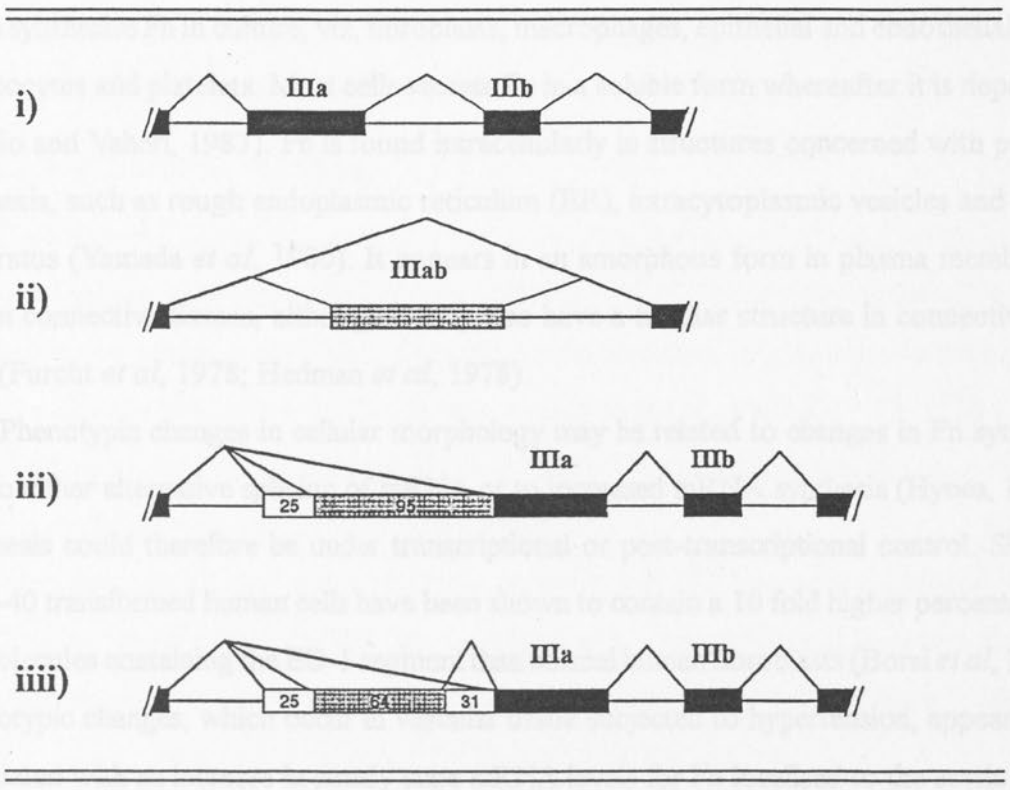


Figure 4.6 - Intron-exon structure of typeIII homologies and regions of variation. Exons are represented by boxes and introns by lines. Patterns of splicing are marked. **i)** Typical type III homology is encoded by two exons, IIIa and IIIb. **ii)** One type III homology is encoded by a single exon (IIIab), which can be included or omitted (ED-1). **iii)** The first exon (IIIa) of the last type III repeat in the rat gene is extended for 120 amino acids (360 base pairs) at its 5'end. This segment encodes one of the difference segments, and can be spliced in three different ways, as shown in **iii)** for the rat gene and in **iiii)** for the human gene. Internal splice sites can delete segments of the exon encoding 25, 64 and 31 amino acids (IIICS). Thus five variants of human Fn are demonstrated.
(Redrawn from Hynes, 1985).

4.1.3 Fibronectin biosynthesis

Fn is synthesised early on in development and is present in embryonic tissues (Zetter *et al*, 1978) but may be absent from the mature forms of some of these tissues eg. cartilage, bone tendon and mature corneal tissue. Apparently the human brain has no Fn except within its vascular walls (Schaechner *et al*, 1978). Fn has been detected in granulation tissue before the deposition of collagen, in rheumatoid synovium in inflammatory conditions and in connective tissue stroma of carcinomas.

All species of vertebrates produce Fn, and immunologically related proteins have been detected in sea urchins and fresh water sponges (D'Ardenne and McGee, 1984). Many cell

types synthesise Fn in culture, viz. fibroblasts, macrophages, epithelial and endothelial cells, hepatocytes and platelets. Most cells secrete Fn in a soluble form whereafter it is deposited (Vartio and Vaheri, 1983). Fn is found intracellularly in structures concerned with protein synthesis, such as rough endoplasmic reticulum (ER), intracytoplasmic vesicles and Golgi apparatus (Yamada *et al*, 1980). It appears in an amorphous form in plasma membranes and in connective tissues, although it may also have a fibrillar structure in connective tissues (Furcht *et al*, 1978; Hedman *et al*, 1978).

Phenotypic changes in cellular morphology may be related to changes in Fn synthesis due to either alternative splicing of mRNA or to increased mRNA synthesis (Hynes, 1986). Synthesis could therefore be under transcriptional or post-transcriptional control. Simian-virus-40 transformed human cells have been shown to contain a 10 fold higher percentage of Fn molecules containing the ED-1 segment than normal human fibroblasts (Borsi *et al*, 1987). Phenotypic changes, which occur in vascular tissue subjected to hypertension, appear to be associated with an increase in steady state mRNA levels for Fn localised to the aortic tissue (Takasaki *et al*, 1990). Alternatively spliced variants of Fn are present in aortic tissue, and following vascular injury there is a switch to the synthetic variant (Glukhova *et al*, 1989).

Aortic rings that maintain cell to cell and cell to ECM interactions in a more physiological manner than that in cell culture, offers a system for determining the factors regulating Fn biosynthesis (Glukhova *et al*, 1989).

The approximately sixfold increase in the biosynthetic rate of Fn after 21 days of treatment with DOC/salt (to induce hypertension) is quantitatively similar to the difference reported in Fn steady-state mRNA levels (Saouaf *et al*, 1991). The newly synthesised Fn being measured in these studies has a relatively slow turnover and provides evidence that transcriptional events influence Fn biosynthesis in the aorta. Using a MAb to the alternatively spliced form of cFn containing the EIIIA (ED-1) insert, significant increases in endogenously produced material were shown (Saouaf *et al*, 1991), although infiltration of plasma Fn secondary to DOC/salt treatment may still occur. Immunodetectable Fn correlated more closely with the biosynthetic data than with steady-state mRNA, suggesting the possibility of post-transcriptional control as well.

The regulatory role of growth factors on Fn synthesis may be relevant in hypertension, as it is in wound repair. TGF-β mRNA levels were increased in rats treated with DOC/salt,

suggesting the possibility of modulation of aortic ECM, but there is no definitive study showing that TGF-β modulates aortic gene expression *in vivo* (Saouaf *et al*, 1991).

4.1.4 Fibronectin receptors

The understanding of the molecular structure of Fn has enabled investigators to examine its interactions with cell surface proteins (Hynes, 1985). The identity of the cell surface binding sites or receptors for Fn has been difficult to elucidate, largely because the binding of Fn to cells is of relatively low affinity, with a dissociation constant $Kd = 3.10^{-7}$ M (platelets), and $Kd = 8.10^{-7}$ M (fibroblasts) (Hynes, 1985). This may be due to the multivalency of the Fn molecule with its many binding domains.

Fn receptors are found among the integrin family of RGD receptors containing the β1 and β2 types, which differ in MWT, glycosylation and composition and therefore differ in their affinities and fine specificities (Takada *et al*, 1987; Hemler, 1988), (Figure 4.7a). The α3–β1 (VLA-3) integrin is a receptor for Fn, collagen and laminin.

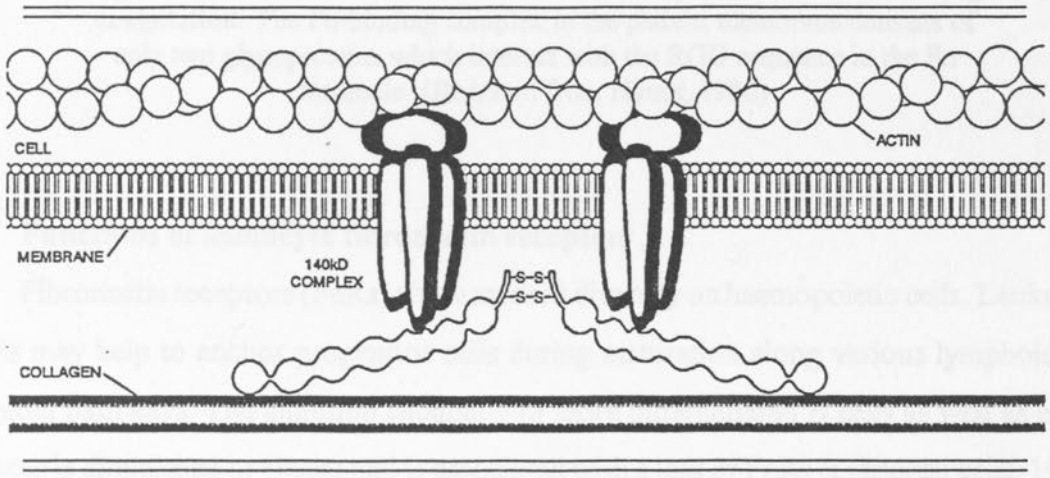


Figure 4.7a - Fibronectin mediates cell adhesion to other proteins.
While linking cells to extracellular matrix collagen, each Fn subunit binds to the 140kD integrin complex, and another integrin complex inside the membrane links Fn and actin filaments within the cell. At their extremities the Fn subunits bind to collagen. (Redrawn from Hynes, 1986)

Platelets express αIIb-βIII receptors designated GPIIb/IIIa for Fn, vitronectin, fibrinogen and von Willebrand factor (Leptin, 1986; Parise and Phillips, 1986). The RGD-containing domain of Fn binds to the IIIa subunit. α5-β1 is found on platelets, variably on lymphocytes and possibly on monocytes and macrophages (Figure 4.7b). Thymus and bone

marrow tissues, human placenta as well as myeloid cell lines also express VLA-5. Integrin VLA-4 recognises a cell attachment sequence located in the alternatively spliced CS-1 variable region of Fn (Nojima *et al*, 1990). Fn has two distinct cell binding sites, which may lead to interaction with related but distinct receptors (Hemler, 1988).

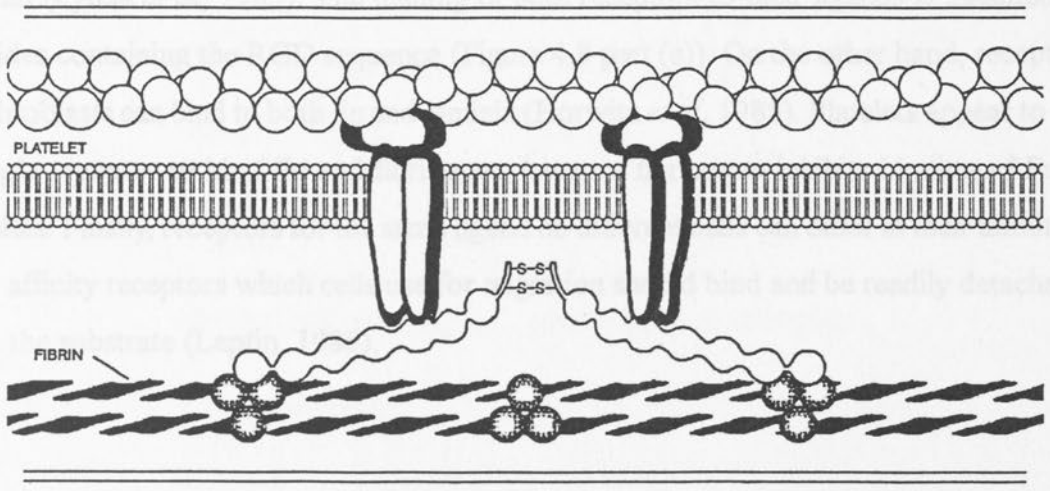


Figure 4.7b - Fibronectin mediates adhesion of platelets to fibrin during coagulation. The Fn-binding complex in the platelet membrane consists of only two glycoproteins which interact with the RGD sequence in the Fn molecule. (Redrawn from Hynes, 1986)

Functions of leukocyte fibronectin receptors

Fibronectin receptors (FnRs) show marked diversity on haemopoietic cells. Leukocyte FnRs may help to anchor progenitor cells during maturation along various lymphoid and myeloid pathways. The adhesion capability of more differentiated B cells as well as erythrocytes is diminished or absent and is associated with a loss of FnRs (Giancotti *et al*, 1986). This supports the hypothesis that FnRs facilitate maturation of haemopoietic cells, perhaps by anchoring cells until they are ready to emigrate (Dexter *et al*, 1977). FnRs also contribute to monocyte activation in response to the inflammatory state eg FnR-triggering activates the monocyte CR3 (Mac-1) receptor, enabling it to bind the C3bi complement protein (Bevilaqua *et al*, 1981).

VLA-3 is quite strongly expressed on malignant cells (Hemler, 1988). Metastatic haemopoietic cells may use VLA-3 for invasive attachment, as this process of cellular migration can be inhibited by RGD peptides in an *in vitro* model system (Figure 4.8 part (c)) (Humphries *et al*, 1987). VLA-3 levels increase on rapidly growing cells and ECM

components can upregulate VLA-3 in certain cell lines.

Cells can carry separate receptors with different specificities, or they can use one receptor to bind to more than one ligand. An example of the first case is seen in osteosarcoma cells, which have two different receptors for Fn and vitronectin, neither of which binds laminin (Pytela *et al*, 1985). The binding of both receptors to their ligands is inhibited by peptides containing the RGD sequence (Figure 4.8 part (c)). On the other hand, receptors on fibroblasts can bind to both Fn and laminin (Horwitz *et al*, 1985). Platelets appear to use a single receptor to bind Fn and fibrinogen, because fibrinogen inhibits binding of Fn to platelets. Finally, receptors for the same ligand on different cells can differ in their affinities. Low affinity receptors which cells use for migration should bind and be readily detachable from the substrate (Leptin, 1986).

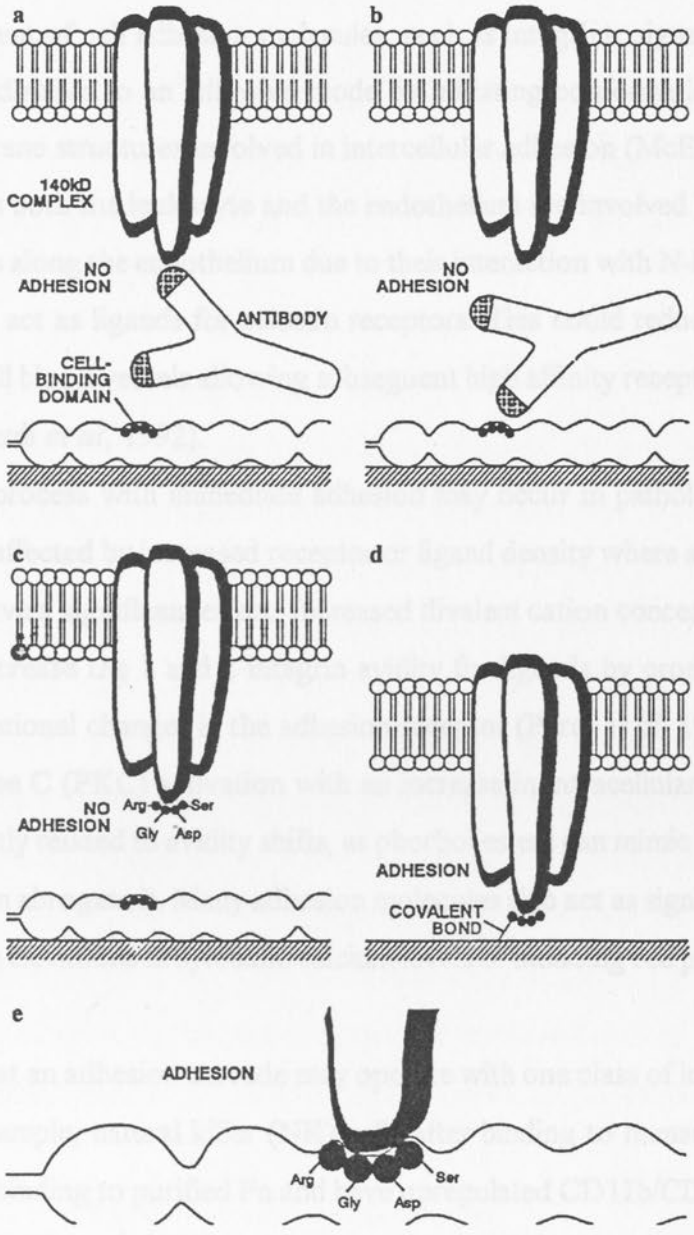


Figure 4.8 - Cell adhesion via the RGDS sequence of fibronectin and a 140kD complex in the cell membrane. a) Antibody to the 140kD integrin complex, b) antibody to the cell binding domain of Fn and c) a synthetic peptide sequence mimicking the active site of the cell binding domain of Fn all block cell adhesion, d) the RGDS peptide alone can promote cell adhesion when coupled to a surface, and e) represents the adhesive interaction between the 140kD complex and the RGDS sequence in the cell binding domain. (Redrawn from Hynes, 1986)

Receptor activation

Leukocytes appear to become adhesive only when activated or triggered by external stimuli. Engagement of cell adhesion molecules, such as integrins, signals the cell to convert from a nonadhesive to an adhesive mode by initiating conformational changes in a number of membrane structures involved in intercellular adhesion (McEver, 1991). In addition selectins on both the leukocyte and the endothelium are involved in the initial "rolling" of leukocytes along the endothelium due to their interaction with N-linked or O-linked sugars which can act as ligands for selectin receptors. This could reduce the flow of the leukocytes in small blood vessels allowing subseguent high affinity receptor-ligand interactions to occur (Pardi *et al*, 1992).

A one-step process with immediate adhesion may occur in pathological conditions. Adhesion will be affected by increased receptor or ligand density where a two to three-fold increase would have a significant effect. Increased divalent cation concentration eg at sites of injury could increase the 1 and 2 integrin avidity for ligands by promoting energy-dependent conformational changes in the adhesion receptor (Pardi *et al*, 1992).

Protein kinase C (PKC) activation with an increase in intracellular calcium ion concentration is directly related to avidity shifts, as phorbol esters can mimic the process, while PKC inhibition can abrogate it. Many adhesion molecules also act as signal transducers, for example, inducing elevations in cytosolic calcium levels or inducing cell proliferation (Pardi *et al*, 1992).

It appears that an adhesion cascade may operate with one class of interaction modifying others, for example, natural killer (NK) cells after binding to human endothelial cells display enhanced binding to purified Fn and have upregulated CD11b/CD18 (MAC-1) surface expression (Pardi *et al*, 1992).

4.2 PHYSIOLOGY OF FIBRONECTIN

The roles of intact fibronectin (Fn) are associated with its binding domains. Fn functions include:

4.2.1 Adhesive Functions

a) Attachment sites

A number of studies demonstrate the adhesive functions of Fn (D'Ardenne and McGee,

1984, review). Fn has been observed to agglutinate formalinised sheep red blood cells (RBC) and increase aggregation of dissociated chick embryo and baby hamster kidney cells. Addition of both pFn and cFn to cell cultures promotes adhesion of most cells to a substrate via the cell attachment domain which contains the RGD sequence and promotes cell spreading (Figure 4.9). Addition of Fn to rounded transformed cells causes flattening and adherence to a substrate. Fn is assembled *in vitro* into fibrils which form a meshwork for cell attachment. Fn has attachment sites for *S.Aureus* and other bacteria, as well as for acute phase proteins such as CRP and amyloid P (Vartio and Vaheri, 1983).

b) Fibronectin and cytoskeleton formation

The loss of surface Fn on mitotic cells is accompanied by a loss of microfilament actin bundles (Hynes and Bye, 1974). Incubation of transformed cells with Fn results in re-organisation of intracellular actin bundles. There are apparently transmembrane linkages (by means of integrins) between actin filaments and Fn fibrils, which are colinear and separated by dense submembranous plaque (D'Ardenne and McGee, 1984, review) (Figure 4.7a).

Fn and vinculin are also related across the cell membrane (Geiger, 1979). Vinculin is a smooth muscle protein in adhesion plaques at the termini of actin filaments. Spreading cells are found to progressively remove Fn from small areas and displace it towards the cell centre (Avnur and Geiger, 1981). The Fn-free areas frequently coincide with cell contacts containing vinculin. Thus, Fn has a dynamic role in attachment and motility. A 135/140 kDa complex aids in transmembrane binding of actin to Fn at the cell surface. This is the VLA-5 integrin or receptor for the RGD sequence of Fn (Hynes, 1986; Akiyama *et al*, 1989).

c) Promotion of cell migration in morphogenesis

Fn has been detected in developing embryonic tissue and appears to help direct cells to their destinations during embryogenesis, either by attracting migrating cells by chemotaxis, or by being part of a specific pathway guiding migrating cells by contact guidance (Mosher,1984). Boucaut (1984) injected a decapeptide containing the RGDS sequence into embryos, and observed inhibition of cell migration in amphibian gastrulae, and the avian neural crest.

d) Trophoblast contact

Oncofetal Fn, which has a specific domain not found in adult tissue or plasma, aids trophoblast contact with extraplacental ECM. Fetal Fn is recognised by MAb FDC-6 (Lockwood, 1991; Matsuura *et al*, 1985). The integrin receptors for Fn, α5-β1 and α3-β1, play a role in the regulation of cytotrophoblast invasion of the decidua and myometrium of the uterus during placentation (Zhou *et al*, 1993).

4.2.2 Immune-cell related functions

TNF-α induced tyrosine phosphorylation depends on adhesion of PMNL to extracellular matrix proteins such as Fn, and participates in the transduction of signals that direct the cells to spread on a matrix and undergo a respiratory burst (Fuortes *et al*, 1993). TNF-α treated PMNL have decreased cAMP levels, reorganise their actin-based cytoskeleton to form focal adhesions, spread out, discharge proteases from their granules and mount a prolonged respiratory burst. Each of these responses depends on expression by the PMNL of adhesion receptors of the CD11/CD18 or β-2 integrin family, and requires that the cells make contact with a surface coated with ECM proteins, plasma proteins or other cells (Fuortes *et al*, 1993).

Responses of cells to cytokines or growth factors are often modulated by interactions of the cells with the ECM. Bevilacqua *et al* (1984), identified spreading on a Fn-coated surface as a stimulus for tyrosine phosphorylation of a 120 kDa protein in 3T3 cells. Adherence to Fn fragments, or cross-linking of the β1 integrin VLA-4 with antibodies, induced tyrosine phosphorylation of a pp105 peptide in T-lymphocytes.

b) Monocyte-macrophage functions

Fn is required for optimal macrophage immobilisation by migration inhibitory factor (MIF). Fn stimulates production of a cell growth factor by monocytes and macrophages and enhances expression of Fc receptors by monocytes and macrophages (Bevilacqua *et al*, 1981).

Normal blood monocytes have no detectable Fn mRNA and no measurable Fn secretion (Yamaguchi *et al*, 1987). As monocytes mature to become macrophages mRNA becomes detectable and Fn secretion is measurable. Active macrophages such as alveolar

macrophages have detectable mRNA and Fn secretion. Lipopolysaccharide (LPS) and immune complexes cause alveolar macrophage mRNA and Fn levels to decrease. However, in idiopathic pulmonary fibrosis alveolar macrophages produce increased amounts of mRNA and Fn (Yamaguchi *et al*, 1987).

i) Opsonic activity

Fn has been shown to opsonise tissue debris for phagocytosis by the reticulo-endothelial system (RES) for example, in the uterine lumen after parturition (Martin *et al*, 1988; Blumenstock *et al*, 1978). This is associated with a significant fall in pFn levels, which suggests a consumptive process (Martin *et al*, 1988).

The RES is composed principally of sessile macrophages within the liver (Kupffer cells), lung and spleen (Martin *et al*, 1988). They contribute to the systemic defence by removing blood-borne bacteria and endotoxin, collagenous tissue debris, injured cells, immune complexes and products of disseminated intravascular coagulation (DIC). Tolerance to experimental burn, trauma, and sepsis has been directly correlated with the ability of the RES to clear injected test particles from the blood.

Tissue debris removal

The distribution of intravenously injected fluorescence isothiocyanate (FITC)-labelled pFn can be monitored in rats with abdominal skin lesions (Martin *et al*, 1988). Debris is clearly coated with FITC-Fn at injury sites within two hours, implying that circulating Fn is available for opsonisation. Locally produced Fn may also be present. Macrophages at injury sites internalise Fn-FITC labelled debris by phagocytosis.

In vitro, Fn promotes phagocytosis of gelatin-coated particles by macrophages in a time, concentration and temperature dependent manner, with heparin as a cofactor. It has been shown that aggregated cell membranes are not phagocytosed unless opsonised with Fn. Fn degradation products are shown to augment the opsonin-independent pathway of phagocytosis by monocytes by enhancing C3bi-mediated uptake (Pommier *et al*, 1983). Binding of Fn to C1q also enhances binding of complexes to phagocytic cells. PMNs were not seen to phagocytose Fn-opsonised debris (Martin *et al*, 1988).

4.2.3 Mitogenic functions

Extracellular matrix Fn appears to be mitogenic for the immune and haemopoietic systems (Levesque *et al*, 1991). Attachment and spreading of epithelioid cell lines is promoted by Fn and is necessary for cytokinesis and growth induced by serum (Levesque *et al*, 1991). Fn in serum-free cultures can replace competence growth factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) to allow fibroblasts to divide. It can also stimulate the mitogenic effect of erythropoietin on erythroid progenitors. A mitogenic domain cannot, however, be distinguished from the cell adhesion domains of the Fn molecules. Mitogenic effects are inhibited by peptides containing RGDS, in contrast to the effects of fibrinogen.

Immobilised, but not soluble, Fn has recently been shown to promote activation and proliferation of naive and memory T cells synergistically with immobilised monoclonal antibodies against CD3, whereas they have no effect when used separately. This effect is mediated via the T-cell integrins $\alpha4$-$\beta1$ (more on the memory T-cells) and $\alpha5$-$\beta1$, which respectively recognize the GPEILDVPST and the RGD sequences of Fn. ECM proteins are now known to have domains that display some homology with growth factors and their receptors. Type III Fn repeats and immunoglobulin-like domains have been found in adhesion proteins, cytokine receptors for IL-2, IL-3, IL-4, IL-6, IL-7, TNF, erythropoietin, granulocyte- and granulocyte-monocyte-CSF, prolactin, and proteins of the immunoglobulin superfamily. This suggests a common ancestral gene for these three superfamilies (Levesque *et al*, 1991). Memory T-cells express three to four times more VLA-4, VLA-5 and VLA-6 than do naive cells and thus bind more efficiently to Fn and laminin (Levesque *et al*, 1991).

4.2.4 Wound healing

Following vascular injury Fn has a pivotal role in haemostasis in that it acts as a chemoattractant for fibroblasts, macrophages and neutrophils (Postlewaite *et al*, 1981; Norris *et al*, 1982) and aids their migration into the clot (Cheng *et al*, 1988). Fn also opsonises denatured collagen and facilitates clearance of non-viable tissue by enhancing the activity of macrophages which in turn are stimulated to produce Fn (Reese *et al*, 1983).

During wound healing a large number of fibroblasts and other cells migrate into the affected area. They travel through the cross-linked fibrin clot using Fn as a matrix (Grinnell,

1980). Fn is cross-linked to itself, (by means of two potential binding sites near the carboxy- and amino-termini) and to fibrin by Factor XIII, and is therefore covalently incorporated into the fibrin matrix (Mosher *et al*, 1980). Fn makes up approximately 4.5% of the clot mass (Mosher *et al*, 1980). There are three sources of Fn available at the wound, namely, pFn deposited as part of the clot, that which accumulates due to increased vascular permeability, and cFn produced by platelets and endothelial cells of blood vessels in response to injury.

Blood platelets also play a role in clot formation (Parise and Phillips, 1986). They adhere to the collagen at the edges of the disrupted vessel and clump together to form a plug. Later they mediate clot retraction by contracting the fibrin matrix. These functions obviously require platelets to adhere tightly to the matrix. Addition of Fn to platelets *in vitro* results in firmer adhesion to the substrate, causing platelet spreading. It is likely that Fn is also necessary *in vivo* for full platelet adhesion. Platelets activated by factors released by other platelets at the clotting site, develop specific binding sites for Fn (Hynes, 1986).

Fn in platelets is associated with -granules and the platelet surface (Ginsberg *et al*, 1979). Thrombin initiates and promotes the release of platelet Fn and increases its surface expression. Collagen may also stimulate Fn release. Plasma Fn binds to thrombin-stimulated platelets but not to resting platelets or ADP- or epinephrine-stimulated platelets (Plow and Ginsberg, 1981). Binding, mediated by integrins, is specific and saturable and requires divalent ions.

It seems likely that exogenous Fn supplements the activity of pFn (Cheng *et al*, 1988). Exogenous Fn may be the source of additional chemotactic fragments, which would result in the more rapid accumulation of monocytes and fibroblasts at the site of injury. In addition, the affinity of the Fn receptor on macrophages is relatively low, so additional coating of debris may increase the avidity of binding to the macrophages (Cheng *et al*, 1988).

Exogenous Fn can speed up the repair process in injuries or ulcers of the cornea of the eye (Hynes, 1986). Experiments with rats showed that excised lesions treated with Fn soon after the initial injury healed more rapidly than paired control lesions treated with a carrier alone (Cheng *et al*, 1988). Fn-treated wounds in essential fatty acid deficient rats healed slightly slower than Fn-treated wounds in normally fed rats. This indicates that fatty acids may play a role in Fn-accelerated healing. Subsequent experiments have shown that circulating levels of Fn are inversely correlated with prostaglandin levels (Cheng *et al*, 1988).

4.3 EFFECTS OF FRAGMENTATION ON FIBRONECTIN FUNCTION

As described earlier, Fn is highly susceptible to proteolysis by enzymes such as plasmin, trypsin, cathepsins D and G and elastase. Fn fragmentation patterns can be deduced by SDS-PAGE analysis of Fn digests performed *in vitro*, as well as from some cryoprecipitate preparations from human plasma (Ruoslahti *et al*, 1981). Fibronectin fragments (FnDP) can alter normal Fn functions, either by enhancing or inhibiting them (Mosher, 1984). Certain fragments or groups of fragments have been shown to have unique functions, or to have synergistic effects when in suitable concentrations with intact Fn. Although most studies to be discussed have been performed *in vitro*, they may help to explain the effects of Fn fragmentation *in vivo* in both healthy and disease states. FnDP functions most frequently described include chemotaxis, immunosuppression, transformation of virus-infected cells and opsonic activity.

4.3.1 Chemotactic activity and immunosuppression

a) Chemotaxis

Neutrophil chemotaxis has been shown to be inhibited by FnDPs experimentally (Easter *et al*, 1988). Intact Fn had no significant effect on neutrophil migration, whereas an elastase digested Fn mixture enhanced migration by 28%. An isolated 30 kDa Fn fragment inhibited chemotaxis by 53% ($p<0.01$). The effect was reversible by addition of gelatin-Sepharose 4B beads to the 30 kDa fragment eluate, as well as by neuraminidase and antibodies to suppressor active peptide. Thus the 30 kDa fragment is able to bind to gelatin and is glycosylated. The glycosylation of this fragment is potentially responsible for its inhibitory effect on chemotaxis.

Phorbol myristate acetate (PMA) stimulated calf pulmonary artery endothelial cells have also been shown to secrete a mixture of Fn fragments. A non-heparin-binding fragment of 120kDa which contains the cell-binding site was shown to be chemotactic for human neutrophils (Odekon *et al*, 1991). The RGD sequence found in the cell binding domain is unglycosylated and has very little cysteine as compared to the gelatin binding domain. These properties may be necessary for recognition of adhesion molecules (integrins) on the neutrophil surface and help to modulate chemotaxis.

Neutrophils migrate through the endothelial cell basement membrane barrier to sites of inflammation and injury in two steps. Initially the neutrophils adhere to the endothelial surface, a process initiated by IL-1β and TNF-α (Moser *et al*, 1989). IL-1β does not induce the release of chemoattractants, however, the activation of plasminogen (plg) at these sites may lead to the fragmentation of Fn, which may activate neutrophils. Neutrophil degranulation with release of elastase and lactoferrin has been shown to be induced by an 11kDa fragment containing the cell adhesion domain of Fn, but not by intact Fn (Wachtfogel *et al*, 1988).

b) Immunosuppression

Mixed lymphocyte reactions (MLR) were shown to be enhanced by intact Fn by 42%, whereas the elastase digest and the purified 30 kDa fragment suppressed the MLR by 19% and 41% respectively. The immunosuppressive effects were not due to cytotoxicity as cells were viable at >95% (Easter *et al*, 1988).

Easter *et al* suggest that excess or uncontrolled serum proteases may contribute to the generation of inhibitory or inflammatory peptides which cause immunosuppression following various forms of trauma. Increased elastase activity has been described in serum from burn patients and correlates with higher suppressor active peptide levels and neutrophil chemotaxis inhibition by burn patient's serum. The 30 kDa fragment is similar to suppressor active peptide and may thus be indicative of the origins of suppressor active peptide in trauma victim serum.

4.3.2 Phagocytic activity

In vitro studies have been done showing both depression and enhancement of phagocytosis by FnDP. Two studies (Czop *et al*, 1981; Pommier *et al*, 1983) showed enhancement of monocyte phagocytosis of particulate material by the activation of the alternative complement pathway by Fn fragments. Pommier's group only used sheep erythrocytes sensitised with IgG, while Czop *et al* used rabbit RBCs (Er), mouse RBCs (Em), and sheep RBCs (Es) sensitised with IgM, C4-3b and IgG in different combinations.

Czop's group showed, using Fn preparations from several human donors that those with less intact Fn (440kDa) and an excess of fragments of 190, 170, 70 and 40 kDa, enhanced the activity of monocytes ingesting zymosan for Em and Er by 2-10 times, but no

effect on the phagocytosis of the IgG sensitised Es cells. This was not in accord with Pommier's data. They also showed that Fn bound to the particles rather than to the monocytes before ingestion. They isolated the active component from 38% of the initial Fn preparation, which contained the fragments at 190, 170, 70 and 40 kDa.

Trypsin digest of inactive Fn preparations rendered them more active, i.e. fragmentation is essential for the generation of the phagocytosis-enhancing principle. Prolonged digests, containing lower MWT fragments, however, were inactive, implying that the active component had been degraded.

Pommier *et al* found that intact Fn (440 kDa), and not a 180 kDa fragment, enhanced monocyte phagocytosis of Es-IgG, but that Fn bound to the monocytes rather than the Es. Fn did not increase the number of monocyte membrane receptors for Fc of IgG, but stimulated monocytes to ingest already opsonised particles more avidly.

In contrast, Erlich *et al* (1981), describe depression of phagocytosis by plasmin degradation products of pFn which relates to the clinical picture of DIC and depression of RE function. Plasmin activation occurs in DIC and results in fragmentation of Fn and fibrinogen to FnDPs and FDPs respectively. Erlich *et al* hypothesise that isolated FnDPs depress phagocytosis by competing with the activity of intact Fn.

Erlich's group used both an *in vivo* colloid clearance assay in rats and an *in vitro* macrophage phagocytosis assay. Rats were injected with gelatinised ^{51}Cr RBCs to induce DIC and FnDPs at a concentration equivalent to 50% degradation of endogenous Fn. Hepatic uptake of RBCs was significantly depressed ($P<0.02$), as was blood clearance of foreign particles ($P<0.04$).

Macrophage uptake was still depressed at a 1:4 (FnDP:Fn) ratio. However when FnDP concentration was increased to twice that of intact Fn there was an upward trend in phagocytosis. FnDPs without any intact Fn were incapable of supporting phagocytosis.

FnDPs were prepared by 6 and 24 hour plasmin digests of Fn passed through gelatin-Sepharose. Gelatin-binding fragments were of 195, 135 and 85 kDa and non-binding fragments of 450, 420, 330 and 260 kDa. Inhibition of phagocytosis may depend not only on the amount of breakdown products but also on the extent of degradation or size of fragments. RE depression was ameliorated by injecting an inhibitor of plasmin, tranexamic acid, prior to induction of DIC.

In vitro studies suggest a stoichiometric relationship between Fn and FnDPs in phagocytic depression. Phagocytic depression attained *in vivo* is only modest, therefore FnDPs may be of greatest significance during Fn depletion and at local sites of high FnDP concentration (Erlich *et al*, 1981).

4.3.3 Cell adhesion

Fn mediated cell attachment and spreading can be competitively inhibited by intact, soluble Fn at a 10-fold excess, as well as by Fn fragments (0.5-1mg/ml) in a noncytotoxic, reversible manner (Yamada *et al*, 1984; Hayman *et al*, 1985). A plasma purified 75 kDa trypsin digest fragment with cell binding activity (not heparin, collagen or fibrin binding activity) as well as synthetic peptides containing the RGD sequence inhibited cell spreading in a dose dependent manner. The inhibition was specific as a 60kDa collagen binding fragment, and a range of other peptides including fibrinopeptide A and B, did not inhibit cell spreading. Concanavalin A mediated cell spreading was not inhibited by Fn or the 75 kDa fragment.

4.3.4 DNA synthesis

Cathepsin D digests of Fn at 1-2g/ml initiate DNA synthesis in serum-deprived quiescent cultures of normal hamster fibroblasts, while intact Fn does not. (Humphries *et al*, 1983). This activity was inhibited when the digest was incubated with either gelatin, heparin or Fn, therefore it is uncertain which domain of Fn is responsible for the stimulation of DNA synthesis. The elevated levels of cellular proteases at sites of tissue damage suggest that Fn fragmentation could be a trigger for the initiation of cell regeneration.

4.3.5 Inhibition of endothelial cell growth

Heparin-binding fragments derived from the amino-and carboxy-terminal regions of human plasma Fn appear to be relatively specific inhibitors of the growth of bovine aortic endothelial cells in culture as assessed by counting cells remaining attached to the substrate in the presence of these Fn fragments (Homandberg *et al*, 1986). Inhibition appeared to be reversible, dose-dependent and occurred at nanomolar concentrations of fragments. Intact

plasma Fn had very little inhibitory activity suggesting that this effect *in vivo*, if it occurs, may be triggered by proteolysis.

The heparin-binding molecules affect cell growth, including protamine, platelet factor, endothelial cell growth factor and tumour-derived capillary endothelial growth factor. The 29 kDa Hep I/Fib I domain, which consists of five large loops formed by a disulphide bridge (Figure 4.6), was one of the most active inhibitory fragments (Homandberg *et al*, 1986).

Other Fn fragments generated by digestion with cathepsin D and found to have significant inhibitory activity on endothelial cell growth include a 35 and a 25 kDa fragment which are the Hep II and Fib II binding domains respectively (Homandberg *et al*, 1986). The 150 kDa digest fragment is the largest fragment with significant inhibitory activity. This activity is probably due to the presence of the 35 kDa Hep II domain. The 120 kDa fragment, which is missing the 35 kDa domain, was not inhibitory. Other large fragments which contained active domains were much less active than their smaller subfragments.

The 190 kDa fragment and native Fn exist as dimers and this may render the active inhibitory sites inaccessible. Native Fn has binding affinity between the amino-terminal 29 kDa fragment and the 35 kDa segment. The 35 kDa segment, as well as the 25 kDa Fib II domain, bind the counterpart segments in the other subunit and may account for monomer-monomer interaction in native Fn and the 190 kDa dimer. The intramolecular interactions in native Fn may reduce their accessibility as inhibitory domains (Homandberg *et al*, 1986).

4.4 FIBRONECTIN IN PREGNANCY AND PREECLAMPSIA

4.4.1 Fibronectin distribution in the placenta

a) Normal pregnancy

Fn is deposited in the ECM of the placental tissues. The distribution of Fn changes during development of the chorionic villi during normal pregnancy. In the first trimester Fn, demonstrated by staining with fluorescent Fn antibody, can be seen in trophoblastic basement membrane and connective tissue of villi. The villous stroma shows a loosely organised network of fibrillar fluorescence. Neither syncytiotrophoblast nor cytotrophoblasts stain with the antibody. In contrast, strong fluorescence is found in the pericellular matrices

of nonvillous trophoblasts, the cells forming cytotrophoblastic cell columns and the cytotrophoblastic shell. Decidual tissue is only faintly stained (Pijnenborg *et al*, 1991).

In the second trimester, Fn is seen around fetal vessels and some villous stroma. At term, fetal vessel wall staining is even more densely distributed in a laminar fashion. Fn disappears from the trophoblastic membranes with progression of pregnancy, although the membrane increases in thickness. Thus, compositional changes are taking place. Pericellular matrices of nonvillous trophoblasts of early chorionic villi contain Fn, while villous trophoblasts do not. Numerous mitotic figures in cytotrophoblastic cell columns indicate a high rate of proliferation. This is consistent with the finding that actively proliferating, undifferentiated cells produce increased amounts of Fn. Trophoblasts may lose this ability as they mature (Pijnenborg *et al*, 1991).

Changes in Fn distribution during the course of pregnancy suggest that Fn may be produced by different cell types and may play different biological roles at different stages. For example, Fn of early chorionic villi may act as a tissue supporting material as well as a modulator of proliferation and organisation of trophoblastic tissues (Yamaguchi *et al*, 1985). During pregnancy fetal Fn which differs from plasma Fn in carbohydrate structure (Friedman *et al*, 1992) can be detected in maternal plasma in concentrations that increase with the duration of pregnancy. However, there is no significant difference between preeclampsia and normal pregnancy fetal Fn concentrations to reflect the reduced trophoblast invasion in preeclampsia (Friedman *et al*, 1992)

b) Hypertensive pregnancies

In placentae of hypertensive pregnancies the most intense staining for Fn is found in spiral arteries demonstrating acute atherosis. Areas of fibrinoid necrosis and intervillous fibrin are also positively stained (Pijnenborg et al, 1991). Preeclamptic placentae at term also demonstrate Fn mainly around the fetal vessels, while trophoblastic membranes lack Fn staining. Vessels in the villi have significantly less intensity of staining compared to normal placentae (Anunciado et al, 1987).

ECM proteins may act as attachment factors for trophoblast. Trophoblast giant cells have thick Fn deposits at their periphery. The cells may secrete Fn themselves. Fn accumulations may occur in conditions of tissue injury (Vaheri *et al*, 1983) probably reflecting tissue repair. This may reflect the raised plasma Fn in preeclampsia (Pijnenborg *et al*, 1991). Enmeshment of trophoblasts by Fn is suggested to limit their migration and contribute to mechanisms that stop the invasion process (Zhou *et al*, 1993).

4.4.2 Plasma fibronectin levels in pregnant women

It is generally accepted that plasma Fn levels are raised during pregnancy although initial studies gave conflicting results (Bykowska *et al*, 1985). This was probably due to the variety of assay methods used to measure plasma Fn (Table 4.1) and to the heterogeneity of the Fn isoforms being measured. Fn may react differently in immunoturbidimetric assays compared to electroimmunoassays depending on whether the molecule is in the native, intact state or in a degraded state (Bykowska *et al*, 1985). Some studies showed no difference between control and pregnant women's plasma Fn levels (Ganrot, 1972; Brubaker *et al*, 1992), with no influence of gestational age (Molnar *et al*, 1988). However, other studies using a rapid immunoturbidimetric assay showed that pFn levels rose significantly throughout pregnancy when measured in the first, second and third trimester, at delivery and up to 6 weeks post partum, and were significantly greater than plasma concentrations of non-pregnant women at all stages (Hess *et al*, 1986). Maternal pFn returned to normal by 8 months postpartum (Hess *et al*, 1986).

Third trimester pFn levels measured by nephelometry (Thurnau *et al*, 1987; Brubaker *et al*, 1992) and electroimmunoassay (Eriksen *et al*, 1987) were shown to be significantly raised in normal pregnancy compared to non-pregnant controls, and between normal pregnancy and patients with preeclampsia. These results suggest that pFn levels may serve as a biochemical marker of disease (Thurnau *et al*, 1987; Stubbs *et al*, 1984; Eriksen *et al*, 1987).

Functional collagen-binding Fn ELISA values were highly correlated with gestational age in normal pregnancy, although the Fn levels between 26 and 38 weeks gestation were lower than non-pregnant levels. By term the Fn concentration had increased to normal levels (Brubaker *et al*, 1992). Low Fn levels may reflect the presence of a dysfunctional collagen binding isoform of Fn in pregnancy which becomes functional at term, or the presence of a factor in the circulation which alters collagen binding (Brubaker *et al*, 1992).

The increase in pFn levels during pregnancy may be representative of a reduction in immune tolerance associated normally with pregnancy (Barber *et al*, 1977). However, it is possible that the rise is reflective of the nonspecific increase in α- and β-globulins that occurs maximally during the third trimester of pregnancy (Hess *et al*, 1986). pFn functions as a nonspecific opsonin in the activation of the host immune system before the synthesis of specific immunoglobulins (Mosher, 1980). Another possiblity is that increased pFn concentrations may result from increased synthesis or decreased catabolism during placental development (Eriksen *et al*, 1987).

An unanswered question is whether cFn quantification may provide a marker as a mid-trimester screening test for preeclampsia. Lazarchick *et al* (1986) reported that elevations in total Fn may be predictive. As total pFn primarily reflects release of hepatic Fn, measurement of the endothelial cell Fn isoforms might provide an earlier and more specific marker of gestational proteinuric hypertension, as demonstrated by Lockwood and Peters (1990). However, the overlap of second trimester values in these preliminary studies suggests that cellular Fn is unlikely to be clinically useful as a cross-sectional predictor of preeclampsia.

Fn µg/ml			
Assay Type	**Non-Pregnant**	**Pregnant**	**Preeclampsia**
Electroimmunoassay			
Eriksen *et al*, 1987	368 (324-400)	453 (374-710)	662 (597-844)
Ballegeer *et al*, 1989		75% SD 20% (of normal plasma levels)	190%SD36%
Nephelometry			
Thurnau *et al*, 1987	897 SD 60	1129 SD 99	1687 SD 101
Brubaker *et al*, 1992	ND	338 SD 47	873 SD 95
Immunoturbidimetry			
Molnar *et al*,1988	342-350.6	same as non-pregnant	
Brubaker *et al*, 1984	ND	292 SD 87	770 SD 225
Calvin *et al*, 1990	ND	459 SD 232	226 SD 89 (M&S)*
Lazarchick *et al*, 1986	ND	238 SD 70	597 SD 173 (M&S)*
ELISA			
Hess *et al*, 1986	262.6 SD 6.4	297.6 SD 17	ND
Lockwood *et al*, 1990		325 (250-400)	401 (343-459)
Brubaker *et al*, 1992		258 SD 47	450 SD 47
*M&S: mild and severe patient plasma Fn levels combined ND: not done			

Table 4.1 - Comparison of fibronectin concentrations measured by means of different assays in non-pregnant, healthy pregnant and women with preeclampsia.

Raised levels of Fn receptors were detected in plasma from patients with preeclampsia compared to those of normal pregnant women, and even higher levels in patients with abruptio

placentae, with or without preeclampsia. Immunohistochemistry revealed moderate to strong staining for Fn receptors on decidual cell membranes compared to the weak staining in controls, staining was stronger with abruptio placentae. They hypothesis that the plasma Fn receptor levels may reflect production of Fn receptor in the placenta and that marked increases in plasma Fn receptor levels may predict preeclampsia or abruptio placentae (Kanayama and Terao, 1992)

4.4.3 Neonatal plasma fibronectin levels

Fn concentrations in healthy term infants are about one half that of healthy adults, while healthy premature infants (including small-for-gestational-age (SGA) infants) have significantly lower concentrations as determined by competitive ELISA than healthy term infants (Yoder *et al*, 1983). Premature infants with respiratory distress syndrome (RDS) or asphyxiation have significantly lower Fn concentrations than healthy premature infants (Barnard and Arthur, 1983) as do newborns with sepsis (Molnar *et al*, 1988). Yoder *et al* showed a significant correlation between plasma Fn concentration and gestational age and weight of neonates, which may be related to the development of RES function. Low pFn concentration may be related to increased susceptibility to bacterial infections in neonates which could relate to a dependency on prenatal transfer of maternal antibody for humoral protection (Stiehm *et al*, 1980). Perinatal asphyxia and RDS further depress plasma Fn concentrations and this may be associated with the altered capillary permeability in these syndromes.

4.4.4 Amniotic fluid fibronectin

Amniotic fluid is known to contain a heavily glycosylated isoform of Fn (Ruoslahti *et al*, 1981). Cells derived from amniotic fluid synthesise and secrete Fn in tissue culture (Chen *et al*, 1976; Crouch *et al*, 1978). It is currently unknown whether the structural differences in amniotic fluid Fn have a significant effect on functional activity. Nonenzymatic glycosylation *in vitro* has been demonstrated to inhibit the binding of Fn to connective tissue components and basement membranes (Cohen and Ku, 1984; Tarsio *et al*, 1985).

Amniotic fluid samples obtained from 54 women with a gestational age range of from 15-40 weeks demonstrated a significant decrease in Fn levels (measured by ELISA) with increasing gestational age (Harris *et al*, 1988). Second trimester Fn levels were higher (145.3±14.2μg/ml) than those reported by Chen *et al* (1976) and Crouch *et al* (1978) (±70μg/ml). Hess *et al* (1986) found no change in amniotic fluid Fn levels between second (32.5μg/ml) and third (33.5μg/ml)

trimesters, similar to the third trimester values (27.1±5.4μg/ml) of Harris *et al*. Harris *et al* had a larger sample size and paid particular attention to sample collection and storage. Diminished concentrations of amniotic fluid Fn in the latter stages of pregnancy may represent either decreasing synthesis by amniocytes or a dilutional effect from fetal urine (Harris *et al*, 1988).

4.5 CONCLUSIONS REGARDING THE ROLE OF FIBRONECTIN IN PREECLAMPSIA

The conclusion from this literature review is that fibronectin (Fn) appears to be a consistent marker of endothelial damage and activation of coagulation and therefore is a potential marker for preeclampsia as evidenced by increased levels of the cellular epitope of Fn in preeclamptic plasma. Increased plasma Fn receptor levels may also be predictive for preeclampsia, with or without abruptio placentae. Fibronectin deposition has been demonstrated to be increased in the placentae of preeclamptic patients and to be present at sites of atherosis with fibrin. Fibronectin has been shown to play a role in the coagulation process in the formation of crosslinked fibrin, as a substrate for wound healing and platelet aggregation at sites of endothelial injury, and as an opsonin for removal of tissue debris by monocyte-macrophages.

Fibronectin fragments have been shown to be produced by proteases, of which plasmin is especially relevant in the coagulation and lysis systems. Fibronectin fragments appear to have functions distinct from those of the intact molecule, including enhancement of chemotaxis for neutrophils and fibroblasts, transformation enhancing activity, DNA synthesis by fibroblasts and immunosuppression, and inhibition of opsonisation and cell attachment.

These observations suggest that fibronectin , and possibly fibronectin fragments, contribute in a distinct manner to the pathophysiology of preeclampsia, which led to the formation of an hypothesis to be examined in this thesis.

FIBRONECTIN: BIOLOGICAL AND IMMUNOLOGICAL ROLE IN PREECLAMPSIA

SECTION II

EXPERIMENTAL SECTION

CHAPTER 5 - EXPERIMENTAL SECTION

FIBRONECTIN - BIOLOGICAL AND IMMUNOLOGICAL ROLE IN PREECLAMPSIA

5.1 HYPOTHESIS

The aetiology of preeclampsia involves sequential and multifactorial processes. An alteration in the normal biology of the vascular endothelium in maintaining coagulation haemostasis, appears to be a major event in the disease process.

An initial event in this pathalogical process is exposure of endothelial cells to TNF- and IL-1, proposed to be generated by immunological processes (Vince *et al*, 1995). These cytokines are essential for the conversion of the endothelium from an anticoagulant to a procoagulant surface (Nawroth and Stern, 1986; Bevilaqua et al, 1984; Nawroth et al, 1986; Colucci et al, 1983). Concomitantly, endothelial cells acquire adhesive properties for leukocytes due to upregulated expression of endothelial cell adhesion molecules by these cytokines (McEver, 1991; Springer, 1990). This inducible procoagulant and proadhesive activity of the endothelium appears to be related to the DIC, the deposition of fibrin and extracellular cellular matrix proteins such as Fn along the endothelium, and the development of atherosclerosis observed in the preeclamptic syndrome (Robertson *et al*, 1967). The acquirement of procoagulant activity by endothelial cells is the result of changes in the synthesis and surface expression of endothelial proteins such as tissue factor (Nawroth and Stern, 1986; Bevilacqua et al, 1984; Nawroth et al, 1986; Colucci et al, 1983), tPA (Loskutoff and Edgington, 1977) and PAI-1 (Colucci et al, 1985; Erneis and Kooistra, 1986). The proadhesive activity of the endothelium is a consequence of the generation of thrombin from prothrombin during the final stage of a complex cascade of coagulation factor-mediated events initiated by endothelial cell-expressed tissue factor (Stern et al, 1983; Heimark and Schwartz, 1983; Stern et al, 1985). Thrombin not only upregulates receptors on the endthelium for platelets and leukocytes but converts fibrinogen to fibrin (Zimmerman et al, 1985; Bizios et al, 1986). Deposition of Fn on the endothelial surface is promoted by transglutaminase-mediated crosslinking to fibrin.

Although Fn has been implicated in the aetiology of preeclampsia (Taylor et al, 1991), its exact role is unclear. Increased circulating Fn concentrations could arise from several different events. Cytokines, released from macrophages and lymphocytes in response to the initial stimu-

lus may cause an acute phase response with increased hepatic synthesis of Fn. Circulating Fn concentrations are also influenced by Fn released from both the extracellular matrix, through the action of neutrophil proteases released during adherence of neutrophils to the endothelium (Wachtfogel et al, 1988), and from activated endothelial cells (Lockwood and Peters, 1990).

Fn released at the site of tissue injury could well have a profound effect on the ensuing pathology through the reported ability of Fn to influence cell adhesion (D'Ardenn and McGee, 1984). Fn is known to be sensitive to proteolytic cleavage by plasmin (generated at the site of injury) (Chen et al, 1977), suggesting too that FnDP may be of importance in modulating the pathology. These products could well competitively inhibit the binding of intact Fn to the extracellular matrix, disrupt the endothelial surface and influence endothelial cell activation. In addition, FnDP may disturb the normal opsonic function of plasma Fn (Brown, 1983), thus contributing to the multiple organ dysfunction described in severe disease.

An understanding of the possible role of Fn and Fn fragmentation in the course of preeclampsia may be of use to the clinician in careful management of patients.

5.2 AIMS OF THIS THESIS

i) To determine plasma and serum Fn concentrations in patients with preeclampsia, as well as levels of coagulation and fibrinolytic factors including plasma PAI-1, tPA and serum FDP, thought to be elevated in preeclampsia.

ii) To determine serial serum Fn, FDP and plasma tPA concentrations in patients in the third trimester and relate these to gestational age. (Note: Plasma Fn levels were not measured in this phase of the study, as serum levels showed greater differences between control and patient groups). Further to this, to determine whether these prospective, serial measurements can distinguish between patients with mild disease and those who progress to severe disease.

iii) To determine the structural and functional integrity of plasma Fn of the patient groups and controls.

iv) To evaluate the effect of FnDP on the measurement of Fn concentration by ELISA, and on opsonic activity, as measured by gelatin-latex agglutination assays.

v) To investigate the ability of FnDP to modulate vascular endothelial cell integrity using tissue culture techniques and immunohistochemistry.

5.3 STUDY DESIGN

Patients with symptoms and signs of preeclampsia (gestational proteinuric hypertension) were compared to a group of healthy, normotensive control pregnant women matched for age, race, parity and gestational age (in the third trimester). Patients were recruited from those attending Groote Schuur Hospital for antenatal care. The Peninsula Maternity and Neonatal Service serves a mostly indigent population where a high incidence of severe preeclampsia is found. Informed consent was obtained from all the women before blood samples were taken, and the study protocol was approved by the Ethics and Research Committee of the University of Cape Town.

Coagulation factors were all measured by enzyme-linked immunosorbent assays (ELISA). tPA and PAI-1 were assayed with commercially available ELISA kits according to manufacturer's instructions. FDP were measured using an "in-house" ELISA (Kruskal et al, 1987). An ELISA for Fn measurement was developed as part of this thesis. The aim was to produce an assay which would be sufficiently sensitive to be used to detect Fn in tissue culture samples and plasma samples from a broad spectrum of patients. For the development of this ELISA i) Fn was purified from normal plasma and ii) polyclonal and monoclonal antibodies to Fn were raised.

In order to determine the structural and functional integrity of Fn in the plasma of patient groups and controls, Fn was separated from patient and control plasma by gelatin-sepharose chromatography. This Fn was subjected to SDS-PAGE and examined for the presence of FnDP products using the Western blot technique and probing with the Fn monoclonal antibody developed for the ELISA.

The effect of FnDP (prepared by plasmic digestion of pure Fn) on the measurement of Fn by ELISA was examined.The influence of FnDP on the measurement of plasma Fn opsonic activity was also examined using a gelatin-latex agglutination assay.

In vitro assays to monitor the ability of Fn and FnDP to disrupt the endothelium were performed using human umbilical vein endothelial cell (HUVEC) cultures. Release of ^{51}chromate (^{51}Cr) from HUVECs challenged with Fn and FnDP purified from patient and control plasma was measured to assess their effect on endothelial cell stability. Disruption of HUVEC monolayers by these products was monitored by phase contrast microscopic examination.

To investigate the mechanism involved in HUVEC disruption by Fn or FnDP, a fibronectin matrix was labelled with ^{125}iodine (^{125}I) and HUVEC monolayers were grown to confluence on the matrix. Intact Fn and FnDP (prepared by 1h plasmic digestion or individual purification) diluted in culture medium were then added to the cells. Degradation of the Fn matrix was assessed by release of radioactivity into the culture medium. Modulation of endothelial cell activity was also monitored by uPA release from the cells.

CHAPTER 6 - EXPERIMENTAL SECTION

DEVELOPMENT OF ENZYME LINKED ABSORBENT ASSAYS (ELISA) FOR FIBRONECTIN AND FIBRIN(OGEN) DEGRADATION PRODUCTS

6.1 PURIFICATION OF FIBRONECTIN

6.1.1 Background

Early Fibronectin (Fn) purification techniques used precipitation steps with fibrinogen or heparin followed by ion exchange chromatography (Mosesson *et al*, 1975; Kleinman *et al*, 1979). Plasma fibronectin (pFn) is currently purified by means of affinity chromatography on gelatin-Sepharose (Engvall *et al*, 1977; Vuento and Vaheri, 1979; Furie and Rifkin, 1980; Ruoslahti *et al*, 1982). Gelatin-Sepharose affinity chromatography makes use of the affinity of Fn for collagen and allows for isolation of electrophoretically pure Fn in one step, with very good yields from plasma, cell culture media and cell extracts (Ruoslahti *et al*, 1982). Fn from plasma and cell cultures has also been isolated on anti-Fn columns (Vuento *et al*, 1977).

6.1.2 Methods and results

i) Blood collection

For purification of stock Fn 45-90 ml blood samples were collected by venepuncture and transferred into test-tubes containing 5-10 ml of a solution of 3.8% sodium citrate (anticoagulant) and 0.025M ε-aminocaproic acid (EACA), a protease inhibitor which binds to plasminogen (plg). Fn was also isolated from 500ml aliquots of fresh frozen plasma obtained from the Groote Schuur Hospital Bloodbank. 5mM benzamidine was added to the plasma immediately after centrifugation and plasma was filtered (Whatman's No. 1 filter paper) to remove insoluble lipid. For purification of patient Fn, blood samples of 9 ml were collected from patients by venepuncture and transferred into test tubes with 1 ml anticoagulant solution as for stock Fn purification.

Citrated specimens were centrifuged at 2500 rpm for 10 min. within 4 hours of collection, and the plasma was transferred into cryotubes for storage at -70°C until use.

ii) Gelatin-sepharose affinity chromatography

The non-denaturing affinity chromatography method of Vuento and Vaheri (1979) was adapted for use.

Gelatin-sepharose affinity matrix was prepared by coupling gelatin to cyanogen bromide (CNBr)-activated Sepharose 4B according to the manufacturer's instructions. 5mM benzamidine was added to all buffers to prevent proteolysis and 0.02% sodium azide was added to the buffers to prevent bacterial contamination.

A 1.5 x 13 cm column (Amicon) was equilibrated with equilibrating buffer (0.05M Tris-HCl, pH 7.5). 50 ml plasma was loaded and washed through the column with equilibrating buffer until the UV monitor recording (280 nm) returned to zero. 1M NaCl was added to the equilibration buffer and the column washed again. Fn was then eluted with 2M arginine in equilibrating buffer without 1M NaCl in 1 ml aliquots. The column was regenerated with 8M urea.

The eluate fractions containing the protein peak were analysed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The protein peak was dialysed into phosphate buffered saline (PBS), pH 7.3 or Tris-HCl, pH 7.5 using Spectrapor 4 dialysis membrane (MWT cut-off 12-14 000) and the protein concentration was determined by means of the Biorad method (Bradford, 1976). Measurement of the optical density (OD) at 280 nm against an arginine buffer blank was not a satisfactory procedure for determination of protein concentration, as commercially available arginine contains impurities that interfere with UV absorbance.

iii) Cellular fibronectin purification

Human fibroblasts were cultured in 10% fetal calf serum in Minimum Essential Medium (MEM) until the monolayer was confluent on the surface of the flask (250ml Falcon). The monolayer was washed with PBS, pH 7.3 and incubated for 24 hours with 5% Fn-free plasma (effluent from gelatin-Sepharose purification of Fn) in MEM. The culture supernatant fluid was harvested for purification of cellular Fn released by the fibroblasts.

25 ml harvest fluid was loaded onto a gelatin-sepharose column (1.5 x 10 cm) which had been equilibrated with 0.05M Tris-HCl, pH 7.5. Cellular Fn was eluted in 2 ml aliquots with 2M arginine in equilibrating Tris buffer and the protein peak was dialysed (Spectrapor 4) into PBS, pH 7.3.

Both plasma and cellular Fn preparations were subjected to SDS-PAGE (Laemmli, 1970) and Western blotting (Towbin, 1979) with a monoclonal antibody against Fn to determine their purity.

iv) Preparation of fibronectin fragments

A number of Fn fragments were selected for purification. The 29 kDa hep1 amino-terminal domain and the 31 kDa fib1 carboxy-terminal fragment (see Figure 4.2, chapter 4) were isolated by affinity chromatography using successive gelatin- and heparin-Sepharose matrices (Pardo *et al*, 1983).

Fn (1mg) was digested (15 min room temperature) with trypsin (1000:1, enzyme:protein). Digestion was stopped with EACA (0.025M). The digest containing cleavage products of 200, 180, 31 and 29 kDa was dialysed into Tris-HCl pH 7.5 and loaded onto gelatin-Sepharose (3.5 ml column volume) with equlibrating buffer (Tris-HCl, pH 7.6). The two high MWT fragments bound to gelatin and were thus removed. The flow-through was collected and the peak was loaded onto heparin-Sepharose (3.5 ml volume) for isolation of the 31 kDa and the 29 kDa fragments. The 31 kDa fragment does not bind to heparin, therefore the effluent containing the 31 kDa fragment was collected in Tris-HCl, pH 7.5. The 29 kDa fragment bound to heparin and was eluted from the column with 0.15M NaCl in Tris-HCl buffer (Figure 6.1a and b).

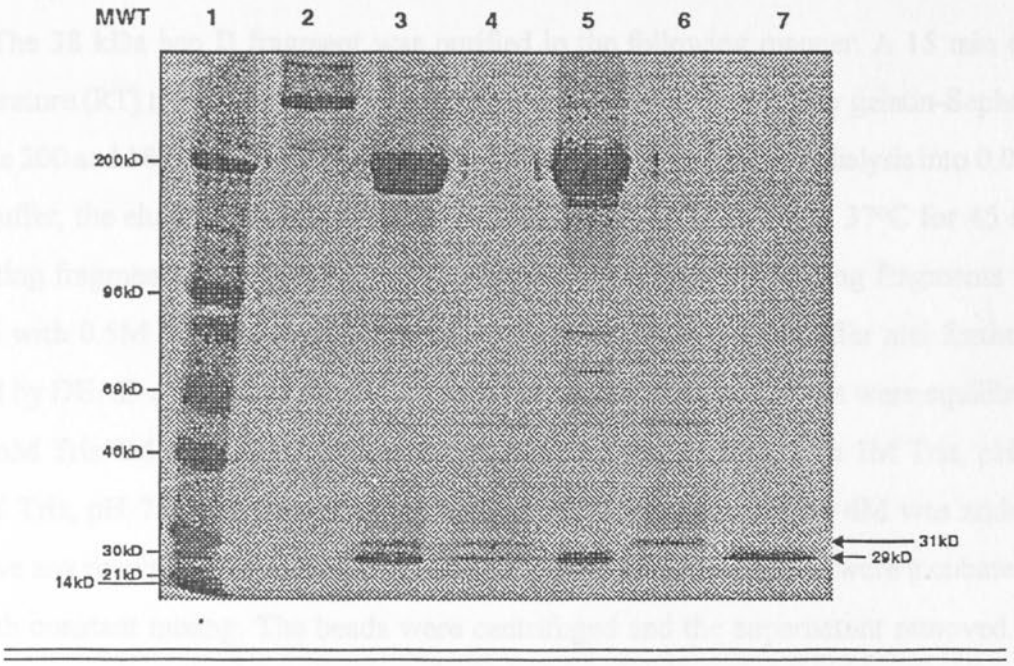


Figure 6.1a - SDS-PAGE gel depicting purification of the 29 kDa fibronectin (Fn) fragment. Lane 1: MWT markers, lane 2, intact Fn, lane 3, 15min trypsin digest of Fn, lane 4, gelatin-Sepharose flow through, lane 5, gelatin-Sepharose eluate, lane 6, heparin-Sepharose flow through, lane 7, 29kDa Fn fragment.

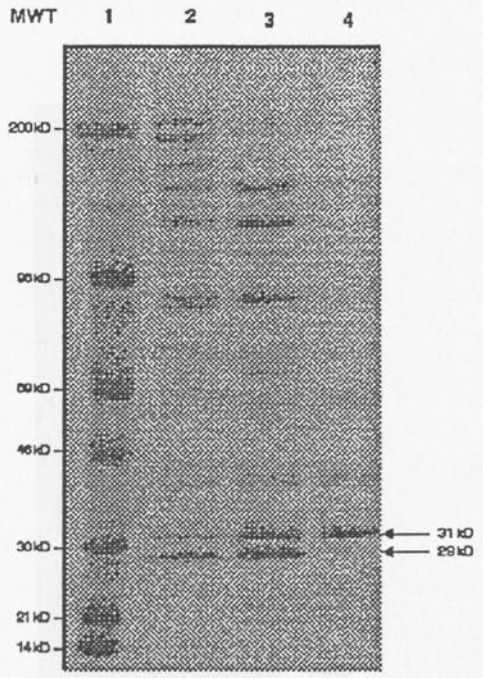


Figure 6.1b - SDS-PAGE gel depicting purification of the 31 kDa fibronectin fragment. Lane 1, MWT markers, lane 2, 15 min trypsin digest of Fn, lane 3, gelatin-Sepharose flow through, lane 4, heparin-Sepharose flow through containing pure 31 kDa fragment.

The 38 kDa hep II fragment was purified in the following manner. A 15 min room temperature (RT) trypsin digest (1000:1, enzyme:protein) was passed over gelatin-Sepharose and the 200 and 180 kDa fragments eluted with 4M-urea. Subsequent to dialysis into 0.025M Tris buffer, the eluted protein was digested with trypsin (1:100 w/w at 37°C for 45 min). Resulting fragments were applied to heparin-sepharose. Heparin-binding fragments were eluted with 0.5M NaCl in Tris buffer, dialysed against 10mM Tris buffer and further re-solved by DEAE-cellulose chromatography. The DEAE-Sephacel beads were equilibrated in 10mM Tris/4M urea, pH 7.0 (without inhibitors) after washing with 1M Tris, pH 7.0, 50mM Tris, pH 7.0 and measuring the conductivity. Solid urea up to 4M was added to dissolve any precipitate formed during dialysis. 10 mg protein/ml beads were incubated for 1h with constant mixing. The beads were centrifuged and the supernatant removed. The unbound material which contained the 38 kDa fragment was dialysed against water then freeze-dried (Pardo *et al*, 1987) (Figure 6.2c.)

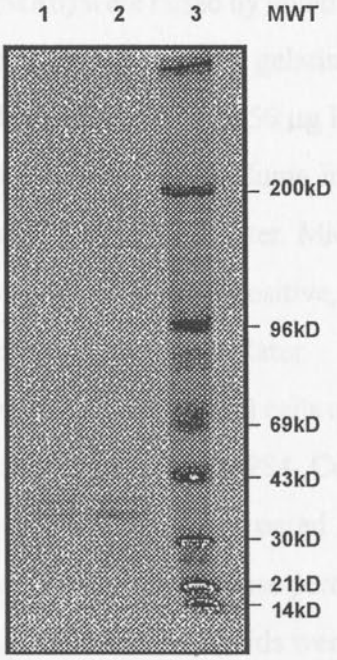


Figure 6.1c - SDS-PAGE gel depicting purified 38 kDa fibronectin fragment.
Lane 1, DEAE-Sephacel effluent, lane 2, 38kDa fragment, lane 3, MWT markers.

6.2 PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES TO FIBRONECTIN

6.2.1 Background

Possibly the most important factor in establishing an ELISA is the production of suitable polyclonal (PAb) and/or monoclonal (MAb) antibodies. The facility of antibody production is determined by the immunogenicity of the antigen, i.e. its ability to invoke an immune response in the immunised animal. Fn, with a MWT of 440 kDa, appears to be a good immunogen, as judged by a number of studies in which both PAbs and MAbs have been successfully raised (Atherton and Hynes, 1981; Borsi *et al*, 1987; Carnemolla *et al*; 1987 Click, 1985; Matsuura, 1985; Ruoslahti *et al*, 1981; Zardi *et al*, 1980).

Studies of the different isoforms of Fn and the structural integrity of the molecule, as well as its distribution, have mostly been possible due to the production of MAbs specific for certain epitopes or sequences, the expression of which is regulated by the alternative splicing of mRNA (Paul *et al*, 1986).

6.2.2 Methods and results

i) Immunisation, hybridoma cloning and ascites production

Monoclonal antibodies (MAb) were raised by immunisation of BALB/c mice (Kearney, 1984) with Fn isolated from human plasma by gelatin-Sepharose chromatography. The mice were initially immunised intradermally with 50 μg Fn in PBS homogenised in an equal volume of complete Freunds adjuvant (total volume injected was 500 μl). This was repeated in incomplete Freunds adjuvant a week later. Mice were bled and serum was tested for the presence of antibodies specific for Fn. If positive, the mice had a booster of 50μg Fn in PBS, and their spleens were removed a week later.

Spleen cells were fused with SP2 (myeloma) cells using polyethylene glycol 4000 and hybridomas cultured as described by Kearney, 1984. Culture fluids were screened for the presence of Fn antibodies by ELISA using Fn-coated microtitre wells blocked with 1% fetal calf serum in PBS, pH9 with a goat anti-mouse peroxidase labelled detection antibody (Engvall and Perlman, 1971). Positive culture fluids were cloned. One clone, CJ1, producing the highest titre of antibody (ELISA positive at dilutions >1/2000) was selected and subcultured to produce enough cells to inject into mice for the production of ascites to enhance the antibody titre (Kearney, 1984). 5-10^6 cells in 0.5 ml PBS were injected into each of 6 mice which were tapped for ascites approximately three days later, once the abdomens had swollen. The IgG fraction of the ascites was purified by protein A affinity chromatography (according to supplier's instructions). Purified IgG was stored at -70°C in small aliquots.

ii) Characterisation of monoclonal antibody CJ1

The specificity of the MAb in hybridoma supernatant fluid G6 was tested by comparing its avidity for Fn and fibrinogen (to test for cross-reactivity) in an ELISA. A known MAb specific for fibrinogen, F11 (in-house), was used as a control. A Western blot was performed by transferring Fn and fibrinogen from a 14-5% SDS-polyacrylamide gel onto nitrocellulose (Towbin *et al*, 1979) and probing with the MAb CJ1 in a blocking buffer (2% casein, 0.3% Tween 20 in 0.1M NaCl, 0.05M Tris/HCl, pH 7.4) to compare the specificity of CJ1 for these proteins.

Further characterisation of purified ascites, CJ1, was performed using the MAb as a probe for Fn in the following Western blots :

1) Fn (purified and in serum) reduced with β-mercaptoethanol (βME).

2) Fn digested with plasmin (using 500U urokinase/mg plg [0.32 mg to 10 mg Fn]).

3) cellular Fn produced by human fibroblasts in culture.

4) human plasma and serum.

6.2.3 Results

The harvest fluid supernatant CJ1 was specific for Fn. There was no cross-reactivity of CJ1 with fibrinogen in the Western blot (Figure 6.2), or of F11 with Fn in the ELISA.

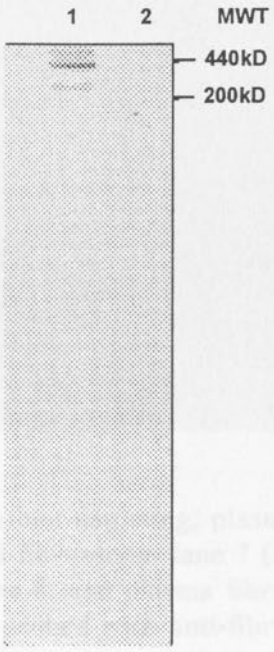


Figure 6.2 - A Western blot depicting fibronectin (1) and fibrinogen (2) blotted onto nitrocellulose and probed with antifibronectin (CJ1) MAb. MWTs indicated on the right.

Western blotting results

i) The MAb, CJ1, recognises non-reduced, purified Fn or Fn in serum, but does not recognise Fn or any Fn fragments (produced by plasmin digestion or present in serum) after reduction of disulphide bonds with 2% β-ME (Figure 6.3).

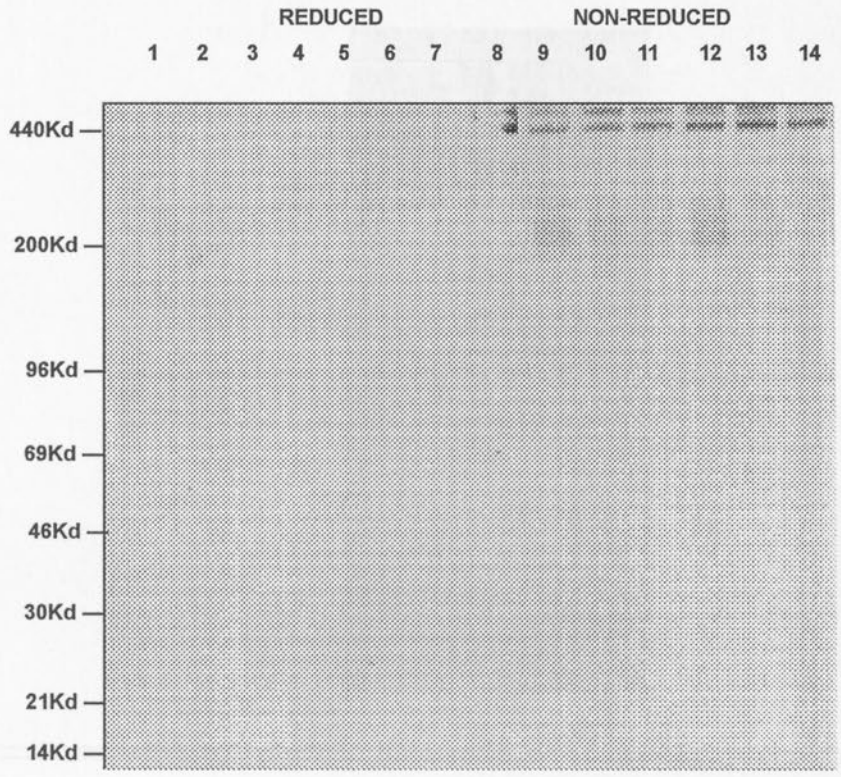


Figure 6.3 - A Western blot depicting, plasma fibronectin samples lanes 1-6, and purified fibronectin, lane 7 (reduced with β-ME), purified fibronectin, lane 8, and plasma fibronectin samples, lanes 9-14 (non-reduced), probed with anti-fibronectin MAb, CJ1.

ii) Fn digested with human plasmin for 1 to 5 hours produced fragments which migrated at MWTs ranging from approximately 280kDa to 28kDa. (Figure 6.4). CJ1 appears to recognise the fragments with apparent MWT of 280, 260, 240, 220, 200, 180, 150, 100, 80, 70 and 28kDa (Figure 6.4).

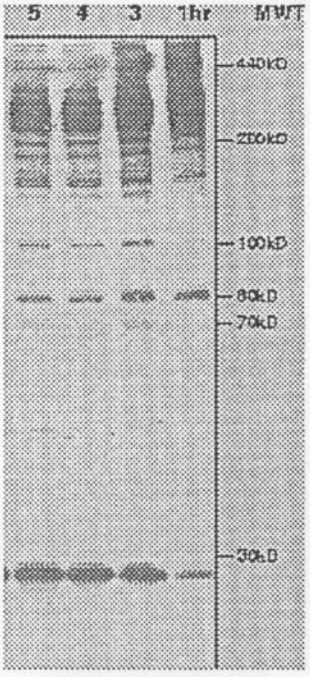


Figure 6.4 - A Western blot depicting fibronectin digested with plasmin for 1 - 5 hours, lanes 1 - 4. MWT are indicated on the right.

iii) Cellular Fn (cFn) was recognised by CJ1. CJ1 appeared to recognise the intact 440kDa molecule and a Fn monomer migrating at approximately 260kDa, a larger subunit than that of pFn monomers at 220 and 240kDa (Figure 6.5). Mab IST-9, specific for the ED domain (a gift from Professor Zardi) was used to confirm that the Fn purified from fibroblast cultures was cFn (Figure 6.6). IST-9 did not react with plasma Fn.

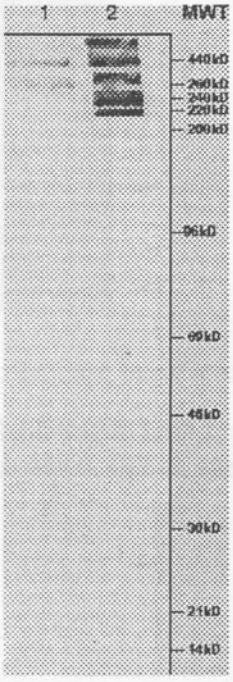


Figure 6.5 - A Western blot depicting in lane 1, cellular fibronectin, lane 2, plasma fibronectin probed with MAb,CJ1. MWTs are indicated on the right.

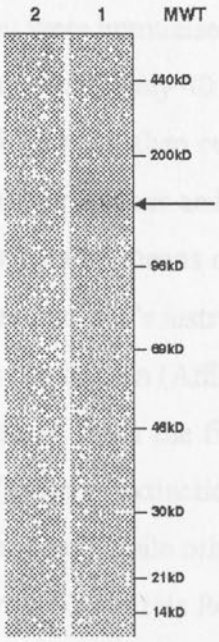


Figure 6.6 - A Western blot depicting in lane 2, plasma fibronectin, lane 1, cellular fibronectin, probed with MAb, IST-9, specific for cellular fibronectin.

6.3 POLYCLONAL ANTIFIBRONECTIN ANTIBODY (ANTISERUM) PRODUCTION AND CHARACTERISATION

6.3.1 Background

Antiserum (immunoglobulin) can be raised in an animal immunised with a particular purified protein. Once the antibody titre is sufficiently high the animal is bled. The IgG can be purified from the serum relatively easily and provides a useful tool for detection of the protein it was raised against. The importance of a polyclonal antibody (PAb) lies in its ability to recognise the entire antigen rather than one specific epitope. Thus, if the protein is degraded it will still be detected by the PAb, whatever the conformation of the molecule, and independently of any one specific epitope. For this reason PAbs are useful as capture antibodies in the ELISA.

6.3.2 Methods and Results

Rabbits (New Zealand white) were immunised with 500 μg human Fn as described by Kirsch *et al* (1975) after which approximately 40 ml blood was collected. The blood was left at room temperature to clot for 1 hour, then centrifuged at 2500 g for 30 minutes. The serum was removed, aliquotted into cryotubes and frozen at -70°C for later purification of the IgG. IgG was purified from serum by means of affinity chromatography with DEAE-Affigel Blue according to the manufacturer's instructions.

4 ml serum was loaded onto the column (Affigel blue) (1 x 4 cm) and an approximate yield of 2.5 mg/ml IgG was obtained from the first aliquot eluted, as estimated by UV absorption at 280nm and 260nm (using an extinction coefficient for Fn of 1.3). IgG washes through at the front of the effluent peak, while other serum proteins bind to the Affigel.

A Western blot of plasma probed with this PAb revealed that it was binding to Fn as well as to albumin which migrates at MWT 69 kDa, so a further purification step was performed. An albumin-Sepharose affinity column was prepared by coupling 150 g bovine serum albumin (BSA) to 5 gm swollen CnBr-Sepharose beads blocked with ethanolamine.

3 ml Affigel-blue purified IgG was loaded on the column (1 x 6 cm).. The effluent was collected in 0.05M Tris-HCl buffer, pH 7.5. The yield of polyclonal IgG was 1.6 mg/ml in 5 ml. When tested on Western blots of plasma the PAb no longer recognised albumin and was specific for intact Fn (Figure 6.7).

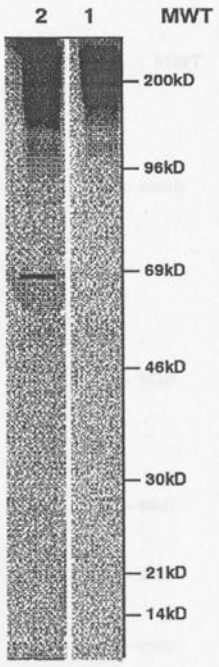


Figure 6.7 - A Western blot depicting plasma probed with polyclonal antibody (PAb) to fibronectin. Lane 2, Affigel blue purified PAb. Lane 1, albumin-Sepharose purified PAb. MWTs indicated on the right

Specificity and cross reactivity of the antiserum was assessed by Western blotting. The anti-Fn PAb was able to detect Fn and plasmin digested Fn fragments, either non-reduced or reduced with β-ME (Figure 6.8). This PAb recognises Fn in tissue sections by immunohistochemistry (figures in chapter 9) in a manner similar to that reported for Fn using standard antisera.

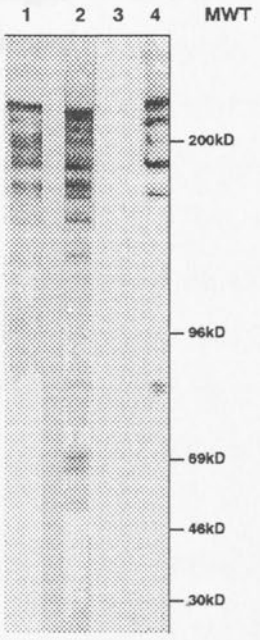


Figure 6.8 - Fibronectin digest (lanes 1 and 3 reduced with βME, lanes 2 and 4, non-reduced) blotted onto nitrocellulose and probed with anti-fibronectin PAb (lanes 1 and 2), and MAb, CJ1 (lanes 3 and 4).

6.4 DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF FIBRONECTIN

6.4.1 General principles of enzyme linked immunoassay (ELISA).

Enzyme-linked immunoassays are now firmly established as providing a precise quantitative technique for the rapid determination of concentrations of various antigenic substances and antibodies (Sharpe *et al*, 1976; Tijssen, 1985; Blaschke *et al*, 1990; Ruoslahti *et al*, 1982) and as such, play an integral role in any diagnostic, clinical service. It was decided to develop and monitor an "in-house" ELISA system in order to establish a set of normal, healthy, control plasma Fn values. This would provide a baseline for further measurements and comparisons of Fn levels in patients. As the scope of this thesis includes a large number of Fn assays, commercially available ELISA kits were not a viable proposition in terms of their expense. A non-competitive, double antibody sandwich technique was developed in preference to a competitive technique. The general principle of the "sandwich" ELISA is summarised by the following set of reactions:-

Non-Competitive ELISA:

1st Antibody + Antigen = 1st Antibody-Antigen Complex

(rabbit antihuman PAb) *(human)*

\+

2nd Antibody

(mouse antihuman MAb)

↓

Ab1-Ag-Ab2

\+

Peroxidase-conjugate-labelled Ab

(antimouse)

↓

Ab1-Ag-Ab2-PC-Ab

\+

substrate + H_2O_2

The non-competitive, double antibody technique is based on the principle of using a capture antibody on the solid phase which is specific for the antigen being measured. Samples are diluted so that all of the antigen in solution is able to complex with the capture antibody. A second antigen-specific antibody is added in excess in solution and binds to the complex already formed. The greater the ratio of antigen to antibody in the complex, the greater the concentration of the second antibody which binds to the complex.

A peroxidase-conjugate (P-C)-labelled antibody, specific for IgG of the animal species in which the preceding antibody was raised, is used as a detection antibody. The peroxidase on this antibody will react with a chromogenic substrate added in the presence of hydrogen peroxide to give a colour reaction. The stronger the colour reaction, the higher the concentration of antigen in the sandwich in the non-competitive ELISA.

The validity of an assay is dependent upon the identical behaviour of the standard and the antigen in unknown samples. The unknown sample must be capable of producing a "dose-response curve", when assayed at different dilutions, identical with the antigen curve produced by the standard. A corollary to this requirement for validity is the minimisation of non-specific effects on the antibody-antigen reaction.

6.4.2 Method (After Ruoslahti *et al*, 1982)

1. The wells of a 96 well round-bottom microtitre plate (Microwell module U-16) were coated with 50 µl purified rabbit anti-human Fn PAb (described previously), diluted in PBS-9 to approximately 5µg/ml and incubated at 4°C overnight (O/N).

2. Wells were blocked with 200 µl 2% BSA in PBS-9 for 1-2h at 23°C to prevent non-specific binding to wells.

3. 50 µl/well of Fn standard (purified as described), control and test sera were were added at desired dilutions in ELISA diluent (0.5M NaCl, 0.01M $Na_2HPO_4.12H_2O$, 0.5% Triton X-100) with 2% BSA and incubated for 1hour (h) at 23°C on an ELISA shaker.

4. Wells were washed 7-10 times with Tris-saline-Tween (0.1M NaCl, 0.05M Tris, pH 8.0, 0.5 ml/L Tween 20).

5. 50 µl/well Fn MAb was added at 1/5000 dilution in ELISA diluent and incubated for 1h at 23°C on shaker.

6. Wells washed as before. 50 µl/well goat anti-mouse immunoglobulin (IgG, IgA, IgM) peroxidase-conjugate at 1/25 dilution of stock in ELISA diluent was added and incubated for 1h at 23°C on shaker.

7. Wash step repeated.

8. 40mM ABTS substrate (2.2 azino-bis(ethyl-benzthiazoline sulphonic acid) in citrate buffer (0.1M sodium citrate, pH 4.0) at 1/25 dilution plus 1:100 volume of H_2O_2 (30% hydrogen peroxide) was added to the wells. The colour reaction was developed for 10-15 minutes.

9. The optical density (OD) was measured in a Titertek Multiscan MCC/340 ELISA reader at 414 nm absorbance against a blank.

10. Linear regression analysis was performed using the standard curve to obtain results for the samples. Each sample was tested in duplicate at three different dilutions.

6.4.3 Results

Optimum antibody and antigen titres: Optimal concentrations of mouse monoclonal anti-Fn and rabbit anti-Fn polyclonal IgG, sample antigen and goat antimouse peroxidase conjugate were determined with checkerboard titrations or dilution curves.

curve derived by using freshly prepared human Fn (Figure 6.8.1).The assay was able to detect 0.078 μg of Fn in 1 ml of solution and a linear standard dose response curve was obtained between 0.156 μg/ml and 5 μg using a linear regression equation, where r =/>9.0.



Figure 6.8.1 - A standard dilution curve using human fibronectin. Substrate absorbance (OD) depicted on Y-axis and amount of Fn in solution on X-axis.

Specificity of the assay: MAb CJ1 has been shown to be highly specific for Fn and not to cross-react with fibrinogen or albumin as detailed above.

Intra-assay variation or reproducibility: The mean +/-2 SD calculated for 10 readings of 1 sample at two dilutions had a reproducibility of 95%.

Inter-assay variation: Calculated values for an internal standard used in 10 different runs of the ELISA were used to calculate the inter-assay variation, which did not exceed 5%.

Normal control serum and plasma Fn levels were established by testing 10 apparently healthy control sera and matching plasma samples at three different dilutions in duplicate. The published normal plasma Fn range is approximately 300-400 μg/ml. Plasma from patients with

trauma (reportedly low) or preeclampsia (reportedly high) were compared with normal levels (Table 6.1) as were sera from patients with cirrhosis or portal vein thrombosis (POVT) with reportedly low Fn levels (Table 6.2). Dilutions of between 1 in 40 and 1 in 320 were used to cover the measurable concentration range (6.24-800 μg/ml) for patient normal and samples. Dilutions of between 1 in 2 and 1 in 20 were used for other fluid samples eg. ascites and tissue culture SNFs.

	Normal pFn (μg/ml)	Trauma pFn (μg/ml)	Preeclampsia pFn (μg/ml)
Mean	312.9	237	376
SD*	14	85.6	28.2

Table 6.1 - Comparison of plasma fibronectin (pFn) levels in normal controls and patients.

	Normal serum sFn (μg/ml)	POVT* sFn (μg/ml)	Cirrhosis sFn (μg/ml)
Mean	89	60.7	66.6
SD*	14.6	27.7	35.6

* POVT denotes portal vein thrombosis

* SD denotes Standard Deviation

Table 6.2 - Comparison of serum fibronectin (sFn) levels in normal controls and patients as measured by sandwich ELISA.

6.5 DISCUSSION

Purified Fn was needed for immunisation of animals in which MAb and PAb antibodies were to be raised. It was also needed as a standard antigen for the ELISA assay and a MWT marker for comparison with patient plasma Fn by means of SDS-PAGE and Western blotting techniques. In addition, Fn was used to prepare Fn digests for use in a number of immunoassays and biological assays.

Most of the procedures required the purified Fn to be intact, immunologically and biologically active and suitable for tissue culture procedures. Benzamidine was added to plasma directly after centrifugation as a precaution against proteolytic degradation of Fn. The purification procedure of Vuento and Vaheri (1979) was adapted. This procedure uses arginine as an eluting agent in preference to urea which is the more common choice. Urea, in comparison to arginine, is reported to be a harsh elution agent and may not protect Fn from degrading (Ruoslahti *et al*, 1982), although the yields of Fn using arginine are lower than the yields using urea elution (Vuento and Vaheri, 1979). Washing of the matrix with 1M NaCl before elution is also an improvement over earlier methods as this wash removes non-specific gelatin-binding material and thus reduces contamination of the protein.

Fn preparations were more than 90% homogeneous as determined by SDS-PAGE. A small amount of degradation of the 440 kDa Fn dimer into monomeric subunits of 240 and 220 kDa was invariably evident. cFn has an extra domain which is spliced out of the plasma isoform of Fn. It therefore migrates more slowly than pFn, and has a correspondingly higher MWT.

The specificity of the antibody CJ1 to Fn is based on the following criteria: 1) It binds to purified human plasma Fn in the ELISA system, 2) but not to fibrinogen. 3) Western blotting analysis of plasma proteins shows that the MAb reacts specifically with a protein of MWT analogous to that of human plasma Fn (440kDa) as well as with Fn fragments produced by enzymatic cleavage, but not with fibrinogen.

CJ1 appears to recognise an antigenic determinant or conformational epitope which is common to most of the Fn fragments formed by plasmin digestion (240, 220, 200, 180, 100, 80, 70 and 30/28 kDa) as presented in the model of Ruoslahti *et al*, 1981 (Figure 4.4, Chapter 4). The fact that CJ1 does not recognise Fn subunits or Fn fragments after reduction with β-ME suggests that the conformation recognised is provided by the disulphide bonds found in all type I and II repeats which occur mainly near the termini of the dimeric molecule (domains 1, 2 and 6) Figure 4.2, Chapter 4).

Zardi *et al* (1980) have produced somatic cell hybrid MAbs recognising epitopes common to both pFn and cFn. While MAbs able to distinguish between the plasma and cellular forms of Fn have been reported (Atherton and Hynes, 1981; Keen *et al*, 1984; Matsuura and Hakomori, 1985), the exact localisation within the Fn molecule of their epitopes has never been possible (Carnemolla *et al*, 1987). As the Fn molecule comprises many repeating subunits of types I, II and III, this is not surprising.

MAbs recognising specific sequences of the Fn molecule, which can be included or omitted from the molecule depending on the pattern of mRNA splicing (regions ED and IIICS, Figure 6.2) have now been produced using β-galactosidase-Fn fusion proteins expressed in *Escherichia coli* (Carnemolla *et al*, 1987).

Although the exact epitope(s) recognised by CJ1 has not been determined, this MAb is nonetheless a useful tool for studying the presence, distribution and structural integrity of the Fn molecule in plasma and tissues of patients with disease states in which Fn may play an important role, such as preeclampsia. The capacity of CJ1 to detect such a wide range of Fn fragments produced by enzymic cleavage is advantageous in distinguishing the extent of fragmentation of Fn in different patients. MAbs used in the Western blotting technique also provide a much more sensitive technique for detection of protein than does Coomassie Blue staining of SDS-PAGE gels.

Our results for Fn levels in normal, healthy control plasma compared favourably with those generally reported in the literature (300-400 μg/ml). This indicates that the assay is detecting Fn within the range of values expected. The Fn levels measured in serum are lower due to Fn uptake into the clot during serum production. The double antibody ELISA system developed was used to assay Fn in plasma and serum samples from a number of different groups of patients eg. cirrhotics, trauma patients (reported to be reduced), patients with preeclampsia (reportedly raised) as well as normal, healthy controls.

Influence of assay technique on accuracy of measured fibronectin (Fn)

A number of different techniques including turbidimetry, nephelometry, electroimmunoassay as well as ELISA assays using collagen, gelatin, Fn or anti-Fn IgG as the capture antibody, have been used to determine Fn levels. The range of Fn values for similar groups of control and patient samples measured, using these different techniques, differs quite considerably (Table 6.3) (Bykowska *et al*, 1985). This may be due to measurement of different parameters by different

techniques eg. bioactivity, immunoreactivity or protein-complex formation by turbidimetry. Different Fn concentrations have been measured on the same samples using different types of assays (Bykowska *et al*, 1985; Brubaker at al, 1992). Some researchers have proposed that measured levels may be falsely low due to the presence of dysfunctional Fn in some samples (Brubaker *et al*, 1992) or falsely elevated levels of Fn due to fragmentation of the molecule (Stubbs *et al*, 1984).

Assay	Author	Fn (µg/ml)
Electroimmunoassay	Eriksen *et al*, 1987	368 (324-400)
Nephelometry	Thurnau *et al*, 1987	897 SD 60
Immunoturbidimetry	Molnar *et al*, 1988	342-350.6
ELISA	Hess *et al*, 1986	262.6 SD 6

Table 6.3 - Comparison of fibronectin (Fn) concentrations measured by means of different assays in healthy women.

6.6 ENZYME-LINKED IMMUNOSORBENT ASSAY FOR FIBRIN- AND FIBRINOGEN-RELATED ANTIGENS (FRA/FDP) IN SERUM

6.6.1 Background

Recent coagulation research has concentrated on the development of specific ELISAs capable of detecting epitopes present on certain fibrin or fibrinogen derivatives. MAbs have been developed against a variety of specific epitopes on fibrin, fibrinogen or their fragments and are thus able to differentiate between fibrin and fibrinogen degradation products (FDP). In order to avoid problems associated with measuring fibrin and fibrinogen, and fibrin and fibrinogen degradation products in serum, antibodies reacting specifically with the γ-γ site of cross-linked fibrin derivatives, but not with fibrinogen, have been developed, allowing for sensitive fibrin fragment measurement in plasma (Lahiri *et al*, 1981; Elms *et al*, 1983; 1986; Soria *et al*, 1983).

However, little attention has been given to the development of assays for measuring the entire spectrum of fibrin- and fibrinogen-related antigens (FRAs). Due to the diagnostic and

prognostic role that FRA/FDP measurements may play in certain diseases associated with DIC and certain cardiac, liver and thrombo-embolic diseases, the necessity has arisen for the development of an ELISA that is able to measure FDPs rapidly and that is simple to perform.

A non-competitive, four-layered, "sandwich" enzyme linked immunoassay has previously been developed in our laboratory for fibrin- and fibrinogen-degradation products, based on the use of MAb, F11, directed against the entire spectrum of fibrin and fibrinogen fragments in serum (Kruskal, 1987). The methods, results and discussion following are summarised from Kruskal J (1987).

6.6.2 Methods

The following antibodies were obtained for the development of the sandwich ELISA:

Goat anti-human fibrinogen IgG (capture antibody).

Mouse anti-human FDP MAb, F11, used as a detection antibody was a gift from Dr I Garcia of the Department of Clinical Science and Immunology, UCT Medical School.

Goat antimouse IgG, IgA, IgM peroxidase-conjugate (second detection antibody).

Human fibrinogen (standard) was purified by ammonium sulphate precipitation as described by Kalvaria *et al* (1987). Purity was confirmed on SDS-PAGE and the final purified protein was dialysed into 0.05M sodium phosphate buffer, pH 9.0 containing 0.15M sodium chloride.

Serum samples were prepared by clotting samples in a mixture containing thrombin (bovine) 20 NIH units and soya bean trypsin inhibitor (approx 3600 NIH units), and incubated for 4h at 4°M as recommended by Merskey *et al*, (1972). Clarified serum samples were transferred to cryotubes and stored at -70°C until assay.

The assay technique was performed according to the standard method described in Section 6.4 using the appropriate antibodies and serum samples.

6.6.3 Results

Specificity of antigen binding of the goat-polyclonal and mouse monoclonal anti-fibrinogen IgG were assessed by Western blotting and immunodiffusion in agar gel. The goat-polyclonal and mouse monoclonal anti-fibrinogen IgG bound to all the principal high and low molecular weight degradation fragments of fibrinogen and cross-linked fibrin, including fibrinogen, D-monomer, D-dimer, fragments X and E and non-crosslinked fibrin monomers (Figure 6.9a).

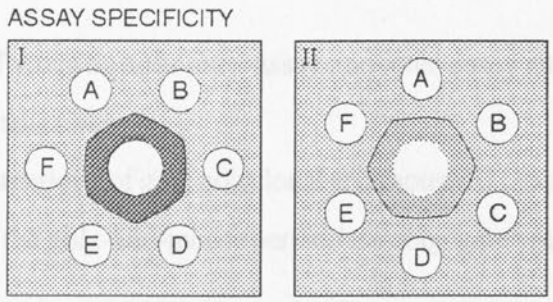


Figure 6.9a - Assessment of specificity of antigen detection of the ELISA. Goat anti-fibrinogen IgG (I) or mouse anti-FDP monoclonal IgG (II) were placed in the central wells of an agar immunodiffusion gel. Fibrinogen (A), D-monomer (B), D-dimer (C), fibrin monomer (D), fragment X (E) and fragment E (F) were placed in the surrounding wells. Diffusion and immunoprecipitation within the gel were allowed to take place for 48h after which the gels were washed and stained. (Redrawn from Kruskal, 1987).

Human albumin, bilirubin, plg, transferrin and Fn were used to demonstrate the lack of nonspecific binding, and the immunoreactivity with goat polyclonal and mouse monoclonal anti-human fibrinogen IgG. Nonspecific binding to these antibodies by the abovementioned serum proteins did not occur and no lines of immunoprecipitation between these proteins and antibodies were produced in agar gel (Figure 6.9b).

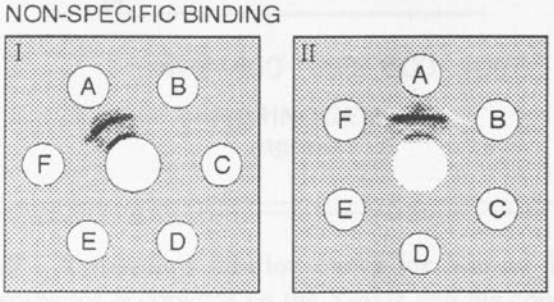


Figure 6.9b - Assessment of nonspecific binding and interference with antigen detection with the ELISA. Goat anti-fibrinogen IgG (I) or mouse monoclonal antifibrinogen IgG (II) were placed in the central wells of an agar immunodiffusion gel. Fibrin digest (positive control) (A), Fn (B), plasminogen (C), albumin (D), transferrin (E) and bilirubin (F) were placed in the surrounding wells. Diffusion and immunoprecipitation within the gels were allowed to take place for 48h after which the gels were washed and stained.(Redrawn from Kruskal, 1987).

ELISA Results

The coefficient of variation for intra-assay and inter-assay measurements did not exceed 4.8% and 5.4%, respectively.

Optimum concentrations of goat polyclonal and mouse F11 monoclonal anti-fibrinogen IgG, sample antigen and the goat-anti-mouse peroxidase conjugate were determined with serial dilution curves.

Results of FDP measurements are expressed in μg/ml of solution relative to a calibration curve derived by using freshly prepared human fibrinogen.

Limits of detection: The assay was able to detect 3.9 ng of fibrinogen in 1 ml of solution and a linear standard dose response curve was obtained between 6 and 600 ng (Figure 6.10).

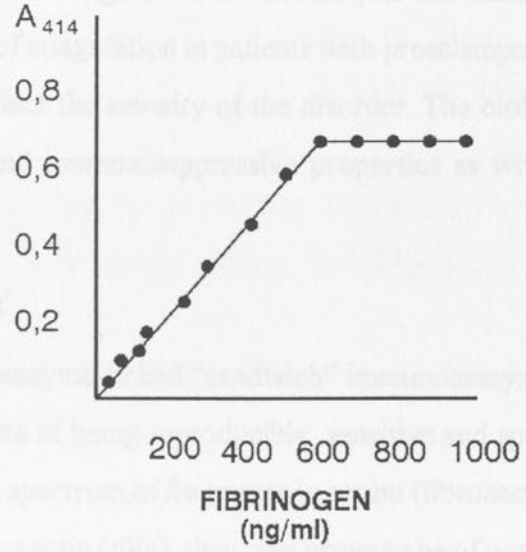


Figure 6.10 - A standard dilution curve for human fibrinogen.
Substrate absorbance is depicted on the Y-axis and the concentration of fibrinogen in solution on the X-axis. (Redrawn from Kruskal, 1987)

FDP values were determined in 10 apparently healthy control subjects. The range in these subjects was 0.093-0.288 μg/ml compared with a normal laboratory range of 0.086-0.237 μg/ml as determined by FDP radio-immunoassay (Kruskal *et al*, 1987). Plasma samples from patients with preeclampsia needed to be diluted between five- and twenty-fold in order to compare with a standard curve.

6.6.4 Discussion

A sensitive, specific and reproducible immunoassay for measuring FDPs in serum has been developed in this laboratory. The technique is simple to perform and since results may be obtained within four hours, it is useful in a clinical setting. Since the adsorbed goat anti-fibrinogen is able to detect and capture a wide spectrum of FDP's, which are all recognised by the mouse monoclonal anti-human fibrinogen, this assay does not differentiate between the breakdown fragments of fibrin and fibrinogen. By using panspecific antibody for antigen capture purposes, problems may arise where high levels of fibrinogen or fibrinogen fragments are present in the test sample as these would compete with fibrin derivatives for binding.

FDP measurements have clinical relevance as prognostic indicators for conditions associated with abnormal coagulation and fibrinolysis and therefore provide an additional marker for activation of coagulation in patients with preeclampsia. The presence of FDP in patient serum may reflect the severity of the disorder. The biological effects of FDPs include anticoagulant and immunosuppressive properties as well as chemotactic activity (Arnesen, 1977).

6.7 SUMMARY

The quantitative enzyme-linked "sandwich" immunoassays described here meet with the clinical requirements of being reproducible, sensitive and specific. Since they are both able to measure a wide spectrum of fragments in serum (fibronectin (Fn), fibrin and fibrinogen) and in plasma fibronectin (pFn), they may prove to be of use in disorders characterised by abnormalities in the thrombotic and fibrinolytic pathways. Moreover, since the assays are simple to perform and can provide results within hours of the blood samples being obtained, they may provide an advantageous alternative to currently available less-sensitive assays for examining coagulation and fibrinolytic parameters.

CHAPTER 7 - EXPERIMENTAL SECTION

COAGULATION ASSOCIATED MARKERS IN PREECLAMPSIA

7.1 BACKGROUND

Preeclampsia, a major cause of maternal death, may be characterised by hypertension and proteinuria alone or may progress to disturbed coagulation and multiorgan failure. The reason for this progression is unclear, and since the condition can only be reversed by termination of pregnancy, there is a need for reliable indicators of severity.

Various tests have been proposed to detect early preeclampsia including serum uric acid levels, platelet counts, haematocrit levels (indicator of plasma volume), serum antithrombin III, 2-antiplasmin, factor VIII and serum iron levels, the angiotensin infusion test, and the rollover test (Redman, 1976; Saleh *et al*, 1994). However, none of these markers are specific diagnostic indicators of the disease. Plasma levels of cellular Fn (Lockwood *et al*, 1990) and plasma Fn (Stubbs *et al*, 1984) have been suggested to be predictive of preeclampsia before the onset of hypertension and Fn assays appear to be more specific than most other easy to perform, laboratory tests in predicting preeclampsia (Saleh *et al*, 1994).

Vascular endothelial cell activation is thought to be central to the sequence of events in the progression of preeclampsia (Roberts *et al*, 1992). Activation of the endothelium results in activation of the coagulation cascade and some patients show evidence of a localised, compensated intravascular coagulopathy (Perry and Martin, 1992). Characteristic pathological findings of fibrin and platelet thrombi in numerous organs of preeclamptic patients support the likelihood of a hypercoagulable state being associated with the disease (Perry and Martin, 1992). Markers of platelet activation and consumption, and of coagulation activation can be detected weeks before the onset of overt symptoms of disease (Beaufils *et al*, 1985). Markers of platelet activation include β-thromboglobulin, platelet factor 4 and platelet calcium flux (Zemel *et al*, 1992) but tests for these factors are probably not yet in common use. Circulating levels of coagulation factors, including factor VIII (FVIII) or von Willebrand factor (vWf), fibronectin (Fn) and plasminogen activator inhibitor-1 (PAI-1), secreted by endothelial cells may be raised. An imbalance in the levels of PAI-1 and PAI-2, with an increase in PAI-1 antigen and activity and a decrease in PAI-2 antigen and activity has been used as a marker of placental damage

(Estelles *et al*, 1989).

There are few serial studies showing correlations of coagulation factor concentrations with gestational age or disease progression. This study attempted to define a predictive marker/s which distinguishes mild, non-fulminant preeclampsia from severe, fulminant preeclampsia. A prospective serial study was carried out to measure circulating levels of tissue plasminogen activator (tPA), PAI-1, fibrin(ogen) degradation product (FDP), and Fn in patients with preeclampsia and matched control subjects. Statistical methods were used to determine whether there was a relationship between any of the factors measured and gestational age or disease progression.

7.2 PATIENTS AND METHODS

7.2.1 General design of the study

The study was conducted in two phases. In the first phase patients with severe preeclampsia and a comparable number of normotensive, pregnant controls matched for age, race, parity and gestational age were recruited. Blood samples were collected and prepared for assays of Fn, tPA, PAI-1 and FDP levels. In the second phase patients presenting with preeclampsia were recruited and managed conservatively until delivery for either severe disease or fetal distress. These patients were studied serially with twice weekly estimations of serum Fn, plasma tPA and serum FDPs until the time of delivery.

Mild gestational proteinuric hypertension (preeclampsia) was defined as a blood pressure of 140/90mmHg and a 24 hour urinary protein >/= 0.3g developing during pregnancy.. Disease fulmination was considered imminent in any patient with acute severe hypertension (a sustained blood pressure 160/110 mmHg), or any patient with organ dysfunction (hepatic, neurological or renal) ascribed to preeclampsia. Blood pressure readings were taken as the fourth Korotkoff sound with the patient in a lateral recumbent position.

Specifically, the development of imminent eclampsia, with symptoms of headaches of recent onset and/or visual disturbances (Davey, 1988), a serum creatinine > 80mmol/l and progressively rising, or thrombocytopaenia, with or without elevated hepatic enzymes (AST and ALT rising beyond the reference range on two or more consecutive specimens), were regarded as manifestations of worsening disease or fulmination.

7.2.2 Data Collection

In both phases of the trial, demographic data including maternal age, gestational age at recruitment and parity were collated. Mean 24 hour systolic and diastolic pressures were de-

rived from 6 hourly observations recorded at the time each specimen was collected. Routine investigations of each patient included a 24 hour urinary collection for urinary protein quantitation (Biuret Method), (or Dipstix with greater than 2+ protein being considered significant in patients who were not hospitalised long enough for a 24 hour urine), serum biochemistry (including renal and liver function tests [SMAC II Auto Analyser] and full blood count [Coulter Full Blood Count Analyser]). At delivery placental weight and birth weight were recorded.

i) Phase 1

21 patients were recruited as fulminant cases on the basis of the above criteria. All such patients were 20 weeks pregnant or more. The pregnant control group (n=21) were all normotensive at the time of recruitment and were also followed up until delivery to exclude the later onset of preeclampsia. A single blood sample was obtained from each woman.

ii) Phase 2

Patients, diagnosed with preeclampsia, were recruited from those admitted to the antenatal wards of Groote Schuur Hospital, Cape Town. These patients remained hospitalised until the time of delivery and were managed by one physician who was responsible for clinical assessment without knowledge of the trial data. Data, as detailed for phase 1, were collected on each patient. Fn, tPA, FDP concentration and platelet counts were performed on each patient twice weekly until delivery. Delivery was expedited either because of incipient disease fulmination or because of fetal growth retardation and/or fetal distress. Data collection was analysed according to the criteria for delivery (delivery for maternal [fulminant group] or fetal [non-fulminant group] indications). Patients were conservatively managed and delivery was mostly by caesarian section. None of the patients or controls were in labour at time of blood sampling.

Severe GPH Patient Data:

Age	16-40 years, median 30 years
GA	24-35 weeks, median 32 weeks
Parity	0-6, median 1
Race	13 coloured, 8 black
Proteinuria	1.22g-6g/24h, median 3.15g/24h
Platelets	120-440 x 10^9/l, median 246 x 10^9/l

iii) Blood collection and preparation of serum

Blood samples of 9ml were collected by venepuncture and transferred into test tubes containing a solution of 3.8% sodium citrate (anticoagulant) and 0.025M ε-aminocaproic acid (EACA), a protease inhibitor. 5ml blood was collected into EDTA test tubes, and 10ml into untreated test tubes for clotted blood. Citrated samples were processed as recommended by Merskey *et al* (1972) (Chapter 6).

7.2.3 Laboratory investigations

i) Fibronectin and FDP Assays

Fn antigen in serum and citrated plasma, as well as serum FDP were measured using double antibody sandwich ELISA assays as described in Chapter 6. Rabbit polyclonal and murine monoclonal antibodies were used for capture and detection of antigen. Purified human Fn (Chapter 6) and fibrinogen (Kalvaria *et al*, 1983) standards were used. Each serum/plasma sample was tested in duplicate at three different dilutions (which would be accommodated by the standard curve) and results obtained by linear regression analysis.

ii) Tissue plasminogen activator (tPA) antigen assay

A standard sandwich ELISA double antibody technique was performed on plasma samples using the Imubind-5-tissue plasminogen activator ELISA kit.

iii) Plasminogen activator inhibitor type 1 (PAI-1) antigen assay

Plasma samples were assayed with an Imubind-5 ELISA kit.

7.2.4 Statistical analysis

Phase 1: Means, standard deviations of individual variables and standard errors of the means are reported for samples along with the sample sizes. The Students t-test was used for comparison of the means with the corresponding p-values. $P<0.05$ were considered significant. Ninety-five percent confidence intervals for the unknown differences of means (diff $\pm$ t.SE [diff]) were also obtained.

The t-statistic approach and the related method of confidence intervals do at least partially depend on the validity of distributional assumptions, such as normal (Gaussian) distribution of the random variables. As a precaution against possible violation of that assumption, non-parametric distribution-free tests were used to confirm the existence of location changes (e.g. differences in means) between groups.

Phase 2: The fulminant and non-fulminant preeclampsia groups include individuals for whom multiple observations were available over time. For patients with preeclampsia multiple observations allowed parallel linear regressions (different intercepts but common slope and common variance) to detect linear changes in some responses with respect to increasing gestational age.

Correlation coefficients were determined using Pearsons correlation test.

7.3 RESULTS

i) Phase 1

There are significant differences between means for the 21 control vs 21 patients with fulminant preeclampsia for serum and plasma Fn, FDP, tPA and PAI-1 (Figure 7.1a-e and Table 7.1). Care was taken to eliminate possible gestational age (GA) effects on the observed difference of means by careful matching. Testing in the control group shows no linear effect of GA on any of these variables.

The difference in Fn concentrations between normal pregnant controls and patients with preeclampsia appeared to be greater when measuring serum as opposed to plasma concentrations, therefore serum was used for Fn assays in Phase 2 in preference to plasma.

A significant positive correlation was found between birth weight and placental weight in the fulminant group, $r = 0.86$; $p = 0.003$. The fulminant group had a no correlation between PAI-1 levels and birth weight ($r = -1.1$;NS) and between PAI-1evels and placental weight ($r = -1.1$; NS) similar to results reported by Estelles *et al* (1989). [NS denotes non-significant]

	FDP µg/ml	tPA ng/ml	sFn µg/ml	pFn µg/ml	PAI-1 ng/ml
Normotensive control pregnant (21)	1.92 SEM 0.5	7.17 SEM 0.8	120 SEM 15.2	355 SEM 26.0	60.0 SEM 17.1
Severe preeclampsia (21)	7.55 SEM 2.0 $p<0.05$*	27.98 SEM 2.1 $p<0.00001$*	221 SEM 15.2 $p<0.0001$*	376 SEM 28.2 $p<0.005$*	174 SEM 49.2 $p<0.02$*

Table 7.1. Concentration of fibrin(ogen) degradation products (FDP), tissue plasminogen activator (tPA), serum fibronectin (sFn), plasma fibronectin (pFn) and plasminogen activator inhibitor-1 (PAI-1) in patients with severe preeclampsia and age and gestational age matched normotensive pregnant control subjects. Results are based on one sample per patient (n=21) or control (n=21)

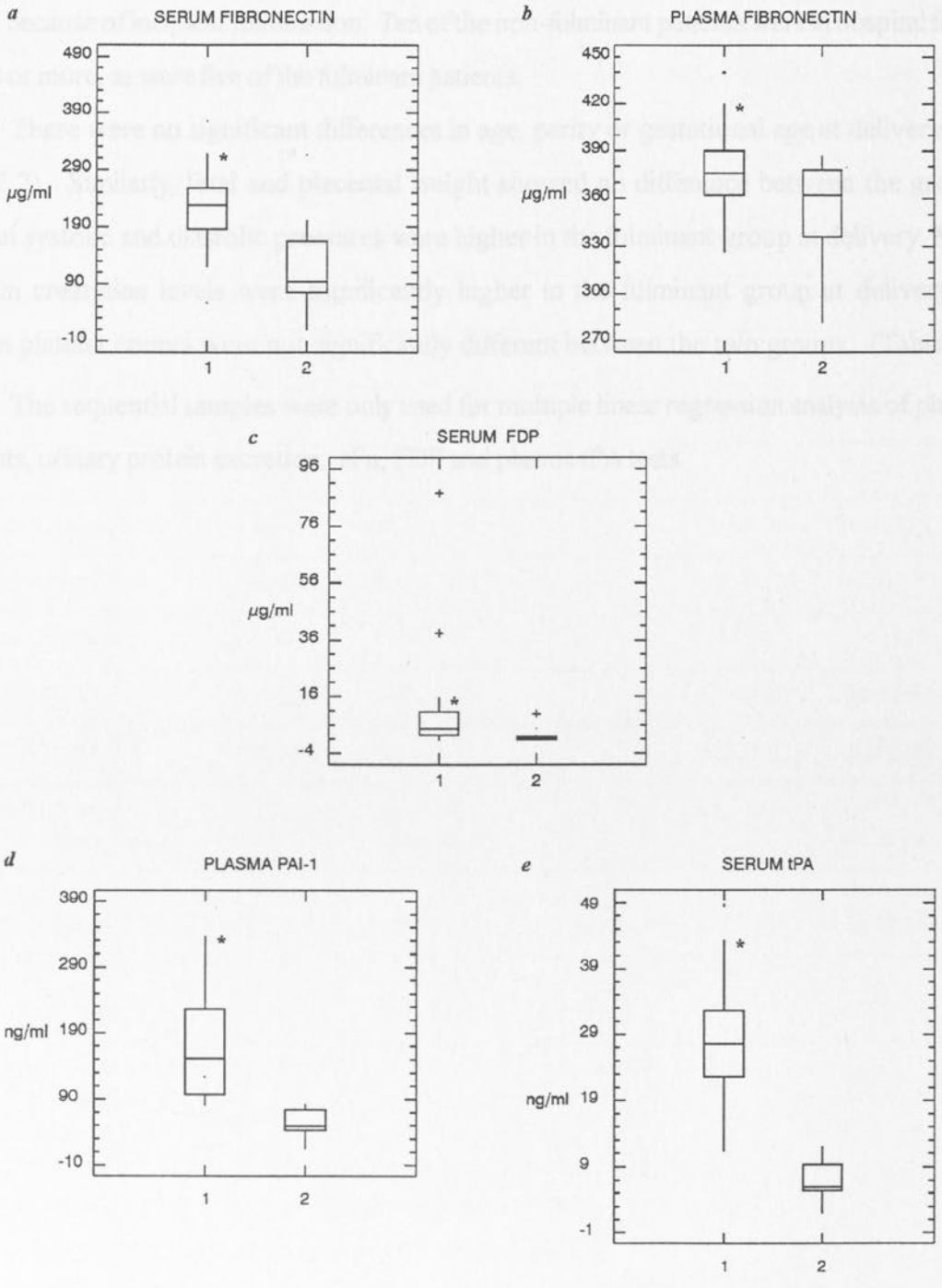


Figure 7.1 - Box and whiskers plots. (box=1 SD from mean; vertical lines=2 SD; horizontal lines=medians). Depicting concentrations of a) serum and b) plasma Fn, c) serum FDP, d) serum tPA and e) plasma PAI-1 in (1) patients with severe preeclampsia and (2) normal pregnant controls. + denotes outliers; * denotes a statistically significant difference from control group (see Table 7.1)

ii) Phase 2

Of the 22 patients recruited, half were delivered for fetal distress and the rest were delivered because of incipient fulmination. Ten of the non-fulminant patients were in hospital for 10 days or more, as were five of the fulminant patients.

There were no significant differences in age, parity or gestational age at delivery (Table 7.2). Similarly, fetal and placental weight showed no difference between the groups. Mean systolic and diastolic pressures were higher in the fulminant group at delivery. Mean serum creatinine levels were significantly higher in the fulminant group at delivery but mean platelet counts were not significantly different between the two groups. (Table 7.2)

The sequential samples were only used for multiple linear regression analysis of platelet counts, urinary protein excretion, sFn, FDP and plasma tPA tests.

	FULMINANT		NON-FULMINANT	
	Recruitment	Delivery	Recruitment	Delivery
Number	11	—	11	—
Age (Years)	22		25	
Average Range	(20 - 36)		(17 - 34)	
Parity	0 (0 - 4)		0 (0 - 4)	
SBP (mmHG)	150 (SD 22)	152 (18)	137 (6)	138 (7)*#
DBP (mmHG)	99 (14)	103 (11)	91 (6)	91 (11)*
Protein (g/l)	5.02 (4.49)	4.77 (5.48)	2.37 (1.8)	4.61 (3.98)
Urea (mmol/l)	4.2 (0.87)	4.95 (1.5)	3.9 (1.34)	5.15 (1.9)
Creatinine (μmol/l)	75.9 (12)	84.1 (10.9)	73.2 (9.86)	70.5 (11.0)*
Urate (mmol/l)	0.4 (0.06)	0.35 (0.09)	0.36 (0.04)	0.41 (0.05)
Platelets (10^9/l)	228 (56)	234 (85)	258 (60)	274 (81)
AST (IU/l)	19.3 (9.1)	26.1 (13.7)	17.4 (4.4)	24 (11.6)
ALT (IU/l)	10.8 (2.6)	14.6 (7.5)	12.5 (9.6)	17.5 (12)
Delivery Gest. Age (wks)		31 (3.2)		32.2 (3.3)
Birth Weight (kg)		1.44 (0.52)		1.61 (0.81)
Placental Weight (gram)		331 (105)		411 (156)
IUGR <10th percentile		1/11		6/11

Data are shown as mean (± standard deviation) values

* denote $p<0.05$ for fulminant vs non-fulminant delivery

Table 7.2 - Comparison of data on pregnant subjects with fulminant and non-fulminant preeclampsia based on initial measurement at recruitment and final measurement prior to delivery.

iii) Fibrinogen degradation products (FDP), tissue plasminogen activator (tPA) and Fibronectin (Fn) concentrations

Blood samples were analysed sequentially according to gestational age from recruitment through to delivery. Certain patients in the fulminant group had only single observations because the patient required delivery before one week had elapsed. Multiple observations (total = 78 observations) were available on 19 patients. A common slope variance model was fitted to this data searching for evidence of linear regression as a function of gestational age.

In the fulminant group a marked relationship between serum FDP concentration and gestational age was evident (Table 7.3). This relationship amounted to a change of 12.38 (SEM 4.40) μg FDP/ml serum/week ($p = 0.007$). Analysis of the tPA-gestational age relationship revealed similar changes: 6.82 (SEM 2.17) ng tPA/ml plasma/week ($p = 0.003$). There was also a positive correlation between FDP and tPA levels at recruitment ($r = 0.89$; $p = 0.002$). It is important to note that prediction intervals for observed rates have not been calculated. These intervals would be much wider than the corresponding confidence intervals. There was no relationship between serum Fn and gestational age.

In contrast, the non-fulminant preeclampsia data showed no evidence of any linear changes in FDP, tPA or Fn levels with increasing gestational age (Table 7.3).

	Mean Serum		Mean Plasma	
	FDP μg/ml/week	**p-value**	**tPA ng/ml/week**	**p-value**
Non-fulminant preeclampsia	0.09 SEM 1.07 0.95 CI SEM 2.23	0.935	1.02 SEM 0.53 SEM 1.10	0.59
Fulminant preeclampsia	12.38 SEM 4.40 0.95 CI SEM 8.32	0.0007*	6.82 SEM 2.17 SEM 4.51	0.003*

* = significant linear regression between gestational age and plasma tPA or serum FDP
CI = confidence interval
SEM = standard error of the mean

Table 7.3 - Serum fibrinogen degradation products (FDP) and plasma tissue plasminogen activator (tPA): Linear regression with gestational age.

Parallel measurements did not reveal a fall in platelet counts or an increase in urinary protein excretion in those patients who subsequently progressed to severe disease.

7.4 DISCUSSION

Fulminant preeclampsia is a progressive disorder reversed only by termination of pregnancy. The high maternal morbidity and occasional mortality associated with severe forms of this condition are an absolute indication for immediate delivery. Prolonging the pregnancy beyond 28-32 weeks gestational age may benefit the fetus and is the goal of management in milder cases where the condition is characterised by hypertension and proteinuria alone. Unfortunately, the course of the disease is often unpredictable and physicians are forced to rely on the degree or rate of increase in hypertension, the amount of proteinuria, the platelet count and tests of renal and hepatic function, all of which are relatively late signs of progression. Better diagnostic predictors for patients at risk are needed to ensure better management.

Since endothelial damage is thought to occur at an early stage (Roberts *et al*, 1992) we have examined markers of such injury in the hope that these may prove to be useful indicators of severity. The data from this study supports previous work suggesting that circulating Fn levels are significantly higher in pregnancies complicated by preeclampsia (Stubbs *et al*, 1984). Similarly FDP, PAI-1 and tPA levels were found to be elevated in patients with preeclampsia.

7.4.1 Fibrinogen degradation products (FDP) and tissue plasminogen activator (tPA)

FDP and tPA levels increased in relationship to gestational age in those patients delivered because of worsening maternal disease (fulminant group). The findings with regard to FDP and tPA levels lend additional support to the contention that endothelial damage (with disordered coagulation and fibrinolysis) plays a central role in the pathogenesis of preeclampsia.

Of importance is the interpretation of the estimated rates of change of serum FDP and plasma tPA (as determined by parallel linear regression analysis) in predicting increased probability of progression to severe disease. When FDP rates of change greater than 2.5μg/ml per week are sustained through several observations on the same individual, there will be an increas-

ing probability of fulmination. Similarly, sustained tPA rates of change of 2.2 ng/ml or more per week support an increasing probability of incipient fulmination. Similarly, sustained rates of change below the stated FDP and tPA rates will be associated with decreased probability of progression and, in the absence of other indications, suggest that pregnancy may not need to be terminated. The finding that neither a fall in platelet count nor an increase in the degree of proteinuria reliably predict progression to severe disease further emphasises the possible importance of the above conclusions. The observation that both FDP and tPA rates of change are of the requisite order in the same patient is to be expected. Since these criteria represent different components of the fibrinolytic process, the use of both criteria increases the predictive power of one criterion alone.

7.4.2 Fibronectin (Fn)

Despite the overall elevation in Fn concentration in patients with preeclampsia as compared to controls, serum Fn concentrations showed no increase with gestational age in patients with fulminant preeclampsia and do not appear to predict the increasing severity of the disease. This confirms the reports by Brubaker *et al*, (1990). The difference between serum and plasma Fn levels between and within the patient and control groups is difficult to interpret. It is known that Fn is taken up during clot formation, but the percentage uptake is variable (Mosher, 1980). This accounts overall for the lower serum Fn levels. However, the results show that the mean serum levels in controls are only 34% of the plasma levels, i.e. 66% uptake, while the serum levels in patients are 59% of plasma levels, i.e. 41% uptake. There are two possible reasons for the discrepancy. Either Fn uptake into the clot is impaired in patient plasma, or plasma Fn levels in patients have been underestimated by ELISA.

Plasma levels of cellular Fn released by damaged endothelial cells have been shown to be significantly higher in patients with preeclampsia than in pregnant controls (Lockwood *et al*, 1987). Cellular Fn could be a component of the Fn measured in the plasma of patients with preeclampsia, with or without fulminant disease, in this study. The lack of increase in Fn levels with disease progression could be due to Fn consumption through the course of the disease. This could occur as a result of its physiological role in promoting platelet adherence, fibrin deposition and opsonisation of debris. Thus Fn levels in patients with preeclampsia might reflect the balance between production and consumption, and any progressive increase in synthesis or secretion may not be detected by our assay. Another

possibility is that Fn may be degraded at sites of vascular damage by the action of plasmin that is not inhibited by antithrombin III or PAI-2 from the placental bed (de Boer *et al*, 1989) at the site of a localised coagulopathy (Vassalli *et al*, 1991).

7.4.3 Plasminogen activator inhibitor-1 (PAI-1)

Plasma PAI-1 activity is reported to be increased in pregnancies with extensive placental infarction and plasma PAI-1 levels apparently correlate with the severity of placental damage, thus being a marker of placental function (Estelles *et al*, 1989). Both platelets and placental endothelium contribute to increased PAI-1 levels (Gilabert *et al*, 1990). Placental villous syncytiotrophoblasts from women with preeclampsia and women with intrauterine growth retardation have shown increased immunoreactivity in PAI-1 and mRNA transcripts and increased PAI-1 expression. (Estelles *et al*, 1994). Localised elevated PAI-1 levels may play a role in the initiation of placental damage.

i) Placental weight and birth weight

The correlation between placental weight and birth weight in the fulminant preeclampsia group may indicate the effect of placental insufficiency on intrauterine growth (IUG). Low placental weight correlates with IUGR (as assessed by APGAR scores and birth percentiles) and low birth weight.

7.5 CONCLUSION

This is the first description of a serial increase in fibrinogen degradation products (FDP) and tissue plasminogen activator (tPA) levels with gestational age in fulminant preeclampsia. The rate of increase of fibrinogen degradation products and tissue plasminogen activator should clearly not be considered in isolation but may provide clinicians caring for patients with preeclampsia with a valuable additional prognostic index which may assist in difficult decisions. Our data indicate that preeclampsia is unlikely to progress in patients with static levels of fibrinogen degradation products and tissue plasminogen activator and suggest that pregnancy be maintained in these patients when fetal viability is at stake.

The lack of relationship between plasma fibronectin (pFn) and gestational age in fulminant preeclampsia patients has been the indication for further investigations of possible structural modifications of this glycoprotein which might have functional implications.

CHAPTER 8 EXPERIMENTAL SECTION

FIBRONECTIN FRAGMENTATION IN SEVERE PREECLAMPSIA

8.1 BACKGROUND

In the previous chapter, plasma fibronectin (pFn) levels were found to be significantly higher than serum fibronectin (sFn) levels, however, the difference between control and preeclampsia sFn levels was greater than that between control and preeclampsia pFn levels. These differences may be due to different amounts of fibronectin (Fn) and fibronectin degradation products (FnDP) being taken up during clotting (Mosher, 1980). Serial estimations of sFn levels in severe cases of preeclampsia failed to show a linear relationship to gestational age. These results suggest that isolated Fn levels may not be a good indicator of severity of disease. A similar lack of relationship has been found by Brubaker *et al* (1992) (determination by nephelometry), Saleh *et al* (1988) (determination by nephelometry) and Lockwood and Peters (1990) (determination by ELISA). Plasma levels of cellular Fn (cFn) (Lockwood and Peters, 1990) and pFn have been found to correlate with gestational age in a functional competitive collagen-binding ELISA (Brubaker *et al*, 1992). High Fn levels may occur due to early endothelial activation in preeclampsia, but these levels may not be maintained in severe disease for various reasons. Decreased hepatic synthesis of pFn due to severe impairment of liver function may account for this or possible degradation of Fn by endothelial associated activation of plasmin (Calvin *et al*, 1990). Disseminated intravascular coagulopathy (DIC) in severe preeclampsia may remove Fn from plasma by incorporation of Fn into fibrin by crosslinking with factor XIII, or be utilised by means of platelet aggregation processes in patients with haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome.

This chapter addresses the integrity of Fn in plasma samples from patients with mild and severe preeclampsia and the possibility that such fragmentation may interfere with the measurement of Fn in plasma. It has been suggested that the structural integrity of the Fn molecule and its functional capacity may be impaired by the pathophysiological processes occurring in preeclampsia (Brubaker *et al*, 1992). Specifically, the objective was to determine whether fibronectin fragments are present in significant amounts in plasma from patients with preeclampsia and to assess their effects on the measurement of Fn concentration by ELISA.

8.2 PATIENTS AND METHODS

8.2.1 Patients

Citrated blood samples were collected from patients with mild and severe preeclampsia diagnosed as described in chapter 7 and from matched normotensive, pregnant controls.

8.2.2 Laboratory Investigations

i) SDS-PAGE and Western blotting

Plasma samples were diluted 1/5 with deionised, double-distilled water. Alternatively, Fn was purified from 2 ml plasma samples from each subject (using a 5ml gelatin-sepharose affinity column as previously described, chapter 6) and then eluted from the gelatin affinity matrix with 2M arginine.

The integrity of Fn in the plasma and purified samples was determined by 5-14% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Electrophoresed proteins were electroblotted onto nitrocellulose (Towbin *et al*, 1979) which was then blocked with 2% casein, and probed with mouse antihuman Fn MAb, CJ2 (described in chapter 6) followed by biotinylated anti-mouse IgG. Visualisation of the antigen-antibody binding was with peroxidase labelled streptavidin and 4-chloro-1-napthol substrate. Western blots of plasma samples were scanned to assess the ratio of intact to fragmented Fn using a densitometric scanner (Hoefer Scientific). The blots were photocopied onto transparent sheets to eliminate background scatter and facilitate more accurate densitometric scanning. Some of the fainter bands are lost in the copy process. However, the ratio of these bands to those of intact Fn can be considered almost negligible.

ii) Plasmic Digestion of Fibronectin

Purified normal human Fn (Chapter 6) was digested at 37°C using urokinase-activated porcine plasminogen. Aliquots at 1mg/ml were removed at 1, 3, 4 and 5 hours and the digestion stopped by adding aprotinin (Midran), at 20U/100 mg protein and aminocaproic acid (0.025M final concentration).

iii) Double antibody sandwich ELISA for fibronectin

Intact pure Fn at 1mg/ml was diluted to obtain a standard titration curve with detection limits between 0 - 10µg/ml. All samples were diluted to fall within this range. The ELISA assay had a 5% intra-assay variation for 10 samples measured in triplicate. (Method described in Chapter 6.)

To monitor the effect of the presence of Fn fragments on the estimation of intact Fn in this assay system, intact Fn (2.5μg/ml) was assayed in the presence of Fn (7.5, 3.75, 1.87 & 0.94μg/ml) that had been digested by plasmin for 1, 3 & 4 hours.

The effect of Fn fragments on Fn estimation in plasma was also studied. Ten samples of normal human plasma were assayed for endogenous Fn concentration. The Fn in these samples was also quantitated in the presence of plasmin digested Fn (3h) at 40, 125 or 250μg/ml.

iv) Gelatin-latex assay for fibronectin (Fn) opsonic activity

The assay was performed according to the method described by Check *et al* (1979). Gelatin-latex particles ($1x10^8$/ml) were prepared using the carbodiimide reaction (Williams and Chase, 1967). Blank tubes contained gelatin-latex beads and buffer, but no heparin. Plasma samples (50μl) were added to gelatin-latex beads in heparinised Hanks balanced salt solution (150μl suspension) and incubated at 37°C for one hour. After addition of Hanks (4ml) to stop the reaction, beads were allowed to sediment at 4°C overnight. Supernatant fluids (SNF) were transferred to glass tubes and turbidity read on a Micronephelometer type 236. Percentage opsonic activity was calculated using the following equation: [(1 - meter reading of sample SNF) / (meter reading of blank SNF) x100] and reported as opsonic activity per μg Fn.

The nephelometer reading of supernatants of completely agglutinated tubes was 0. Predelivery plasma samples (neat) from normotensive pregnant controls, patients with mild and severe preeclampsia were prepared to compare the opsonic activity of the three groups.

8.3 RESULTS

8.3.1 Integrity of Fn by means of SDS-PAGE, Western blotting and Densitometric Scanning.

i) Fn and FnDP detection by SDS-PAGE and Western blot

Fn from 4 control gestational plasma samples, 4 severe preeclamptic patient plasma samples, and pure Fn digested with plasmin (4h), are demonstrated in a Western blot probed with anti-fibronectin MAb CJ1 (Figure 8.1). Fn from control gestational and severe preeclamptic plasma migrated to an apparent molecular weight of 440 kDa and Fn subunits of apparent molecular weights of 240-220 kDa. FnDP with apparent molecular weights of 330, 260, 180 and 150 kDa were detected in plasma from both control pregnant women and patients with preeclampsia. Plasma from patients with severe preeclampsia contained additional FnDP mi-

patients with preeclampsia. Plasma from patients with severe preeclampsia contained additional FnDP migrating at apparent molecular weights of 280, 135, 85, 82,80 and 70 kDa. Plasmin digest of Fn generated FnDP of similar molecular weights to those observed in the plasma of severe preeclamptic patients (Figure 8.1). Patient Fn levels in serum and plasma are presented in Table 8.1.

	Normotensive Controls		Severe preeclampsia	
	Serum Fn µg/ml	Plasma Fn µg/ml	Serum Fn µg/ml	Plasma Fn µg/ml
1	93	387	263	375
2	158	378	238	371
3	188	375	202	371
4	180	369	257	379

Table 8.1. Fibronectin levels in serum and plasma from patients whose samples were used for SDS-PAGE and Western blotting in Figure 8.1.

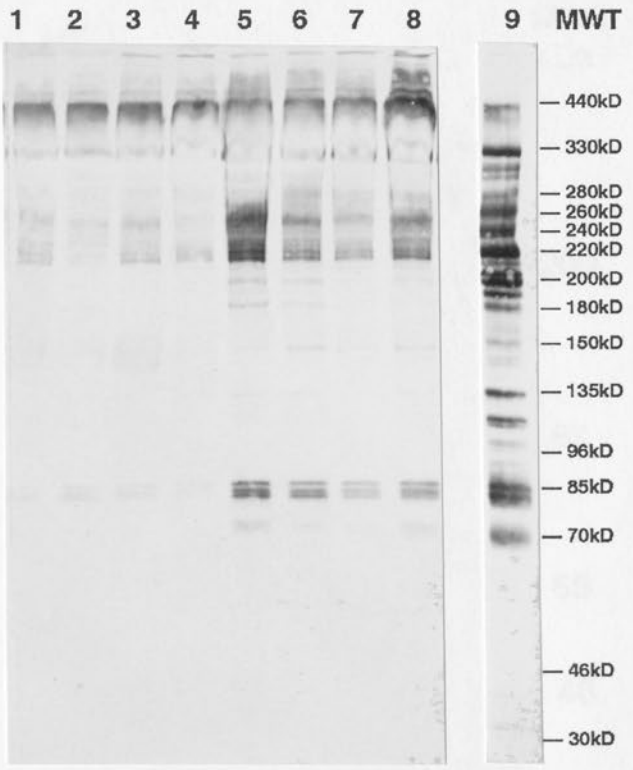


Figure 8.1 A Western blot depicting plasma fibronectin and fragments in samples of normotensive control subjects (lanes 1-4), patients with severe preeclampsia (lanes 5-8), and Fn digest (4h) lane (9) Molecular weights are indicated on the right.

Fn isolated by gelatin-sepharose affinity chromatography from control gestational, non-fulminant preeclamptic (mild) and fulminant preeclamptic (severe) patient plasma showed a similar pattern of Fn fragmentation on Western blots (Figure 8.2). Fn isolated from plasma samples of these three groups migrated to an apparent molecular weight of 440 kDa and Fn subunits of apparent molecular weights of 240-220 kDa. Intact Fn and FnDP with apparent molecular weights of 180 and 150 kDa were detected in plasma from control pregnant women and patients with preeclampsia. Plasma from representative severe preeclamptic patients contained additional FnDP migrating at apparent molecular weights of 135, 120, 100, 82, 80, 70, 38 and 28 kDa. Patient data are presented in Table 8.2.

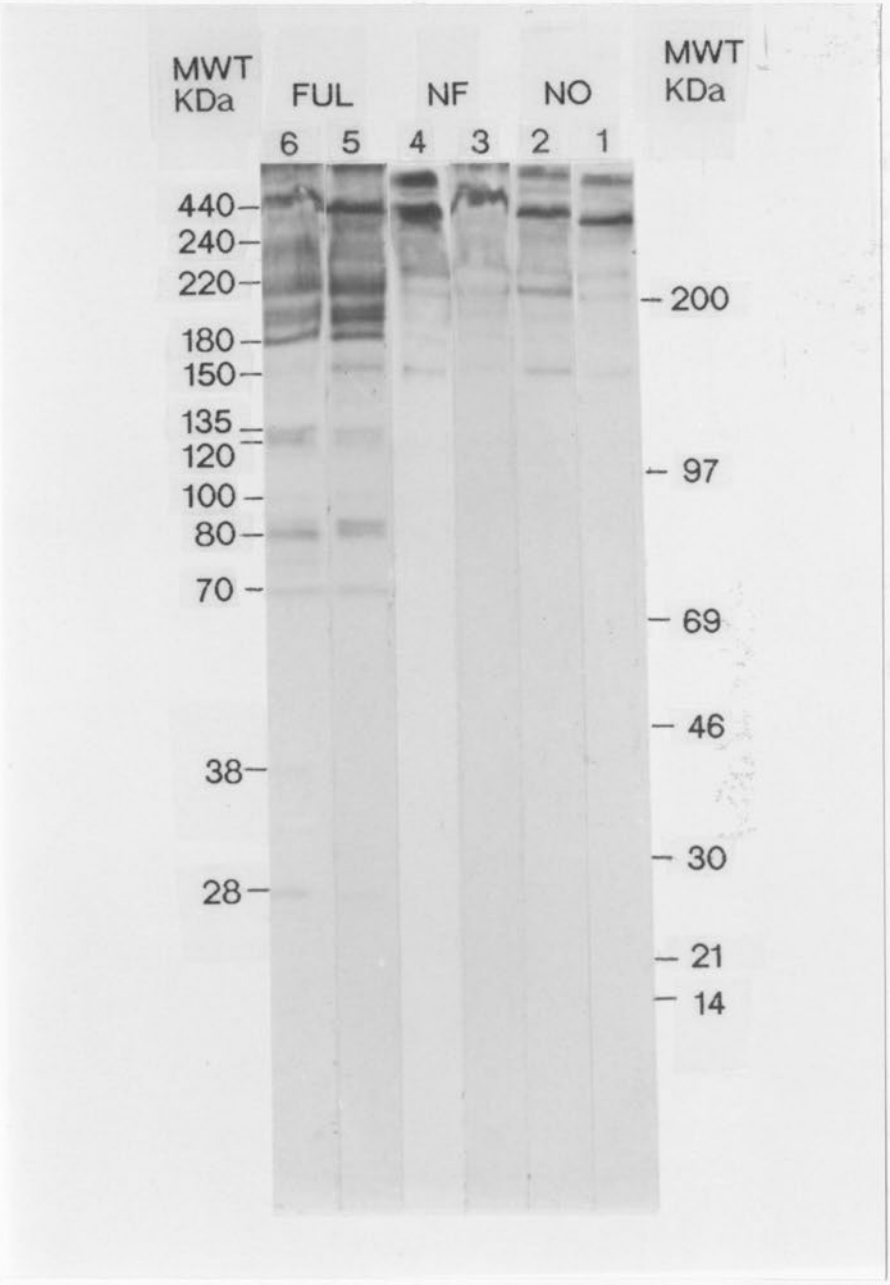


Figure 8.2. A Western blot showing fibronectin (Fn) and specific degradation fragments (FnDP) isolated from plasma from representative normotensive pregnant women (lanes 1 and 2), patients with mild preeclampsia (lanes 3 and 4), and severe, fulminant preeclampsia (lanes 5 and 6). Relative molecular weight markers are indicated on the right of the figure and approximate molecular weights of Fn and FnDP are indicated on the left.

Patient group	Number	BP (mmHg)	GA#	Fn*μg/ml	Proteinuria+
		all at time of sampling			
Normal pregnancy	1	110/60	28	112	Absent
	2	120/70	31	73	Absent
Non-fulminant preeclampsia (mild)	3	140/90	27	176	++++
	4	130/90	30	105	+
Fulminant preeclampsia	5	155/104	28	209	+++
(severe)	6	160/100	28	317	++++

+ Amount of proteinuria as measured with Multistix (Lindow and Davey, 1992):

Range: + — ± 200-800mg, ++ – ± 400-1800mg, +++ – ± 800-4000mg ++++ – ± 800-4000mg.

* Fn μg/ml: Predelivery sFn levels measured by ELISA

GA: Gestational age

Table 8.2. Clinical data of Patients in Figure 8.2.

ii) Densitometric scanning

Plasma samples analysed by direct SDS-PAGE and Western blotting (Figure 8.1) were scanned (Hoeffer densitometer) to assess the percentage fragmentation of Fn in the sample (Figure 8.3). Samples from control pregnant women (n = 4) had 9.37 SD 6.7 % fragmented Fn (<400kDa) as measured by the densitometer (Figure 8.1). The severe preeclamptic patient samples (n = 4) had 62.57 SD 7.0% fragmented Fn (p = 0.003). The most predominant fragments in the patients samples migrated to an approximate molecular weight of 80kDa, where 2 or 3 bands could be detected in this region of the gel. These bands comprised 11.5% (n = 4) of the total Fn measured (Figure 8.3).

The mean recorded total units (TU) Fn per group:- Controls, TU= 10707 SD 2110 (n=4), and severe GPH, TU = 40497 SD 16153 (n=4). Thus, control plasma contained approximately 26% the amount of Fn contained in preeclamptic plasmas.

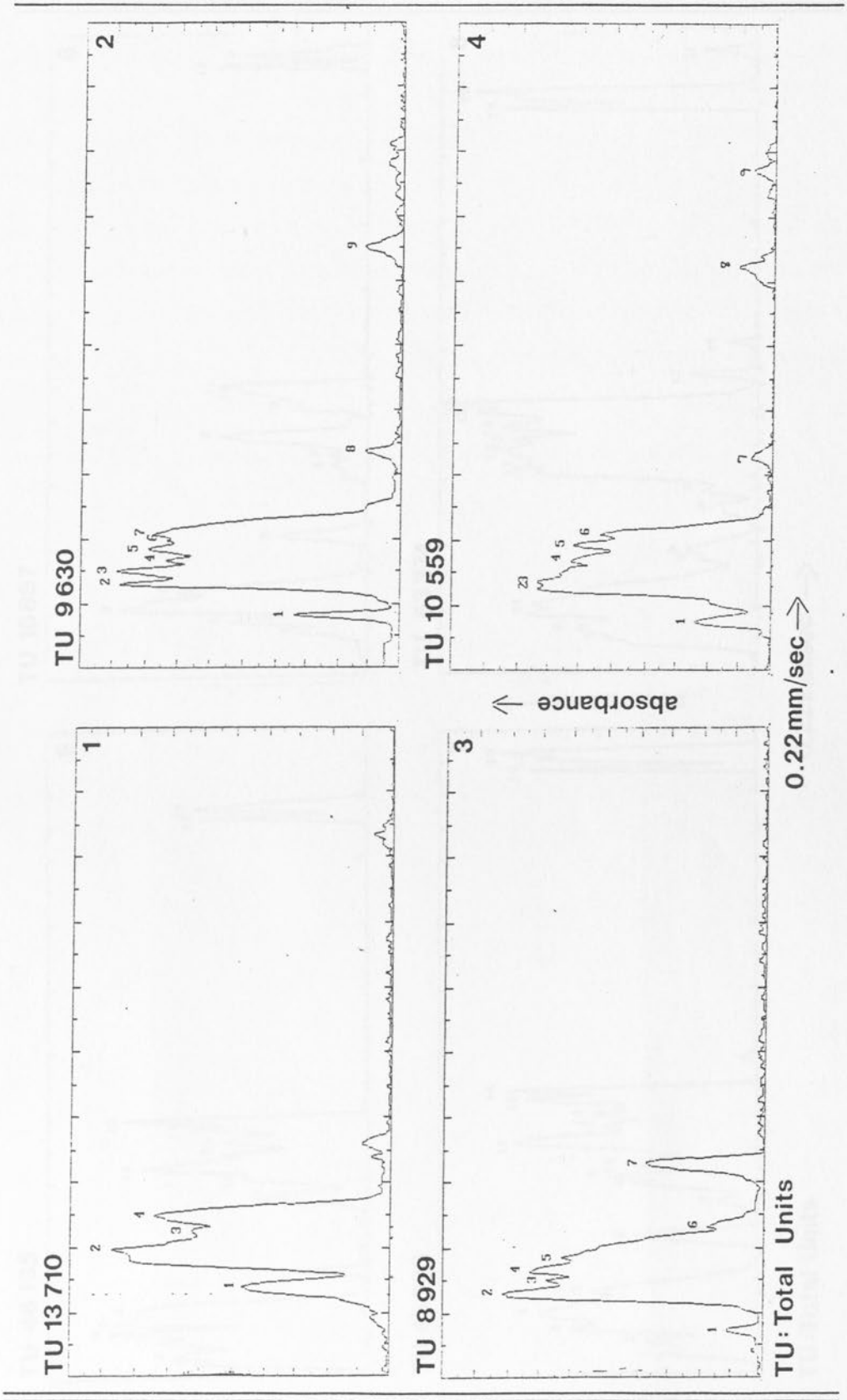


Figure 8.3a - Densitometric scanning graphs from Figure 8.1 used to determine relative areas under the peaks corresponding to Fn and fragments in control plasma (lanes 1-4) at relative MWTs as indicated.

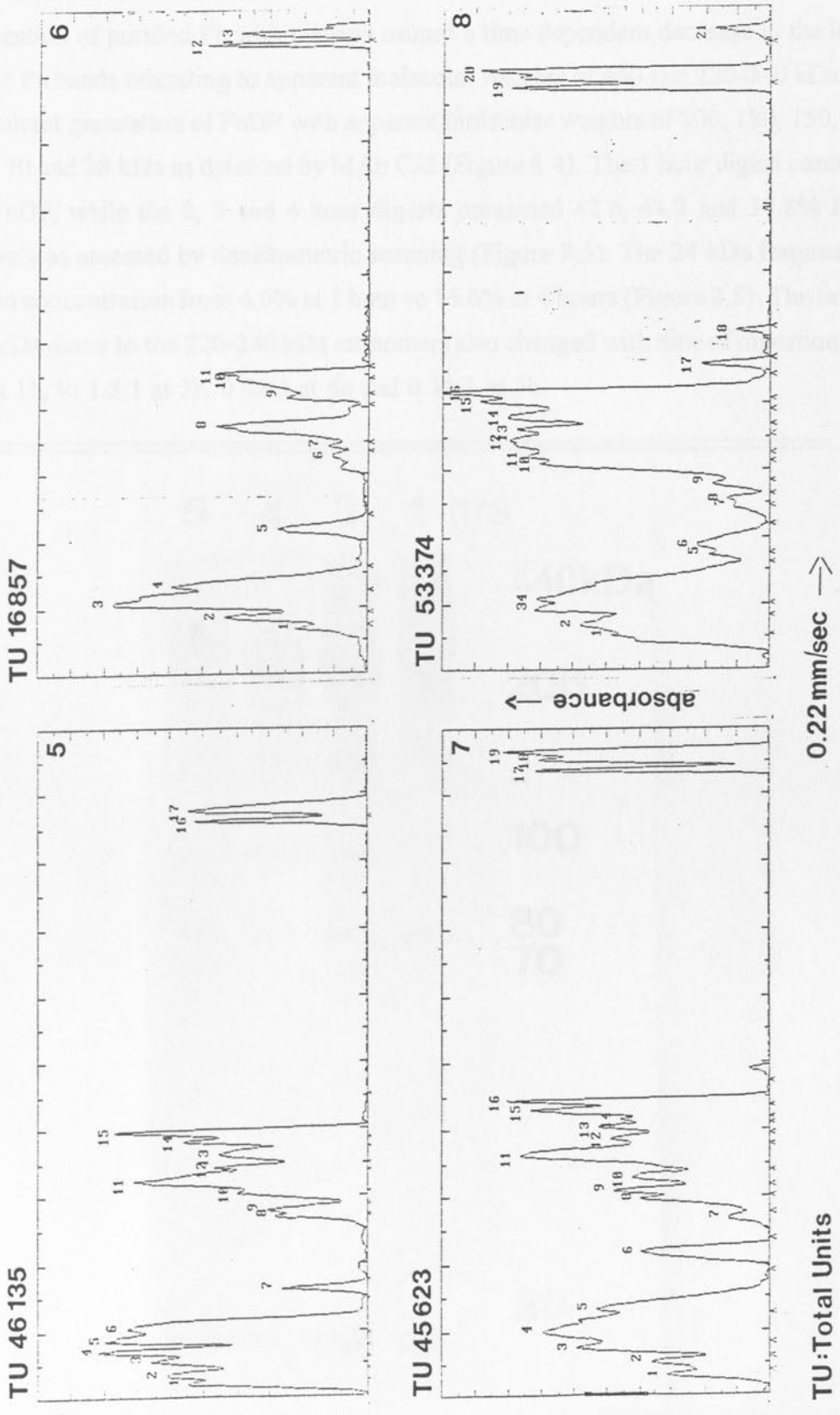


Figure 8.3b - Densitometric scanning graphs from Figure 8.1 used to determine relative areas under the peaks corresponding to Fn and fragments in plasma from patients with severe preeclampsia (lanes 5-8). MWTs as indicated.

iii) Comparison with Plasmin Digest of Fibronectin

Digestion of purified Fn with plasmin caused a time dependent decrease in the intensity of the Fn bands migrating to apparent molecular weights of 440 and 220-240 kDa, and a concomitant generation of FnDP with apparent molecular weights of 200, 180, 150, 135, 100, 80, 70 and 28 kDa as detected by MAb CJ2 (Figure 8.4). The 1 hour digest contained 23.5% FnDP, while the 2, 3 and 4 hour digests contained 42.6, 44.3 and 37.8% FnDP respectively as assessed by densitometric scanning (Figure 8.5). The 28 kDa fragment increased in concentration from 4.6% at 1 hour to 16.6% at 4 hours (Figure 8.5). The ratio of the 440 kDa dimer to the 220-240 kDa monomers also changed with time of digestion from 1.72:1 at 1h, to 1.3:1 at 3h, 0.42:1 at 4h and 0.36:1 at 5h.

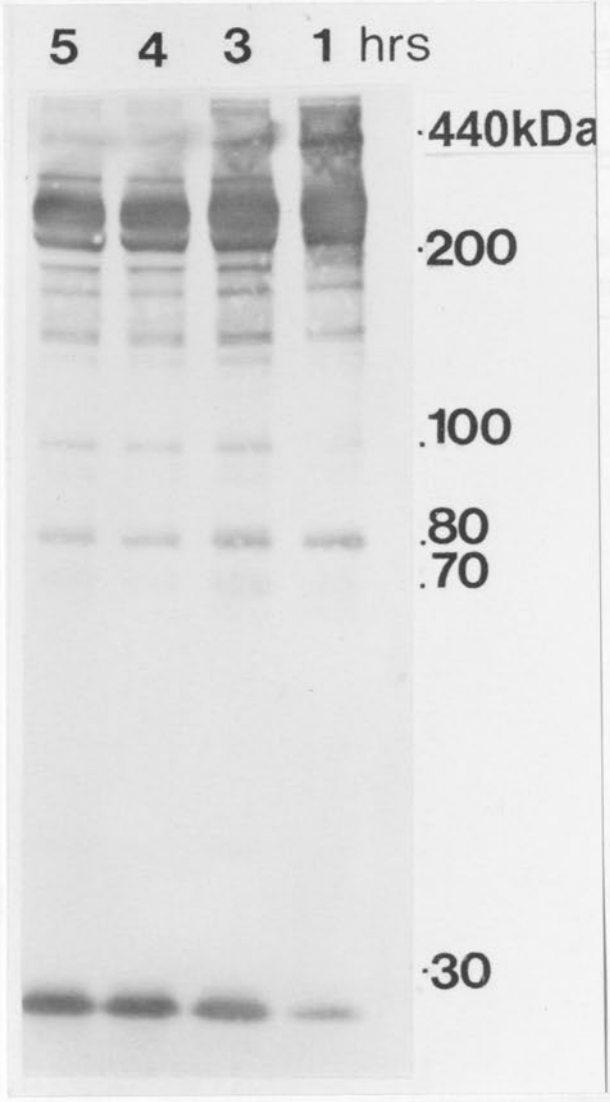


Figure 8.4. A Western blot depicting Fn fragmentation after digestion with plasmin for 1 - 5 hours. Approximate molecular weights indicated on the right.

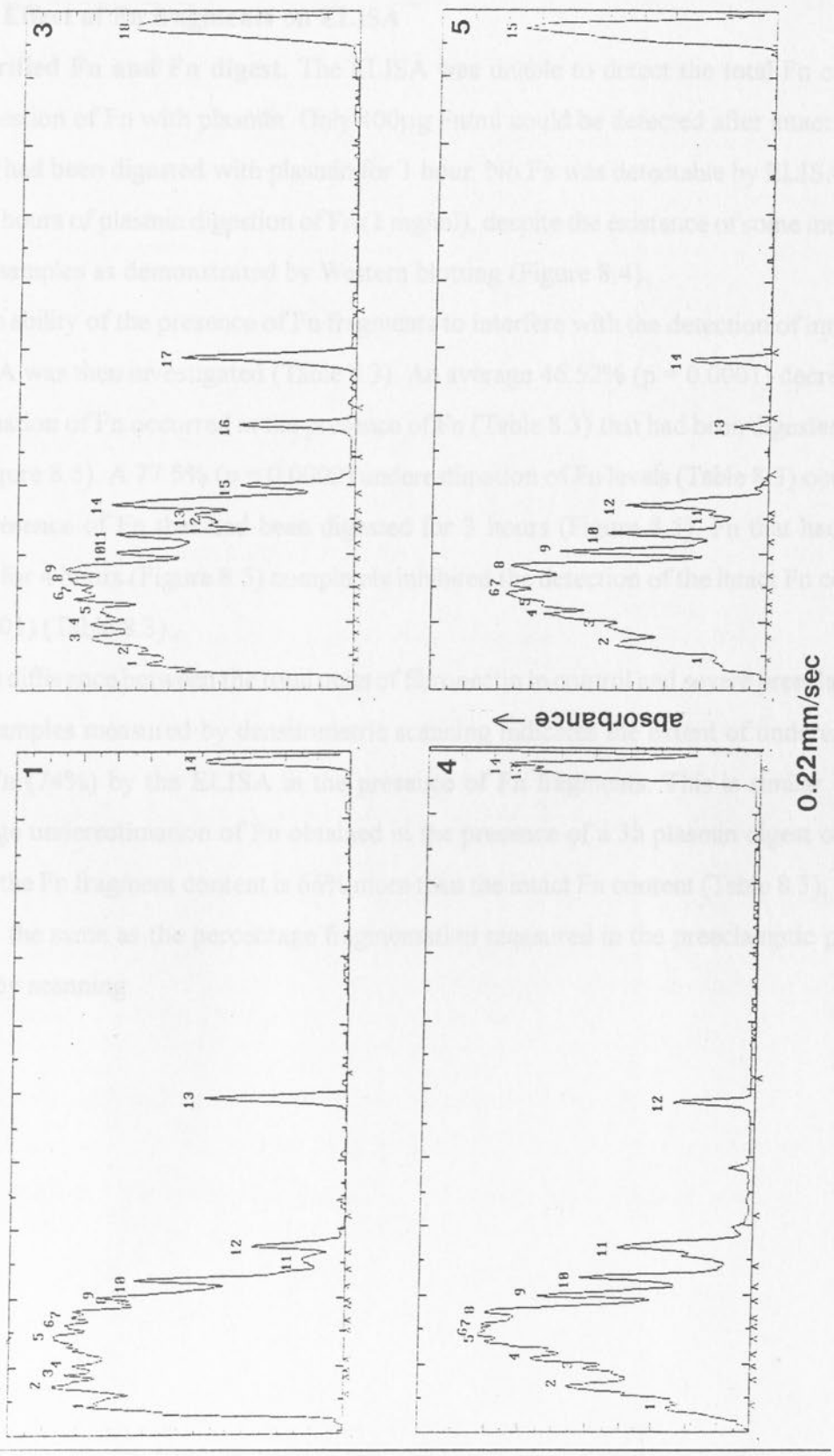


Figure 8.5 Densitometric scanning graphs depicting peaks corresponding to bands in figure 8.4 at relative molecular weights as indicated at the top of the figures.

iv) Effect of Fn fragments on ELISA

Purified Fn and Fn digest. The ELISA was unable to detect the total Fn content after digestion of Fn with plasmin. Only 400μg Fn/ml could be detected after intact Fn, at 1 mg/ml had been digested with plasmin for 1 hour. No Fn was detectable by ELISA after 3, 4 or 5 hours of plasmin digestion of Fn (1 mg/ml), despite the existence of some intact Fn in these samples as demonstrated by Western blotting (Figure 8.4).

The ability of the presence of Fn fragments to interfere with the detection of intact Fn by ELISA was then investigated (Table 8.3). An average 46.52% ($p = 0.0001$) decrease in the estimation of Fn occurred in the presence of Fn (Table 8.3) that had been digested for 1 hour (Figure 8.5). A 77.5% ($p = 0.0002$) underestimation of Fn levels (Table 8.3) occurred in the presence of Fn that had been digested for 3 hours (Figure 8.5). Fn that had been digested for 4 hours (Figure 8.5) completely inhibited the detection of the intact Fn content ($p < 0.0001$) (Table 8.3).

The difference between the total units of fibronectin in control and severe preeclampsia plasma samples measured by densitometric scanning indicates the extent of underestimation of Fn (74%) by the ELISA in the presence of Fn fragments. This is similar to the percentage underestimation of Fn obtained in the presence of a 3h plasmin digest of pure Fn when the Fn fragment content is 66% more than the intact Fn content (Table 8.3), which is almost the same as the percentage fragmentation measured in the preeclamptic plasma samples by scanning.

i) Measurement of intact Fn in the presence of digested Fn.

A) Intact Fn ug/ml	B) Added (digested) Fn ug/ml	Expected Total (A+B) Fn ug/ml	Assayed Fn (μg/ml) in the presence of digested Fn C) Digest time			
			0h	1h	3h	4h
2.5	7.5	10	9.64	5.2	3.2	0
2.5	3.75	6.25	6.25	3.3	1.5	0
2.5	1.87	4.37	4.7	2.2	0.7	0
2.5	0.94	3.44	3.7	2.0	0.6	0

Note: Fibronectin (**B**) digested, for times as indicated (**C**), was added to intact fibronectin (**A**) and then measured by ELISA.

ii) Percentage underestimation of Fn in the presence of Fn digests (0h, 1h, 3h, 4h)

	Fn digest time			
	0h	1h	3h	4 h
	3.6%	48%	68%	100%
	0%	47.2%	76%	100%
	0%	49.7%	84%	100%
	0%	41.2%	82%	100%
Mean	0.9%	46.52%*	77.5%*	100%*
SD	1.8%	3.69%	7.18%	0%
p-values	0.39	0.0001	0.0004	0.000002

Note: The effect of digested fibronectin on the quantitation of fibronectin, as measured in **i, above**, is expressed as % underestimation at each timepoint. The mean underestimation and SD at each timepoint was calculated as a percentage.

* significantly different from Fn estimation in the absence of digested Fn.

Table 8.3 The effect of Fn fragments on the estimation of intact pure Fn by ELISA.

Fn and FnDP in plasma: The effect of Fn fragmentation on the detection of total Fn in normal plasma is presented in Table 8.4. Inhibition of pFn estimation by Fn digested for 1hour was 24±8% (n=4) in the presence of 40μg/ml Fn digest, 36±5% (n=3) in the presence of 125μg/ml Fn digest, and 44±7% (n=3) in the presence of 250μg/ml Fn digest (Table 8.4). Suppression with only 250μg/ml FnDP was significantly higher than with 40μg/ml ($p<0.05$)

Sample	a) Digested Fn μg/ml	b) Plasma Fn μg/ml	c) Assayed Fn μg/ml (a+b)	d) Underestimation %
1	40	425	281	34
2	40	352	300	15
3	40	263	201	26
4	40	164	133	20
			Mean underestimation (1-4) : 23.75 SD 8.1%	
5	125	271	176	35
6	125	226	130	42
7	125	82	56	32
			Mean underestimation (5-7) : 36.33 SD 5.1%	
8	250	603	301	50
9	250	446	251	44
10	250	144	91	37
			Mean underestimation (8-10) : 43.66 SD 6.5%*	

* Significantly greater underestimation than with 40μg/ml digested Fn.

Table 8.4 The effect of Fn fragments on the estimation of pFn from normal healthy controls. ELISA measurements of pFn (b) in the absence and (c) in the presence (a+b) of digested Fn (1h) at 40, 125, 250 μg/ml (a). Underestimation (d) of pFn in the presence of digested Fn (a) is presented as a percentage (b-c/b x100).

v) Opsonic Activity

Opsonic activity measured by ability of Fn to bind to gelatin-latex particles and precipitate was compared in plasma of control pregnant women and patients with mild and severe preeclampsia and expressed as the ratio of percentage gelatin-latex agglutination to Fn concentration (Table 8.5). While there was no difference between the opsonic activity of mild and severe preeclampsia plasma, both patient groups had significantly lower ratios of opsonic activity relative to Fn concentration than that of the control group ($p<0.008$).

Control pregnant			Mild preeclampsia			Severe preeclampsia		
% OA	Fn μg/ml	Ratio	% OA	Fn μg/ml	Ratio	% OA	Fn μg/ml	Ratio
73	96	0.76	61	254	0.24*	67	228	0.30*
(72-75)	(85-173)	(0.42-0.84)	(61-72)	(214-318)	(0.22-0.29)	(56-74)	(169-319)	(0.23-0.34)

OA: opsonic activity
Ranges in brackets
* **Ratios** (OA/μg Fn) significantly different from control group ($p<0.008$) as determined by Wilcoxon Rank Sum Test for unpaired samples.

Table 8.5 Opsonic activity per mg Fn in patients and controls.

8.4 DISCUSSION

8.4.1 Fibronectin fragmentation

This study has demonstrated the novel observation that plasma obtained from patients with preeclampsia contains FnDP and that the degree of Fn fragmentation appears to increase with severe disease. The ratio of high to low molecular weight (MWT) fragments also changes with disease progression, with increasing amounts of low MWT fragments occurring in more severe cases. The apparent MWTs of Fn fragments seen in patient plasma are similar to those obtained by plasmic digestion of purified pFn.This suggests that Fn degradation could be by plasminolysis, possibly at sites of endothelial cell injury, where generation of plasmin is reported to be elevated due to activation of the coagulation cascade (Roberts *et al*, 1991).

Release of neutrophil elastase at sites of vascular injury has been reported to occur and to be responsible for cleavage of Fn to produce a 120kDa, non-heparin-binding and unglycosylated fragment which enhanced chemotaxis (Odekon *et al*, 1991), and a 30kDa gelatin-binding, glycosylated fragment which inhibited migration of neutrophils (Easter *et al*, 1988).

8.4.2 ELISA results

It has been suggested, but not proven, that Fn fragmentation may contribute to the elevated Fn levels in preeclampsia (Stubbs *et al*, 1984). In our studies in vitro, FnDPs in fact result in an underestimation of intact Fn measurement by ELISA. The extent of Fn fragmentation achieved by increasing digestion periods had a significant effect on the estimation of the Fn concentration. This may be related to both the size and the concentration of the fragments. Alternatively the fragments may competitively inhibit monoclonal antibody (MAb)-binding to antigen-antibody complexes in the ELISA due to the stoichiometric relationship of fragments to intact Fn molecules. However these fragments may be recognised by the MAb in Western blots where the fragments are separated, due to their different migration rates during electrophoresis, or their epitopes may assume a different structural conformation in the solid phase (SDS-PAGE or Western blot). There is also the possibility that at least some of the fragments have a low affinity for the MAb in the ELISA assay. Thus, the effects of Fn fragmentation might offer one explanation for the observed lack of a progressive rise in Fn levels with progression of the disease (Ballegeer *et al*,1989; Calvin *et al*, 1990; Lockwood and Peters, 1990).

Divergent measurements of Fn concentration have been obtained in two types of assays, namely electroimmunoassay and immunoturbidimetry, using Fn digested with enzymes such as trypsin, kallikrein and plasmin in vitro (Bykowska *et al*, 1985). These discrepant results are explained as a decrease in affinity of degraded Fn for Fn antibodies (Bykowska *et al*, 1985).

Digestion by trypsin and kallikrein leads to a progressive decline in Fn detectability by immunoturbidimetry to zero values, but is associated with an increase in the height of rockets in the Laurells electroimmunoassay. Plasmin mediated Fn fragmentation results in overestimation of the Fn content by the electroimmunoassay and, at high enzyme concentrations, provokes an underestimation of Fn by the turbidimetric technique. The decline in

Fn reactivity in the immunoturbidimetric assay coincides with the disappearance of high MWT FnDP as seen in SDS-PAGE. The increase in the height of rockets in the electroimmunoassay is highest when fragments of low MWT prevail in the SDS-PAGE pattern.

Considerable differences in measurement of intact and degraded Fn have also been reported for ELISA assays (Blaschke *et al*, 1990; Brubaker *et al*, 1992). It has been suggested that the use of two different ELISA assays (coating microtitre plates with collagen versus Fn antibody as capturing agents) for the same samples may be useful in distinguishing native from degraded Fn by means of the difference between the two assay results (Blaschke *et al*, 1990) as would concomitant use of the immunoturbidimetric and electroimmunoassays.

8.4.3 Opsonic Activity

Opsonic activity (as measured by the ability of Fn to bind to gelatin-latex beads) of control plasma was significantly greater than that of patients with both mild and severe preeclampsia. Non-gelatin-binding FnDP in preeclampsia patient plasma such as the 70 and 30-28kDa fragments could account for the observed inhibitory effects on the opsonic activity of mild and severe preeclampsia plasma. In addition, the Lower molecular weight fragments 135, 120, 85, 80 kDa detected in plasma samples from mild and severe preeclampsia patients (of similar MWT to inhibitory gelatin-binding fragments described by Erlich *et al* (1981)) may also competitively inhibit binding of intact Fn to gelatin-coated beads and therefore reduce opsonic activity (Brown *et al*, 1989).

A limited amount of plasmin digestion has been suggested to be necessary to activate the opsonic process (Czop *et al*, 1981). However, if Fn digestion is uncontrolled due to a deficiency of plasmin inhibitors such as antithrombin III or PAI-2, which occurs in preeclampsia (Weiner, 1988; Yoshimura *et al*, 1985), lower MWT fragments inhibitory to opsonic activity may be generated.

8.5 CONCLUSION

The inability to show a correlation between Fn levels in plasma and disease progression appears from our results, to be due to degradation of Fn, which leads to an underestimation of the concentration using the sandwich ELISA technique. This may confirm the finding of impaired collagen binding (functional assay) by Brubaker *et al* (1992); Fn fragmentation at sites of vascular endothelial damage may affect the biological activity of intact Fn in the subendothelial matrix and may contribute to the perpetuation of the endothelial damage occurring in preeclampsia.

Fibronectin fragments which have been shown to cause opsonic dysfunction have been suggested to account for the depressed phagocytosis and accumulation of particulate matter in the blood vessels, and therefore may contribute to the poor perfusion and organ dysfunction which occurs in preeclampsia (Erlich *et al*, 1981). The possible biological effects of FnDP in the disease state will be discussed in the next chapter.

CHAPTER 9 - EXPERIMENTAL SECTION

DEGRADATION OF FIBRONECTIN IN ASSOCIATION WITH VASCULAR ENDOTHELIAL DISRUPTION IN PREECLAMPSIA

9.1 INTRODUCTION

Various *in vitro* studies support the concept that generalised endothelial cell dysfunction may be induced by serum factors from patients with preeclampsia (Rodgers *et al*, 1988; Endresen *et al*, 1992; Branch *et al*, 1992; Taylor *et al*, 1991a). Focal disruption of vascular endothelium has been observed in the walls of the spiral arteries of placental bed biopsies from these patients (Khong *et al*, 1992). This disruption might be the result of endothelial cell activation and conversion of the endothelium to a procoagulant state which is characterised by increased release of fibronectin (Fn), von Willebrand factor (vWf) (Piovella *et al*, 1992), and plasminogen activator inhibitor-1 (PAI-1) (Gilabert *et al*, 1990).

Saksela and Rifkin, (1988) have demonstrated that plasminogen activators (PA's), plasminogen activator inhibitors (PAIs) and plasmin systems may play a role in remodelling of the extracellular matrix and in regulating cell-cell and cell-substrate adhesion. Fibrinogen fragment D in similar models has been reported to modulate plasmin generation and function, resulting in disorganisation of endothelial cell monolayers *in vitro* (Dang *et al*, 1985; Ge *et al*, 1992). In the previous chapter the presence of Fn fragments (FnDP) in plasma from patients with severe preeclampsia was demonstrated. It is tenable that Fn might be degraded at activated endothelial cell surfaces and that FnDP might affect endothelial cell integrity.

Proteolytically degraded FnDP have been shown to stimulate plasminogen activation and generate the serine protease plasmin (Stack and Pizzo, 1993). This stimulation may be associated with the structural conformation and binding capacities of the relevant Fn domains. Heparin-binding fragments derived from the amino-and carboxy-terminal regions of human plasma fibronectin (pFn) appear to be relatively specific inhibitors of the growth of bovine aortic endothelial cells *in vitro* (Homandberg *et al*, 1986). Inhibition of endothelial growth was reversible, dose-dependent and occurred at nanomolar concentrations of fragments. Intact pFn had very little inhibitory activity suggesting that this effect *in vivo*, if it occurs, may be triggered by proteolysis.

The 29 kDa Hep I/Fib I amino-terminal domain was one of the most active endohelial growth-inhibitory fragments in the studies of Homandberg *et al* (1986). Tyrosine modification, reduction of the disulphides as well as proteolytic digestion of this amino-terminal fragment to 20 and 8 kDa subfragments did not affect this inhibitory activity, although the modified fragment and subfragments did not bind heparin. Lysine, tryptophan and carboxyl group modifications largely abolished heparin-binding and decreased this inhibitory activity to less than 25% of the 29 kDa fragment controls. This suggests that the endothelial growth-inhibitory activity of the 29kDa heparin-binding Fn fragment is not conformation-dependent, and can be expressed by an 8kDa and a 20kDa carboxy-terminal segment (Homandberg *et al*, 1986).

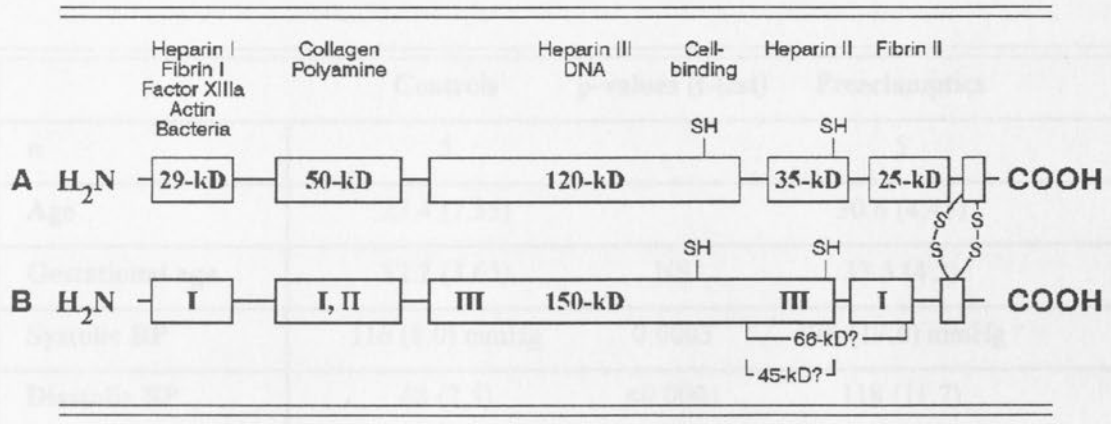


Figure 9.1 - Schematic representation of the fibronectin domain structure. (Redrawn from Homandberg *et al*, 1986)

Other Fn fragments found to have significant inhibitory activity on endothelial cell growth include a 35/38 and a 25/31 kDa fragment (Figure 9.1) which are the Hep II and Fib II domain-containing fragments respectively (Homandberg *et al*, 1986). The 29 and 31kDa fragments have a common feature in that they are comprised entirely of type I repeats, five and three repeats respectively, which confer fibrin-binding affinity on these domains. The 31 kDa fragment may be present in only one of the Fn subunits and only binds to fibrin at physiological NaCl concentrations (Homandberg *et al*, 1986). The heparin-binding activity common to the 29 and 38kDa fragments did not confer the inhibitory effect on endothelial cell growth (Homandberg *et al*, 1986).

The aim of this study was to investigate the ability of Fn and FnDP generated both *in vivo* in preeclampsia and *in vitro* by plasmin digestion and chromatographic separation, to

disrupt endothelial cell attachment *in vitro*. Modulation of plasminogen activator (PA) and plasmin activity of endothelial cells was also addressed.

9.2 MATERIALS AND METHODS

9.2.1 Patient plasma studies

i) Patients and controls

5 patients with severe preeclampsia and 5 normotensive control pregnant women were matched for gestational age as determined by menstrual dates or early ultrasound extrapolation (Table 1). Patients were diagnosed according to ISSHP criteria (Davey and MacGillivray, 1988) as described in Chapters 1 and 7.

	Controls	p-values (t-test)	Preeclamptics
n	5		5
Age	27.4 (7.55)		30.6 (4.45)
Gestational age	32.2 (3.65)	NS	33.5 (4.3)
Systolic BP	116 (8.0) mmHg	0.0003	176 (19.6) mmHg
Diastolic BP	68 (7.5)	<0.0001	118 (11.7)
Urine	Clear		3+ (R 2-4)
Birth Weight	3092 (331)g	0.015	1720 (883)g
5' Apgar	9 (R: 8-10)		9 (R: 6-10)

SD: Standard deviations from the mean are presented in brackets
Associated conditions:
Controls: 2 normal pregnancies
1 prelabour rupture of membranes
1 mitral stenosis, normal pregnancy
1 SLE in remission, normal pregnancy
Severe Preeclamptic group: 2 patients with preeclampsia developing at term,
(1 with antepartum haemorrhage at 32 weeks)
2 imminent eclamptics (one HELLP* syndrome)
1 eclamptic patient.
* **HELLP**: Haemolysis, Elevated Liver enzymes, Low Platelets.

Table 1 - Demographic data of representative control pregnant women and severe preeclamptic patients.

ii) Purification of plasma fibronectin (pFn)

Blood samples were collected from the preeclamptic patients prior to delivery, while control blood samples were collected from normotensive women at matching gestational ages to the patients. (This study unfortunately did not control for labour). All blood samples were collected as described previously and Fn, together with gelatin-binding FnDP present in the plasma, was then purified by gelatin-sepharose affinity chromatography as described in Chapter 6.

For some experiments Fn was isolated from plasma from normal, healthy subjects using this technique (Chapter 6), and used either in the intact state or as FnDP after digestion with plasmin (0.32mg activated plasmin/10mg Fn) for 1h at 37°C. Digestion was stopped with aprotinin (20U/100mg protein) and EACA (0.025M). Protein concentrations were determined by the BioRad method (Bradford, 1976) after dialysis against phosphate buffered saline (PBS).

iii) SDS-PAGE and Western blot technique

The purity and extent of fragmentation of the isolated Fn and Fn digest samples was analysed by non-reduced, 5-14% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) followed by Western blotting (Towbin *et al*, 1979). Following electrophoresis, proteins were electroblotted onto nitrocellulose, and then probed with mouse antihuman Fn monoclonal antibody, CJ1, followed by biotinylated anti-mouse IgG. Visualisation of the antigen-antibody binding was with peroxidase labelled streptavidin and 4-chloro-1-napthol substrate.

9.2.2 Umbilical cord studies

i) Fibronectin (Fn) release from human umbilical vein organ cultures

The integrity of the Fn released from umbilical cords from patients with severe preeclampsia was compared to that of controls. For this four umbilical cords from normotensive pregnant women and four from pregnant women with severe preeclampsia were obtained either by vaginal delivery or caesarean section. The cords are subjected to traction by both modes of delivery. Cords were clamped and cut from the placentae, immersed in phosphate buffer and delivered to the laboratory with minimal delay. Cord sections were cut (three x1cm sections per cord) and prepared for culture immediately. Cord sections were washed with

PBS to remove blood and clots. The veins were dissected from the Wharton's jelly, cut open to expose the maximum surface area to the culture medium, and cultured in 24 well Costar plates in 1ml serum-free minimal essential medium (MEM) at 37°C in humidified air with 5% CO_2 (method adapted from Saouaf *et al*, 1991). Aliquots of 200µl of culture medium were collected at 24h and mixed with 200µl of 2% SDS-sample buffer and analysed by non-reduced, 5-14% gradient SDS-PAGE (Laemmli, 1970). Detection of Fn and FnDP in the separated proteins was by Western blot as described above (Towbin *et al*, 1979).

ii) Immuno-histochemistry

Ten umbilical cords from normotensive controls and patients with severe preeclampsia were collected as described above. Short sections of 4-5 cm were wrapped in tinfoil, frozen and stored in liquid nitrogen and used for immunohistochemistry. Cross-sections of frozen cords were stained by means of the immunoperoxidase technique with polyclonal rabbit anti-human-Fn antibody (Chapter 6) at dilutions of 1/500, and a marker for endothelial cell cytoplasmic Factor VIII related antigen, anti-Factor VIII monoclonal antobody, at dilutions of 1/100. The streptavidin method was used to visualise the antigen-antibody reaction.

9.2.3 HUVEC studies

i) Human Umbilical Vein Endothelial Cell (HUVEC) Culture

All cell cultures were carried out at 37°C, in humidified air with 5% CO_2. Vascular endothelial cells were harvested from normal umbilical cords according to the method of Jaffe *et al* (1973). One cord yielded sufficient cells for each primary culture. Briefly, the umbilical vein was cannulated, rinsed with PBS, and the endothelial cells were detached by a 15 min incubation (37°C) with collagenase (0.25 mg/ml in PBS). After washing, the cells were resuspended in MEM containing 20% heat inactivated fetal calf serum supplemented with benzylpenicillin, 100 units/ml and streptomycin, 100µg/ml. Primary cultures were set up by plating cells on gelatin coated petri dishes. Purity of the HUVEC cultures was confirmed by their homogeneous, pavement-like morphological appearance as well as by >95% positive labelling of cytospins with factor VIII antibody using the immunoperoxidase technique.

ii) Labelling of HUVEC with ^{51}Chromium (^{51}Cr)

After two to three days in primary culture, cells were detached with 0.05% trypsin/0.02% EDTA, (1 min, 37°C), centrifuged and resuspended in 1ml MEM. ^{51}Cr was added at

were washed and resuspended at 10^6 cells/ml in MEM with 20% fetal calf serum. 100µl aliquots were dispensed into uncoated microtitre wells and grown to confluence overnight. Efficiency of labelling was assessed by counting radioactivity in 10µl cells before dispensing.

iii) Release of ^{51}Chromium (^{51}Cr) from HUVEC in the presence of fibronectin (Fn) and fibronectin degradation products (FnDP)

Intact purified Fn, Fn digested with plasmin (FnDP) (1h) *in vitro*, or Fn with FnDP isolated from plasma from normotensive pregnant subjects (n=5) and patients with severe preeclampsia (n=5) as described above (Table 1) were added to washed, confluent, ^{51}Cr-labelled HUVEC (100 000 cpm/well) to give final Fn concentrations of 100, 50 and 25 µg/ml in serum-free MEM in a total volume of 200µl. The ^{51}Cr content of the supernatant was assessed at 6, 12 and 24 hour by gamma-counting. The mean of triplicate wells was calculated and the ^{51}Cr released from HUVEC in the presence of Fn or FnDP was compared to the ^{51}Cr released from cells cultured with MEM alone.

HUVEC incubated with or without Fn and FnDP were examined by phase-contrast microscopy for morphological changes at 6, 12 and 24h *in vitro*.

iv) Subendothelial ^{125}I-Fn degradation by HUVEC

Fibronectin was iodinated at 1µCi/g as described by Fraker and Speck (1978). Microtitre plates were coated with ^{125}I-Fn (200,000 cpm/well) in the presence of 75µg/ml unlabelled Fn (no gelatin) in a total volume of 100µl PBS/well for 2-4h at RT. Plates were washed with MEM 2x before plating with HUVEC harvested after primary culture at 10^6/ml. Trypsin (0.1mg/ml, 2h) treatment of three wells revealed a total of 22 000 cpm to be plated per well. At confluence cell monolayers were washed twice with MEM and either intact, purified Fn (75µg/ml final concentration) or FnDP (75µg/ml) obtained by digesting intact Fn, was added. The total volume in the wells was 200µl. These incubations were set up with or without aprotinin (200U/ml final concentration) or plasminogen (0.1µM final concentration). HUVEC in 200µl MEM served as a control. Each incubation was set up in triplicate and radioactivity released into the medium was monitored at 2, 4 and 8 hours.

v) Preparation of fibronectin fragments

A number of Fn fragments were selected for purification. The 29 kDa hep1 amino-

v) Preparation of fibronectin fragments

A number of Fn fragments were selected for purification. The 29 kDa hep1 amino-terminal domain and the 31 kDa fib1 carboxy-terminal fragment were isolated by affinity chromatography using successive gelatin- and heparin-Sepharose columns (Pardo *et al*, 1983).

The 38 kDa hep II fragment was purified from 15min trypsin digest products subjected to gelatin-Sepharose affinity chromatography. 200 and 180 kDa fragments, eluted with 4M-urea were dialysed into 0.025M Tris buffer and digested with trypsin (1:100 w/w at 37°C for 45min). Resulting fragments were applied to heparin-sepharose. Heparin-binding fragments were eluted with 0.5M NaCl in Tris buffer, dialysed against 10mM Tris buffer and further resolved by DEAE-cellulose chromatography (Pardo *et al*, 1987). The DEAE-cellulose column was equilibrated in 10mM Tris/4M urea, pH 7.0 (without inhibitors) after washing beads with 1M Tris pH 7.0, 50mM Tris pH 7.0 and measuring the conductivity. 10mg protein/ml beads were incubated for 1h with constant mixing, after which the beads were centrifuged and the supernatant, (containing the unbound 38kDa Fn fragment, was removed .

vi) Subendothelial ^{125}I-Fn degradation by HUVEC in presence of selected FnDP

In order to determine which particular domain of the fibronectin molecule is responsible for the disruptive effect seen in endothelial cell cultures microtitre plates were coated with ^{125}I-Fn (200,000 cpm/well) in the presence of 25μg/ml unlabelled Fn (no gelatin) and plated with HUVECs as described above. Purified Fn (0.75μM final concentration), Fn digest (1h), FnDP including the purified 29kDa, 31kDa, 38kDa fragments (0.75μM), and a 10 amino acid peptide containing the cell attachment RGD sequence (15μM) (Sigma, St Louis, MO), were added to the wells. These incubations were set up with or without aprotinin (200U/ml final concentration) or plasminogen (0.1μM final concentration). Each incubation was set up in triplicate and radioactivity released into the medium was monitored at 2, 4 and 8 hours. Morphological changes were also recorded photographically.

vii) Measurement of urokinase plasminogen activator (uPA)

A sandwich Imubind-5 ELISA kit was used to measure uPA concentrations in supernatant culture media collected from the HUVEC grown on ^{125}I-Fn coated wells in both the experiments described above. uPA was measured, as opposed to tPA, as it is associated with cell-substrate contact points (Murata et al, 1991) and may be important in mediating cell detachment (Ge et al, 1992).

9.2.4 Data analysis

Data are presented in Tables 1, 2, 3 and 4. Patient demographic data, represented as means and standard deviations, were compared by Student's t-test. The Kruskal-Wallis one-way analysis by ranks (non-parametric) and multirange analysis for multiple samples (Statgraphics 5.0) were used to compare values between groups for the *in vitro* assays. Kruskal-Wallis non-parametric analysis does not assume normality, while multiple range analysis allows for comparisons of multiple samples. Statistical significance was defined as $p<0.05$.

9.3 RESULTS

9.3.1 Fibronectin (Fn) and fibronectin degradation product (FnDP) detection by SDS-PAGE and Western blot

Fn isolated by gelatin-sepharose affinity chromatography from normal plasma, control gestational and severe preeclamptic patient plasma, and Fn digested with plasmin (1h), subjected to SDS-PAGE and Western blotting with anti-fibronectin MAb CJ1 showed similar results to those presented in Chapter 8. Fn isolated from normal plasma migrated to an apparent molecular weight of 440 kDa and Fn subunits of apparent molecular weights of 240-220 kDa. Plasmin digests of Fn generated FnDP of similar molecular weights to those observed in the plasma of severe preeclamptic patients (Figure 9.2).

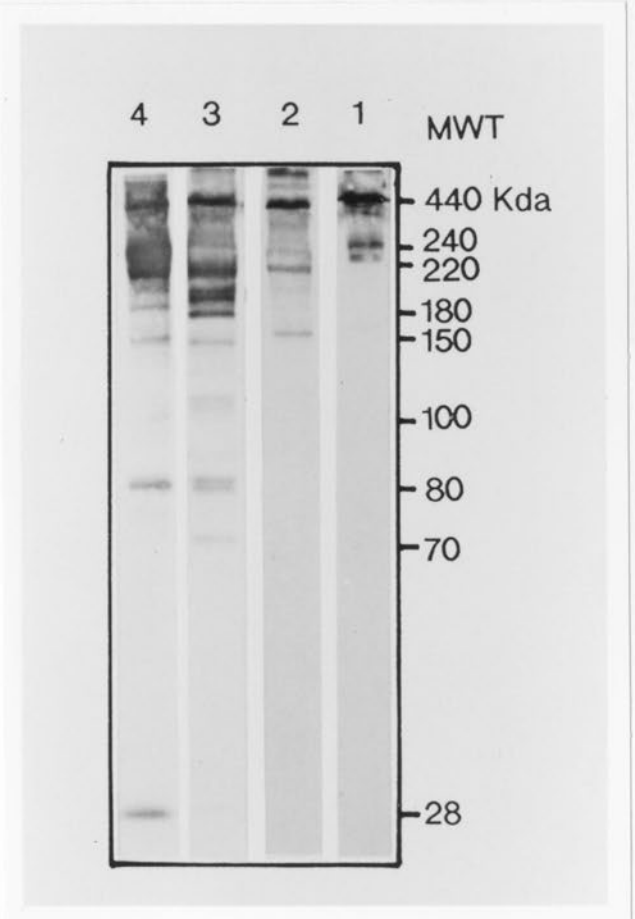


Figure 9.2 - A Western blot probed with monoclonal antibody CJ1 for fibronectin. Depicting 1) intact Fn, 2) Fn and FnDP purified from plasma from a representative control pregnant woman, 3) Fn and FnDP from plasma from a severe preeclampsia patient, 4) FnDP generated by plasmin digestion of Fn for 1h.

9.3.2 Umbilical cord studies

i) Fibronectin (Fn) and fibronectin degradation product (FnDP) release by umbilical cord vein cultures

The release of Fn from cord vein sections into culture supernatant was monitored by Western blotting (Figure 9.3). Fn released from cord vein sections from normotensive women appeared intact, while cord vein sections from patients with severe preeclampsia produced both Fn and FnDP which migrated to molecular weights of approximately 70kDa and 21kDa. Densitometric scanning of the Western blot indicated that these FnDP accounted for approximately 60% of the total Fn loaded in the track (Figure 9.3, lane 3).

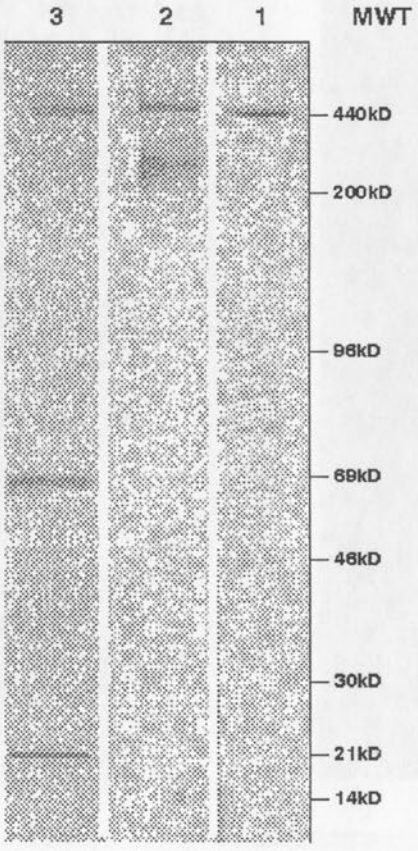


Figure 9.3 - A representative Western blot probed with monoclonal antibody CJ1 comparing Fn and Fn fragments in supernatant fluids. Cultures of umbilical cord sections from a control pregnant woman (lane 2) and a patient with severe preeclampsia (lane 3). 5μg purified Fn standard (lane 1). MWT indicated on the right.

ii) Immunostaining of umbilical vein cross sections

Normal cord sections showed weak staining with Fn antibody in subendothelial intimal tissue, whereas sections from patients with severe preeclampsia appeared to show strong immunoreactivity, indicating higher concentrations of Fn in the preeclamptic tissues [Figures 9.4, parts (a) and (b)]. Factor VIII antibody immunoreactivity was strong in the sections prepared from normal cords, demonstrating integrity of the umbilical vein endothelium. Cross sections prepared from umbilical cord veins from patients with severe preeclampsia, when stained for factor VIII, revealed an irregular endothelial layer with sloughing or detachment of cells from the subendothelium [Figure 9.4 parts (c) and (d)].

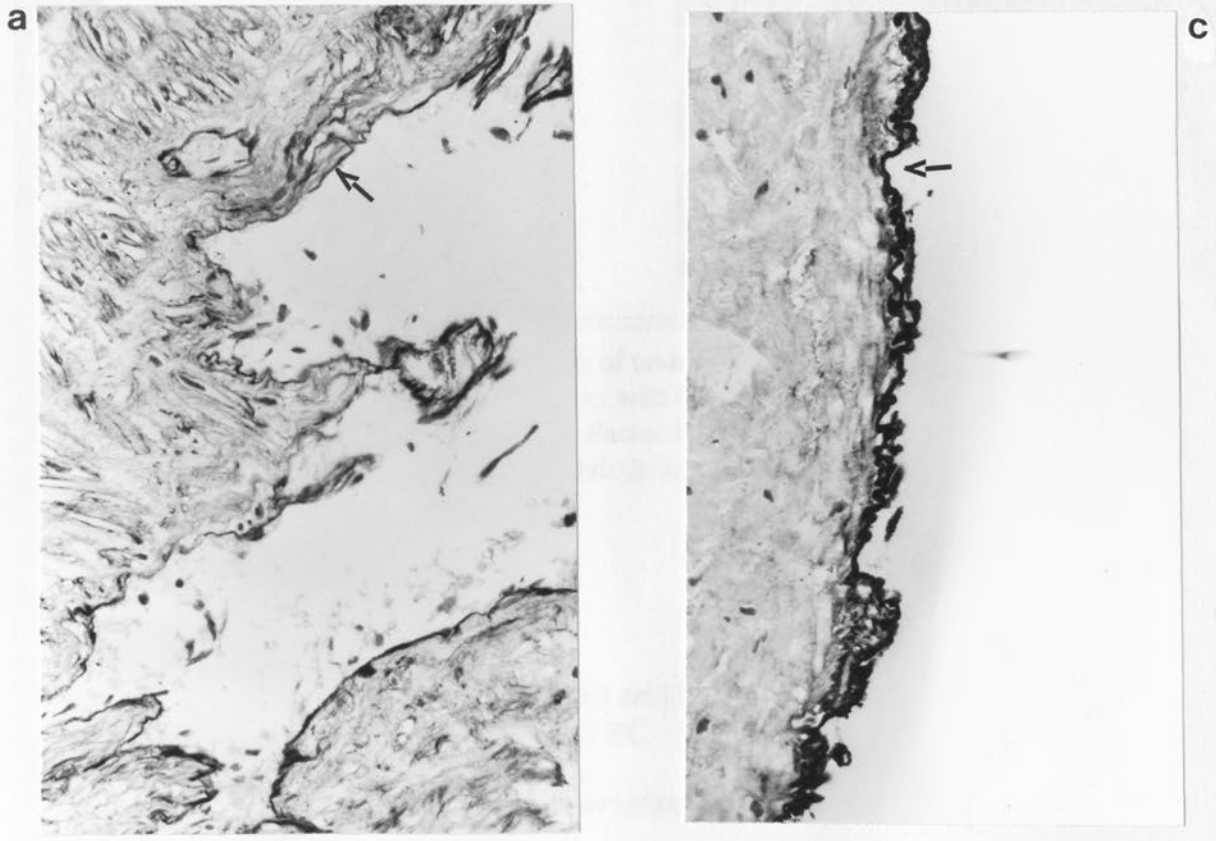


Figure 9.4(i) - Cross-section (c/s) (x360) of umbilical cord from a representative control pregnancy subject subjected to immunohistochemistry: with (a) Fn polyclonal antibodies, counterstained with haematoxylin. (Arrow indicates weak subendothelial Fn staining). (c) Factor VIII monoclonal antibodies, immunoperoxidase counterstained (x360). (Arrow indicates intact endothelial layer).

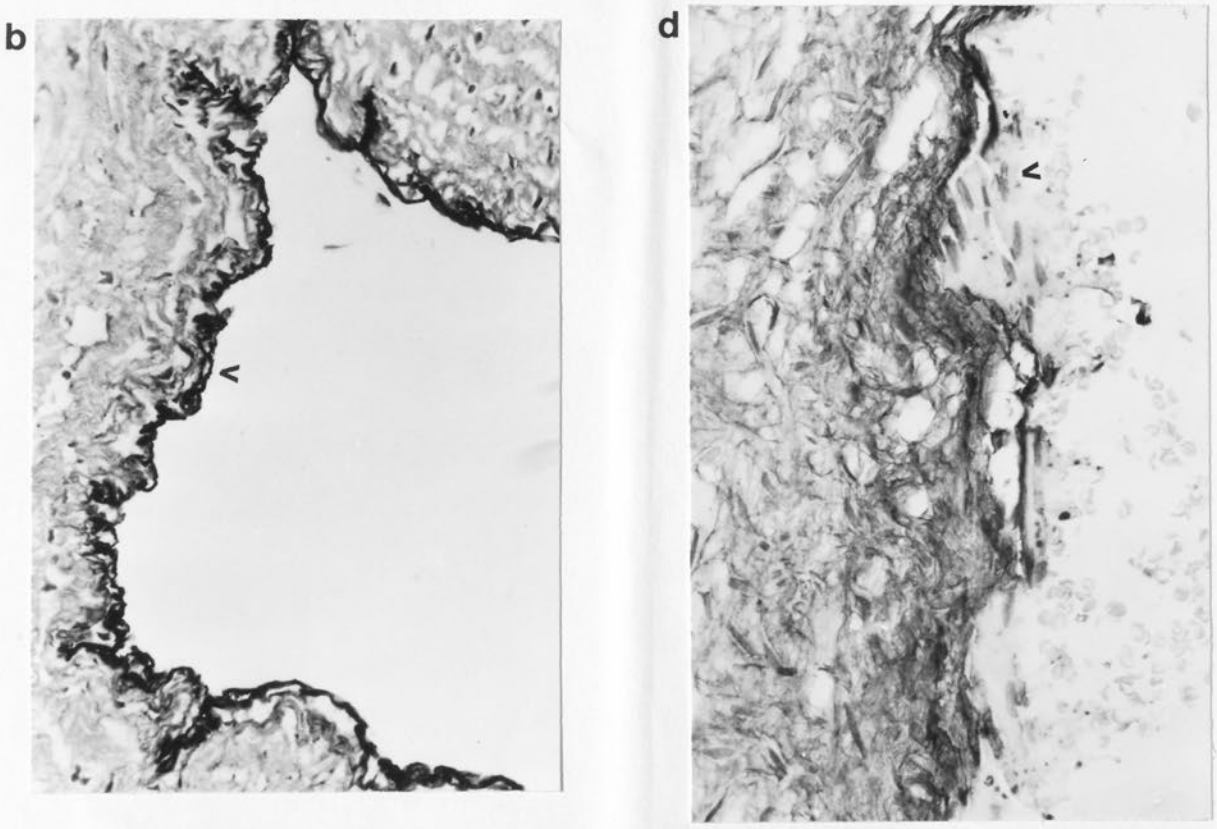
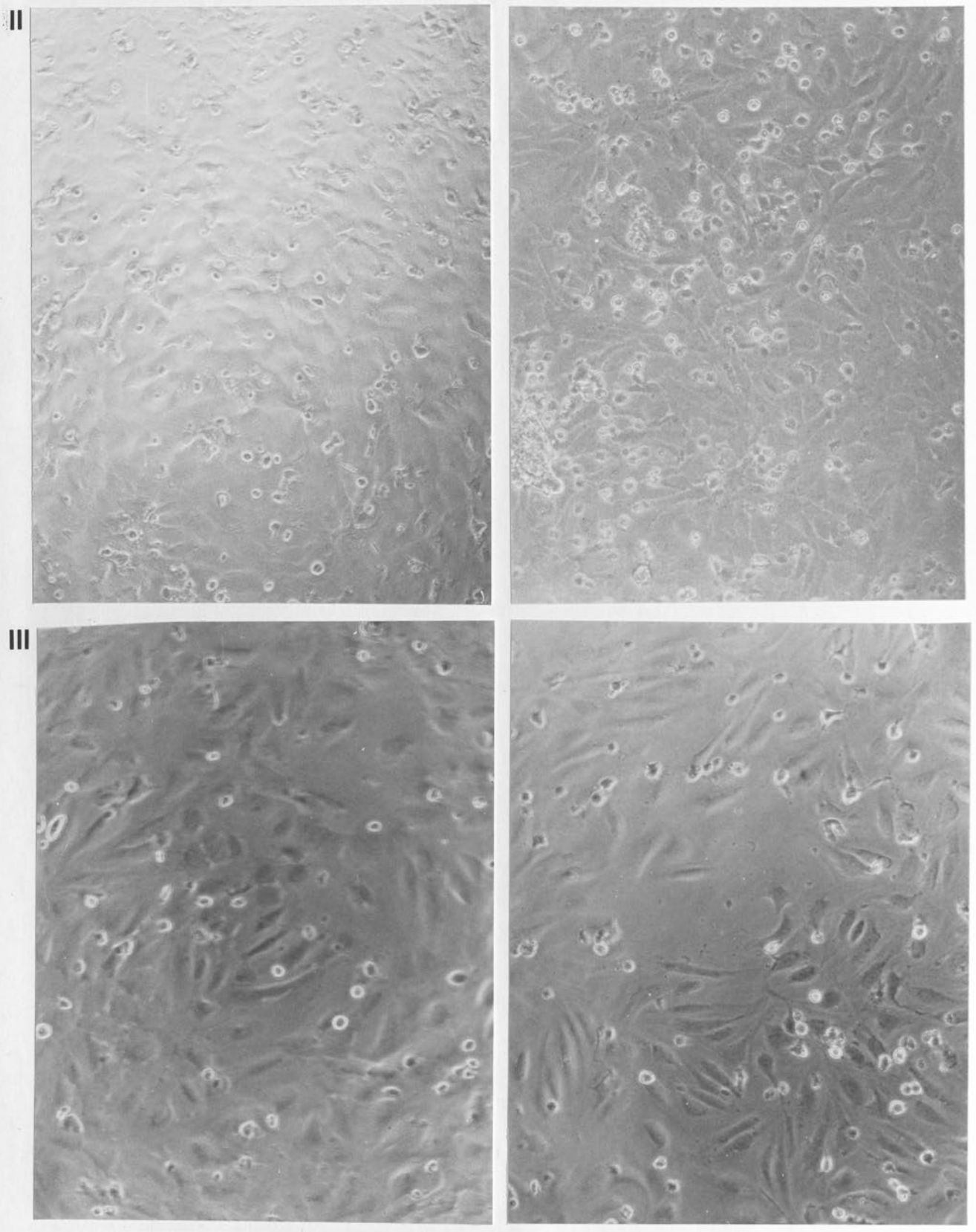


Figure 9.4(ii) - Cross-section (x360) of umbilical cords from representative preeclamptic patients stained as above: with (b) Fn polyclonal antibodies, (arrow indicates strong staining), and d) with Factor VIII monoclonal antibodies, (arrow indicates irregular, disrupted endothelium with sloughing of cells).

9.3.3 HUVEC studies

i) Effect of plasma fibronectin (pFn) and fibronectin degradation product (FnDP) on release of ^{51}Cr from HUVEC

^{51}Cr released from HUVEC into supernatant was calculated as a percentage of the total cellular radioactivity per well (100 000 cpm). The ^{51}Cr released from endothelial cells incubated in MEM alone (baseline) for 12h was 7.7±2.5% of the total radioactivity. No significant alteration in the release of ^{51}Cr from HUVEC occurred in the presence of either i) Fn isolated from plasma from normal pregnant women or ii) from severe preeclampsia patients, iii) pure Fn or (iv) FnDP (1h plasmin digest).


II
III
12h
24h

ii) Phase contrast microscopy of HUVEC

Microscopic examination revealed that there was no disruption of the monolayers in the presence of MEM alone, or in the presence of Fn from plasma from control pregnant subjects. Endothelial monolayers became increasingly disrupted with time in the presence of Fn from plasma of patients with severe preeclampsia (Figure 9.5). Further investigation of this phenomenon was done by observing the effect of FnDP prepared by plasmin digestion (1h) of pure Fn, on the integrity of HUVEC monolayers. Plasmin generated FnDP were found to disrupt HUVEC monolayers in a concentration dependent manner (Figure 9.6). Cell retraction and elongation occurred giving rise to gaps in the monolayer. Rounding up and detachment of cells from the monolayer was observed to a greater extent when FnDP were present in the medium, than in the presence of intact Fn or MEM alone (Figure 9.6). This disruptive effect of FnDP appeared to reach a plateau between 100-200μg FnDP/ml.

i

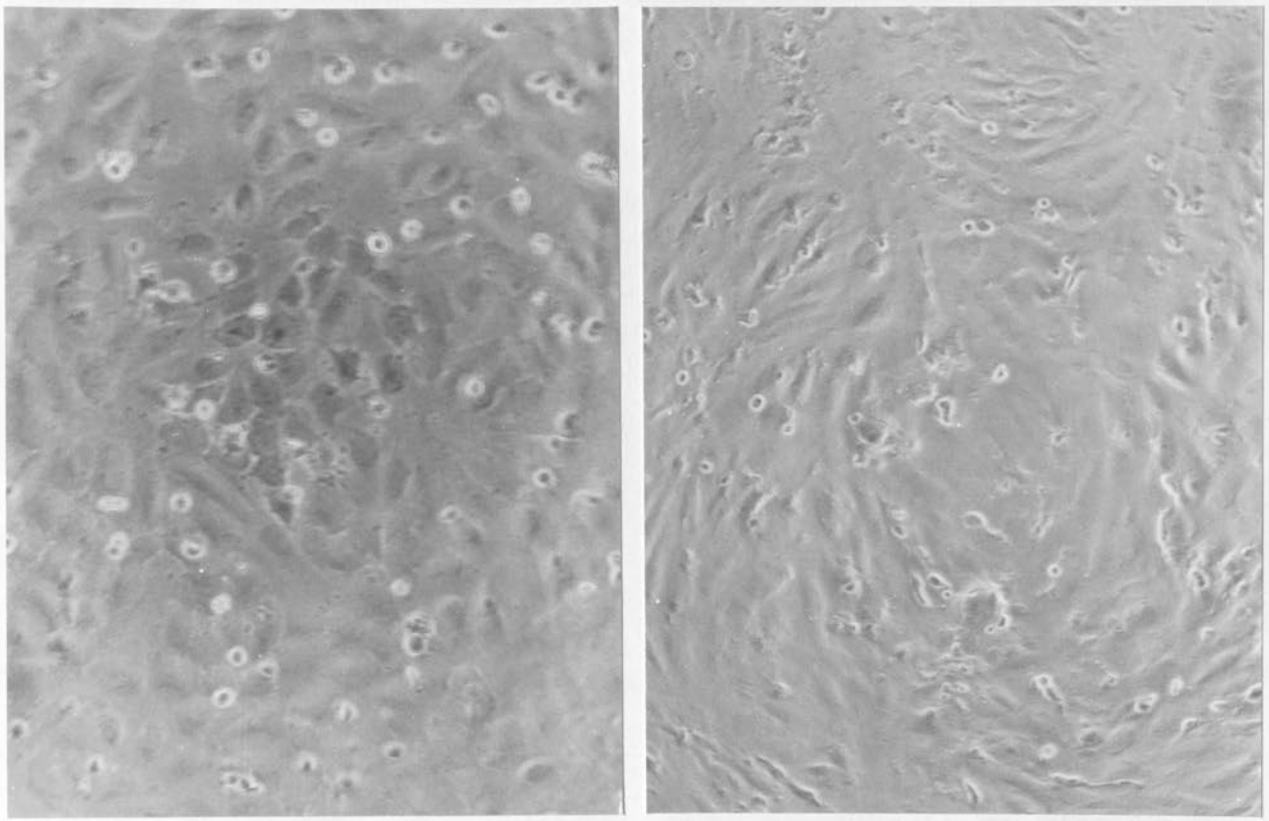


Figure 9.5 - (Above and on facing page)
Effects of FnDP on HUVEC morphology. HUVECs incubated in the presence of i) MEM alone after 12 and 24h incubation, ii) Fn from a control pregnant subject (12h, 24h), iii) Fn from a patient with severe preeclampsia (12h, 24h) (x100).

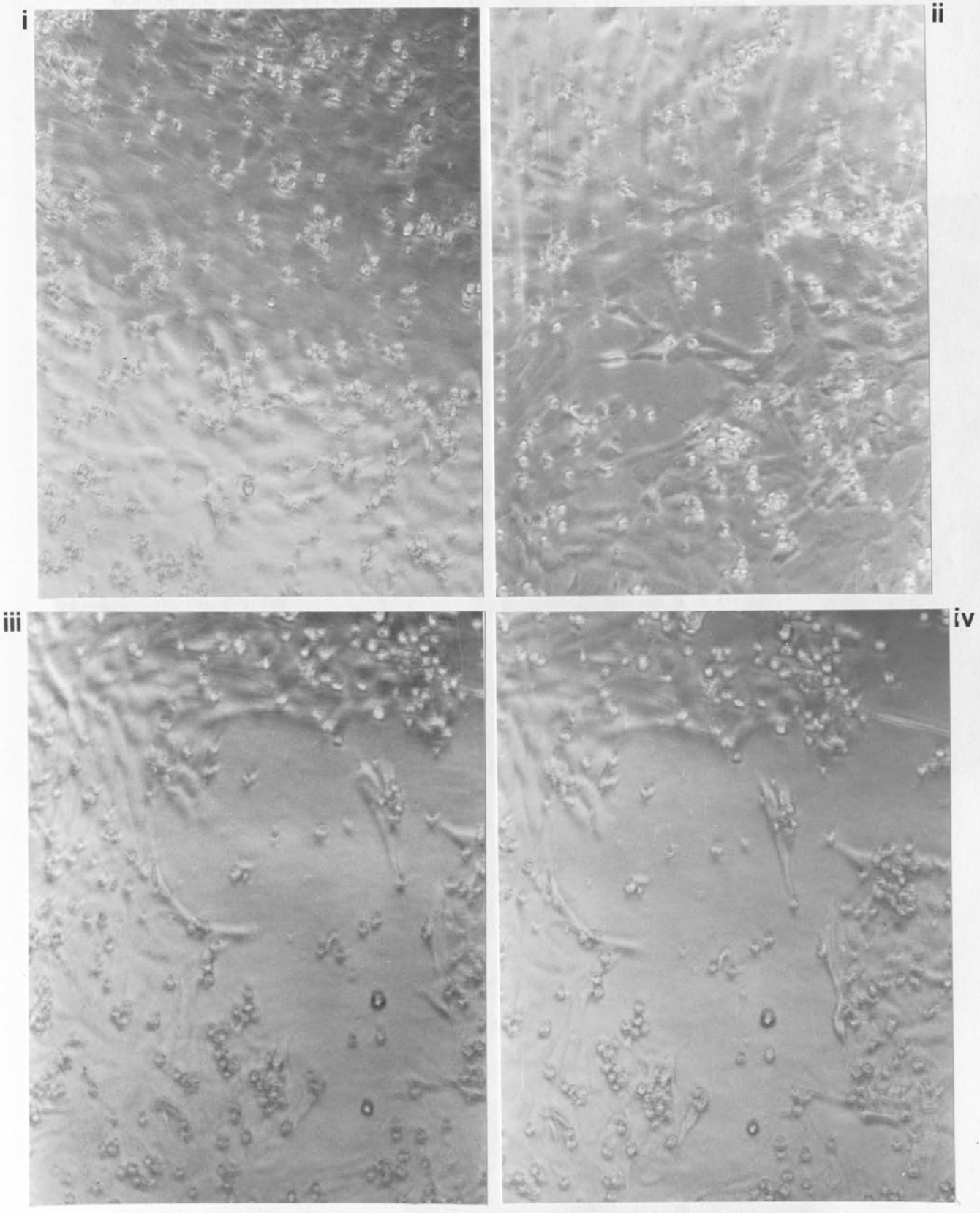


Figure 9.6 - Dose-response effects of purified Fn digested with plasmin (FnDP) on HUVEC morphology. HUVECS incubated in the presence of (i) MEM and in the presence of FnDP at: ii) 50μg/ml; iii) 100μg/ml; iv) 200μg/ml for 12h, (x40).

Individually purified Fn fragments had the following effects on morphology: a marked degree of gap formation occurred within 2h incubation in the presence of the 29kDa fragment, there was minimal gap formation observed in the presence of both the 31 and 38kDa fragments, while the RGD sequence caused no gap formation as for the Fn control (Figure 9.7).

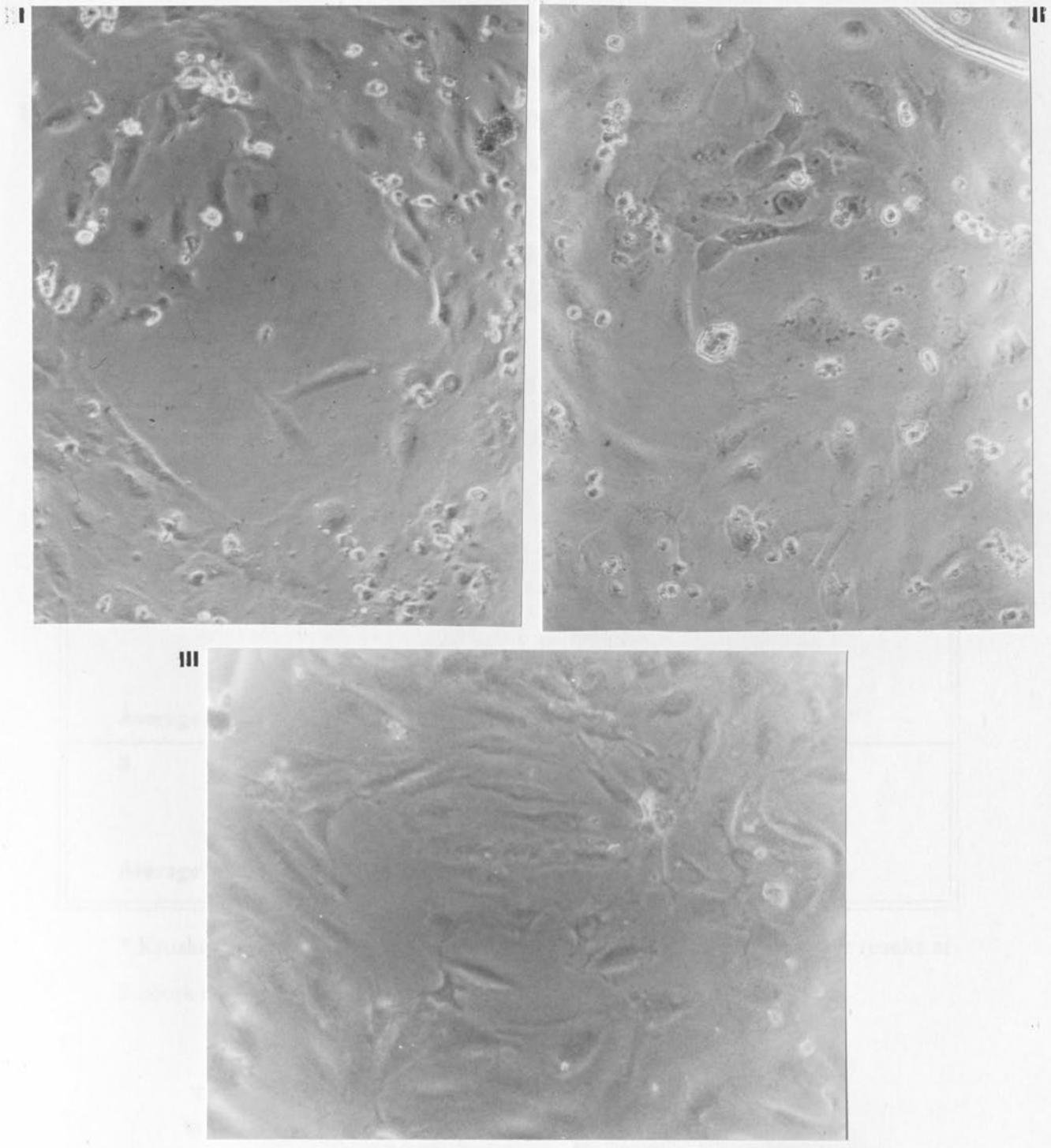


Figure 9.7 - Effects of selected FnDP on HUVEC morphology: i) 29kDa; ii) 31kDa; iii) 38kDa.

iv) Effect of pure fibronectin (Fn) and plasmin-generated FnDP on subendothelial ^{125}I-labelled fibronectin

Plasmin activity, generated as a consequence of increased uPA activity, was investigated by monitoring the radioactivity released into the medium of HUVEC cultured on ^{125}I-Fn in the presence of pure Fn and plasmin-generated FnDP. Incubation of HUVEC with intact Fn (75μg/ml) for 2h resulted in 60% more release of ^{125}I-Fn into the supernatant than that released into the supernatant by HUVEC incubated in MEM alone (Table 9.3). When this incubation was extended beyond 2h there was no significant difference between ^{125}I-Fn released in the presence or absence of Fn even with added plasminogen (Table 9.3). HUVEC cultured in the presence of FnDP (75μg/ml) caused further subendothelial ^{125}I-Fn to be released into the medium in a time-dependent manner. At 2, 4 and 8h the FnDP-induced release of subendothelial ^{125}I-Fn was greater than that of Fn-induced release (Table 9.3). Addition of plasminogen to wells containing Fn digest augmented the release of ^{125}I-Fn (vs FnDP without plasminogen) (Table 9.3). Pretreatment of cells with aprotinin before addition of FnDP inhibited ^{125}I-Fn release at these time points (Table 9.3). Three separate experiments gave comparable results.

Time (hours)	MEM	Fn	FnDP	Fn+plg	FnDp+plg	FnDP+Apr
		(75µg/ml)				
2	404	644	940	604	1444	728
	472	844	1008	628	1484	940
	612	904	1128	764	1488	1020
Average rank*		5.333	2.666	10.166	14.00	7.83
4	1396	1416	2296	1360	3138	1888
	1512	1736	2556	1572	3556	2144
	2045	1936	2744	1720	3988	2168
Average rank*		4.666	2.666	11.00	14.00	7.666
8	2860	2948	4664	2892	7296	3732
	3000	3536	4922	3184	9136	3756
	3568	3924	5300	3412	9264	4464
Average rank*		5.00	2.666	11.00	14.00	7.333

* Kruskal-Wallis one-way analysis by ranks (non-parametric) showed the results to be significantly different at each time point: **2h** : p=0.02; **4h** : p=0.01; **8h** : p=0.01. (Results for "MEM" samples were not included in the analysis.)

Using ANOVA the following factors were compared. P<0.05 was considered significant:

	2h	4h	8h
Fn vs FnDP	P=0.076	P<0.014*	P<0.012*
Fn vs Fn + Plg	P<0.229	P=0.47	P=0.39
FnDP vs FnDP + Plg	P<0.0014*	P<0.02*	P<0.006*
FnDP vs FnDP+Apr	P=0.27	P<0.04 *	P<0.03*
Fn+Plg vs FnDP+Plg	P<0.008*	P<0.002*	P<0.001*

Table 9.3 - Subendothelial ^{125}I-Fn degradation by HUVEC during incubation with Fn and FnDP (with or without plasminogen (Plg) or aprotinin (Apr)). The results at each time point are the radioactivity (cpm) present in 200µl supernatant medium from each sample in a single experiment.

v) Effect of purified fibronectin degradation products (FnDP) on subendothelial ^{125}I-labelled fibronectin (Fn)

A significant release of radioactivity above that of Fn was seen at 2h in the presence of the 1h Fn digest and the 38kDa. At 4 and 8h all fragment samples except the RGD peptide released significant amounts of radioactivity from the subendothelial ^{125}I-Fn layer (Table 9.4a). To detect whether this release of radioactivity was due to plasmin activity the release

of radioactivity was monitored in the presence of plasminogen and aprotinin as explained above. Addition of plasminogen to wells containing FnDP (1h digest) and the 29kDa fragment caused a significant augmentation of release of ^{125}I-Fn (vs cpm without the addition of plg) (Table 9.4b), while pretreatment with aprotinin inhibited ^{125}I-Fn release in wells containing these same samples at 8h points (Table 9.4c). Addition of plasminogen or aprotinin to the wells containing the 38kDa and 31kDa FnDP did not have this effect. Three separate experiments gave comparable results.

a) Mean cpm	**2h**	**4h**	**8h**
Fn	1131	1402	2175
1h Fn digest	2237*	4177*	4955*
31kDa	1099	1633*	2403*
29kDa	1097	1659*	3027*
38kDa	1494*	2148*	2749*
RGD	1150	1593	2044
* denotes significant difference from Fn control			
b) Mean cpm with plg	**2h**	**4h**	**8h**
Fn	1662*	1717*	2431
1h Fn digest	3076*	5568*	7244*
29kDa	1256	1843	3596*
RGD	1318	1581	2255
* denotes a significant difference from cpm in the absence of added plg (see a)			
c) Mean cpm with apr	**2h**	**4h**	**8h**
Fn	1177	1472	2249
1h Fn digest	1109*	1508*	2248*
29kDa	1024	1479	2503*
RGD	1083	1441	2144
* denotes statistically significant difference between cpm with and without apr (see a) by multiple range analysis (Statgraphics 5.0).			

Table 9.4 - Effect of individual FnDP on ^{125}I-Fn release

9.4 DISCUSSION

This study has demonstrated that FnDP present in the plasma of patients with severe preeclampsia, may potentiate the vascular endothelial dysfunction which has been described as an early event in the course of the development of the disease (Taylor *et al*, 1991b). The observation that some of these fragments are generated by umbilical vein cells is thus of pathogenetic interest. If any of the patients were in labour at the time of blood sampling, an increase in plasmin-mediated Fn fragmentation may be evident as labour tends to be associated with increased tPA antigen and activity (Bremer et al, 1994).

We detected increased deposition of Fn in the subendothelial intima together with disruption and sloughing of focal areas in the endothelium of umbilical cord veins from patients with severe preeclampsia. Umbilical vein cultures from severe preeclamptic patients released Fn and FnDP of predominantly 70kDa and 21kDa molecular weight. Fragments of 70kDa have been reported to be immunoreactive (Czop *et al*, 1981).

Our experiments demonstrate that the addition of FnDP from approximately 70% of plasma samples from patients with severe preeclampsia in this study, or plasmin-generated FnDP, to HUVEC is associated with morphological changes *in vitro*. This phenomenon appears to be associated with Fn fragments of less than 150 kDa as fragments of Fn in control plasma (180 and 150 kDa) did not exhibit this activity. We have shown HUVEC morphological changes with Fn fragments of 29kDa, 31kDa and 38kDa, although the effect with the latter two fragments was not as marked as that by a Fn digest or the 29kDa fragment. Fn domains of 35kDa (equivalent to our 38kDa fgragment), 29 and 25kDa (equivalent to our 31 kDa fragment) have been described as inhibitors of bovine artery endothelial cell growth in culture (Homandberg *et al*, 1986), although the heparin-binding activity of the fragments was not responsible for this inhibition (Homandberg *et al*, 1989. The 38 kDa heparin-binding fragment immediately precedes the 31 kDa domain on its amino end (Figure 9.1) and contains 380 residues arranged in three type III homology regions of approximately 90 residues each, and a 67-amino acid carboxy-terminal segment which is a region of heterogeneity found in at least one of the Fn subunits (Kornblihtt *et al*, 1984).

The mechanism of action of the FnDP from plasma from severe preeclamptic patients and the 29kDa plasmin generatd FnDP appears to be that they upregulate endothelial cell uPA release (associated with cell-matrix contact points) which converts plasminogen to

plasmin. The increased plasmin activity appears to cause degradation of the subendothelial Fn matrix, resulting in focal disruption of the monolayer. This concept is supported by the observation that subendothelial Fn degradation was partially inhibitable by aprotinin (a plasmin inhibitor) and enhanced by the addition of plasminogen to HUVEC in the presence of FnDP. Metalloproteinases might account for the non-aprotinin-inhibitable proteolytic activity, as ligation of the Fn receptor on fibroblasts has been shown to induce metalloproteinase expression (Werb *et al*, 1989). The morphological changes by the 31kDa and 39kDa fragments do not appear to be due to the generation of plasmin and could possibly be due to the generation of other proteases.

Comparison of the time course of plasmin generation, subendothelial Fn degradation and endothelial cell gap formation indicates that proteolytic cleavage occurs initially at cell-cell contact points followed by cell-matrix adhesion sites, which then causes endothelial cell detachment and gap formation. The decrease noted in uPA concentration with increasing incubation time indicates the possible production of plasminogen activator inhibitors (PAI) by the cells.

9.5 CONCLUSION

Fragment D of fibrinogen has similar effects to those described by us on gap formation in endothelial monolayers (Ge *et al*, 1992). It is therefore tenable that FnDP and fragment D of fibrinogen, both generated by disseminated intravascular coagulation and pericellular proteolysis, may predispose the vasculature to progressive loss of endothelial integrity and the denudation seen in the placental and maternal blood vessels in preeclampsia. As plasminogen activator inhibitor-2 (PAI-2) levels may be low in placentae of preeclampsia patients (de Boer *et al*, 1989) plasminogen activators (PAs) will not be efficiently neutralised and this may promote Fn fragmentation. Release of these products into the circulation may account for the acceleration of the disease process to the severe state and for the continuation of the coagulation disturbances at the endothelial surface.

CHAPTER 10

CONCLUSIONS

The following points sum up what this thesis has contributed to the growing body of knowledge about the puzzle of the preeclamptic syndrome. Based on the development of monoclonal antibodies (MAb) and polyclonal antibodies (PAb) specific for fibronectin (Fn) and in-house ELISAs for the measurement and detection of fibronectin and its degradation products as well as for fibrinogen degradation products (FDP), this thesis has achieved the following:

This study has shown that circulating concentrations of the coagulation parameters, plasminogen activator inhibitor-1 (PAI-1), fibronectin, fibrinogen degradation product and tissue plasminogen activator (tPA), are significantly increased in patients with severe preeclampsia when compared with normotensive, age- and gestational age-matched pregnant controls. Of importance, was the finding that patients who develop severe preeclampsia, show a progressive increase in tissue plasminogen activator and fibrogen degradation product levels with time and it has been suggested that this observation, when considered together with other clinical parameters, could provide a valuable, additional prognostic index. The observed raised serum levels of PAI-1, fibronectin, fibrinogen degradation product and tissue plasminogen activator support the contention that endothelial cell damage, disordered coagulation and fibrinolysis play a role in the pathogenesis of the condition. The finding therefore, that fibronectin levels in the sera from patients with the preeclampsia syndrome did not increase with disease progression was surprising however, since endothelial damage is associated with degradation of extracellular matrix and the release of fibronectin. Investigation of the integrity of the fibronectin in the plasma from patients with preeclampsia revealed fibronectin fragmentation, the extent of which was dependent on the severity of the syndrome. Plasma from patients with severe preeclampsia had larger amounts of low molecular weight fragments than plasma from patients with mild preeclampsia. The size of the fragments observed in these plasma samples suggested that fibronectin degradation could have occurred by plasminolysis. The fibronectin degradation product (FnDP) present

in these patient plasma were found to significantly inhibit the opsonic activity of intact fibronectin and the determination of fibronectin concentration by ELISA. This latter finding may explain the inability to detect increasing plasma fibronectin concentrations with disease progression.

This study shows that fibronectin fragments are able to disrupt endothelial cultures *in vitro*. Disruption of HUVEC monolayers was observed microscopically when incubated with either fibronectin degradation products from severe preeclamptic plasma, fibronectin degradation products prepared by plasmic digestion of fibronectin and a 29 kDa fibronectin degradation product heparin-binding fragment from the amino-terminus of the fibronectin molecule. This disruption was not related to damage or lysis of the cells, as there was no significant release of ^{51}Cr from ^{51}Cr-labelled HUVEC monolayers, when challenged with these fibronectin degradation products. HUVEC monolayer disruption appears to be the result of degradation of the subendothelial matrix, possibly at contact points between cells and the subendothelial matrix, through the action of plasmin, generated from plasminogen by urokinase plasminogen activator (uPA), released from HUVEC, in the presence of fibronectin degradation products. Although it appears that urokinase plasminogen activator inhibitors are also produced during incubation of HUVEC with these fibronectin degradation products, there is sufficient unihibited urokinase plasminogen activator for the generation of plasmin for proteolysis of the subendothelial matrix to occur .

It is tenable from the results of these *in vitro* experiments, investigating the interaction of fibronectin degradation products with HUVEC monolayers, that fibronectin plays a role in the progression of the syndrome of preeclampsia through its ability, when degraded, to disrupt the endothelium. This disruption is associated with the generation of plasmin as a result of plasminogen activation by urokinase plasminogen activator. Endothelial cell production of urokinase plasminogen activator, together with other proteins such as coagulation factors and plasminogen activator inhibitors, occurs during the conversion of the endothelium from an anti-coagulant surface to a procoagulant surface, a process governed by a complex series of reactions. The deposition of fibronectin, through crosslinking to deposited fibrin on the vascular endothelium, is also a consequence of the conversion of

the endothelium to a procoagulant surface. We have shown by immunostaining that cord sections from patients with preeclampsia have a disrupted endothelium and high concentrations of fibronectin as compared to sections from normal cords. The initial event(s) in the preeclamptic syndrome which eventually gives rise to the procoagulant state of the endothelium is extremely complex and undefined, but may involve, prior to endothelial damage, stimulation of the endothelium by cytokines such as IL-1 and TNF-α generated from lymphocytes and mononuclear phagocytes. However it appears from the results of this study, that the generation of fibronectin degradation products, following endothelial damage, may perpetuate further damage to the endothelium. Escape of these products into the maternal circulation may be postulated to give rise to maternal endothelial cell damage and to the generalised organ failure observed in preeclampsia.

COAGULATION DISTURBANCES AND FIBRONECTIN FRAGMENTATION IN PREECLAMPSIA

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COAGULATION DISTURBANCES AND FIBRONECTIN FRAGMENTATION IN PREECLAMPSIA

APPENDICES

APPENDIX I

ABBREVIATIONS

AA	arachidonic acid
aa	amino acid
Ab	antibody
Ag	antigen
apo	apolipoprotein
ATP	adenosine triphosphate(also ADP)
bp	base pair
BSA	bovine serum albumen
βTG	beta-thromboglobulin
C	complement
°C	degrees Celcius
Ca	cancer
cDNA	complementary DNA
CFA	complete Freund's adjuvant
cFn	cellular Fn
CIG	cold insoluble globulin
Cr^{51}	$chromium^{51}$
Ci	Curie(s)
cpm	counts per minute
CR	complement receptor
CRP	C-reactive protein
COOH	carboxy-terminal
cys	cysteine
Da	dalton (kDa kilodalton)
DEAE	diethylaminoethyl
DIC	disseminated intravascular coagulopathy
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTE	dithioerythritol
DTH	delayed type hypersensitivity
DTT	dithiothreitol
ECM	extracellular matrix
EDRF	endothelial derived relaxing factor
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
E/T	effector/target ratio
FCS	foetal calf serum
Fn	fibronectin
sFn	serum Fn
pFn	plasma Fn

FnDP	fibronectin degradation product
FnR	fibronectin receptor
FDP	fibrin/fibrinogen degradation product
FITC	fluorescein isothiocyanate
FGF	fibroblast growth factor
FHF	fulminant hepatic failure
g	gram
mg	milligram
ng	nanogram
pg	picogram
µg	microgram
GA	gestational age
GH	gestational hypertension
GPH	gestational proteinuric hypertension
GSH	glutathione, reduced
h/hr	hour
HBSS	Hank's balanced salt solution
HDL	high density lipoprotein
HELLP	haemolysis, elevated liver enzymes, low platelets
Hepes	N-2-hydroxyethylpiperazine-N1 2-ethane sulfonic acid
HLA	human histocompatability leucocyte antigens
HUVEC	human umbilical vein endothelial culture
IFN	interferon
Ig	immunoglobulin (IgG, IgM)
IL	interleukin (IL-1, IL-6)
IU	international units
I^{125}	iodine^{125}
kb	kilobase
Kd	dissociation constant
Ki	inhibition constant
LDL	low density lipoprotein
l	litre(s)
dl	decilitre
ml	millilitre
µl	microlitre
LPS	lipopolysaccharide
m	metre(s)
mm	millimetre
M	molar
MAb	monoclonal antibody
MEM	minimal essential medium
MHC	major histcompatibility complex
mol	mole(s)
MWT	molecular weight
Mr	relative molecular mass
mRNA	messenger RNA
n	number in study, group

N	normal (concentration)
NAD	nicotinamide adenine dinucleotide
ND	not determined
NH_2	amino terminal
No.	number
NO	nitric oxide
NS	not significant
OD	optical density
%	percent
P	probability
PAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1 (also PAI-2)
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCS	portacaval shunt
PCT	portacaval transposition
PDGF	platelet derived growth factor
PEG	polyethylene glycol
Pf_4	platelet $factor_4$
PGE	prostaglandin (also PGF, etc)
PH	partial hepatectomy
PI	phagocytic index
pi	isoelectric point
PMA	phorbol myristate acetate
PMN	polymorphonuclear leucocyte
PMSF	phenylmethylsulfonyl fluoride
PO_2	partial pressure of O_2
PTT	partial prothrombin time
r	correlation coefficient
RBC	red blood cell
RES	reticulo-endothelial system
RGD	arg-gly-asp
RIA	radioimmunoassay
rpm	revolutions per minute
RNA	ribonucleic acid
s	second(s)
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SOD	superoxide dismutase
sp act	specific activity
Staph	Staphylococcus
t1/2	half-life
TCA	trichloroacetic acid
TEF	transformation enhancing factor

TF	tissue factor
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
TPR	total peripheral resistance
Tris	tris (hydroxymethyl)-aminomethane
t test	Student's t test
TXA	thromboxane A
U	units
uPA	urokinase plasminogen activator
VLA	very late antigen (VLA1,2,3,4,5)
VLDL	very low density lipoprotein
vol	volume
vWf	von Willebrand factor
wk	week
wt	weight
yr	year

APPENDIX II

MATERIALS AND SUPPLIERS

Unless otherwise stated, all reagents were of analytical grade.

American Diagnostica Inc.

Imubind Tm-5 tPA ELISA kit, Imubind uPA ELISA kit, Imubind PAI-1 and PAI-2 ELISA kits.

Amersham U.K.

Anti-mouse Ig (Biotinylated species specific), Rainbow Molecular Weight Markers 20 000 - 300 000 MW, Chromium 51, Iodine125.

Bayer-Miles (Pty) Ltd., Isando, South Africa.

Bovine serum albumin (Fraction V),

Becton Dickinson and Co., Lincoln Park, New Jersey, USA.

Falcon Micro Test flexible assay plates, petri dishes and other sterile tissue culture plasticware eg. 1,5 and 10ml pippettes, 15ml and 50ml test-tubes.

Bio-Rad Laboratories, D 8000, Munchen, West Germany.

Bio-Rad dye reagent concentrate, Affigel Blue Gel.

BDH Chemicals Ltd., Poole, England.

Acetic acid, Acrylamide, Ammonium persulphate, Ammonium sulphate, Bromophenol blue, 3.3'di-aminobenzidine tetrahydrochloride, Ethanol, Ethanolamine, Glycerol, Glycine, Hydrochloric acid, Hydrogen peroxide, Imidizole, Methanol, Sodium azide, Sodium hydrogen carbonate, Sodium hydroxide, Tris (hydroxymethyl methylamine), Trypan blue, NN'Methylene-bisacrylamide, NNN'N'-Tetramethylethylene (TEMED), Urea.

Cooper Biomedical, Inc. West Chester, P.A., U.S.A.

Cappel peroxidase conjugated IgG fraction goat anti-rabbit (heavy and light chain specific), Goat anti-human fibrinogen IgG.

Dako-Immunoglobulins, 22 Guldborgveg, KD-20000, Copenhagen F. Denmark.

PAP complex, goat anyi-rabbit IgG.

Difco Laboratories, Detroit, Michigan, U.S.A.

Complete Freund's adjuvant, Incomplete Freund's adjuvant.

E.I. du Pont de Nemours and Co. Inc., Wilmington, Delaware, U.S.A.

Photra graphic arts film (C-41).

Gibco Ltd., Paisley, Scotland.

Aminopterin, Hypoxanthine, Penicillin, Streptomycin.

Highveld Biologicals.

Foetal calf serum, Hanks Balanced Salt Solution, Minimal Essential Medium, RPMI 1640 tissue culture medium.

Kimble, Illinois.

Glass Test tubes, 12x75mm.

Leo Pharmaceuticals, Denmark.

Urokinase.

Merck, Darmstadt, Germany.

Amido black, Arginine, Citric acid, 4-chloro-1-napthol, Dimethylsulphoxide, disodium hydrogen phosphate, Ethylenediamintetra-acetic acid disodium salt, Gelatin, Hydrochloric acid, Potassium chloride, Sodium acetate, Sodium carbonate, Sodium chloride, Sodium dodecyl sulphate, Sodium dihydrogen phosphate, Sodium hydrogen carbonate, Tween 20.

Nunc, D.K.4000, Roskilde, Denmark.

96 well Microwell plates (flat-and round-bottomed) and 24 well Multidishes, 1.8ml cryotubes.

Pharmacia Fine Chemicals, Uppsala, Sweden.

PD-10 Sephadex G-25, CNBr-activated sepharose 4B, DEAE-Sephacel.

Schleicher and Schuell, D-3354, Dassel, W.Germany.

Membrane filters, 0.45um, Nitrocellulose paper.

Spectrum Medical Industries, Inc., Los Angeles, U.S.A.

Spectraphor membrane tubing, molecular weight cut-off 12 000 - 14 000 daltons.

Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Avidin-peroxidase, azino-bis(ethyl-benzthiazoline) sulphonic acid (ABTS), Benzamidine, Brilliant Blue R, Carboxy-latex particles, Collagenase, Dithiothreitol, 1-ethyl-3-carbodiimide HCl (ECDI), 2 beta Mercaptoethanol, PAGE Blue, Phenylmethylsulfonyl fluoride, Pristane, R-G-D peptide, Sepharose 4B, Thrombin, DMSO.

Saarchem, Univar.

Potassium thiocyanate, Glacial acetic acid, Trisodium citrate, Hydrogen peroxide.

Titertek, Skatron, Lier, Norway (for Flow Laboratories).

ELISA plate reader.

Whatman Ltd., Maidstone, Kent, U.K.

Filter Paper No. 1.

Zymed Lab. Inc., San Francisco, U.S.A.

Peroxidase conjugated goat anti-mouse IgG,
Goat anti-mouse IgG,IgM, IgA (H + L),
Gilson tips, Eppendorf tubes, multidispenser pipettes.