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VASCULAR DAMAGE IN PRE-ECLAMPSIA :

The role of endothelial dysfunction.

by

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Thesis submitted for the degree of Master of Science to the University of Glasgow.

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SUMMARY

Pre-eclampsia is a multisystem disorder specific to pregnant women and is the major cause of maternal death in the United Kingdom today. The common pathophysiological feature of virtually all manifestations of pre-eclampsia is vascular endothelial dysfunction. This probably results from poor placental perfusion due to defective trophoblast invasion. Platelets and neutrophils play a role in the pathophysiological process; recruitment of these cells is mediated by cell adhesion molecule expression. Intrauterine growth restriction (IUGR) is often associated with pre-eclampsia. It too is associated with neutrophil activation.

The hypothesis examined in this thesis was that cell adhesion molecule expression, potential markers of endothelial damage and neutrophil activation, was increased in pre-eclampsia and IUGR. The following studies were performed:

1. Circulating concentrations of cell adhesion molecules were measured in normal and pre-eclamptic pregnancies.
2. Cell adhesion molecule mRNA and protein expression were determined in placentae from normal, pre-eclamptic and IUGR pregnancies,
3. Circulating concentrations of cytokines, known to activate neutrophils, were measured in normal and pre-eclamptic pregnancy and
4. The effect of the cytokine IL-6 on the expression of the cell adhesion molecules VCAM-2 and E-Selectin on human umbilical vein endothelial cells was determined.

Circulating concentrations of the cell adhesion molecules VCAM-1 and E-Selectin were increased in the maternal circulation of women with pre-eclampsia compared to normal pregnant controls. We found a difference in the expression of E-Selectin between plasma and serum suggesting that the wide range of concentrations of E-Selectin between patients may require large numbers of patients to show a significant trend. We found a higher expression of E-Selectin in serum compared to plasma in normal pregnant patients suggesting that more E-Selectin is shed into serum, after the activation of cells, due to clotting. Immunostaining for platelet endothelial cell adhesion molecule (PECAM) and intercellular adhesion molecule-1 (ICAM-1) was localised mainly to the endothelium of the stem villi, intermediate villi, terminal villi and decidual vessels. ICAM-1 staining was also evident in the stroma and fetal membranes. The placentae sections were negative for VCAM-1 and E-Selectin immunostaining. PECAM, ICAM-1 and ICAM-2 mRNA were all detectable in normal and pre-eclamptic placentae but E-Selectin and VCAM-2 mRNA were undetectable. There were no significant differences in the cell adhesion molecule mRNA expression or immunostaining in pregnancies complicated by pre-eclampsia or IUGR compared to normal pregnant placentae. The concentrations of the cytokines IL-6 and interleukin-1 receptor antagonist (IL-1ra) were significantly higher in patients with pre-eclampsia

compared to normal pregnant patients whilst there were no significant differences in the concentrations of tumour necrosis factor alpha (TNFα), IL-8, granulocyte macrophage-colony-stimulating factor (GM-CSF) and IL-1β. IL-6 induced the expression of E-Selectin and VCAM-2 mRNA and protein on human umbilical vein endothelial cells, which was both time and dose dependent.

The presence of increased concentrations of VCAM-1, E-Selectin, IL-6 and IL-1ra may all contribute to the endothelial damage seen in pre-eclampsia and may explain the mechanism underlying leucocyte activation in this disorder. Management of pre-eclampsia focuses on controlling blood pressure but effective treatment will only come as a result of a clearer understanding of the pathogenesis of the disease.

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DECLARATION

I declare that I have contributed to the experimental work described in this thesis, with help from Dr Fiona Lyall with ELISAs, Mrs Anne Young with immunocytochemistry and Dr Tim Perera (Yamanouchi Research Institute, Oxford), under the supervision of Dr Fiona Lyall, in the Department of Obstetrics and Gynaecology at the University of Glasgow.

The work presented here is original research and has not previously been submitted for a higher degree. I am the author of the thesis and have consulted all the references cited

signed

Fiona Boswell

I certify that the experimental work reported in this thesis was performed by Miss Fiona Boswell at the University of Glasgow and that during the period of the study she has fulfilled the conditions of the relevant regulations governing the degree of Master of Science (MSc)

signed.

Dr Fiona Lyall

Abbreviations

ADMA	N^GN^G dimethylarginine
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CD	cluster of definition
cGMP	cyclic guanosine monophosphate
CLASP	collaborative low dose aspirin study in pregnancy
CO_2	carbon dioxide
CSF-1	colony stimulating factor-1
CT	computerized tomography
CTP	deoxycytidine 5'- triphosphate ammonium salt
DAB	diamino benzidine tetrahydrochloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddH_2O	deionised distilled water
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphoshate
dH_2O	distilled water
DIC	disseminated intravascular coagulation
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
ECGS	endothelial cell growth supplement
EDRF	endothelial derived relaxing factor
EDTA	diaminoethanetetra-acetic acid-disodium salt
EGF	epidermal growth factor
ELAM-1	endothelial leukocyte adhesion molecule-1
ELISA	enzyme linked immunosorbent assay
F	fungizone
FCS	fetal calf serum
FMLP	formyl-methionyl-leucyl-phenylalanine
G	glutamine
GM-CSF	granulocyte macrophage-colony stimulating factor

GTN	glyceryl trinitrite
h	hour
HCL	hydrochloric acid
HELLP	haemolysis, elevated liver enzymes and low platelet count
HRP	horseradish peroxidase
HS	horse serum
5-HT	5-hydroxytryptamine
HUVECS	human umbilical vein endothelial cells
ICAM-1/2	intercellular adhesion molecule-1/2
ICC	immunocytochemistry
IFN(γ)	interferon (gamma)
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin
IgSF	immunoglobulin superfamily
IL-α	interleukin -alpha
IL-β	interleukin-beta
IL-1β	interleukin-1 beta
IL-1ra	interleukin-1 receptor antagonist
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
IUGR	intrauterine growth restriction
kb	kilobases
KDa	kilo Dalton
KDR	kinase-insert domain
LFA-1	lymphocyte function associated antigen
MOPS	2-[N-Morpholino] ethane-sulphonic acid
mRNA	messenger ribonucleic acid
NCAM	neural cell adhesion molecule
NGS	normal goat serum
NHS	normal human serum
NO	nitric oxide
NOS	nitric oxide synthase
OD	optical density
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule-1

PIH	pregnancy induced hypertension
P/S	penicillin/streptomycin
PVP	polyvinylpyrrolidine
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulphate
SE	standard error
SF	serum free (media)
SSC	sodium saline citrate
TBS	tris buffered saline
TE	Tris/EDTA electrophoresis buffer
TEMED	tetramethylethylenediamine
TNFα	tumour necrosis factor alpha
TNF-R	tumour necrosis factor receptor
VCAM-1/2	vascular cell adhesion molecule-1/2
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4
VWF	von Willebrand factor

CHAPTER ONE

INTRODUCTION

Pre-eclampsia is a disorder specific to pregnant women which affects virtually every organ and system in the body. It remains the major cause of maternal death in the United Kingdom today (Dept. of Health et al, 1996). Hypertension and proteinuria, the traditional diagnostic features, represent only two facets of a complex pathophysiological process, the pathological feature being vascular endothelial dysfunction.

1.1 Hypertension in pregnancy

1.1.1 Definition and clinical aspects

Hypertensive disorders are the most common medical complications of pregnancy. There are three types of hypertension in pregnancy (Roberts et al, 1991). The first is hypertension in women who have hypertension predating pregnancy such as essential hypertensives. Secondly, hypertension may develop during pregnancy caused by another medical condition (e.g. phaeochromocytoma). Thirdly, there are women who are normotensive prior to pregnancy, who develop hypertension during pregnancy which subsequently remits within a few months of delivery. These woman have pregnancy induced hypertension (PIH). The most severe form of PIH is pre-eclampsia which occurs in 4-5% of pregnancies in the UK.

The internationally accepted definition of pre-eclampsia is a diastolic blood pressure of >90mm Hg after 20 weeks of pregnancy and proteinuria of >0.3g/24 hours (Davey & MacGillivray, 1988). Other associated findings include oedema, headache, hyperflexia, epigastric pain, fetal distress, placental abruption and increased uric acid levels. Eclampsia, the most severe form of the disease, is characterised by seizures (Friedman et al, 1991).

While microalbuminuria, measured by the albumin/creatinine ratio, may be a practical method to detect early renal involvement in hypertensive pregnancies, it is dependent on posture, time of day and excercise therefore standardisation of collection is important (Thong et al, 1991). A 24 hour urine collection remains the best method of monitoring proteinuria in pregnancy (Lindow & Davey, 1992).

A lack of knowledge regarding the aetiology of the disease contributes to the difficulties in treatment. Treatment involves the control of blood pressure with antihypertensive drugs and anti-convulsants to prevent seizures, but only delivery of the fetus and placenta will result in disease regression (Lowe & Rubin, 1992). Because delivery is often in the late second trimester, or early third, the child will be exposed to the problems

of immaturity. Studies relating the endothelial damage and dysfunction to platelet activation resulted in attempts to prevent or treat pre-eclampsia with anti-platelet therapy in the form of aspirin (Sibai et al, 1993; Loudon et al, 1989; Italian study of aspirin in pregnancy, 1993). Early small trials proved encouraging but the multicentre collaborative low dose aspirin study in pregnancy failed to support the administration of low doses of aspirin (CLASP, 1994). This is more fully discussed in section 1.11.

1.1.2 Effect on the fetus: Hypertension begins in utero

In addition to the hazards of prematurity pre-eclampsia is frequently associated with intrauterine growth restriction (IUGR). IUGR is the most common cause of late antepartum death of a normally formed fetus. Epidemiological evidence suggests that low birthweight is associated with elevated blood pressure in childhood and adult life, and involves a greater risk of mortality from cardiovascular disease (Barker et al, 1992b). Men and woman who had had a relatively low birthweight but a heavy placental weight were at the highest risk of developing hypertension. Barker et al (1992a) also concluded that reduced growth in fetal life was strongly related to high plasma concentrations of fibrinogen and factor VII which could be a persisting response to impaired liver development during a critical early period. Another study by Fall et al (1992) found that babies born 70 years ago, who were breast fed and weaned relatively late, had raised serum concentrations of low density lipoprotein cholesterol and increased death rates from ischaemic heart disease in adult life. A suggested cause of low birthweight is maternal malnutrition but Edwards et al (1993) also suggested that increased fetal exposure to maternal glucocorticoids was an alternative cause. They hypothesised that glucocorticoids, which act during critical periods of prenatal development, may exert effects or imprint responses which could persist throughout adult life. High concentrations of glucocorticoids in the fetus could affect the development of the fetal vasculature, since glucocorticoids modulate vascular tone, thus causing irreversible changes in the vascular structure.

1.1.3 Risk Factors

Pre-eclampsia is regarded as a disease of primigravidae (MacGillivray, 1958). With increasing parity underlying renal disease (or other medical condition) is more likely to be a cause of pre-eclampsia (Fisher et al, 1981). The risk of developing pre-eclampsia is also increased if the patient has a change in partner (Robillard et al, 1994). Pre-eclampsia is

linked to maternal age, there being a rise in incidence of the disease over the age of 35 (Butler and Alberman, 1969). Multiple pregnancies, hydatidiform moles, diabetes mellitus (Howey and Baird, 1982) and a positive family history all increase the risk of developing pre-eclampsia (Chesley, 1978). A late spontaneous abortion, however, significantly reduces the risk of pre-eclampsia in a subsequent pregnancy (Campbell and MacGillivray, 1985).

1.1.4 Genetics of pre-eclampsia

Women have a genetic predisposition to develop pre-eclampsia which is possibly based on a single gene inheritance (Cooper & Liston, 1979; Sutherland et al, 1981; Chesley & Cooper, 1986). However because of the wide variatons in presentation of the condition, the mode of inheritance is still uncertain. The pathogenesis of pre-eclampsia is multifactorial involving coagulation, vascular reactivity or perhaps an immunological reaction between the mother and the fetus (MacGillivray, 1983). Some of these abnormalities may be determined genetically. Ward et al (1993) described a mutation in the angiotensinogen gene which seemed to be associated with an increased tendency to pre-eclampsia whilst Arngrimsson et al (1993) found a distortion in a region close to the angiotensinogen gene. The changes in the renin-angiotensin system in pre-eclampsia include a reduction in the levels of plasma renin activity, angiotensin II and aldosterone. However there are conflicting results reported in this area depending on the population studied (Morgen et al, 1995). The incidence of pre-eclampsia in mothers, daughters, sisters and granddaughters is significantly higher than in mothers-in-law, daughters-in-law and in a control population suggesting that the condition is caused by maternal genes (Sutherland et al, 1981). Kilpatrick et al (1989) found that women who developed pre-eclampsia had an increased frequency of the gene HLA DR4 compared to normotensive pregnant woman. Susceptibility to the inheritance of this gene depended on a combination of fetal and maternal genotypes, involving a single recessive gene shared by the mother and the fetus. More research needs to be undertaken to create a clearer understanding of the genetics of pre-eclampsia.

1.2 Pathophysiological changes in pre-eclampsia

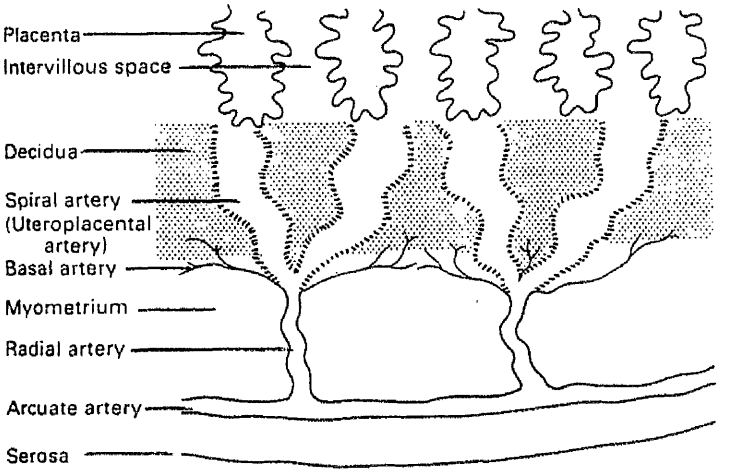
1.2.1 Uteroplacental bed and placenta

Physiological changes in the uteroplacental bed occur during normal and pre-eclamptic pregnancies. In normal pregnancy the maternal spiral arteries undergo a number of changes

as the pregnancy advances. In the first trimester cytotrophoblasts penetrate the spiral arteries in the decidua of the uterus and migrate down these vessels invading the vessel wall. The endothelium is removed by these cells as well as the elastic lamina and muscular coat of the vessel, and they are replaced by fibrinoid material. By the end of the first trimester virtually every spiral artery will have undergone these physiological changes. In the early second trimester a second invasion of cytotrophoblasts occurs which converts the vessels supplying the placenta from muscular arteries into wide mouthed vessels. By doing this the vascular supply to the fetoplacental unit is converted from a high pressure low flow system to a low pressure high flow system. In this way the placenta and thus the fetus receive all the nutrients required for growth. However in pre-eclampsia only about half of the maternal arteries undergo these physiological changes (Khong et al, 1986). Not only is the primary invasion of trophoblasts partially impaired, but also the second invasion fails to occur or is inhibited (see figure 1). Therefore there is reduced placental blood flow which becomes critical with advancing gestation as the demands of the fetus increase. Furthermore, in pre-eclampsia the vessels retain their muscular coat and are sensitive to vasomotor stimuli. There is an increased build up of fibrin, platelets and lipid laden macrophages in some vessels. This defect is called acute atherosis which can lead to partially or completely blocked arteries (Labarrere, 1988).

The narrowed spiral arteries and placental ischaemia are a result of impaired trophoblast invasion. Cytotrophoblast expression of adhesion mlecules in pre-eclampsia is abnormal. Cytotrophoblasts fail to upregulate the expression of one integrin receptor which suggests that some trophoblast cells fail to differentiate properly (Zhou et al, 1993). Damsky et al (1994) reported that later gestation cytotrophoblasts, which have greatly decreased invasive capacity, are unable to upregulate the integrin alpha 1 beta 1, suggesting that this integrin is critical for cytotrophoblast invasion. Genbacev et al (1996) investigated whether lowering oxygen tension affected cytotrophoblast invasion. They found that hypoxia affected the progression of cytotrophoblasts through the cell cycle, their invasiveness being greatly reduced partly caused by alterations in integrin cell-extracellular matrix receptor expression (markers of transitions in the differentiation process). A study by de Groot et al (1996) found that women destined to have pre-eclampsia had lower circulating concentrations of insulin like growth factor binding protein-1 (a stromal protein). This was observed 12 to 26 weeks before the onset of clinical signs of the disease thus providing biochemical evidence that abnormalities of trophoblastic invasion may affect the maternal vascular deportation of the decidual stromal protein. This is further discussed in section 1.9.

A



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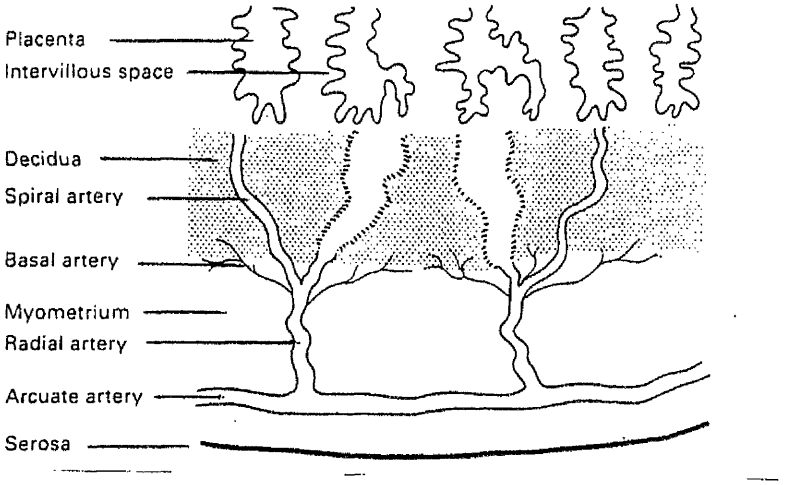


Figure 1. Cytotrophoblast invasion of the spiral arteries

A. Diagram of normal blood supply to the placenta. The radial arteries terminate into two spiral arteries. B. Diagram of abnormal blood supply to the placenta which occurs in preeclampsia. Physiological changes occur in some decidual segments of spiral arteries but are absent in others [reproduced from Khong et al (1993)]

1.2.2 Renin-angiotensin system

The renin-angiotensin system can change significantly in women with PIH. Plasma renin activity, angiotensin II and aldosterone levels are all reduced (Weir et al, 1973). However, a study measuring active renin, angiotensin converting enzyme and angiotensin II in placenta and fetal membranes by Kalenga et al (1996) showed that in moderate pre-eclampsia the activity of the renin-angiotensin system remained essentially normal. Broughton-Pipkin and Symonds (1986) suggested that the reduced perfusion of the uteroplacental bed stimulated the renin angiotensin system to increase blood pressure to improve perfusion, sometimes causing further damage. There may be a genetic link to the involvement of the renin-angiotensin system in pre-eclampsia. Inoue et al (1995) identified a genetic mutation at the site of renin cleavage in the angiotensinogen gene which significantly altered the reactions with renin and angiotensin-converting enzyme. They suggested that this mutation may predispose a carrier patient to the development of pre-eclampsia but because of the complexity of the renin-angiotensin system the physiological consequences of this mutation are not fully understood. The varying severity of disease in study groups proves to be a problem in studying the renin-angiotensin system in pre-eclampsia

1.2.3 Kidney

A number of pathological changes occur in the kidney in women with pre-eclampsia. The glomeruli enlarge and may bulge into the proximal tubule, which narrows the lumen. The changes in the glomeruli are termed glomerular endotheliosis (Spargo et al, 1959) and are found in 70% of women with pre-eclampsia in their first pregnancy. It has been noted that primary changes occur in the endothelial cells, but the mechanisms behind these changes are unclear (McCartney, 1964). The obvious clinical sign of glomerular dysfunction is proteinuria, a measure of disease severity. The increase in plasma uric acid levels precedes the presence of proteinuria and a decline in glomerular filtration rate is also indicative of glomerular damage and dysfunction (Gallery and Gyory, 1979).

1.2.4 Liver

Pre-eclampsia is the most common cause of hepatic pain and abnormal liver function tests in the pregnant patient. Hepatic lesions seen in pre-eclampsia include lake haemorrhage and areas of infarction and necrosis (Sheehan and Lynch, 1973). Haemorrhages arise in the arteries of the portal tract showing evidence of vascular damage. The hepatic lesions may be related to the activation of the coagulation system, endothelial damage and vasoconstriction. Biochemical evidence of hepatic dysfunction and damage is manifested by elevated liver enzymes. Occasionally jaundice occurs and may progress to hepatic failure producing vomiting, epigastric pain and tenderness. Weinstein (1982) derived the acronym HELLP (haemolysis, elevated liver enzymes and low platelet count) to describe 29 patients with severe pre-eclampsia all of whom had these complications. Weinstein argued that HELLP syndrome was distinct from pre-eclampsia, although was part of the disease process. These complications may all be present even when blood pressure is not grossly elevated and management of HELLP syndrome patients usually requires urgent delivery, as in other forms of severe pre-eclampsia.

1.2.5 Brain

Cerebral involvement is the most frequent cause of death in pre-eclampsia/eclampsia (Sheehan and Lynch, 1973). The pathological features include cerebral oedema, cerebral haemorrhage, thrombotic lesions and fibrinoid necrosis, all reflecting vascular damage. Computerized tomography (CT) scanning of the brain in eclamptic patients often shows cerebral oedema but Richards et al (1988) proposed that the oedema was a secondary feature ocurring after seizures.

1.3 The Endothelium

1.3.1 Normal endothelial functions

Endothelial cell injury and altered endothelial cell function plays an important role in the pathogenesis of pre-eclampsia.

The vascular endothelium forms the lining of the circulatory system. Endothelial cells exist in a continuous monolayer which lines all blood vessels and constitutes the interface between the soluble and cellular components of the blood and vascular smooth muscle. The three main functions of the endothelium, particularly relevant to our understanding of pre-

eclampsia, are to maintain the integrity of the vascular system, to control vascular tone and to prevent intravascular coagulation (Friedman et al, 1991).

The endothelium acts as a semi-permeable membrane ensuring that nutrients, hormones and waste products remain intravascular until they are used by their target organs. The endothelial cells normally secrete vasodilatory agents, such as prostacyclin and nitric oxide (NO), but in response to certain stimuli, including hypoxia and vasoactive substances, the endothelial cells secrete vasoconstricting agents thus maintaining vascular tone. The endothelium can render itself thrombogenic by secreting von Willebrand's factor (VWF), platelet activating factor and plasminogen activator inhibitor, which promote local coagulation and repair at the site of injury. These cells also produce prostacyclin and nitric oxide (NO) which inhibit the activation of platelets and neutrophils therefore preventing coagulation and vascular damage (see figure 2).

It has been suggested that problems in the understanding of pre-eclampsia are due to excessive attention being focussed on the blood pressure elevation and less attention to other pathological problems, especially endothelial cell dysfunction (Roberts et al, 1991).

1.3.2 Endothelial dysfunction in pre-eclampsia

There is considerable evidence that endothelial cell injury is present in women with pre-eclampsia. Glomerular endotheliosis and structural changes in the placental bed and uterine boundary vessels provide morphological evidence of endothelial injury (Shanklin a & Sibai, 1989, Roberts et al, 1990). At a biochemical level an increase in VWF antigen, fibronectin and a disturbance in the prostacyclin/thromboxane balance all point to an injured endothelium.

1.3.3 Biochemical Changes

Fibronectin and VWF antigen are two factors known to be released from injured endothelial cells and are found to be increased in blood from pre-eclamptic women (Lockwood & Peters, 1990; Fournie et al, 1981). Roberts et al (1989) suggested that VWF, which mediates platelet adhesion to the endothelium, was a major marker of vascular endothelial cell damage and a change in maternal VWF might be related to the release of a factor or factors toxic to the maternal endothelium, secondary to reduced trophoblastic perfusion. Serum from pre-eclamptic women was thought to contain a factor(s) which was cytotoxic to endothelial cells *in vitro* (Rodgers et al, 1988). Early studies suggested that

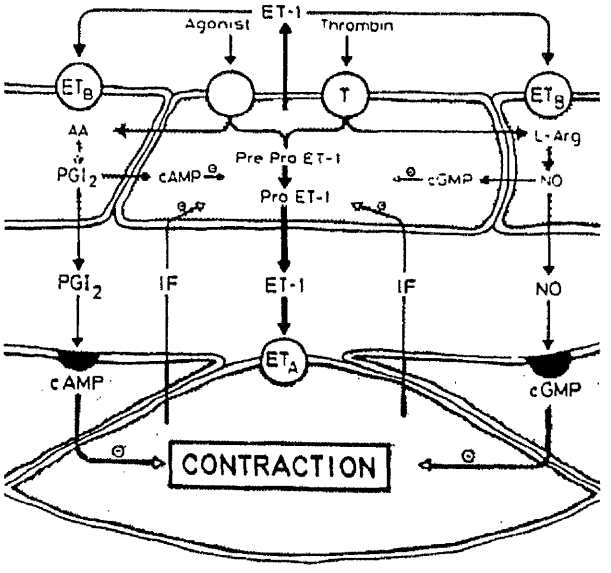


Figure 2. Schematic diagram showing the putative interaction between endothelin-1 (ET-1), endothelium derived nitric oxide (NO) and prostacyclin (PGI_2) within the endothelium and vascular smooth muscle.

Production of ET-1 is stimulated by thrombin and other receptor-operated agonists (O). Released ET-1 can activate receptors on vascular smooth muscle mediating contraction (ET_A-receptors) and on endothelial cells releasing NO and PGI_2 (ET_B-receptors). Increases in cyclic GMP(cGMP) and cyclic AMP (cAMP) in endothelial cells evoked by the latter inhibit the production of ET-1. Also, vascular smooth muscle cells produce a putative inhibitory factor (IF) that reduces ET-1 production. At the level of vascular smooth muscle, both NO and PGI_2 blunt or prevent ET-1 induced contraction (reproduced from Luscher et al, 1993).

poorly perfused placental tissue released a factor(s) into the circulation which injured endothelial cells and set in motion a variety of problems including coagulation, vasoconstriction and intravascular fluid redistribution (Roberts et al, 1989). However, recent studies have shown that sera from pre-eclamptic women are not cytotoxic to endothelial cells. Zammit et al (1996) found that serum from pre-eclamptic women stimulated less endothelin production than serum from normal pregnant women but did not alter prostacyclin production and was not cytotoxic to endothelial cells. In a study by Endresen et al (1995) endothelial cells incubated with sera from pre-eclamptic women did not affect DNA or protein synthesis of the endothelial cells. Baker et al (1996) hypothesised that in previous studies, which suggested that a factor in the maternal circulation caused activation of vascular endothelial cells, a static tissue culture environment was used. They showed that shear stress (a closer reflection of what was occurring *in vivo*) altered the effect of plasma on endothelial cells, showing no differences in the production rates of NO or prostacyclin in cells exposed to pre-eclamptic or normal plasma. The factor responsible for the increase in blood pressure appears to differ between women with pre-eclampsia and those with other hypertensive disorders of pregnancy but this factor has not been identified (Tsukimori et al, 1992).

When endothelial cells are damaged and the release of prostacyclin and NO is decreased, endothelin and endothelium derived constricting factors are released (Zeeman et al, 1992). Dekker et al (1991) studied endothelin levels in plasma from normotensive and pre-eclamptic women and suggested that the increased levels of endothelin in severe pre-eclampsia were a consequence of extensive endothelial damage. Significantly increased levels of endothelin in plasma samples from women with pre-eclampsia were also found by Kraayenbrink et al (1993). However, Wang et al (1994a) found reduced urinary endothelin values in pre-eclamptic pregnancies, which may reflect impaired renal endothelin production, whilst Cervar et al (1996) suggested that pre-eclampsia was associated with a decreased release of endothelin-1 from trophoblastic cells *in vitro*. Gallery et al (1995) found no difference in endothelin levels in cells incubated in serum from normal and pre-eclamptic women. Wang et al (1994b) studied the production of NO and endothelin from placental villi and found no differences in the production rates between normal and pre-eclamptic placentae. Not all studies involving endothelin are consistent and further studies are required to clarify its role.

1.3.4 Oxidative Injury

Further endothelial cell injury is caused by free radicals, which contain unpaired electrons in their outermost orbitals and are therefore extremely reactive. Free radical activity (oxidative stress) increases during normal pregnancy probably due to increased cell turnover (Zeeman et al, 1992). In pre-eclamptic patients the plasma concentrations of free radical oxidation products are higher even before the onset of symptoms. This shows a correlation with blood pressure. The unsaturated lipids and thiol-containing proteins in the cell membranes are susceptible to free radical attack (Wisdom et al, 1991). The unsaturated fatty acids undergo oxidation by the superoxide anion (O_2-), which is cytotoxic to the cells. Neutrophils play an important role in the host defence against microbial infection. One of these defense mechanisms is the production of active oxygens, such as superoxide and hydrogen peroxide, which occur as by products during the degradation of purines to uric acid by xanthine oxidase (Many et al, 1996a). Hypoxia-reperfusion, cytokines and increased substrate availability all increase the activity of xanthine oxidase in pre-eclampsia. Many et al (1996b) suggested that xanthine oxidase may increase the production of uric acid and contribute to the hyperuricemia and oxidative stress of pre-eclampsia. Placental production of lipid peroxides is abnormally increased in pre-eclampsia. The reason for this is not known but Wang and Walsh (1996a) hypothesised that if placental antioxidant enzymes were deficient, lipid peroxides would increase unchecked. They found significantly lower levels of superoxide dismutase and glutathione peroxidase in placental tissue from pre-eclamptics compared to normal pregnant women and speculated that the decreased antioxidant activity could result in increased lipid peroxide levels in pre-eclamptic placentae. Since the placenta secretes lipid peroxides, this could be a source of increased lipid peroxides in the circulation of women with pre-eclampsia (Walsh et al, 1996). Superoxide anions have been shown to inactivate NO and to reduce the production of prostacyclin, thus influencing vascular tone (Tsukimori et al, 1993). Also high concentrations of reactive oxygen species reorientates the arachidonic acid pathway in the cell from the production of prostacyclin towards thromboxane A_2, thus stimulating platelet aggregation and vasoconstriction, causing endothelial cell dysfunction.

1.3.5 Prostacyclin and Thromboxane

The eicosanoid system plays an important role in the mechanisms involved in the development of pre-eclampsia. Prostacyclin (a potent vasodilator, inhibitor of platelet aggregation and stimulator of renin secretion by the endothelium) and thromboxane (a

potent vasoconstrictor and platelet aggregating agent) influence the tone of placental and umbilical vessels *in vitro* (Chaudhuri et al, 1991).There is an increase in thromboxane and a decrease in prostacyclin production in the placenta in pre-eclampsia (Walsh, 1985). In normal pregnancy the placenta produces approximately equal amounts of prostacyclin and thromboxane whilst the pre-eclamptic placenta produces over seven times as much thromboxane as prostacyclin. The resulting imbalance between prostacyclin and thromboxane in pre-eclampsia contributes to the enhanced platelet activity and vascular damage. Factors in the sera of pre-eclamptic women cytotoxic to human endothelial cells could be responsible for the decreased prostacyclin production (Wang et al, 1992). Evidence suggests that elevated blood levels of lipid peroxidation products which disrupt membrane lipids and other cell components cause endothelial injury. Vascular contact with circulating peroxidating products directly cause dysfunction of the vascular endothelium by promoting the peroxidative damage of the endothelial cell membranes and selective inhibition of prostacyclin synthetase (Hubel et al, 1989). On the fetal side the production of prostacyclin from cord vessels is reduced in pre-eclampsia (Downing et al, 1980; Walsh et al, 1985).

1.3.6 Lipids

Normal human pregnancy results in a phenomenal physiological hyperlipidemia which involves a gestational rise in blood triglycerides and cholesterol (Potter and Westel, 1979). Women with pre-eclampsia display additional alterations in blood lipids which reflect on a disorder of lipid and lipoprotein metabolism (Kaaja et al, 1995). Lipid peroxides are increased in pre-eclampsia (Uotila et al, 1993) which could induce hepatic dysfunction, a frequent occurrence in pre-eclampsia. The lipid metabolism of women who develop pre-eclampsia seems to be altered 10 to 20 weeks before the clinical onset of the disease. There are increased concentrations of circulating free fatty acids, increased concentrations of triglycerides, and also alterations in the fatty acid composition of circulating lipids (Lorentzen et al, 1994, 1995). Van den Elzen et al (1996) found a positive association between first trimester serum total cholesterol level and the chance of developing pre-eclampsia. Hubel et al (1996) found that serum triglycerides and free fatty acids were increased in pre-eclampsia, and this correlated with the lipid peroxidation metabolite malondialdehyde. Endresen et al (1994) found that the free fatty acid, linoleic acid, impeded the ability of endothelial cells to produce prostacyclin and cGMP, and to inhibit

platelet aggregation. These findings suggest that dysfunction in lipid and lipoprotein metabolism may contribute to endothelial cell dysfunction in pre-eclampsia.

1.3.7 Nitric Oxide

Endothelial cells modulate vascular tone. Acetyl choline acts on receptors on endothelial cells which release NO. This in turn stimulates guanylate cyclase in vascular smooth muscle to produce cyclic guanosine monophosphate which causes relaxation (Myatt et al, 1991a). NO is a noxious stable, highly reactive free radical gas which was found to be a major messenger molecule that regulated immune function and blood vessel dilation. NO also serves as a neurotransmitter in the brain and peripheral nervous system (Lowenstein and Snyder, 1992). The NO system is widespread and is important in signal transduction and in immunological reactions (Chin et al, 1992). NO is labile, therefore direct measurements are difficult, so nitrates and/or nitric oxide synthase (NOS) are measured in the assessment of NO synthesis. L-arginine is converted to L-citrulline and NO by the enzyme NOS (Palmer and Moncada, 1989). NOS enzymes have been characterized and there may be up to six different isoforms (Pollock et al, 1991). Constitutive calcium-dependent isoforms of NOS are found in endothelial cells and brain and an inducible calcium-independent form is seen in macrophages, neutrophils and also in the endothelium (Knowles & Moncada, 1994). In blood vessels NOS is localised to the endothelial layers in large vessels. There is very little or no NOS in small arterioles and capillaries.

The role of NO in pregnancy has been widely assessed. NOS can produce superoxide as well as NO (Lowenstein and Snyder, 1992). This is significant in that NO is rapidly inactivated by superoxide anions and therefore a decrease in NO release in pre-eclampsia may be caused by an increase in oxygen free radicals (Zeeman et al, 1992). A major role of NO is to prevent the adhesion of platelets to endothelial cells and to prevent platelet aggregation. NO may help to prevent thrombosis formation in the fetus (Varela et al, 1992). In pregnancy, NO is generated in the human fetoplacental circulation contributing to the control of vascular tone and attenuating the actions of vasoconstrictors (Myatt et al, 1991b). A calcium dependent endothelial isoform of NOS has been characterised in the human villous vascular tree (Myatt et al, 1993). Immunohistochemical techniques have shown that endothelial NOS is localised to the endothelium of umbilical arteries and veins, chorionic arteries and veins and to the syncytiotrophoblast but not to the underlying cytotrophoblast and small fetal blood vessels (Buttery et al, 1994). Yallampalli and Garfield (1993) hypothesised that impaired vascular NO synthesis during pregnancy may be an important causal factor in pre-eclampsia. They infused an inhibitor of NOS, L-nitro-arginine methyl

ester (L-NAME), into pregnant rats which caused hypertension and fetal growth restriction, without affecting gestation and concluded that an alteration in nitric oxide synthesis may be one of the factors responsible for pre-eclampsia. Ghabour et al (1995) found endothelial NOS staining in terminal villous vessels in pregnancies complicated by pre-eclampsia and IUGR but not in the terminal villous vessels from normal pregnancies. This contrasts with Morris et al (1995) who found lower NOS activities in villous tissue homogenates from pregnancies complicated by pre-eclampsia and growth restriction and who suggested that a reduction in NOS activity had a role in generating the high resistance fetoplacental circulation. Perfused umbilical vessels have a reduced ability to release NO in response to bradykinin in PIH (Pinto et al, 1991). This was in contrast to a study by McCarthy et al (1993) who found that the NO mediated component of endothelium-dependent relaxation was unaffected. However there was impairment of endothelium-dependent relaxation in arteries of women with pre-eclampsia compared to normotensive women. The reduced production of endothelial derived vasodilators may account for the increase in vascular resistance in pre-eclampsia. Cameron et al (1993) measured urinary levels of nitrites and nitrates (oxidation products of NO) in urine of normal pregnant and hypertensive pregnant women showing no significant difference between the two groups. We have recently shown that total nitrites are increased in the fetoplacental circulation but not in the maternal circulation in pre-eclampsia (Lyall et al, 1995b) and nitrites are also increased in the fetoplacental circulation in pregnancies complicated by IUGR (Lyall et al, 1996). Nobunga et al (1996) found increased levels of nitrate/nitrite in maternal plasma in pre-eclamptics compared to normotensive patients. However Davidge et al (1996) found that urine concentrations of nitrates and nitrites were significantly lower in pre-eclamptic patients compared to normal pregnant patients. They suggested that both plasma and urine nitrate and nitrite levels should be measured to address the possible impact of alterations in renal clearance of plasma NO metabolites from women with pre-eclampsia compared to normal pregnant women. Discrepancies amongst studies may be due in part to this reason, but also to inconsistency in patient populations and disease definitions. Endothelial NOS expression and NO production were increased in endothelial cells exposed to plasma from women with pre-eclampsia (Davidge et al, 1995; Baker et al, 1995). Sooranna et al (1996) suggested that expression of NOS was not different in placentae obtained from normal and pre-eclamptic women whilst Rutherford et al (1995) found increased NOS activity in first trimester placentae and pre-eclamptic and IUGR placentae but decreased NOS activity in the umbilical artery in pre-eclamptic and IUGR complicated pregnancies compared to normal pregnancy. They suggested that this reduction in NOS activity was indicative of endothelial dysfunction. The mechanism that gives rise to this increase in NO remains to be established

but it may be a compensatory mechanism related to defective placentation. Myatt et al (1997a) found an increase in endothelial NOS staining in placentae from patients with pre-eclampsia compared to normal pregnant patients and suggested that this increase in NO production may be due to a response to the increased resistance and poor perfusion in these pathological pregnancies. Myatt et al (1997b) found no difference in the staining of inducible NOS between patients with pre-eclampsia and control pregnant patients. N^G,N^G dimethylarginine (ADMA), an arginine analogue, inhibits NO synthesis and is found in significantly higher concentrations in patients with pre-eclampsia (Fickling et al, 1993). Ramsay et al (1994) studied the effect of intravenous glyceryl trinitrate (GTN), a donor of NO *in vivo,* on uterine artery blood flow in pregnancy and found that there was a decrease in resistance which could be beneficial to women with pre-eclampsia. Inconsistency between studies proves to be a problem in elucidating the role of NO in pre-eclampsia.

1.4 Coagulation System

When vascular damage occurs in pre-eclampsia fibrin is widely deposited, for example in acute atherosis or glomerular endotheliosis, suggesting that the coagulation system is activated (Davies and Prentice, 1992). Rather than being a primary component of the pre-eclamptic process the activation of the coagulation system is more likely to be a secondary event. Douglas et al (1982) found that women with severe pre-eclampsia had elevated levels of fibrinopeptide A, which is a sensitive indicator of coagulation activation and a marker of thrombin generation. In pre-eclampsia there is an increase in factor VIII coagulant activity (Howie et al 1971, 1976) and also an increase in VWF (Redman et al, 1977b) the latter reflecting endothelial damage by mediating platelet adhesion to the endothelium (Roberts et al, 1989). The fibrinolytic system is activated along with the activation of the coagulation cascade in pre-eclampsia. An increase in concentrations of fibrinogen-fibrin degradation products and fibrinogen-fibrin complexes as well as an increase in the fibrinopeptide Bβ 1-42 levels all provide evidence of fibrinolytic activation in severe pre-eclampsia. This can occasionally progress to full blown disseminated intravascular coagulation (DIC) in severe disease. In mild pre-eclampsia these changes are less striking but still occur suggesting that a similar process is occurring but perhaps to a lesser extent (Howie et al, 1971).` Borok et al (1984) suggested that fibrinolysis was more pronounced than fibrin formation in patients with severe pre-eclampsia. An increase in placental plasminogen activator inhibitor in severe pre-eclampsia suggests vascular damage which leads to thrombosis in the vessels in the placental bed (Estelles et al, 1987).

1.5 Platelets

Platelets play a crucial role in the pathophysiology of pre-eclampsia by promoting vascular damage and obstruction which leads to tissue ischaemia and further damage (Greer, 1992).

Normally thromboxane A_2 and prostacyclin function as local hormones and are important in the control of the platelet-endothelial interaction. They act against each other through the regulation of platelet adenylate cyclase, a controller of cAMP production and therefore platelet free calcium concentrations. Thromboxane A_2 inhibits adenylate cyclase, which allows free intracellular calcium to rise. Meanwhile prostacyclin stimulates adenylate cyclase increasing cAMP which reduces free intracellular calcium and inhibits the activation of platelets.

In pre-eclampsia increased platelet reactivity may contribute to the obstruction of the uteroplacental microcirculation by platelet aggregation and by the release of vasoactive substances such as thromboxane A_2. In pre-eclampsia the circulating platelet count is reduced (Redman et al, 1978) which reflects a reduced platelet life span, perhaps caused by a dysfunctional endothelial surface which induces an increased local consumption of platelets (Rakoczi et al, 1979). The reduced platelet count in women who develop pre-eclampsia suggests that increased platelet consumption is an early feature of the disorder. An inverse relationship between the platelet count and fibrinogen-fibrin degradation suggests that the reduction in platelet count is due to increased platelet consumption which is associated with low grade DIC (Howie et al, 1971). In pre-eclampsia there is also an increase in the platelet specific protein β-thromboglobulin, a marker of platelet activation *in vivo* (Redman et al, 1977a; Socol et al, 1985). This correlates with proteinuria and serum creatinine and suggests a link between platelet activation with renal microvascular damage. Zemel et al (1990) measured the intracellular free calcium concentration in maternal platelets and detected a change that preceded the clinical manifestations of pre-eclampsia as early as the end of the first trimester. They showed that the increased sensitivity of platelets to arginine vasopressin may be a useful indicator of the subsequent development of pre-eclampsia.

The platelet content of 5-hydroxytryptamine (5-HT) is reduced in pre-eclampsia. This is a vasoactive amine which is released when platelets aggregate and its loss indicates aggregation of platelets and stimulation of the platelet release reaction *in vivo*. Howie et al (1977) suggested that the platelets become activated in the microcirculation and release their products such as β thromboglobulin and 5-hydroxytryptamine. The platelets then re-

enter the circulation but are exhausted and cannot respond normally to aggregating agents. Platelet reactivity is increased in women with PIH (Greer et al, 1988) but is reduced in women with pre-eclampsia, the latter perhaps due to platelet exhaustion (Loudon et al, 1991). However, there are conflicting results between studies suggesting that platelet reactivity varies depending on the severity of the disease or the stage of the disease.

The changes in the coagulation system and in platelet function supports the concept that low grade DIC occurs in pre-eclampsia. Howie et al. (1976) described a coagulation 'index' of serum fibrin-fibrinogen degradation products, platelet count and plasma factor VIII which correlates with an index of severity of the disease, from mild to severe pre-eclampsia, therefore showing an association of the two conditions. All cases of perinatal death associated with pre-eclampsia had coagulation indices in the most severely abnormal range. These indices reflect the severity and progress of the disease.

1.6 Neutrophil Activation

When inflammatory mediators such as cytokines or thrombin are released neutrophils become activated and roll along the endothelium eventually flattening against the vessel wall. After diapedesis the activated neutrophil releases a variety of substances all mediating vascular damage. These include elastase, which destroys the physical integrity of the endothelial cell and inactivates NOS and cyclooxygenase, so reducing NO and prostacyclin production. Free radicals are also released which produce membrane lipid peroxides, causing lysis of the endothelial cells and increased vascular permeability and reactivity (Harlan, 1987). Leukotrienes are synthesized and released which also increases vascular permeability and promote further neutrophil activation and adherence (Bray, 1983).

Neutrophil elastase, free radicals and leukotrienes may therefore be a mediator of vascular damage and could contribute to the vascular lesion seen in pre-eclampsia. Neutrophil elastase substrates include elastin, collagen, fibrinogen complement and proteoglycans. It can therefore cause substantial tissue damage (Janoff, 1985). Greer et al (1989) found elevated levels of neutrophil elastase in pre-eclampsia but this was confined to the maternal circulation. However neutrophil activation also occurs in the fetal circulation during uncomplicated vaginal delivery. The mechanism behind this is so far unknown but may be due to a disturbance in the placental endothelium during labour (Greer et al, 1991b). Elastase positive neutrophils are found in increased numbers in the decidua of the placental bed (the site where the characteristic vascular lesion acute atherosis is seen) of women with pre-eclampsia (Butterworth et al, 1991). Pre-eclampsia is also

associated with the presence of a neutrophil activator which enhances superoxide production. These mediators include peptide factors or other as yet unidentified metabolic products generated by the pre-eclamptic state (Tsukimori et al, 1993).

The activation of neutrophils in pre-eclampsia is likely to be a secondary phenomenon possibly triggered by immunological mechanisms which are implicated in the aetiology of pre-eclampsia (Stirrat, 1986). Neutrophils also interact with the platelet, coagulation and complement systems therefore indirectly causing vascular damage. There is a significant correlation between plasma neutrophil elastase and VWF which suggests that neutrophil activity may be responsible for the endothelial damage seen in pre-eclampsia (Greer, 1991a). The mechanism of neutrophil activation is unknown but we previously reported that circulating concentrations of cell adhesion molecules and certain cytokines were increased in the serum from pre-eclamptic women (see sections 3.1.2 and 3.3.2). The increased expression of these molecules on the endothelium may represent one of the mechanisms by which neutrophils are attracted to the endothelium where they contribute to endothelial dysfunction (Lyall et al, 1994; Greer et al, 1994).

1.7 Cell Adhesion Molecules

1.7.1 The role of cell adhesion molecules

Before neutrophils penetrate the endothelium and cause vascular damage they are selectively recruited to the endothelium, adhere to it and then move over the luminal surface of the endothelial cells. The attachment and extravasation of leukocytes is controlled by the expression of cell surface adhesion molecules on both the circulating cells and the vascular endothelium (Harlan and Liu, 1992). Increased expression of adhesion molecules by the endothelium could be responsible for the neutrophil activation which occurs in pre-eclampsia.

Cell adhesion molecules play a major role in maintaining the integrity of tissues and in controlling aggregation of leukocytes and platelets (Harlan and Liu, 1992). The endothelium controls the movement of molecules and cells between the blood and sites of injury. Circulating leukocytes (neutrophils, lymphocytes and monocytes) must become attached to a surface before they can migrate chemotactically. This is a crucial early step in mounting an effective inflammatory or immune response. The three general mechanisms of adhesion are the rapid transient upregulation of leukocyte adhesion molecules, the upregulation of endothelial adhesion molecules and more persistent cytokine activation of

endothelial cells, leading to the synthesis of new adhesion molecules (Harlan and Liu, 1992). Endothelial cells lining the vascular endothelium possess leukocyte specific adhesion molecules constitutively and in response to a wide range of inflammatory mediators. The circulating leukocyte displays receptors for these endothelial cell adhesion molecules. The interaction of the cell adhesion molecules and their ligand fixes and flattens the leucocyte to the endothelium. The leukocyte then passes between the endothelial cell junctions and penetrates the basement membrane to the tissues. Normal regulation of endothelial cell adhesion molecules allows the endothelium platelets and neutrophils to promote coagulation and repair at the sites of injury. Therefore an increase in cell adhesion molecules provides an indication of inflammation/immune activity and/or endothelial damage or activation. Each endothelial cell adhesion molecule recognises a subset of leucocytes and is expressed according to an individualised time course in response to a subset of inflammatory mediators, or cytokines, such as interleukin-1(IL-1), interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα).

1.7.2 Cell adhesion molecule families

The three families of adhesion molecules relevant to our area of research are the selectins, the immunoglobulin superfamily (IgSF) and the integrins. The selectins are involved in the earliest phases of a cascade of events leading to leukocyte extravasation. In regions of inflammation leukocytes move towards the edge of the capillary and begin to roll along the endothelium. P-Selectin (GMP-140) initiates the rolling of cells along the vessel wall, (Hogg et al, 1992), and this process is continued by E-Selectin (endothelial leukocyte adhesion molecule-1 or ELAM-1). The role of a third selectin, L-selectin, in leukocyte migration is, as yet, unclear. Each selectin may recognise not one but a number of carbohydrate ligands. Members of the IgSF function at different times during an inflammatory response. They include intercellular adhesion molecules-1 and -2 (ICAM-1 and ICAM-2), the lymphocyte binding vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1(PECAM-1) or CD31. Members of the IgSF originally identified as adhesion molecules have diverse functions and in some cases the adhesion role is of secondary importance (Pigott and Power, 1993). Integrins are membrane glycoproteins expressed on diverse cell types. The activation of the leukocyte integrin receptors occurs after the downregulation of the selectin interactions. Integrin activation is triggered by molecules which are induced rapidly on the activated endothelium, e.g. platelet activating factor. Integrins bind to a wide variety of extracellular matrix proteins

(fibronectin, fibrinogen, laminin, collagen and VWF) and to members of the IgSF. Most integrins bind to more than one ligand (Pigott and Power, 1993).

The cell adhesion molecules of interest in our studies were E-Selectin (ELAM-1), ICAM-1, ICAM-2, VCAM-2 and PECAM-1(CD31).

1.7.3 E-Selectin

E-Selectin is a cell surface glycoprotein, with a molecular weight of 95-115kDa. It is expressed by the cytokine activated endothelium and is involved in mediating the adhesion of blood neutrophils (Bevilacqua et al, 1989). Initial binding of neutrophils by E-Selectin is thought to trigger recruitment and activation of additional adhesion molecules to the site of inflammation (Pigott and Power, 1993). E-Selectin appears on the endothelium approximately 2 hours after exposure to the cytokines interleukin-1 (IL-1) and tumour necrosis factor α (TNFα). It is induced more than 100-fold on human umbilical vein endothelial cells (HUVECS) by IL-1 or TNFα (Osborn, 1990). The ligands of E-Selectin are sialyl Lewis and related fucosylated N-acetyl lactosamines found on leukocyte glycolipids and glycoproteins (Pigott and Power, 1993).

1.7.4 ICAM-1 and ICAM-2

ICAM-1 and ICAM-2 on endothelial cells recognise their leukocyte ligand which enables the adhesion and migration of these cells out of the bloodstream. ICAM-1 contributes to both neutrophil and leukocyte adhesion *in vitro*. It is involved in a variety of other cell to cell interactions including lymphocyte aggregation and lymphocyte-fibroblast adhesion. ICAM-1 is a ligand for lymphocyte function associated antigen (LFA-1). High levels of ICAM-2 present on resting endothelial cells suggests that it is the major ligand for LFA-1. LFA-1 binds to both ICAM-1, which is inducible on the endothelium, and ICAM-2, which is present constitutively (Staunton et al, 1989). ICAM-2 is not upregulated by cytokine stimulation. LFA-1 plays a key role in mediating leukocyte adhesion to the endothelium during inflammatory responses through binding to ICAM-1. ICAM-1 and ICAM-2 account for all LFA-1 dependent adhesion to the either resting or activated endothelium. ICAM-1 is important in adhesion and migration and is the major ligand for CD11/CD18. A CD11/CD18 dependent mechanism is necessary for neutrophil recruitment in response to infection. It is not clear what the roles of ICAM-1 and ICAM-2 are in neutrophil recruitment. However, ICAM-1 mediates adhesion of T-lymphocyte cells with antigen presenting cells or target cells and is involved in T-cell/T-cell and T-cell/B-cell

interactions. ICAM-2 provides a co-stimulatory signal for T-cell activation and therefore may be important where the antigen presenting cells express little or no ICAM-1. ICAM-1 is expressed basally on a variety of cell types including endothelial cells and monocytes but its expression is upregulated in response to specific stimuli such as cytokines. ICAM-1 is induced more than 30-fold on cultured HUVECS by interferon (IFN), IL-1 or TNFα. ICAM-1 is homologous to the neural cell adhesion molecule (NCAM) (Simmons et al, 1988).

1.7.5 VCAM-1

Another member of the IgSF VCAM-1 is expressed on the luminal surface of cytokine-activated endothelium and binds lymphocytes, monocytes and eosinophils to activated endothelium using the integrin VLA-4 (very late antigen-4)(Hogg, 1992; Elices et al, 1990). VCAM-1 expression on endothelial cells is induced by interleukin-1β (IL-1β), interleukin-4 (IL-4), TNFα and IFNγ (Masinovsky et al, 1990; Thornhill and Haskard, 1990). VCAM-1 is present on the cell surface by 3 hours after cytokine stimulation and remains for at least 72 hours in the presence of cytokine. VCAM-1 has 7 immunoglobulin domains whilst an alternatively spliced form of VCAM-1 lacks domain 4 of the 7 immunoglobulin domains (Hession et al, 1991) We have shown that serum concentrations of VCAM-1 and E-Selectin are elevated in pre-eclampsia (Lyall et al, 1994), which may reflect increased expression of these molecules on the endothelium, perhaps also reflecting on the contribution of these molecules to the endothelial dysfunction seen in pre-eclampsia.

1.7.6 PECAM-1 (CD31)

PECAM-1 is expressed by trophoblasts and may act as a mediator at a molecular level of the adhesive interaction between trophoblasts and the endothelium. PECAM-1 is strongly expressed on T-cells adhering to endothelial cells and is constitutively expressed on endothelial cells. The precise role of PECAM-1 is not clear but it may play a role in adhesive events during thrombosis or wound healing and it has been implicated in the formation of cell-cell junctions within the placenta (Leach et al, 1993).

1.8 Cytokines

Cytokines are regulatory molecules which act at short distances. There are numerous cytokines which can upregulate and downregulate adhesion in a very complex system. Cytokines may play a role in endothelial activation and may be responsible for many features of endothelial dysfunction. It is thought that cytokines such as TNFα, interleukin (IL)-6, IL-8, IL-1β, IL-1 receptor antagonist (IL-1ra), a naturally occurring inhibitor of IL-1, and granulocyte macrophage colony stimulating factor (GM-CSF) can trigger neutrophil activation, the expression of VWF and cell adhesion molecules on the endothelium, all resulting in endothelial damage. The two forms of IL-1, IL-α and IL-β, are both important mediators of the inflammatory and immune responses. They are produced primarily by mononuclear phagocytes (Dinarello, 1991a). IL-1 also acts on endothelial cells perhaps contributing to the development of atherosclerotic lesions, which are seen in pre-eclampsia when acute atherosis occurs in the spiral arteries of the placental bed. The naturally occurring inhibitor of IL-1 is IL-ra which is produced by blood monocytes and tissue macrophages and plays an important role in many disease states (e.g. arthritis, diabetes and osteoporosis) [Dinarello, 1993]. IL-1β is involved in neutrophil adherance. IL-6 plays an major role in the mediation of inflammatory and immune responses and is produced by activated endothelial cells, monocytes and macrophages (Wong et al, 1988). We have shown recently that the concentration of the cytokine IL-6 is increased in the maternal circulation in pre-eclampsia (Greer et al, 1994). We have also shown that IL-6 increases the expression of VCAM-1 and E-Selectin on endothelial cells *in vitro* which suggests that IL-6 plays a role in increased cell adhesion molecule expression and also neutrophil activation occurring in pre-eclampsia. An increase in IL-6 and IL-ra suggests that increased cytokine concentrations may be responsible for the endothelial cell adhesion that occurs in pre-eclampsia and furthermore endothelial dysfunction (Greer et al, 1994). IL-8 is a known chemoattractant and activator of neutrophils.

TNFα, a potent proinflammatory cytokine, is involved in inflammation, endothelial cell activation, shock and death (Dinarello, 1991a). Recently it has been suggested that TNFα plays an important role in pre-eclampsia and contributes to endothelial activation. However, Meekins et al (1994) found that circulating TNFα did not contribute to the initiation of endothelial cell activation that could be associated with the development of pre-eclampsia since the rise in circulating TNFα measurements was detected in 36% of cases with established pre-eclampsia, but this occurred after the syndrome was detected clinically. We have also found no significant difference in the concentration of TNFα in the plasma of pre-eclamptics compared to controls (Greer et al, 1994). Chen et al (1996) found

that TNFα mRNA expression was significantly elevated in pre-eclampsia. The half life of TNFα is only a few minutes and therefore it is not consistently present in the circulation (Van der Poll et al, 1990). A single blood sample may therefore fail to detect TNFα. Vince et al (1995) proposed that increased concentrations of TNF-receptors may be a better clinical marker of excessive TNFα release *in vivo,* rather than measurements of TNFα (because of its short half life). Vince et al (1995) hypothesised that in pre-eclampsia one or more factors derived from the placenta stimulate monocytes and/or neutrophils to produce TNFα which in turn causes endothelial dysfunction. GM-CSF is a specific glycoprotein which stimulates the formation of neutrophil macrophage and eosinophil lineage (Metcalf, 1986).

1.9 The Placenta in Pre-eclampsia

During placentation physiological changes in the placental bed normally extend from the decidua into the inner myometrium (Pijnenberg et al, 1981). In pre-eclampsia the physiological changes are restricted to the decidual segments alone (Brosens et al, 1972). A complete absence of physiological changes throughout the entire length of some spiral arteries is seen in pre-eclampsia (Khong et al, 1986). Butterworth et al (1991) found an accumulation of elastase positive neutrophils in the decidua of pre-eclamptic placentae which may represent a secondary phenomenon after tissue ischaemia due to the inadequate maternal vascular response to placentation. It has been previously reported that neutrophil activation occurs in maternal plasma in pre-eclampsia (Greer et al, 1989). The release of neutrophil elastase could contribute to the vascular damage seen in the decidual region of the placenta. Recruitment and extravasation of neutrophils is partly mediated by the cell adhesion molecules expressed on the maternal endothelium and at the uteroplacental bed. We have recently shown that there is no difference in cell adhesion molecule expression in placentae from pregnancies complicated by pre-eclampsia and IUGR suggesting that neutrophil activation is confined to the maternal circulation as was previously reported (Greer et al, 1991a; Lyall et al, 1995a).

Macara et al (1995) demonstrated abnormalities in the development of the placental villous tree in pregnancies complicated by IUGR with and without preeclampsia (the structure of the placenta is shown in figure 3). However, there was no evidence to suggest that this abnormality was due to selective loss of small stem villous vessels but perhaps the abnormality lay within the nonmuscularised capillaries or the peripheral villi. The findings of Jackson et al (1995) were that growth restriction was associated with reduced large

vessel wall thickness but again no reduction was found in the number of vessels per stem villous, suggesting that reduced villous development may contribute to abnormal umbilical artery blood flow in some cases of intrauterine growth restriction. These results contrasted with Wilcox and Trudinger (1991) who suggested that platelet activation within the small vessels of the stem villi played a role in placental insufficiency. Preterm small-for-gestational age fetuses showing abnormal umbilical artery Doppler waveform are at a high risk of perinatal death due to chronic fetal hypoxia. Macara et al (1996) examined the ultrastructure of the IUGR placentae to determine the likely origin of fetal hypoxia. Structural differences found in the terminal villi of the IUGR group, such as reduced cytotrophoblast proliferation and stromal fibrosis, were inconsistent with the view that the placenta is hypoxic in IUGR. They found a thickening in the basal lamina and congestion of the capillaries by erythrocytes both of which would limit oxygen transfer from the intervillous space to the fetus. Burton et al (1996) suggested that placentae adapt to hypoxic conditions by dilating the capillary sinusoids as well as thinning the villous membrane. Krebs et al (1996) found that the terminal villous compartment of the placenta from preterm intrauterine growth restricted pregnancies were maldeveloped, showing elongated villi and a wrinkled trophoblast surface with the occasional covering of a fibrin plaque. These findings may account for the impaired gas and nutrient transfer in this disorder.

The role of growth factors in pregnancy is a rapidly expanding subject. Implantation and growth of the placenta requires extensive angiogenesis to establish the vascular structures involved in exchange. Vascular endothelial growth factor (VEGF) is potentially an important regulator of angiogenesis, particularly during the extensive tissue growth and remodelling that occur in utero. VEGF may also play a role in term placenta when extensive angiogenesis diminished possibly regulating vascular permeability (Sharkey et al, 1993). Clarke et al (1996b) indicated that VEGF exerted a role within the placental villi and the maternal decidua in relation to growth, differentiation and migration of trophoblast, this being mediated primarily through the regulation of the receptor flt-1 (fms-like tyrosine kinase) rather than the KDR receptor (kinase -insert domain receptor). This contrasted with the views of Vuckoviv et al (1996) who suggested that VEGF was controlled through the KDR receptor. The presence of the growth factors, platelet-derived endothelial cell growth factor (PDEGF) and VEGF, in trophoblasts in early pregnancy are indicative of an active role for trophoblasts in the development of the villous vascular network (Jackson et al, 1994; Shiraishi et al, 1996). Insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) are used for cell to cell communication between fetal trophoblasts and maternal decidual cells at the feto-maternal interface for placental development or function (Han et al, 1996). Epidermal growth factor (EGF) produces a dramatic change in morphology and

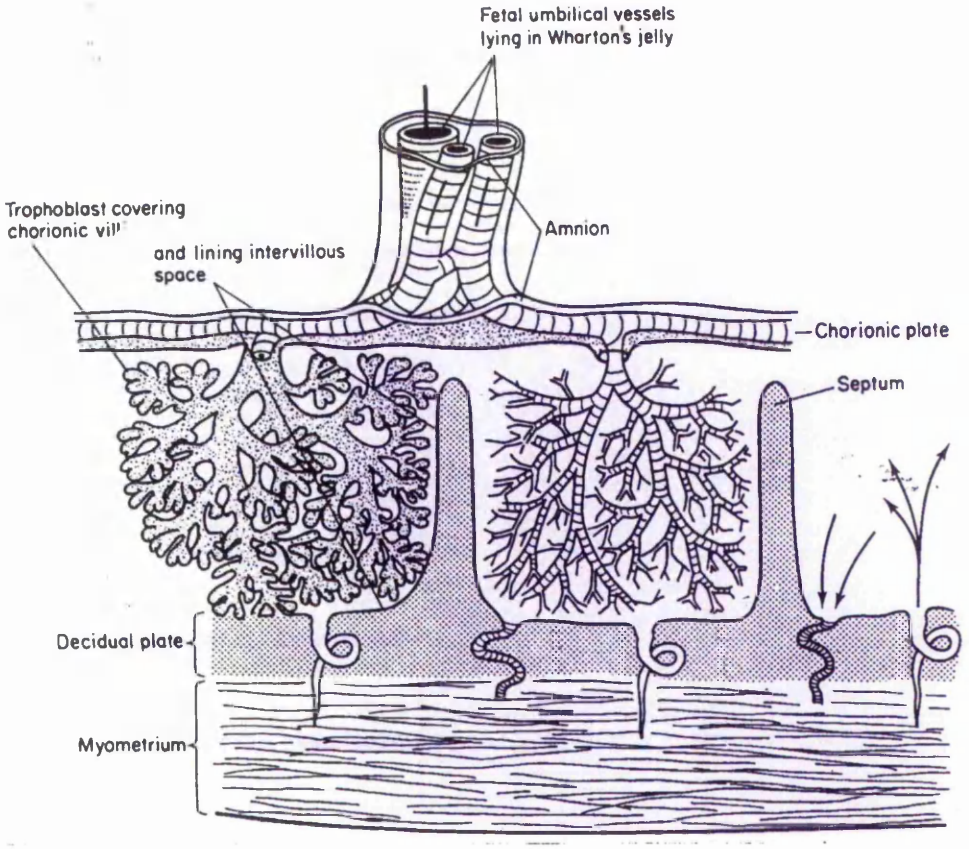


Figure 3. The structure of the placenta

Only the fetal arteries are shown in the mid section, for clarity. The villi are much more finely branched [reproduced from Obstetrics by Ten Teachers (16th edition) editor G.V.P.Chamberlain, 1995, Edward Arnold, London]

an increase in invasive capacity on first trimester cytotrophoblasts but this effect is reduced throughout gestation (Bass et al, 1994). Growth factors have an important part to play in pregnancy and further studies may help to elucidate this role.

1.10 Treatments

When considering the use of drugs in pregnancy clinicians must always be conscious that they are treating two patients. The control of the hypertension may be a useful short term treatment preventing the mother from the risks of high blood pressure such as stroke and allow the pregnancy to be prolonged for optimal timing of delivery. Such therapy may also allow for the administration of steroids to enhance fetal lung maturity. Drugs commonly used to treat hypertension in pregnancy are the adrenoceptor antagonist labetalol, atenolol, methyldopa, calcium channel blockers and hydrallazine. Generally treatment starts with methyldopa or labetalol and a vasodilator such as nifedipine (a calcium channel blocker) or hydrallazine added. There is little evidence to suggest that any drug can affect the disease process once established.

It is very difficult to identify accurately which patients are likely to have eclamptic seizures even though most women have their first seizure whilst in hospital. Anticonvulsants have been used to arrest and prevent seizures. It is likely that the risk of seizures is reduced when blood pressure is controlled. Thus the role of prophylactic anticonvulsants is unclear although they are prescribed for fulminating disease. Diazepam is used to halt seizures. It can be used to prevent further convulsions but its side effects include respiratory depression, poor feeding and hypothermia in the neonate and it impairs the level of conciousness and suppresses respiration in the mother. So diazepam is not ideal for prevention. Other anticonvulsants include chlormethiazole and phenytoin. Chlormethiazole will suppress respiration and has to be carefully titrated against the patients concentration levels. It requires to be given in large volumes of fluid and may cause or contribute to fluid overload. Phenytoin has recently become popular being given intravenously. In the United States, magnesium sulphate is widely used as an anticonvulsant, probably working by reversing vasoconstriction (Belfort et al, 1992). A recent international multicentre randomized trial comparing standard anticonvulsants found that women allocated magnesium sulphate had a 52% lower risk of recurrent convulsions than those allocated diazepam, a 67% lower risk of recurrent convulsions than those allocated phenytoin, and women were less likely to require ventilation, to develop

pneumonia and to be admitted to intensive care facilities than those allocated phenytoin. Babies of women allocated magnesium sulphate were less likely to need intubation or to be admitted to a special care nursery. This provides compelling evidence in favour of the use of magnesium sulphate for the treatment of eclampsia (Duley et al, 1995).

1.11 Anti-platelet Therapy in Prevention of Pre-eclampsia

Pre-eclampsia is caused partly by endothelial dysfunction and is associated with platelet disturbance. Therefore, anti-platelet therapy might have a beneficial action in preventing the progression of the disease. Aspirin is the most effective agent available for clinical use in anti-platelet therapy. Aspirin irreversibly inhibits platelet cyclooxygenase activity, so blocking the synthesis of thromboxane. Small scale studies suggest that aspirin may be effective in the treatment of pre-eclampsia and IUGR. However, one problem is identifying the patients who will require such therapy, especially women with pre-eclampsia where those with severe disease are mainly primigravidae. The biggest potential concern regarding the use of aspirin in pregnancy was the problems caused if aspirin were to reach the fetus where it might impair haemostasis or lead to the closure of the ductus arteriosis. Jacobson et al (1991) studied the transfer of aspirin in the perfused human placental cotyledon but found no effect on the maternal or fetal circulation. A number of studies have shown that low doses of aspirin have a selective effect on maternal platelet function, sparing fetal platelet function and prostacyclin production and having no obvious effect on the ductus arteriosis (Ritter et al, 1987; Loudon et al, 1989). However Sibai et al (1991) found that patients on low-dose aspirin therapy had an increased risk of abruptio placentae which is a matter of concern although this was not found in the larger CLASP study.

There have been a number of studies examining the use of low-dose aspirin in the prevention of pre-eclampsia. Beaufils et al (1985) found a significant reduction in the incidence of pre-eclampsia and improved perinatal outcome. The incidence of growth restriction and fetal loss was also significantly reduced. However this study used small numbers of patients and the prognosis of the groups were not well matched. Another study by Wallenberg et al (1986) found a significant reduction in the development of hypertensive complications in women treated with low-dose aspirin. There was also a lower incidence of growth retardation but this was not significant. Many other small scale studies have been carried out but it is questionable whether these studies were large enough to assess accurately the benefits of aspirin in pre-eclampsia. The Collaborative Low-dose Aspirin Study in Pregnancy (CLASP, 1994), a much larger study, looked at the effects of

60mg of aspirin or a matching placebo in 9364 women. Aspirin reduced the incidence of proteinuric pre-eclampsia by only 12%, which was not significant. There were no significant effects on the incidence of IUGR or of stillbirth and neonatal death. The Italian study of aspirin in pregnancy (1993) recruited over one thousand women at risk of pre-eclampsia and found no differences in the outcomes measured. Sibai et al (1991) administered aspirin to over three thousand women and although there was a 26% decrease in the incidence of pre-eclampsia this was not significant.

In conclusion, it appears that aspirin should not be used routinely in the prevention of pre-eclampsia. It may be have a place in high risk groups such as patients with anticardiolipin antibodies. Identification of patients, especially primigravidae, is a major problem and until objective techniques to identify these patients are available then antiplatelet therapy should not be routinely administered. The failure of antiplatelet therapy is likely to reflect the complex disease process of pre-eclampsia. Rational treatment of pre-eclampsia will not be achieved until the pathogenesis of the disorder is fully understood.

1.12 Conclusions

Pre-eclampsia is a multisystem disorder which affects virtually all maternal organ systems and is manifested clinically as hypertension and proteinuria. The pathophysiological processes result from vascular damage and dysfunction which originate in the uteroplacental bed due to defective trophoblast invasion. The management of pre-eclampsia focuses on controlling blood pressure and monitoring the mother and the fetus to optimize the timing and the mode of delivery. However, effective diagnosis and treatment will come as a result of a clearer understanding of the pathogenesis of the disease.

1.13 Hypothesis

The hypothesis examined in this thesis was that cell adhesion molecule expression, a potential marker of endothelial damage and neutrophil activation, was increased in pre-eclampsia and IUGR. The objectives were to measure the circulating concentrations of cell adhesion molecules and the cell adhesion molecule mRNA and protein expression in placentae from normal and pre-eclamptic pregnancies. We also measured the concentrations of circulating cytokines, known to activate neutrophils, in normal and pre-eclamptic pregnancies and whether any of the cytokines had an effect on cell adhesion

molecule expression on human umbilical vein endothelial cells. These objectives may identify possible mediators of vascular endothelial dysfunction and help to create a clearer understanding of the disease process in pre-eclampsia

CHAPTER TWO

MATERIALS AND METHODS

2.1 GENERAL METHODS

2.1.1 Biochemicals

The following chemicals were purchased from the Sigma Chemical Company (Dorset, England); molecular biology agarose (gelling temperature 36°C), bovine serum albumin, bromphenol blue, dextran sulphate, diethylpyrocarbonate (DEPC), MR12 dowex mixed bed resin, ethidium bromide, 30% w/v hydrogen peroxide, Harris haematoxylin, 2-mercaptoethanol, 3-[N-Morpholino] propane-sulfonic acid (MOPS), polyvinylpyrrolidone (PVP), salmon testes DNA, 2% silane, sephadex G-50, sodium acetate, tetramethylethylenediamine (TEMED), tris chloride, urea.

The following chemicals were purchased from BDH (Poole, England); acetic acid, buffer tablets (pH 4, 7.4 and 9.2), chloroform, decon 75, ethylene diaminetetra-acetic acid disodium salt (EDTA), formaldehyde, glycerol, hydrochloric acid, industrial alcohol, methylated spirit, methylated spirit phenol, propan-2-ol, sodium chloride, sodium citrate, sodium dodecyl sulphate (SDS), sodium hydroxide pellets, wax.

Ethanol, acetone and methanol (Analar quality) were purchased from Hayman Limited (Essex, England).

The following chemicals were bought from DIFCO (Surrey, England); agar, bacto agar, casamino acids, yeast extracts.

Phosphate buffered saline tablets (PBS) were purchased from ICN Flow (Irvine, Scotland).

Ficoll 400 was obtained from Pharmacia (Buckinghamshire, England).

Interleukin-6 was purchased from Genzyme (Kent, England).

2.1.2 General apparatus

2.1.3 Centrifuges

Glasgow Royal Infirmary: MSE Mistral 4L was purchased from Fisons (Sussex, England) and the Damon CRU 5000 centrifuge from Life Sciences (Hampshire, England).

Glasgow Royal Maternity Hospital: The Damon Clini-cool centrifuge was purchased from Life Sciences (Hampshire, England) and the MSE Chilspin from Fisons (Sussex, England).

2.1.4 Miscellaneous

The Scandinova (SLF 111) fridge, Blomberg freezer and the Proline microwave were purchased from Comet (Glasgow, Scotland). The -70°C freezer was purchased from Scotlab (Coatbridge, Scotland). The Corning magnetic stirrer, the Sartorius balance and the whirlimixer were obtained from BDH (Poole, England).
The Stanton fine balance was purchased from Fisons (Sussex, England).

2.1.5 Radiochemicals

deoxycytidine 5'-[α^{32}P] triphosphate triethylammonium salt (dCTP), 3000Ci/ml, was purchased from Amersham Life Science (Buckinghamshire, England).

2.1.6 General solutions and buffers

Distilled Deionised Water (ddH_2O)

All solutions and buffers were prepared using ddH_2O, obtained from a Milli Q Plus water purification system purchased from Millipore S.A. (Molsheim, France). 500ml batches of ddH_2O were autoclaved and stored at 4°C.

Concentrated (20x) sodium saline citrate (SSC)

175.3g sodium chloride and 88.2g sodium citrate were dissolved in 800ml of ddH_2O. The pH was adjusted to pH7 with 5M hydrochloric acid. The solution was made up to 1L, autoclaved and stored at 4°C. The final concentrations of sodium chloride and sodium citrate were 3M and 0.3M respectively.

Broth and agar

5g yeast extract, 5g sodium chloride, 10g casamino acids and 2ml 1M sodium hydroxide were dissolved in 1L of ddH_2O. 15g of bacto-agar was added to the above broth to make the agar. Both solutions were autoclaved immediately and stored at 4°C until use.

0.5M EDTA (pH8)

18.6g EDTA was dissolved in 80ml ddH_2O. The pH was adjusted to pH8 with 10N sodium hydroxide, and made to 100ml with ddH_2O. The solution was autoclaved and stored at 4°C.

Tris EDTA (TE) electrophoresis buffer

2.42g Tris Cl was dissolved in 4ml 0.5M EDTA (pH8) and made to 2L with ddH_2O The pH was adjusted to pH8 and stored at RT. 500ml aliquots of this buffer were autoclaved and stored at 4°C for use as G50 column buffer.

Concentrated (50x) Denhardt's reagent

1g PVP, 1g ficoll and 1g BSA were dissolved in 80ml ddH_2O, stirred gently to avoid frothing and made up to 100ml with ddH_2O. The solution was then aliquoted in 5ml batches and stored at -20°C.

Concentrated (10x) MOPS

41.2g MOPS was dissolved in 1600ml of 0.1M sodium acetate (27.2g in 2L). The pH was adjusted to pH7 with 10N sodium hydroxide. 20ml of 0.5M EDTA was added and the final volume and adjusted to 2L with ddH_2O. The MOPS buffer solution was autoclaved and stored at 4°C.

RNA loading dye

The RNA loading buffer consisted of 800µl formamide, 10µl 10% xylene cyanole, 10µl 10% bromphenol blue, 4µl 0.5M EDTA and 176µl sterile glycerol. 500µl of the loading buffer was added to 500µl glycerol and 500µl deionised formamide. 1 part of 10% ethidium bromide solution (1mg/ml) was added to 2 parts of the dye solution, mixed and stored at -20°C.

Versene solution (pH7.2-7.3)

Versene was purchased from the University of Glasgow, Department of Virology. 80g sodium chloride, 2g potassium chloride, 11.5g sodium hydrogen phosphate, 2g EDTA

and 15ml phenol red (1%) were dissolved in 10L of ddH_2O. The solution was autoclaved and stored at 4°C.

Trypsin solution

Trypsin was purchased from the University of Glasgow, Department of Virology. 8g of sodium chloride, 2ml potassium chloride (19%), 0.1g sodium hydrogen phosphate, 1g dextrose and 3g tris amino methane, were dissolved in 700ml of ddH_2O. 1.5ml of phenol red (1%), penicillin (100,000U) and 0.1g of streptomycin were added to this solution. The volume was made up to 1L with ddH_2O and 2.5g of trypsin was added. The solution was then autoclaved and stored at -20°C.

Trypsin/Versene solution

This solution contained 20ml of versene and 5ml of trypsin.

Deionised formamide

10g dowex resin was added to 500ml formamide. This was stirred for 1h and then filtered. After aliquoting, the deionised formamide was stored at -70°C and used within three months.

Tris buffered saline (TBS) buffer

10N hydrochloric acid was added to 25ml 0.2M tris until a pH of 7.4 was obtained. This was made up to a final volume of 100ml with ddH_2O. The TrisHCl was added to 900ml of 0.9% sodium chloride to give the final solution.

2.1.7 Cell culture reagents and disposable apparatus

Fungizone (Amphotericin B, 250µg/ml)(F), Penicillin (100U/ml) and streptomycin (100µg/ml) (P/S) were purchased from Gibco BRL (Paisley, Scotland).
Dulbecco's modification of Eagles medium DMEM (without L-glutamine, with 4.5g/L dextrose), heat inactivated foetal calf serum (FCS), L-glutamine (G) and heat inactivated horse serum (HS) were purchased from ICN Flow (Irvine, Scotland).
50ml centrifuge tubes, 6-well clusters and 10ml stripettes were purchased from Costar (Buckinghamshire, England).
8-well Labtek chamber slides were purchased from Gibco BRL (Paisley, Scotland).

7ml bijoux containers, 30ml universal containers and 90mm petri dishes were purchased from BDH (Poole, England).
Collagenase (Type II), endothelial cell growth supplement (ECGS), 2% gelatin solution and heparin were purchased from Sigma Chemical Company (Dorset, England).
Sterile 0.22μm filter units were purchased from Millipore S.A. (Molsheim, France.)
All syringes, 18GA cannulas, 10% buffered formaldehyde and 500mg vials of ampicillin were obtained from the Pharmacy, Glasgow Royal Infirmary.

2.1.8 General equipment for cell culture

The following is a list of general equipment used in cell culture;

BSB48 Laminar flow hood:	ICN Flow (Irvine, Scotland).
Gallencamp CO_2 incubator:	Fisons (Sussex, England).
Olympus inverted microscope:	Olympus (London, England).

2.1.9 Molecular biology reagents and disposable apparatus

The Geneclean II kit was obtained from Stratech Scientific (Luton, England). The Oligolabelling kit was obtained from Pharmacia (Buckinghamshire, England). RNAzol™ was purchased from Biogenesis (Bournemouth, England). The blot transfer system, restriction enzymes and reaction buffers were purchased from Gibco BRL (Paisley, Scotland). Microcentrifuge tubes, sterile pastettes and pipette tips were purchased from Alpha Laboratories (Hampshire, England). Hybond-N transfer membranes were purchased from Amersham (Buckinghamshire, England). Blotting pads were bought from Gibco BRL (Paisley, Scotland).
The Magic Maxiprep and Magic Miniprep DNA purification systems were obtained from Promega (Southampton, England). Ultracomp *E. coli* (MC1061/P3) were bought from British Biotechnology (Oxon, England). DH5α competent cells and 1kb DNA ladder were obtained from Gibco BRL (Paisley, Scotland). DNA gel loading dye and the rabbit anti-human von Willebrand factor was purchased from Sigma Chemical Company (Dorset, England). The donkey anti-rabbit fluorescein was bought from Amersham Life Science (Buckinghamshire, England). The IL-6, IL-1RA, IL-6 receptor antibody, GM-CSF, IL-1β, TNFα and IL-8 Quantikine ELISA kits, the cell adhesion molecule antibodies PECAM, E-Selectin, VCAM-1 and ICAM-1 and the VCAM-1, ICAM-1 and E-Selectin ELISA kits were purchased from R+D Systems (Oxon, England). Aspergillus niger glucose oxidase antibody, biotinylated goat anti-mouse immunoglobulins (IgGs),

3,3'-diaminobenzidine tetrahydrochloride (DAB) and peroxidase conjugated streptavidin were purchased from DAKO Ltd (High Wycombe, England). The Vectastain kit and the Vectashield mounting medium was purchased from Vector Laboratories (Peterborough, England). Goat serum was obtained from the Scottish Antibody Production Unit (SAPU: Carluke, Scotland)

2.1.10 General equipment for molecular biology

The power supply, spectrophotometer and the gel electrophoresis apparatus (G100 tank) were purchased from Pharmacia (Buckinghamshire, England).
The microcentrifuge and Grant water bath were obtained from Scotlab (Coatbridge, Scotland).
The Stuart Scientific shaker was purchased from BDH (Poole, England).
The Techne hybridiser and the transilluminator were purchased from Genetic Research Instrumentation (Essex, England).
The UV Stratalinker was purchased from Stratagene (Cambridge, England).
Fuji X-ray film was obtained from Amersham (Buckinghamshire, England).
Hypercassettes were obtained from Appligene (Durham, England).
Silica cuvettes were obtained from FSA (Loughborough, England).
The ^{32}P β emitter monitor was purchased from Mini Instruments (Essex, England). The Histokinette 2000 was purchased from Medical Laboratory Equipment Services (Glasgow, Scotland). The Tissue Tek 3 and green uni-cassettes (to be used with this machine) were purchased from Bayer Diagnostics (Basingstoke, England).

2.1.11 General methods

All items of glassware were washed in solutions of decon 75 and were then rinsed in distilled water and dried at 50°C.

2.1.12 Micropipetting

Volumes of solutions in the range of 1-25µl were transferred accurately using digital pipettes from Scotlab (Coatbridge, Glasgow). 10-1000µl were transferred using Finn-pipettes purchased from Labsystems (Hampshire, England).

2.1.13 pH measurement

Measurements of pH were carried out using a Kent 7020 pH meter purchased from BDH (Poole, England). This apparatus was regularly standardised using a solution of pH4, pH7 or pH9.2 prepared using buffer tablets.

2.2 MOLECULAR BIOLOGY

2.2.1 Antibiotics

The antibiotics used in the transformation of cells are shown in the following table:

Antibiotic	Stock concentration	Working concn.
ampicillin	50mg/ml in ddH_2O	50µg/ml
tetracycline	10mg/ml in ethanol	30µg/ml

Both antibiotics were stored at -20°C.

2.2.2 Transformation of Ultracomp *E. coli.*

Transformation involves treatment of the bacteria with divalent cations to make them temporarily permeable to small DNA molecules. In order to be able to select the bacteria that have been successfully transformed selectable markers are required. The most commonly used selectable markers are genes which confer resistance to antibiotics such as ampicillin and tetracycline (Sambrook, 1989).

The transformation of Ultracomp *E. coli* was carried out following the protocol accompanying the kit purchased from British Biotechnology. The competent cells were thawed on ice and mixed gently when the cell suspension was liquid. The cells were kept on ice at all times. 100µl of cells were aliquoted into a pre-chilled 50ml polypropylene tube and 5µl of a fresh dilution of 500mM mercaptoethanol in ddH_2O was added. This solution was incubated for 10 min and was mixed gently every 2 min. 2µl plasmid DNA solution (approximately 1µg/ml) was added and the tube swirled gently, and placed on ice for 30 min. Heat shock was performed by placing the cells in a 42°C water bath for 75s and then returning to ice for 2 min. 900µl SOC medium (provided with kit) was added and the solution then incubated at 37°C with moderate agitation (225 rpm) for 60 min.

100ml agar was autoclaved, allowed to cool and separated into 2 x 50ml portions. To one portion the appropriate antibiotic was added. 4 plates each containing 25ml of

agar were poured and allowed to set. Using a sterile wire, the *E. coli* were then streaked across agar plates. In some cases, with transformed cells, there was little growth on the agar plate containing the appropriate antibiotic and, in these cases, 100µl of transformed cells were streaked across the agar plate using a sterile bent glass rod. Untransformed cells were used as a control and spread onto 2 plates, with and without antibiotic. The plates were then incubated at 37°C overnight.

The results for a successful transformation were as follows:-

Transformed cells	+	antibiotic	=	growth
Transformed cells	-	antibiotic	=	growth
Untransformed cells	+	antibiotic	=	no growth
Untransformed cells	-	antibiotic	=	growth

The colonies growing on the agar plate containing transformed cells were used for overnight cultures.

2.2.3 Transformation of DH5α *E. coli.*

DH5α competent bacteria, which had been stored at -70°C, were thawed on wet ice. 1µl of the required plasmid DNA (500µg/ml) was added to 100µl DH5α bacteria in a microcentrifuge tube and gently tapped. This solution was incubated on ice for 30 min. The cells were heat shocked by incubation at 42°C for 90s and then incubated on ice for 2 min. After 0.5ml of broth was added the microcentrifuge tube was incubated for 1 hour at 37°C in an orbital shaker at 225 cycles per min. Cells (100µl) were either plated directly onto agar plates or a sterile wire loop was used to streak cells across the agar plates as in the transformation of Ultracomp *E. coli.* (section 2.2.2).

2.2.4 Overnight cultures

3 separate colonies from the agar plate containing transformed cells and the appropriate antibiotic were each added to 10ml of nutrient broth containing the appropriate antibiotic. A control transformation involved the addition of a colony from the plate with untransformed cells without addition of the antibiotic to the nutrient broth. Cultures were shaken at 225 cycles per minute overnight in an orbital shaker at 37°C. No growth was apparent in the control nutrient broth whereas the solution in the other 3 universals was turbid. The cells from these overnight cultures were used for the small scale plasmid DNA preparations.

2.2.5 Plasmids

Details of plasmids used in this study:
The 1.2kb E-Selectin complementary deoxyribonucleic acid (cDNA) in the CDM-7 plasmid was kindly donated by M.P. Bevilacqua, Howard Hughes Medical Institute, University of California, San Diego, La Jolla, California, U.S.A. The plasmid was amplified in MC1061/P3 cells (competent *E. coli.*) and excised using the restriction enzyme Xba 1.

The 1.8kb ICAM-1, 800b ICAM-2, 1.9kb VCAM-2 and 2.5kb PECAM cDNA, all in the plasmid pCDM-8 were all grown in MC1061/P3 cells and excised using the restriction enzymes Xba-1 and HIND-III. These were kindly donated by Dr David Simmons, Cell Adhesion Laboratory, Imperial Cancer Research Fund, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, U.K.

2.2.6 Small scale preparation of plasmid DNA

The components required for this procedure were supplied in the Magic Minipreps DNA Purification System kit.

3ml of the most turbid overnight culture of cells were pelleted using a microcentrifuge (13,000 rpm for 20s at room temperature [RT]). The cell pellet was resuspended in 200µl of Cell Resuspension Solution (50mM Tris HCl, pH 7.5; 10mM EDTA; 100µg/ml RNase A). 200µl of Cell Lysis Solution (0.2M NaOH, 1% SDS) was added and the tube was inverted several times to mix. The cell suspension cleared almost immediately. 200µl of Neutralization Solution (2.55M potassium acetate, pH 4.8) was then added and the tube mixed. Following centrifugation at 10,000 r.p.m. for 5 min at 4°C the clear supernatant was decanted to a new microcentrifuge tube. 1ml of the Purification Resin was added to the supernatant and the two mixed.

For each miniprep a 3ml disposable syringe was required. The plunger from the syringe was removed and the syringe barrel was attached to the minicolumn which was in turn attached to a vacuum pump. The resin/DNA mix was pipetted into the syringe barrel. A vacuum was applied to pull this mix into the minicolumn. 2ml of Column Wash Solution (200mM NaCl; 20mM Tris HCl, pH 7.5; 5mM EDTA-diluted 1:1 with 95% ethanol) was added to the syringe barrel and a vacuum applied. The resin was dried by drawing a vacuum for a further 2 min. The syringe barrel was removed and the minicolumn transferred into a 1.5ml microcentrifuge tube. To remove any residual Column Wash Solution the minicolumn was spun in a microcentrifuge at 10,000 rpm for 20s at RT. The minicolumn was then transferred to a new microcentrifuge tube and 50µl of TE buffer (preheated to 70°C) was added. After 1 min the DNA was eluted by

centrifugation of the minicolumn for 20s at 10,000 rpm at RT. The plasmid DNA was stored at -20°C.

2.2.7 Large scale preparation of plasmid DNA

To 500ml of nutrient broth, 2ml of the overnight culture used for the mini preparation was added, along with the appropriate antibiotic. This solution was shaken at 225 cycles per minute overnight in an orbital shaker at 37°C. The resulting cloudy solution was then used to carry out the Promega Magic Maxipreparation protocol.

The components required for this procedure were supplied in the Magic Maxipreparation DNA purification system kit. 500ml of an overnight culture of *E. coli* were spun at 14,000g for 15 min at 4°C. The cell pellet was resuspended in 15ml of Cell Resuspension Solution and then divided equally into duplicate centrifuge tubes. All subsequent steps were conducted in duplicate. 7.5ml of Cell Lysis Solution was added and the solution mixed gently. The cell suspension appeared clear and viscous. 7.5ml of Neutralization Solution was then added, the solution mixed immediately, and centrifuged at 14,000g for 15 min at 4°C. The supernatants were decanted into new centrifuge tubes (avoiding the white precipitate) and 0.6 x volume of isopropanol (approximately 11.4ml) was added. After mixing, the solution was centrifuged at 14,000g for 15 min at 4°C. The DNA pellet was resuspended in 2ml of TE buffer. Next 10ml of well mixed Purification Resin was added to the DNA solution and transferred to the maxicolumn inserted into the vacuum pump. A vacuum was applied to pull the mix into the column. 13ml of Column Wash Solution was added to the tube containing the DNA/Resin mix and poured into the column and a vacuum applied. A further 12ml of Wash Solution was then added followed by 5ml of 80% ethanol. The resin was dried by drawing a vacuum for 10 min. After removing the column from the vacuum it was placed in the 50ml screw cap tube provided and 1.5ml of preheated (70°C) TE buffer was added and incubated for 1 min. The DNA was eluted by spinning the column in the 50ml tube at 1300g for 5 min at 4°C. The DNA was stored at -20°C.

2.2.8 Freezing transformed cells

The following protocol was used to freeze and store cells for future use;
8.5ml of transformed cells were added to 1.5ml sterile glycerol. The solution was well mixed and then aliquoted into 1ml batches. These aliquots were frozen using an ethanol/dry ice bath and stored at -70°C.

2.2.9 Quantification of DNA and RNA

5µl of the DNA/RNA sample was added to 995µl of ddH_2O in a microcentrifuge tube. This solution was vortexed and transferred into a silica cuvette using a pastette. The absorbance of the solution at 260nm and 280nm was measured using a spectrophotometer. The absorbance of the solution at 260nm and 280nm is directly proportional to the concentration of RNA and DNA present in the sample. The ratio of absorbance values obtained (i.e. A_{260}/A_{280}) is an index of the purity of the RNA sample. The ratio can vary, however, a ratio of 2.0 represents a pure RNA sample with very little protein contamination. The amount of DNA present in 1ml of a sample was calculated by multiplying the absorbance at 280nm by 50 since an optical density of 1 represents 50mg of DNA. The amount of RNA present in 1ml of a sample was calculated by multiplying the absorbance at 260nm by 40 since an optical density of 1 represent 40mg of RNA. The total amount of DNA/RNA present in a sample was calculated by multiplying the above value by the dilution factor.

2.2.10 Digestion of plasmid with restriction enzymes

React 2 (provided with the enzyme) was the reaction buffer required when the cDNA E-Selectin, PECAM-1, ICAM-1, ICAM-2 and VCAM-2 were digested.
To digest E-selectin, 10µl cDNA, 2µl Xba-1, 2µl React 2 (x10) and 6µl ddH_2O were combined and incubated at 37°C overnight.
For the other cDNA, 10µl cDNA, 2µl Xba-1, 2µl HIND III, 2µl React 2 (x10) and 4µl ddH_2O were combined and incubated at 37°C overnight.
After incubation a 10µl aliquot of each sample was electrophoresed on a DNA gel to ensure that the cDNA had been excised from the plasmid.

2.2.11 DNA gels

0.8g of agarose was dissolved in 100ml of TE buffer and the solution was microwaved to dissolve the agarose. The flask was gently swirled every 20s to ensure even dissolution. 1µl of ethidium bromide (10mg/ml) was added to the solution which was then allowed to cool slightly. The solution was poured into a gel plate which contained a plastic comb, was allowed to set and was then placed into a G-100 gel tank and submerged in TE buffer. 2µl of DNA loading dye was then added to each sample before loading into the wells of the gel. The DNA samples were electrophoresed at 60V for 2-3h. All cDNA inserts were excised from the gel using a clean scalpel blade and then extracted from the agarose using the gene cleaning kit.

2.2.12 Gene cleaning

The components required for this procedure were supplied in the Geneclean II kit.

Each gel slice was placed in a microcentrifuge tube which was subsequently filled with sodium iodide. This was placed at 50°C for 5 min until the gel slice had dissolved. (Depending on the size of the gel slice it could take between 5-9 min to dissolve.)

The "glass milk" (silica matrix) was resuspended thoroughly and 10µl was then added to the dissolved gel slice and vortexed. This was incubated on ice for 5 min. The microcentrifuge tube was then spun at 13,000 rpm for 10s at RT. After the supernatant was removed, 200µl of NEW solution (which contained NaCl, Tris, EDTA, H_2O and ethanol) was added to the pellet. This was resuspended thoroughly using a 200µl pipette tip. The microcentrifuge tube was spun again at 13,000 rpm for 10s at RT and the supernatant removed. Another 200µl of NEW solution was added and this step was carried out twice more. 50µl TE buffer was added to the pellet, resuspended thoroughly and incubated at 50°C for 3 min. After a 30s spin at 13,000 rpm at RT in the microcentrifuge the supernatant, which contained the eluted DNA, was removed. A further 50µl TE buffer was then added to the pellet and resuspended well. Again after a 30s spin at 13,000 rpm at RT the eluted DNA was removed in the supernatant.

5µl of gene cleaned DNA and 1µl of 1kb DNA (500bp-12kb) ladder were electrophoresed on an agarose gel to check the correct size of insert required was obtained (figures 4, 5 and 6). The DNA was then aliquoted into 50ng batches and stored at -20°C.

2.2.13 Northern analysis

When preparing RNA from human umbilical vein endothelial cells (HUVECS) RNAzol B (1ml/9.5cm^2)was added directly to the cells. The solution was then triturated and transferred to a microcentrifuge tube containing 100ml chloroform. This tube was vortexed and incubated on ice for 5 min.

When preparing RNA from frozen blocks of placenta the tissue was ground into fine particles using a mortar and pestle, ensuring the tissue was kept frozen, i.e. submerged in liquid nitrogen at all times. Once ground, 2ml of RNAzol B was added for every 0.1g of tissue, and the resulting solution was homogenised (2x10s at setting 8 on the dial). 1ml batches of this solution were added to 100µl aliquots of chloroform, vortexed and incubated on ice for 5 min. The tubes were spun in a microcentrifuge at 13,000 rpm for 15 min at 4°C. The upper layer was removed and an equal layer of isopropanol was added (approximately 500µl). The sample was spun for 30 min at 13,000 rpm at 4°C, after which the supernatant was removed and the pellet drained by inverting the tube on a tissue. 500µl of 70% ethanol was then added to the pellet and the RNA was stored at -70°C until the next step could be performed. The RNA was spun at

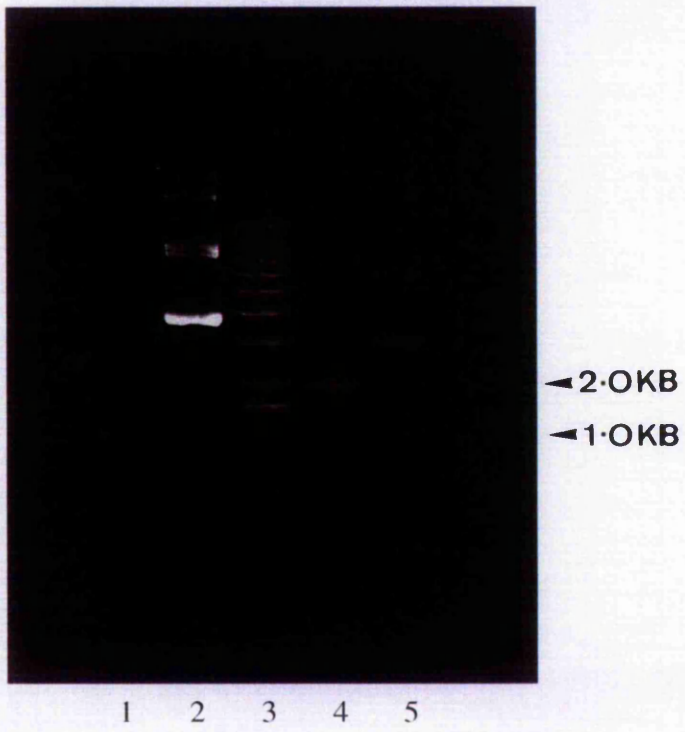


Figure 4. Scan of a DNA gel containing:

Lane 1 - gene cleaned 1.2 kb E-selectin cDNA excised with Xba-1

Lane 2 - uncut E-selectin plasmid

Lane 3 - 1kb DNA ladder

Lane 4 - gene cleaned 1.8kb ICAM-1 cDNA excised with Xba-1 and HIND-III

Lane 5 - uncut ICAM-1 plasmid

The samples were run against a 1kb cDNA ladder and their length calculated by comparing their positions on the gel to the bands of the cDNA ladder

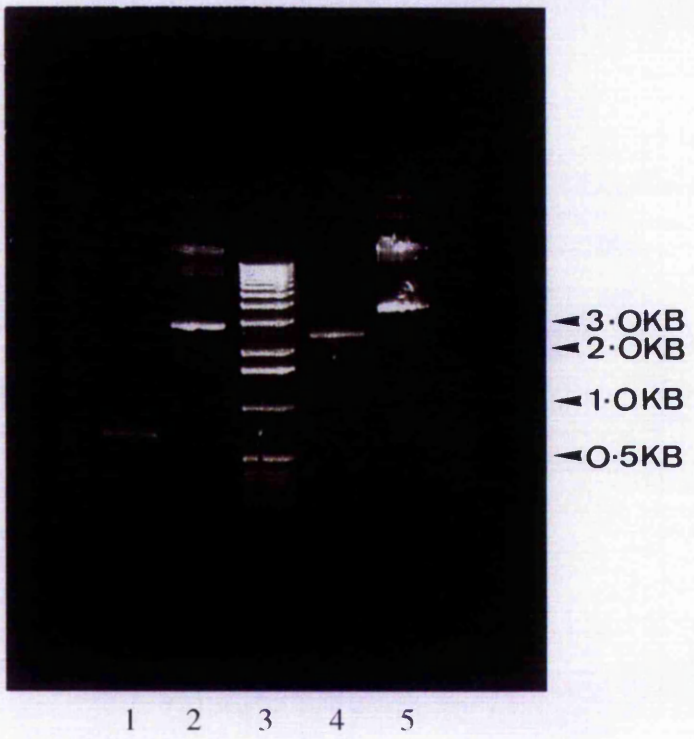


Figure 5. Scan of a DNA gel contaning:

Lane 1 - gene cleaned 800b ICAM-2 cDNA excised with Xba-1 and HIND-III

Lane 2 - uncut ICAM-2 plasmid

Lane 3 - 1kb DNA ladder

Lane 4 - Gene cleaned 2.5kb PECAM cDNA ladder excised with Xba-1 and HIND-III

Lane 5 - uncut PECAM plasmid

The samples were run against a 1kb cDNA ladder and their length was calculated by comparing their positions on the gel to the bands of the DNA ladder.

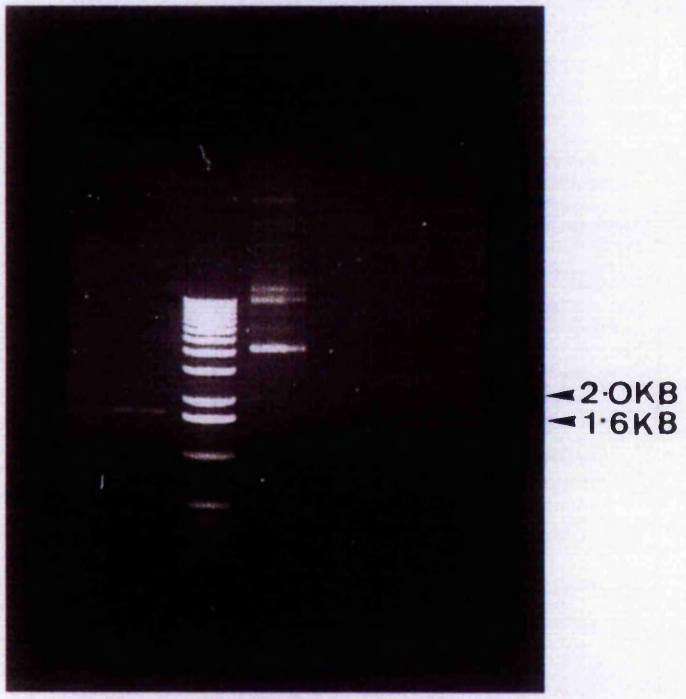


Figure 6. Scan of DNA gel containing:

Lane 1 - gene cleaned 1.9kb VCAM-2 cDNA excised with Xba-1 and HIND-III

Lane 2 - 1kb DNA ladder

Lane 3 - uncut VCAM-2 plasmid

The samples were run against a 1kb DNA ladder and their length was calculated by comparing their positions on the gel to the bands of the DNA ladder.

13,000 rpm for 15 min, and then drained until the pellet was dry. An appropriate amount of ddH_2O was added and the sample was incubated in a waterbath at 65°C for 10 min to dissolve the RNA. The amount of RNA was determined as before. 10µg batches of RNA were aliquoted into microcentrifuge tubes containing 250µl ddH_2O, 500µl ethanol and 25µl 3M sodium acetate (pH 5.2) and stored at -70°C until required for electrophoresis.

In preparation for electrophoresis 10µg of RNA was dissolved in 10µl of ddH_2O (65°C for 5 min). 5µl of the following solution was then added : deionised formamide 1250µl; 37% formaldehyde 400µl; 10x MOPS 0.2mol/l (0.05mol/l sodium acetate, pH7; 0.01mol/l Na_2EDTA) 250µl. The resulting solution was incubated at 65°C for 10 min. 2µl of RNA loading dye was added whilst the samples were kept on ice constantly. The samples were electrophoresed on an RNA agarose gel.

2.2.14 RNA gels

1.2g of agarose was dissolved in a solution containing 73ml of ddH_2O and 10ml of 10x MOPS. The solution was microwaved for 3min, gently swirling every 20s. 17ml of formaldehyde was then added and the solution allowed to cool slightly. The solution was poured into a gel plate containing a plastic comb and allowed to set before it was submerged in 1x MOPS (20mMol/L) in a G-100 gel tank. The samples were electrophoresed at 60V for 3h.

2.2.15 RNA transfer

RNA was transferred onto Hybond N nylon membranes by blotting in 20xSSC (3mol/L NaCl, 0.3mol/L NaCit). The RNA was allowed to transfer overnight. The RNA was crosslinked onto the nylon membrane using ultra violet radiation (40s at $1.2x10^5$ µjoules). The nylon membrane was rinsed for 20s in 5xSSC and small cuts were made at the 18S and 28S marker area on the nylon membrane while being viewed on a transluminator. The nylon membrane was stored at -20°C in sealed plastic sheeting.

2.2.16 Prehybridisation

250µl of salmon testes DNA (100µg/ml) was boiled for 5min and then cooled on ice for 5 min. This DNA was added to 12ml of the prehybridisation solution which contained the following: 2.5ml 10% sodium dodecyl sulphate (SDS), 12.5ml 20xSSC solution, 5ml Denhardt's solution, 5g dextran sulphate and 27ml ddH_2O. 12ml of prehybridisation solution was poured into a hybridisation tube. The nylon membrane containing the RNA was placed in the tube with the RNA side facing upwards. Air

bubbles were removed, very gently, with a plastic 10ml pipette. The hybridisation tube was placed in the hybridisation incubator at 65°C and the nylon membranes were prehybridised for a minimum of 4h.

2.2.17 Random priming cDNA probes

The oligolabelling kit contained the reagent mix and Klenow fragment which were used in this procedure. The reagent mix was composed of a buffered aqueous solution containing deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and random hexadeoxyribonucleotides.

26µl of ddH_2O was added to 10µl of a cDNA probe (already aliquoted into 50ng batches) and was boiled for 5 min. 10µl of the reagent mix, 3µl of CTP and 1µl of Klenow fragment were added and the microcentrifuge tube was spun for 5s to gently mix the solutions. The resulting solution was incubated for at least 1h at 37°C.

The radiolabelled probe was then separated from unincorporated ^{32}P CTP on a G-50 sephadex column. The radioactive cDNA was boiled for 5 min and placed on ice for 5 min.

2.2.18 Hybridisation

The radioactive cDNA was injected into the hybridisation tube and the tubes placed in the hybridisation incubator at 65°C for approximately 18h.

2.2.19 Washing nylon membranes

The nylon membranes were washed in 15ml of 1xSSC (containing 0.5% SDS) for 20 min at 65°C and then further washed at 0.5xSSC (containing 0.5% SDS). Autoradiography was carried out with Fuji X-ray film at -70°C for 24-48h. If necessary a further wash in 0.1xSSC was carried out.

2.3 CELL CULTURE

2.3.1 Human umbilical vein endothelial cell (HUVEC) preparation

All cell culture techniques were carried out in a laminar flow hood.

Umbilical cords were obtained no later than 48h following delivery from Glasgow Royal Maternity Hospital. The umbilical cord was cut from the placenta in a plastic bag in order to avoid spillage and placed in sterile 0.9% sodium chloride solution before being transported to the laboratory. Care was taken not to bend the cord during the preparation to avoid smooth muscle cell accumulation. An 18G venflon cannula was inserted fully into the vein until the green part of the cannula started to enter the vessel. A syringe containing serum free (SF) media (Dulbecco's modification of Eagles medium with 4.5g/L dextrose without glutamine with 100IU/ml penicillin, 100μg/ml streptomycin (P/S), 2 mmol/L glutamine (G) and 2.5μg/ml fungizone (F) added) was attached to the cannula. The cord and cannula were clamped using forceps. The cord was thoroughly flushed through with SF media until the drained liquid was clear with no blood contamination. 0.1% of collagenase (type II) was then added to the vessel. The collagenase solution was allowed to run through the vessel for a few seconds before the other end of the cord was clamped. Collagenase was added until a slight resistance was felt in the syringe. The cord was wrapped in clingfilm and incubated at 37°C for 15 min. The collagenase solution from the cord was collected in a 50ml polypropylene tube. The endothelial cell layer was removed by gently rubbing the cord. The cord was then rinsed through with approximately 20ml of SF media. The contents of the 50ml tube were pipetted into sterile universal containers and centrifuged at 200g for 5 min at 4°C. The supernatant was removed and a further 20ml of SF media added. The universal container was tapped slightly to resuspend the cells. This was then centrifuged at 200g for 5 min at 4°C. Again the supernatant was removed and 6ml of DMEM, containing 10% FCS, 10% HS, P/S, G and F, was added. After gentle trituration the cells were split between two wells of a 6 well cluster plate. The endothelial cells were maintained at 37°C in a 5% carbon dioxide (CO_2) /95% air humidified atmosphere. The media was changed on alternate days until the cells were confluent. When the cells were confluent (after approximately 2-3 days), they were passaged as follows: The medium was removed and 2ml of versene was added to the well for 5s, removed and then 1ml of a versene/trypsin (4:1) mix was added to the well. The plate was then incubated at 37°C for up to 5 min or until the cells became detached from the well bottom (a gentle tap to the bottom of the well aided detachment). Medium was added to the cells, which were then spun at 200g for 5 min at 4°C to obtain the cell pellet. The supernatant was then discarded. The appropriate amount of

medium was added to the cells which were then triturated to obtain a single cell suspension and were then plated out in a 1:3 split ratio onto gelatin coated plates. (Plates were previously coated with 1ml of a 2% gelatin solution, incubated at 37°C for 1h, rinsed thoroughly with SF media and dried for 2h at 37°C). When setting up 8 well Labtek chamber slides with endothelial cells, the wells were treated with gelatin as before, and 350µl of the single cell suspension in DMEM were added to the wells.

After the first passage the endothelial cells were maintained in the presence of endothelial cell growth supplement at 50µg/ml. The endothelial cells were used up to the second passage. Endothelial cells grow to form a cobblestone morphology at confluence (see plate 1). The cells were shown to be endothelial cells by positive staining for von Willebrand factor (VWF) (see plate 2). Confluent HUVECS were fixed in ice cold 50% acetone/methanol (1:1) for 10 min at 4°C. The cells were washed with PBS for 5 min and then incubated with rabbit, anti-human, von Willebrand factor (diluted 1 in 200 in PBS) for 30 min at 37°C. The cells were then washed with PBS for 3, 15 min incubations. Next, the cells were incubated with donkey, anti-rabbit Ig, fluorescein, for 30 min, at 37°C. This was followed by 2, 15 min washes in PBS. The cells were then mounted in Vectashield mounting medium. Control cells were incubated as described above but with the first antibody omitted.

2.3.2 Patient sample collection and preparation

2.3.3 Patient Details

The pre-eclamptic pregnancies in each study were defined as having a persistent diastolic blood pressure > 90mm Hg, with proteinuria > 0.3mg/24h. All patients were normotensive before 20 weeks gestation. Pregnancies complicated by IUGR had estimated fetal weight < 5th centile and Doppler ultrasound of the umbilical artery demonstrated absent end-diastolic flow velocity. Local birthweight centiles were used.

2.3.4 Blood collection tubes

10ml glass tubes were purchased from LIP Equipment Services (Yorkshire, England). The10 ml disposable plastic tubes were obtained from Sarstedt (Leicester, England).

2.3.5 Plasma preparation

Up to 10ml of blood was added into pre-cooled Sarstedt 10ml tubes containing 1ml of 3.2% sodium citrate. The blood was spun at 1,500g for 15 min at 4°C as soon as possible after collection.

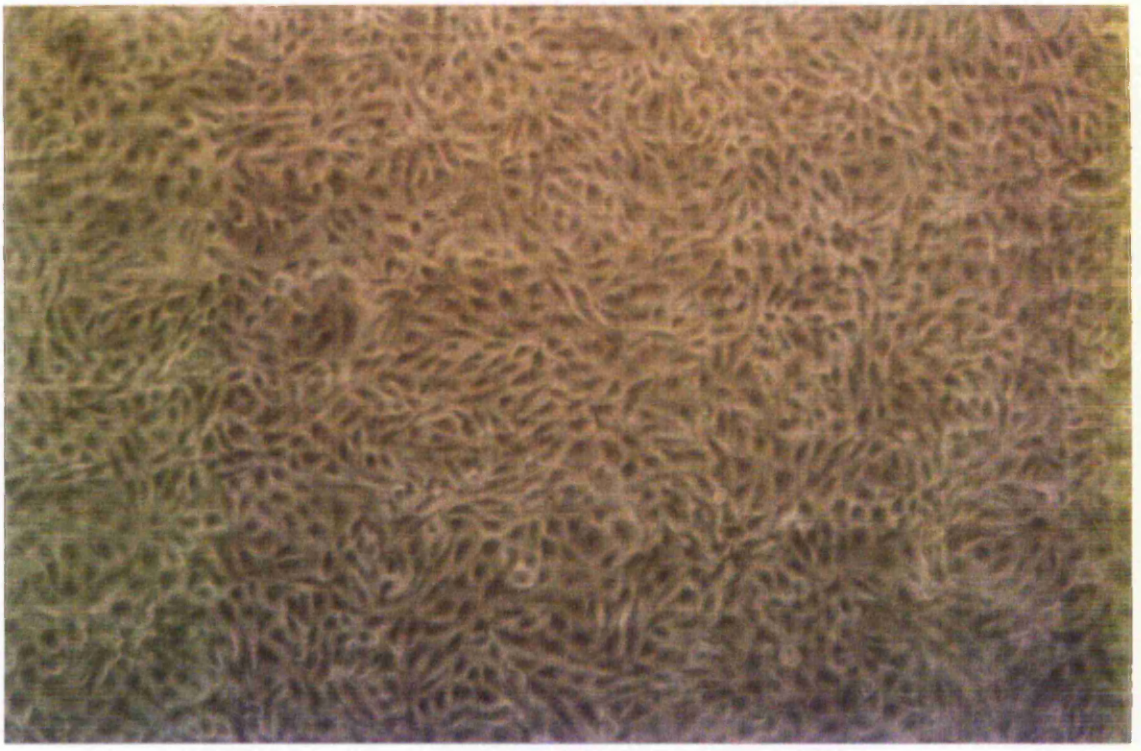


Plate 1. A photograph showing human umbilical vein endothelial cells which were prepared as described in section 2.3.1, and grown in a tissue culture flask for 4 days. The confluent endothelial cells adopt a typical cobblestone appearance.

Plate 2. A photograph of human umbilical vein endothelial cells stained with anti human von Willebrand factor and visualised by immunofluorescence, as described in section 2.3.1.

2.3.6 Serum preparation

A maximum of 5ml of blood was added to 10ml glass tubes. The tubes were allowed to stand at RT for up to 15 min to clot and then centrifuged at 1,500g for 15 min at 4°C.

2.3.7 Plasma and serum storage

After centrifugation the supernatants were removed and aliquoted into 250µl or 500µl batches. The microcentrifuge tubes were pre-cooled on dry-ice or taken straight from the fridge. Aliquots were frozen immediately in dry-ice until they could be placed in the -70°C freezer. The serum was removed with a pasteur pipette and placed into a pre-cooled bijoux and then aliquoted. At all times the blood was kept on ice, unless it was spinning, or clotting for serum.

2.3.8 Collecting maternal blood

40ml of maternal blood was collected from patients before the onset of labour. 2 x 10ml of blood were added to precooled Sarstedt 10ml tubes containing 1ml of 3.2% tri-sodium citrate to give plasma. 4 x 5ml of blood were added to glass tubes, allowed to clot and were processed for serum as previously detailed.

2.3.9 Placentae collection

As soon as the placenta was delivered it was weighed and taken immediately to the laboratory. Blood was collected from the cord root and serum and plasma was prepared as described above. Surfaces and surgical appliances were sterilised using 70% ethanol. While the blood samples were being centrifuged, the placentae samples were collected. Approximately $1cm^3$ blocks of placenta were cut using very sharp scissors taking care that a full section of placenta was cut each time from the chorionic plate through to the basal plate. Each cube of placenta was rinsed 4 times in sterile 0.9% sodium chloride to remove as much blood as possible and then placed directly into liquid nitrogen. Sections of umbilical cord were also rinsed well and frozen immediately. Sections of placenta and cord were placed in 10% buffered formaldehyde for 24h, 50% ethanol for 8h, 70% ethanol for up to 24h and then processed in the Pathology Department, Glasgow Royal Infirmary using the following protocol. Full sections of placentae were cut and placed in green uni-cassettes. The cassette was then placed in the Histokinette 2000 and was then taken through the following solutions: methylated spirits for 1h, methylated spirit phenol for 1h 30min, industrial alcohol for 1h 30min, 2h, 2h, and 2h 30min, industrial alcohol/chloroform (50/50) for 1h, chloroform for 1h 30min, 2h and 2h, and 2 wax treatments for 2h and 3h 30min. The uni- cassettes were then transferred

to metal uni-cassettes and embedded using the Tissue Tek 3.

The tissue frozen in liquid nitrogen was placed in pre-cooled storage cryo-tubes and stored at -70°C until required for experiments.

2.3.10 Immunocytochemistry

10μm sections, from the full thickness of placentae frozen in liquid nitrogen, were cut on a cryostat and mounted on slides. The slides had previously been soaked in acetone for 5 min, 2% silane in acetone for 5 min, washed in water for 30 min and then air dried. The sections were then fixed in acetone for 10 min. When carrying out ICC on HUVECS on 8-well chamber slides the cells were first fixed in acetone for 10 min at 4°C. After air drying for 5 min, PBS was added to the HUVECS which were then stored at 4°C until use. 0.5% hydrogen peroxide in methanol was then added to block endogenous peroxidase activity. Sections/HUVECS were then washed in TBS buffer (5mMol/LTris/Cl, pH 7.4; 0.9% NaCl) and blocked with 20% normal goat serum (NGS) for 30 min at RT. Next the sections were incubated overnight at 4°C with primary cell adhesion molecule antibody diluted in 2% NGS in TBS. The cell adhesion molecule antibodies were monoclonal mouse anti-human IgG_1 subclass (a)PECAM (1:500 dilution), (b) E-Selectin (1:250), (c) VCAM-1 (1:500) &(d) ICAM-1 (1:500). Negative controls were slides incubated with 2% NGS in TBS or with a mouse monoclonal IgG_1 Aspergillus niger glucose, oxidase an enzyme that is neither present nor inducible in mammalian tissue. Sections were next washed in TBS and then incubated for 1h at RT with 1:200 biotinylated goat anti-mouse immunoglobulins in 2% NGS/5% normal human serum (NHS) in TBS. After washing in TBS, sections were incubated for 30 min at RT with 1:400 peroxidase-conjugated streptavidin in TBS then washed again in TBS. Finally immunoreactive cell adhesion molecules, which appear as a brown end-product, were localised by incubating sections in 1mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.02% hydrogen peroxide in TBS for 10 min. The sections were counterstained in Harris haematoxylin.

2.3.11 Measuring cell adhesion molecules using ELISA

The cell adhesion molecules VCAM-1, E-Selectin and ICAM-1 were measured by enzyme linked immunosorbent assay (ELISA). The protocol provided with each kit was followed. This involved the simultaneous reaction of the cell adhesion molecule present in the sample or standard to two monoclonal antibodies which were directed against different epitopes on the cell adhesion molecule. One antibody was coated onto the walls of the microtiter wells and the other was conjugated to the enzyme horseradish peroxidase (HRP). Any cell adhesion molecule present formed a bridge between the

two antibodies. After the unbound material was removed by aspiration and washing, the amount of conjugate bound to the well was detected by the reaction with a substrate specific for the enzyme which yielded a coloured product proportional to the amount of conjugate (and thus cell adhesion molecule in the sample). The coloured product was quantified using a spectrophotometer. OD was plotted against concentration of cell adhesion molecule in the standard wells, was prepared. By comparing the OD of the samples to the standard curve, the concentration of cell adhesion molecule in the unknown samples was determined.

2.3.12 Measuring cytokines using ELISA

The cytokines IL-8, TNFα, IL-1β, GM-CSF, IL-1RA and IL-6 were measured by ELISA. The components required for these procedures were supplied in the Quantikine kits. This assay employed the quantitative immunoenzymometric sandwich technique. A monoclonal antibody specific for the cytokine was coated onto the microtiter plate provided in the kit. Standards with known amounts of the cytokine and the samples were pipetted into the wells and any cytokine present was bound by the immobilised antibody. Any unbound sample proteins were washed away and an enzyme linked polyclonal antibody specific for the cytokine was added to the wells and allowed to bind to the cytokine which was bound during the first incubation. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and a colour developed in proportion to the amount of cytokine which was bound in the initial step. The colour development was stopped and the intensity of the colour was measured using a spectrophotometer. A curve was prepared, plotting the optical density versus the concentration of the cytokine in the standard wells. By comparing the OD of the samples to the standard curve the concentration of the cytokines in the unknown samples was determined.

2.3.13 Expression of VCAM-2 and E-Selectin mRNA and protein expression on endothelial cells *in vitro* following treatment with IL-6

HUVECS were isolated as described in section 2.3.1 and were used on the first passage at confluence in 6-well cluster plates.

To assess IL-6 receptor expression on the cells an anti-human IL-6 receptor antibody was used at 1:1000 dilution. Immunostaining was detected using the ABC method with a Vectastain kit according to the manufacturers instructions.

mRNA: The growth medium was replaced with the same medium but with the horse serum omitted. This medium contained a range of IL-6 concentrations (0, 10,

100, 500, 1000U/ml) and was incubated with the cells for 4h at 37°C (The stock solution of IL-6 was made up in 0.1% BSA in DMEM). This timepoint was chosen since pilot experiments showed maximal induction at this time. The cytokine IL-1β was used at a concentration of 5U/ml as a positive control. A negative control was a well of cells which was incubated in the normal growth medium. The RNA was extracted as detailed in section 2.2.13. The VCAM-2 and E-Selectin mRNA were assessed by Northern blotting using a 1.9kb VCAM-2 cDNA and 1.2kb E-Selectin cDNA respectively.

ICC: The growth medium was replaced with the same medium as detailed for mRNA. The endothelial cells were treated with 500µl/ml IL-6 for 0, 4, 8, 24h. At the end of the incubation, the cells were fixed in acetone and stained using the peroxidase labelled streptavidin method (section 2.3.10). Again the primary antibody was the monoclonal mouse antibody human IgG1 subclass VCAM-1 (1:500) and E-Selectin (1:250). The negative controls were cells incubated with mouse monoclonal IgG1 Anti aspergillus niger glucose oxidase (section 2.3.10).

2.4 Statistics

The values for circulating concentrations of cell adhesion molecules were analysed using the Students *t*-test.

The bands on the autoradiographs showing the cell adhesion molecule expression in placentae were quantified with a scanning densitometer and an arbitrary densitometry unit was obtained for each band. The mean and SE arbitrary densitometry units were calculated for each cell adhesion molecule and compared using ANOVA.

The chi squared test was used to analyse the data for the intensity of staining of cell adhesion molecule expression on placenta sections.

The circulating cytokine concentrations were compared using the Mann-Whitney U test, whilst the clinical characteristics of the patients were compared using the unpaired Student *t* -test.

The chi squared test was used to analyse the densitometry units obtained for the bands on the autoradiograph from the experiments involving the treatment of HUVECs with IL-6.

CHAPTER THREE

RESULTS

3.1 Circulating concentrations of cell adhesion molecules in normal and pre-eclamptic pregnancies

Circulating concentrations of cell adhesion molecules are thought to be indicative of endothelial expression and also endothelial damage. Increased expression of cell adhesion molecules is also thought to be associated with leukocyte activation (Harlan and Liu, 1992). Soluble forms of cell adhesion molecules are detectable in normal serum and plasma.

The objective of this study was to measure soluble cell adhesion molecule expression in serum and plasma from normal pregnant women and in women with pre-eclampsia to determine whether circulating cell adhesion molecules, indicating cell adhesion molecule expression on the endothelium, might reflect a feature of endothelial damage in pre-eclampsia and so contribute to neutrophil activation.

3.1.1 Patient details

Sixteen women with pre-eclampsia and 18 women with uncomplicated pregnancies were studied. None of the women had any evidence of urinary tract infection or chorioamnionitis and none were close to labour at the time of sampling. Venous blood samples were obtained from the patients and serum was prepared as described in methods. The patients were matched for maternal age and gestational age at the time of sampling (Table 1).

3.1.2 Cell adhesion molecules serum results

The cell adhesion molecules VCAM-1, E-Selectin and ICAM-1 were measured in maternal serum by ELISA. The pre-eclamptic patients had significantly higher concentrations of the cell adhesion molecule VCAM-1 compared to the control group (Table 2). However there were no significant differences in the adhesion molecules ICAM-1 or E-Selectin in pre-eclampsia compared to normal pregnancy (Table 2).

3.1.3 Follow up study

Susequent to this work, a study by Fickling et al (1995) suggested that E-Selectin was significantly increased in patients with pre-eclampsia compared to normal pregnant and non-pregnant patients. We therefore performed a follow up study to determine whether plasma and serum concentrations of E-Selectin were similar and could the differences in results be explained by whether serum or plasma was used. By the time this follow up

study was performed we had increased the number of blood samples collected for our study groups.

3.1.4 Patient details

The number of patients in this study were increased to 32 pre-eclamptics and 30 normal pregnant women. Venous blood samples were obtained from all patients and plasma was prepared as described in the methods chapter.

3.1.5 Cell adhesion molecule plasma results

E-Selectin concentrations were significantly higher in the plasma samples from women with pre-eclampsia compared to the normal pregnant controls (Table 3). Values for E-selectin were found to be lower in plasma when compared with the serum samples. When we analysed the E-Selectin concentrations in serum from the women recruited in the original study (16 pre-eclamptics and 18 normal pregnant women) there were no significant differences between the two groups.

Variable	Normal	Pre-eclampsia
	n = 18	n = 16
Age (years)	23 ± 3	26 ± 6
Gestational age at sampling (weeks)	31 ± 3	33 ± 4
Platelet count (x 10^9/l)	n/a	220 ± 57
Plasma urate (mmol/l)	n/a	0.36 ± 0.06

Table 1. Characteristics of primigravid women with and without pre-eclampsia.

Data are shown as mean ± SD values.

Concentration of cell adhesion molecule (ng/ml)	Normal n = 18	Pre-eclampsia n = 16
VCAM-1	560.2 ± 47.9	841.9 ± 49.7*
ICAM-1	187.3 ± 15.8	148.8 ± 11.9
E-Selectin	45.8 ± 4.6	49.8 ± 7.2

*P<0.001

Table 2. Cell adhesion molecule concentrations in serum from women with and without pre-eclampsia

Mean (SE) values were compared by Student's *t* test.

Concentration of cell adhesion molecule (ng/ml)	Normal n = 30	Pre-eclampsia n= 32
E-Selectin	29.11 ± 2.84	41.90 ± 2.76

Table 3. E-Selectin concentrations in plasma samples from women with and without pre-eclampsia. Data is shown as mean ± SE.

P <.005 compared to normal pregnant group.

3.2 Cell adhesion molecule mRNA and protein expression in placentae from normal pregnant patients compared to patients with pre-eclampsia or IUGR

Our work leading up to this study had shown that VCAM-1 and E-Selectin were increased in the maternal circulation of women with pre-eclampsia. Although previous data had suggested that neutrophil activaton was confined to the maternal circulation in pre-eclampsia we carried out experiments to determine if there were altered cell adhesion molecules on endothelial cells, trophoblasts or decidua in placenta from women with pre-eclampsia. Such expression on trophoblast or decidua could explain the neutrophil activation, placental damage and increased neutrophil content of decidua in pre-eclampsia.

3.2.1 Patient details

Placentae from 15 pregnancies complicated by pre-eclampsia (gestation at delivery 34.3 ± 0.85 SE weeks), 10 pregnancies complicated by pre-eclampsia and IUGR (gestation at delivery 32.97 ± 0.72 SE weeks) and 7 pregnancies complicated by IUGR (gestation at delivery 31.18 ± 2.9 SE weeks) were matched for gestation with normal placentae from pregnancies delivered early for reasons such as placenta praevia, previous caesarean section and breech presentation (gestation at delivery 34.6 ± 0.86 SE weeks). All the patients were matched for maternal age, parity and smoking habit. None of the women in the study had evidence of urinary tract infection or chorioamnionitis.

3.2.2 mRNA expression of cell adhesion molecules in placentae

Northern analysis of cell adhesion molecule mRNA expression in normal placentae is shown in plate 3. PECAM mRNA was the most abundant species present. Numerous species of PECAM were detectable, the two major transcripts being approximately 3.5 and 3.7 kb. ICAM -1 cDNA was expressed as two bands, one at 3.3kb and another weaker band at 2.4kb. The expression of ICAM-2 was higher than ICAM-1, the cDNA hybridising to a 1.4kb mRNA transcript. Both the ICAM-1 bands and the ICAM-2 band have previously been reported to be present in cultured endothelial cells by Staunton, Dustin and Springer (1989). The 1.2kb E-Selectin and 1.9kb VCAM-2 mRNA were both undetectable by northern blotting in placentae from all normal placentae. When comparing the expression of all the cell adhesion molecules mRNAs in placentae from pregnancies complicated by pre-eclampsia, IUGR or pre-eclampsia and IUGR to placenta from normal pregnancies, there were no significant differences (Table 4).

3.2.3 Cell adhesion molecule protein expression in placentae

Strong immunostaining for PECAM was apparent throughout the vascular tree in normal placentae. Strong staining was localized to the endothelium of stem villi (plate 4A), the intermediate villi (plate 4B) and the terminal villi (plate 4B). PECAM was also found in the endothelium of the decidual vessels. Staining was not apparent in the fetal membranes. Like PECAM, ICAM-1 staining was scattered throughout the vascular tree, again in the endothelium of the stem villi, intermediate villi and terminal villi (plate 4C and4D). Faint staining occurred in the stroma, perhaps indicating macrophage staining (plate 4D). Otherwise there was no extravascular expression in trophoblast or decidua. The endothelium of all the vessels were negative for both VCAM-1 and E-selectin immunostaining(plate 4E and 4F respectively). There were no significant staining differences in the immunostaining for PECAM, ICAM-1, VCAM or E-Selectin in placentae complicated by pre-eclampsia or IUGR (Table 5).

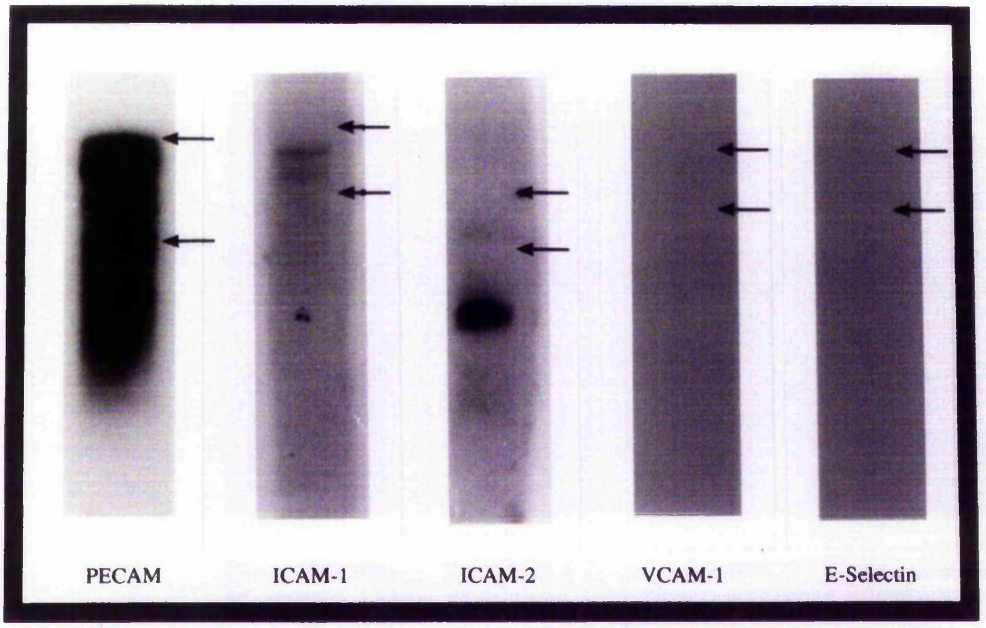


Plate 3. Northern analysis of cell adhesion molecule expression in placentae. RNA was extracted from the placentae, run on agarose gels and blotted onto nylon membranes. The membranes were then hybridised with a cDNA probe as described in sections 2.2.13 - 2.2.19, washed to a final stringency of 0.5 x SSC at 65^{o}C. Autoradiography was performed with Fuji x-ray film overnight at -70^{o}C. The 28S and 18S rRNA bands are marked by the arrows.

	Control (n = 10) Densitometry units	Pre-eclampsia (n = 15) Densitometry units	Pre-eclampsia + IUGR (n = 10) Densitometry units	IUGR (n = 7) Densitometry units
PECAM	9.7 ± 1.3	9.3 ± 0.97	9.1 ± 1.1	8.9 ± 0.5
ICAM-2	8.5 ± 0.8	8.8 ± 0.8	8.3 ± 0.5	8.7 ± 0.7
ICAM-1 (3.3kb)	2.2 ± 0.4	2.4 ± 0.4	2.2 ± 0.5	2.6 ± 0.6
ICAM-1 (2.3kb)	1.5 ± 0.2	1.5 ± 0.2	2.0 ± 0.5	1.8 ± 0.4
VCAM	0	0	0	0
E-Selectin	0	0	0	0

Table 4. Scanning densitometer analysis of cell adhesion molecule expression in placentae.

Bands on the autoradiographs were quantified with a scanning densitometer and an arbitrary densitometry unit was obtained for each band. The mean and SE arbitrary densitometry units were calculated for each cell adhesion molecule and compared using ANOVA.

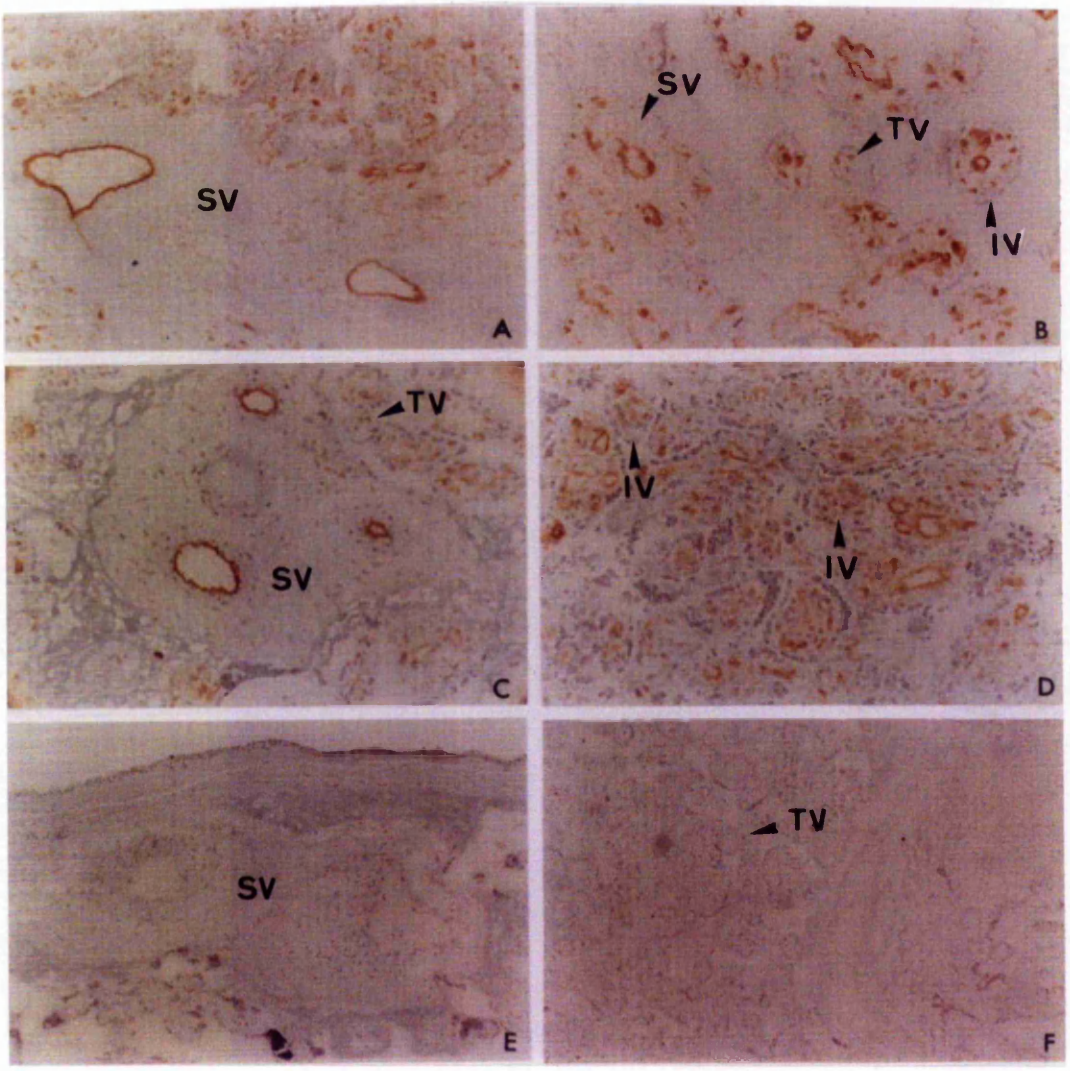


Plate 4. Photomicrographs of the cell adhesion molecule immunostaining in the vascular tree of a placenta from a normal pregnancy. PECAM staining was localized to the endothelium of the stem villi (SV)[A] , the intermediate villi (IV)[B] and the terminal villi (TV)[B]. Like PECAM, ICAM-1 was scattered throughout the vascular tree, in the endothelium of the stem villi, intermediate villi and terminal villi (C and D). ICAM-1 staining also occurred in the stroma (D). The endothelium of all the vessels was negative for both VCAM-1 and E-Selectin (E and F respectively).

Diagnosis	Normal	Pre-eclampsia	Pre-eclampsia + IUGR	IUGR
No. samples	10	15	10	7
No. fields scored per section	20	20	20	20
Endothelial staining				
PECAM	+	+	+	+
ICAM	+	+	+	+
E-Selectin	-	-	-	-
VCAM	-	-	-	-
Stromal staining				
PECAM	-	-	-	-
ICAM	+	+	+	+
E-Selectin	-	-	-	-
VCAM	-	-	-	-
Mean intensity endothelial staining				
PECAM	+++	+++	+++	+++
ICAM	+++	+++	+++	+++
Mean intensity of stromal staining				
ICAM	+	+	+	+

Table 5. Immunolocalization of cell adhesion molecules in placentae.

Twenty fields were examined for each section by an investigator blinded to the tissue identity and scored for location of staining of the endothelium or stroma (+ or -) and for the intensity of the staining (+, ++ or +++). Statistical analysis was performed using the χ^2 test. The mean scores for each group are shown.

3.3 Circulatory cytokines in normal pregnancy and pre-eclampsia

Our previous studies, including the demonstration of increased cell adhesion molecules, suggested that neutrophil activation occurs in pre-eclampsia. Leukocytes (including neutrophils) are stimulated and inhibited by a large number of agents, including cytokines which may act directly on neutrophils or indirectly via stimulation of cell adhesion molecules. In this study, we aimed to determine whether there was evidence of increased circulating concentrations of candidate cytokines (i.e. those known to activate neutrophils) in plasma from women with pre-eclampsia.

3.3.1 Patient details

Twenty primigravidae with pre-eclampsia and 15 primigravidae with uncomplicated pregnancies were studied. None of the women had any evidence of urinary tract infection or chorioamnionitis and none were close to labour at the time of sampling. Venous blood samples were obtained from all patients and plasma was prepared as described before. The patients were matched for gestational age. Maternal age, gestation at the time of sampling, gestation at delivery, birthweight and placental weight were compared using the unpaired Student *t* test (Table 6). There were no significant differences between the groups studied in terms of maternal age, parity and gestational age at the time of blood sampling. However, the mean gestational age at delivery, the mean birthweight and the mean placental weight were all significantly lower in the pre-eclamptic group compared to the normal group.

3.3.2 Cytokine results

The concentration of the cytokines IL-6, Il-1ra, TNFα, IL-8, GM-CSF and IL-1β in plasma are shown in table 7. Interleukin-6 concentrations were significantly higher in the patients with pre-eclampsia compared to the normal patients ($P < 0.01$), as were the concentrations of IL-1ra ($P < 0.01$). For the other cytokines measured there were no significant differences between pre-eclamptics compared to normal patients. The concentration of cytokines detected in all the samples was above the lower limits of detection of the assays.

Variable	Normal	Pre-eclampsia
Number of primigravidae	15	20
Age (years)	24.6 ± 4.5	29.5 ± 7.4
Gestational age at sampling (weeks)	32.6 ± 3.7	33.05 ± 3.7
Gestational age at delivery (weeks)	39.2 ± 2.2	33.3 ± 3.5*
Placental weight (g)	731.83 ± 170.51	427.36 ± 173.45*
Birth weight (kg)	3.51 ± 0.6	1.83 ± 0.87*
Platelet count ($x10^9$/L)	-	241 ± 66
Plasma urate (mmol/L)	-	0.36 ± 0.07
Number < 5th percentile	0	8

Table 6. Clinical characteristics of women with and without pre-eclampsia.

Data are presented as mean ± standard deviation. * $P < .0001$ compared with the normal pregnant group (unpaired Student *t*-test).

Cytokine	Cytokine levels (pg/ml) in control subjects n = 15	Cytokine levels (pg/ml) in pre-eclamptic subjects n = 20
IL-6	2.06 (1.44 - 3.63)	2.56 (1.48 - 5.06)*
Il-1ra	142.00 (36.84 - 603.10)	251.85 (96.25 - 806.50)*
TNFα	11.96 (9.6 - 16.39)	14.09 (8.33 - 41.06)
Il-8	44.46 (34.90 - 67.86)	50.52 (37.71-60.28)
GM-CSF	121.3 (90.67-167.6)	125.8 (86.12 - 183.2)
IL-1β	2.01 (1.64 - 2.62)	2.08 (1.52 - 3.19)

Table 7. Cytokine concentrations in plasma from women with and without pre-eclampsia.

Data are presented as mean cytokine concentration (range).* $P < .01$ compared with the normal pregnant group (Mann-Whitney U test).

3.4 Expression of VCAM protein and mRNA on HUVECS *in vitro* following treatment with IL-6.

Our previous experiments have shown that both IL-6 and VCAM-1 and E-Selectin are increased in pre-eclampsia. These data raised the possibility that IL-6 may, at least in part, be responsible for the increased expression of cell adhesion molecules seen in this condition. The aim of the next set of experiments was to determine whether IL-6 had an effect on the expression of the cell adhesion molecules VCAM and E-Selectin on human umbilical vein endothelial cells.

Firstly we established that endothelial cells expressed IL-6 receptors. Immunocytochemistry was carried out in cultured endothelial cells with an IL-6 receptor antibody. To confirm that the results obtained were not due to the *in vitro* conditions, parallel experiments were carried out on cryosections of normal placenta. Plate 5 shows that both endothelial cells *in vitro* and endothelial cells in the vessels of the placenta stained positively for the IL-6 receptor antibody.

3.4.1 Protein VCAM-1 results

The VCAM-1 protein was almost undetectable on untreated HUVECS. Following incubation with 500U/ml IL-6 (which produced maximum effect), there was a significant increase in VCAM-1 immunostaining at 4h which remained elevated at 8h and declined by 24h. By 24h, the VCAM-1 protein expression was comparable to that at 4h (plate 6). No staining was detected on the negative controls. The same results were achieved in 3 separate experiments with 3 different cell cultures.

3.4.2 mRNA VCAM-2 results

Unstimulated HUVECS expressed little or no VCAM-2 mRNA. Following treatment with 10U/ml, 100U/ml, 500U/ml, and 1000U/ml IL-6 for 4h, there was a dose dependent increase in VCAM-2 mRNA expression which reached maximal levels at 1000U/ml (plate 7). 3 separate experiments showed the same results.

3.5 Expression of E-Selectin protein and mRNA on HUVECS *in vitro* following treatment with IL-6

3.5.1 Protein E-Selectin results

The E-Selectin protein was undetectable on untreated HUVECS. Following incubation with 500U/ml IL-6, there was a significant increase in E-Selectin protein expression at 4h which remained elevated at 8h and declined by 24h, with maximum expression at 4-8h (plate 8). A maximum effect was found with 1000U/ml IL-6. No staining was detected on the negative controls. The same results were achieved on 3 separate occasions.

3.5.2 mRNA E-Selectin results

E-Selectin mRNA expression was undetected on untreated HUVECS. After treatment with 10U/ml, 100U/ml, 500U/ml and 1000 U/ml IL-6 for 4h, there was a dose dependant increase in E-Selectin mRNA expression, which reached maximal levels at 500U/ml (plate 9). 3 separate experiments showed the same results.

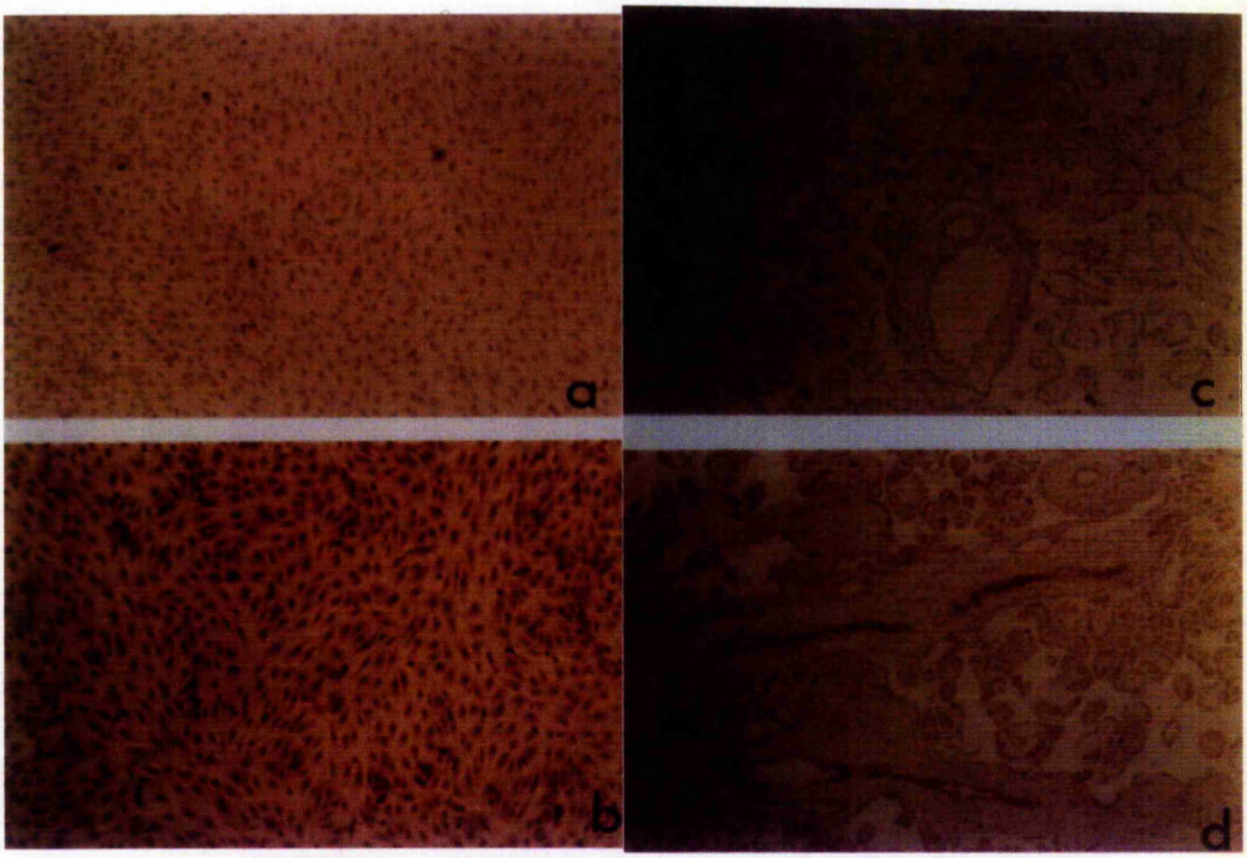


Plate 5. Endothelial cells express IL-6 receptors both *in vivo* and *in vitro* . Immunocytochemistry was performed as described in section 2.3.13. Immunolocalization of IL-6 receptor protein on HUVECS *in vitro* is shown in plate (b) and on a term human placenta cryosection in plate (d). Plates (a) and (c) show *in vitro* and *in vivo* immunostaining respectively when the primary antibody was omitted.

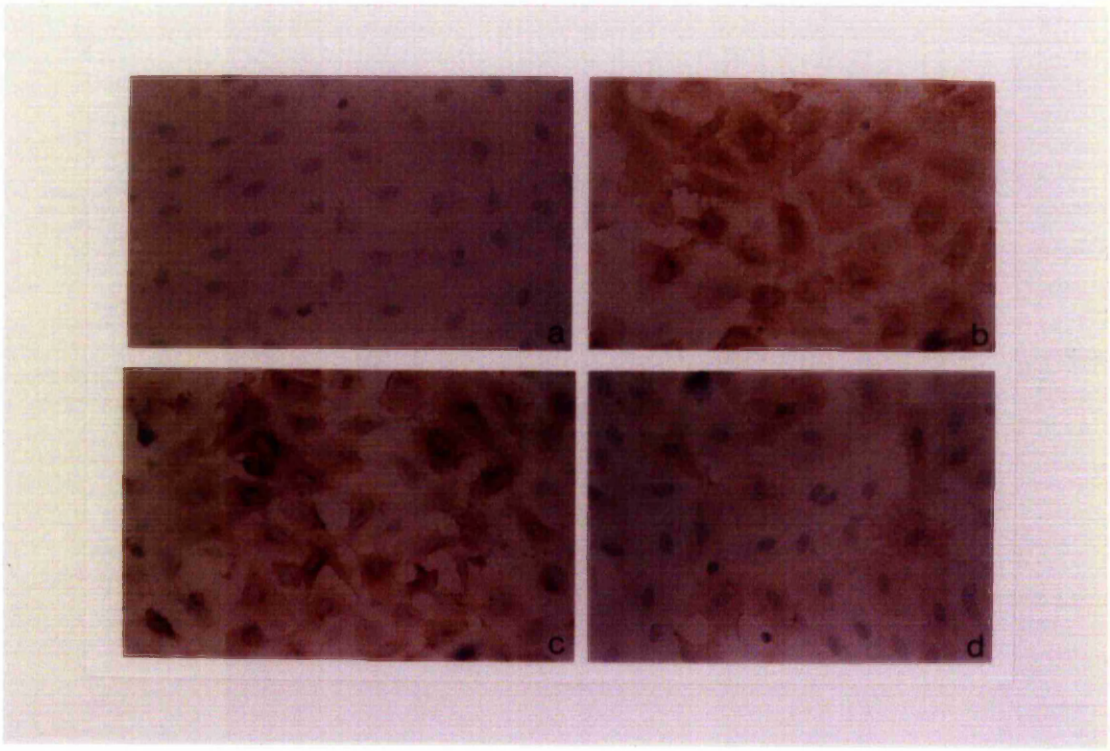
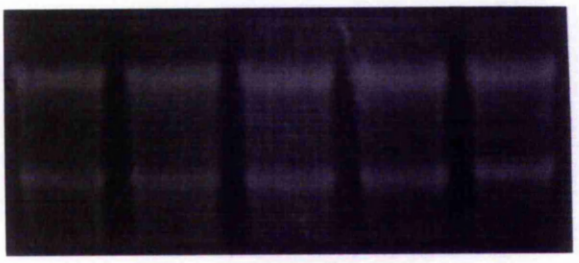
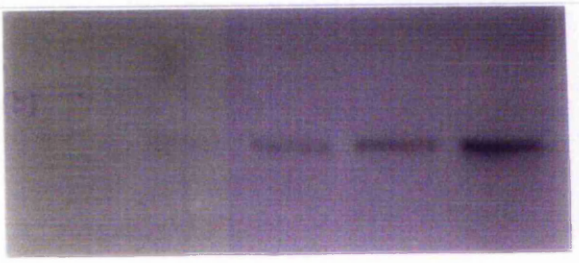


Plate 6. Immunocytochemistry was performed as described in section 2.3.13. These photographs show VCAM-1 immunostaining on HUVECS after incubation with 500U/ml IL-6 at time 0 (a), 4h (b), 8h (c), and 24h (d).

(a)



(b)



0 10 100 500 1000 U/ml

(c)

IL-6 U/ml	0	10	100	500	1000
Densitometry units	0*	0.85 ± 0.2*#	3.4 ± 0.5#‡	6.3 ± 1.2‡	8.5 ± 1.1

* $P < 0.05$

\# $P < 0.05$

‡ $P < 0.05$

Plate 7. A photograph of a gel showing even loading of RNA (10µg/lane) and intact 28S and18S rRNA bands(a). A representative autoradiograph showing VCAM-2 mRNA expression in HUVECS after incubation with 10U/ml, 100U/ml, 500U/ml and 1000U/ml IL-6 for 4h(b). After incubation with IL-6 the RNA was extracted from the cells, run on an agarose gel and blotted onto a nylon membrane. The membrane was hybridised with a 1.9kb VCAM-2 cDNA probe as described in section 2.2.17 and then washed to a final stringency of 0.1 x SSC at 65°C. The autoradiograph was exposed to Fuji x-ray film for 3 days at -70°C. Bands on the autoradiographs were quantified with a scanning densitometer and an arbitrary densitometry unit was obtained for each band (c). The mean and SD densitometry units are shown for 3 separate experiments.

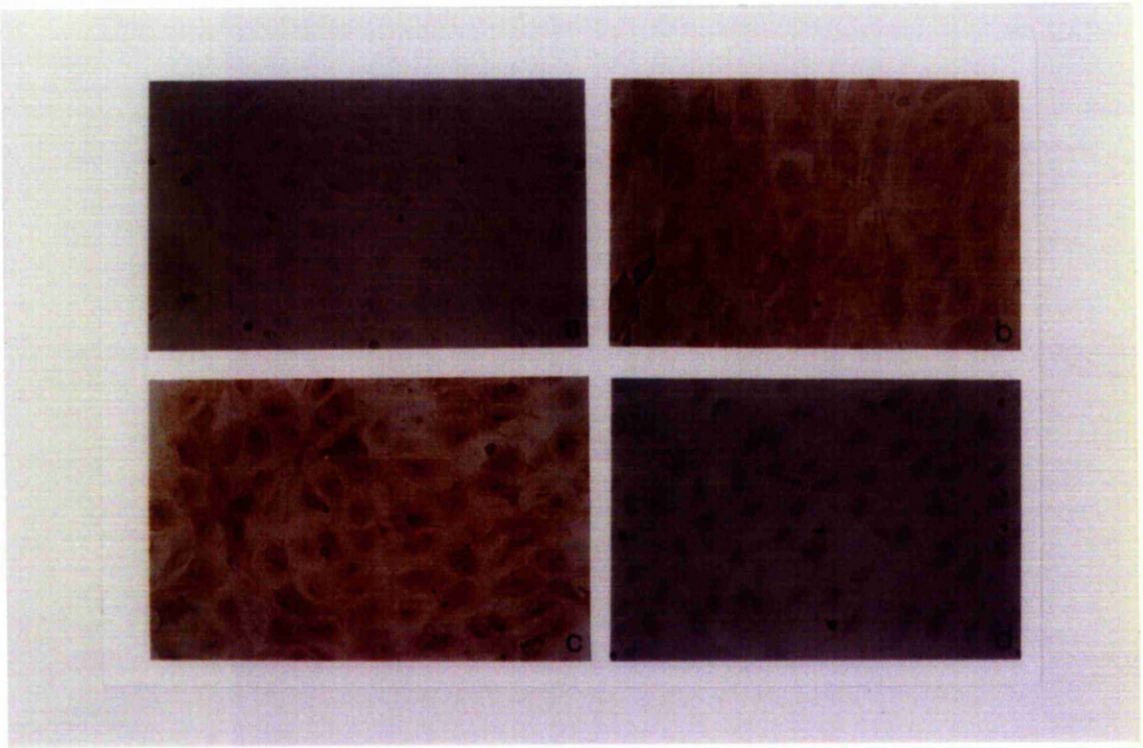
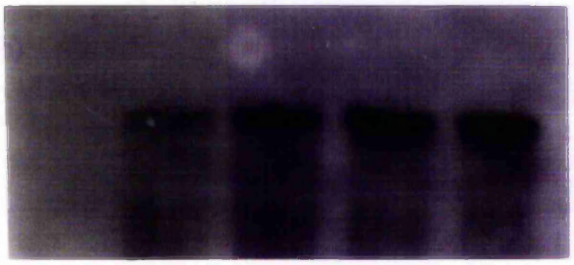


Plate 8. Immunocytochemistry was performed as described in section 2.2.13. These photographs show E-selectin immunostaining on HUVECS after incubation with 500U/ml IL-6 at time 0 (a), 4h (b), 8h (c) and 24h (d).

(a)

(b)



0 10 100 500 1000 U/ml

c.

Il-6 U/ml	0	10	100	500	1000
Densitometry units	0*	4.1 ± 0.1*#	8.5 ± 0.7#	9.1 ± 1.1	9.7 ± 0.5

* $P < 0.05$

\# $P < 0.05$

Plate 9. A photograph of a gel showing even loading of RNA (10µg/lane) and intact 28S and18S rRNA bands (a). A representative autoradiograph showing E-Selectin mRNA expression in HUVECS after incubation with 10U/ml, 100U/ml, 500U/ml and1000U/ml IL-6 for 4h (b). After incubation with IL-6 the RNA was extracted from the cells, run on an agarose gel and blotted onto a nylon membrane. The membrane was hybridised with a 1.2kb E-Selectin cDNA probe as described in section 2.2.17 and then washed to a final stringency of 0.1 x SSC at 65°C. The autoradiograph was exposed to Fuji x-ray film for 3 days at -70°C. Bands on the autoradiographs were quantified with a scanning densitometer and an arbitrary densitometry unit was obtained for each band (c). The mean and SD densitometry units are shown for 3 separate experiments.

CHAPTER FOUR

DISCUSSION

4.1 Introduction to discussion

Endothelial damage and dysfunction are common to all the pathophysiological features of pre-eclampsia. Increased circulating concentrations of cell adhesion molecules and cytokines may indicate leucocyte-endothelial attachment and activation resulting in damage to the endothelium.

4.2 Cell adhesion molecules in pre-eclampsia

4.2.1 VCAM

VCAM-1 is a 90-110kDa glycoprotein expressed on the surface of activated endothelium and on a variety of other cell types, including tissue macrophages and bone marrow fibroblasts. The maternal serum, from the women with pre-eclampsia we studied, had significantly higher levels of soluble VCAM-1 compared to the control group. This may reflect increased expression of VCAM on the endothelium and perhaps represents a possible mechanism by which leucocytes are attached to the endothelium, where they contribute to the endothelial dysfunction seen in pre-eclampsia (Lyall et al, 1994). VCAM-1 may be cleaved or shed from the cell surface but the mechanism by which this occurs is not known. VCAM-1 therefore may be upregulated by a mechanism which is triggered by an effective inflammatory response in the maternal circulation in pre-eclampsia. No evidence of VCAM-2 mRNA or protein expression was found in the placentae of women with pre-eclampsia, IUGR or control pregnant women. This suggests that increased VCAM expression may be confined to the maternal circulation in pre-eclampsia. Feng et al (1996) found higher levels of soluble VCAM-1 in cord blood compared to maternal blood in uncomplicated term gestations in labour. This may be a consequence of the process of labour which is itself an inflammatory process. This might also be the mode of leucocyte activation described by Greer et al (1991a).

4.2.2 ICAM-1 and ICAM-2

ICAM-1 and ICAM-2 bind the leucocyte beta2 integrin, LFA-1, via the immunoglobulin domains 1 and 2. ICAM-2 consists only of immunoglobulin domains 1 and 2, so closer cell-cell contact is required for ICAM-2 binding whilst ICAM-1 also binds to Mac-1 via domain 3 (another beta2 integrin on leucocytes) (Zimmerman et al, 1992). In our study there were no significant differences in the serum levels of soluble ICAM-1 between women with pre-eclampsia and normal pregnant women, which agrees with a study by Fickling et al (1995). In the placenta, we found weak expression of ICAM-1 mRNA in two bands, whilst a stronger ICAM-2 mRNA band was expressed. Since ICAM-1 is

induced on endothelial cells by certain cytokines, and ICAM-2 is expressed constitutively on endothelial cells, monocytes and lymphocytes (Staunton et al, 1989) and is not affected by cytokines, we would expect the expression of ICAM-2 to be greater. ICAM-2 expression was greater than ICAM-1 expression in each of the groups we studied. ICAM-2 may play a role in normal lymphocyte recirculation so strong expression would be expected in normal placentae. ICAM-1 staining was found in the endothelium of the stem villi, intermediate villi and terminal villi and also in the stroma (Lyall et al, 1995a). Labarrerre and Faulk (1994) found ICAM-1 expressed on endovascular cytotrophoblasts in abnormal pregnancies (including women with pre-eclampsia, IUGR and women with a history of secondary recurrent spontaneous abortion) but not in placentae from normal pregnancies. ICAM-1 and ICAM-2 have established roles in endothelium/neutrophil and endothelium/platelet interactions. Neutrophils and platelets are activated in pre-eclampsia but there was no increase in expression of these cell adhesion molecules in the maternal serum or in the placenta from patients with pre-eclampsia. Thus ICAM-1 and ICAM-2 are unlikely to be significant factors in the neutrophil activation and endothelial dysfunction of pre-eclampsia.

4.2.3 E-Selectin

E-Selectin, a member of the Selectin family of adhesion molecules, has a molecular weight of 95-115kDa. E-Selectin expression is protein synthesis-dependent, peaking after 4-6 hours stimulation by the cytokines IL-1 or TNFα and declining to basal levels by 24 hours (Needham et al, 1991). In our study, there were no significant differences in the serum levels of soluble E-Selectin between women with pre-eclampsia and normal pregnant women. However, our follow-up study, with an increase in patient numbers found that soluble E-Selectin plasma concentrations were significantly higher in women with pre-eclampsia. The differences in E-Selectin concentrations between plasma and serum may be due to the serum containing no coagulant, which could be important for the expression of E-Selectin.

We found no evidence of E-Selectin mRNA or protein expression in the placentae studied. Although we have shown an increase in soluble E-Selectin in the maternal circulation in pre-eclampsia there was no increase in this cell adhesion molecule in the placentae from patients with pre-eclampsia or IUGR. E-Selectin may therefore play a role in the recruitment and subsequent activation of neutrophils in the maternal circulation in pre-eclampsia with expression in the maternal circulation rather than in the placenta. Austgulen et al (1997) found that maternal plasma levels of soluble ICAM-1, soluble VCAM-1 and soluble E-Selectin were increased in pre-eclamptic pregnancies. The changes in the soluble cell adhesion molecule concentrations of VCAM-1 and E-Selectin in our study support the idea that changes in endothelial cell function are important in the

aetiology of pre-eclampsia.

4.2.4 PECAM

PECAM is a 120-130KDa glycoprotein expressed in large amounts on endothelial cells at intercellular junctions, and on T cells. Recently PECAM has been shown to play an important role in the extravasation of natural killer cells into sites of inflammation (Berman et al, 1996). It is expressed in smaller amounts on platelets and most other leucocytes, including monocytes and neutrophils (Vaporciyan et al, 1993) and is required for transendothelial migration of leucocytes through intercellular junctions of vascular endothelial cells (Muller et al, 1993). In this study, numerous species of PECAM mRNA were detected and PECAM protein expression was found constitutively on the endothelium of the stem villi, intermediate villi and terminal villi as well as the endothelium of the decidual vessels. PECAM was not seen in the fetal membranes. PECAM has an established role in endothelium/neutrophil and endothelium/platelet interactions, but there was no evidence of increased expression of PECAM in the placentae from patients with pre-eclampsia or IUGR. Because PECAM was found in abundance in control placentae, it is possible that the immunocytochemistry and Northern blotting methods were not sensitive enough to show a difference between control and pre-eclamptic placentae. Measuring each species of PECAM mRNA may have shown a difference between the two groups. However, the abundant expression in normal pregnancy suggests that PECAM expression is not a major factor in the pre-eclamptic process.

Ruck et al (1994) investigated first trimester human decidua, from normal pregnant women, for cell adhesion molecules involved in the interactions between the various maternal and fetal cell populations. ICAM-1 and VCAM-1 staining was observed in the stromal cells. Both PECAM and ICAM-1 were observed on the endothelium of a large number of blood vessels whilst VCAM-1 stained positive on the endothelium of arterioles, venules and a few capillaries. Weak E-Selectin staining was seen in a few arterioles and venules on the endothelium. In our study we found PECAM and ICAM-1 on the endothelium of decidual vessels but found no VCAM-1 or E-Selectin staining in the trophoblast or stromal tissue. Ruck et al (1994) found positive staining for both E-Selectin and VCAM-1 in first trimester decidua whilst we found no VCAM-1 or E-Selectin staining in term placentae suggesting that there may be a mechanism that downregulates these cell adhesion molecules in the later stages of normal pregnancy.

There were no differences in cell adhesion molecule immunostaining or mRNA expression in placentae from pregnancies complicated by pre-eclampsia or IUGR. These results suggest that cell adhesion molecules have a normal physiological role in the fetal circulation which does not alter in pregnancies complicated by pre-eclampsia or IUGR.

4.3 Cytokines

Our previous work has shown that the cell adhesion molecule VCAM-1 is elevated in the serum of women with pre-eclampsia. Increased expression of adhesion molecules by the endothelium could be responsible for the neutrophil activation which occurs in pre-eclampsia. Cytokines could be responsible for features of endothelial dysfunction, including neutrophil activation and certain cytokines stimulate endothelial cells from their normal anticoagulant state to a procoagulant state with increased adhesiveness for leucocytes and platelets to the endothelium. Cytokines contribute to the maintenance of normal pregnancy. TNFα and GM-CSF probably influence trophoblast growth and differentiation in early pregnancy since their receptors are present on trophoblasts (King et al,1995). Burrows et al (1994) suggested that during implantation the vascular invasion by trophoblasts was regulated by the expression of the appropriate adhesion molecules, which permitted interaction between endovascular trophoblast and decidual endothelial cells. GarciaLloret et al (1994) found that placental fibroblasts underlying the trophoblast epithelium produced GM-CSF and CSF-1 and suggested that a network of cytokines played a significant role in the morphological and functional development of the human placenta. We know that in pre-eclampsia the primary invasion of the spiral arteries in the uteroplacental bed is impaired. Cytokines are involved in cytotrophoblast differentiation in normal pregnancy so perhaps a disturbance in the cytokine concentration has a detrimental effect on the differentiation process, resulting in the development of pre-eclampsia. We found significantly higher concentrations of the cytokines IL-6 and IL-1ra in patients with pre-eclampsia compared to normal pregnant patients. There were no significant differences in the concentrations of TNF-α, IL-8, GM-CSF and IL-1β between the two groups.

4.3.1 IL-1/IL-1ra

IL-1 is a polypeptide produced during infection, injury or immunologic challenge. There are two molecular forms IL-1α and IL-1β and despite only a 26% amino acid homology both forms induce a wide variety of biological changes (Dinarello & Savage, 1989). Acute atherosis occurs in the spiral arteries of the placental bed in pre-eclampsia, which may be a result of IL-1 targetting endothelial cells and contributing to the development of these atherosclerotic lesions (Dinarello et al, 1993). The potent effects of IL-1α and IL-1β are modulated by the naturally occurring inhibitor IL-1ra (Dinarello et al, 1991b). IL-1ra has been cloned, it is produced in recombinant organisms, and is thought to play an important role in the normal physiological or pathophysiological states by functioning as a natural IL-1 receptor antagonist (Arend et al, 1990). Inhibition of the cleavage step is

required to generate an active form of IL-1 is a potential mechanism for reducing IL-1 activity (Cerretti et al, 1992). IL-1ra competes with the binding of IL-1 to its cell surface receptors (Seckinger et al, 1987) but does not result in signal transduction. A 50% inhibition of IL-1 induced biological responses requires amounts of IL-1ra up to 100 fold in excess of the amounts of IL-1α or IL-1β present. IL-1ra may be potentially useful as a therapeutic agent for the treatment of IL-1 mediated diseases in humans (Eisenberg et al, 1990). IL-1ra administration to animals significantly reduces the severity of disease. IL-1ra can block endotoxin-induced IL-8 production which may be a major component of the anti-inflammatory property of IL-1ra (Porat et al, 1992). The increase in IL-1ra, in the present study may be due to the upregulation of IL-1ra to reduce the inflammation, mediated by IL-1, in the peripheral circulation in pre-eclampsia. IL-1ra may be blocking the activity of IL-1. As IL-1ra is approximately 100 fold less effective at binding to IL-1 receptor, substantially higher quantities are required to compete with the small concentration of IL-1. As IL-1 has a local effect, and we measured systemic concentrations, the difference in concentration may be too low to be picked up by the assay. IL-1ra may therefore act as a surrogate measure of IL-1β as well as being an important factor in antagonising the effect of IL-1.

4.3.2 IL-6/TNFα

IL-6 may be an important regulator of the host defence response and its production by endothelial cells may contribute to the pathogenesis of various inflammatory and immunologic diseases. Gunn et al (1996) found that in normal pregnancy the concentration of IL-6 in amniotic fluid and fetal membranes increased after the onset of labour suggesting that during labour the fetal membranes are the main source of IL-6. Decreased levels of IL-6 were found in the amniotic fluid of patients with pre-eclampsia suggesting a role for IL-6 in the pathophysiology of pre-eclampsia (Silver et al, 1993). Increased circulating concentrations of IL-6 may be a consequence of increased TNF production (Jirik et al, 1989). IL-6 is involved in the modulation of TNF production (Akira et al, 1993) and stimulation of TNF-R shedding (Aderka et al, 1993). There are two specific receptors for TNF which have molecular weights of 55kDa and 75kDa, called p55TNF-R and p75TNF-R (Tartaglia and Goeddel, 1992). By binding circulating TNF, the soluble receptors are thought to prevent target cell activation and provide a protective mechanism against TNF overproduction (Aderka et al, 1993). *In vivo* soluble TNF-R may play a role in modulating the activity of TNF, since each receptor is shed as a soluble protein that specifically binds to TNF, inhibiting its activity (Van Zee et al, 1992). The source of the circulating p55TNF-R and p75TNF-R is not known but Porteau and Nathan (1990) suggested that the receptors may be derived from activated neutrophils. This would agree with reports that activated neutrophils occur in pre-

eclampsia, resulting in increased TNF-R concentrations in the disease state. There is increased expression of TNFα mRNA in leucocytes from patients with pre-eclampsia which may be associated with the TNF 1 allele whose frequency is markedly increased in pre-eclamptic patients. A disturbance of TNFα may therefore contribute to the endothelial damage seen in pre-eclampsia (Chen et al, 1996). Wang and Walsh (1996b) found increased concentrations of TNFα protein and TNFα mRNA in pre-eclamptic placentae compared to controls. They suggested that the increase of TNFα was associated with increased lipid peroxidation since TNFα induces oxygen free radicals which are involved in the initiation of lipid peroxidation. Meekins et al (1994) suggested that TNFα does not contribute to the initiation of endothelial cell activation that may be associated with the development of pre-eclampsia but may arise as a consequence of the pathological processes of pre-eclampsia. Meekins (1995) confirmed this hypothesis by showing that TNFα was increased in women with pre-eclampsia, only after the disease had been diagnosed. TNFα was not increased in patients who were sampled before they developed pre-eclampsia compared to normal pregnant women. Kupferminc et al (1996) found increased levels of soluble TNF-R in amniotic fluid and maternal plasma samples from patients with severe pre-eclampsia which returned to normal control levels after 24 hours post partum. They found increased IL-6 concentrations in maternal plasma before the onset of labour, again providing a link between increased levels of TNF and IL-6, both contributing to endothelial cell dysfunction. Opsjon et al (1995) found an increase in p55TNF-R in the maternal serum from patients with pre-eclampsia before the initiation of labour compared to the normal pregnant controls. Vince et al (1995) found a significant correlation between the levels of IL-6 and TNF in pre-eclamptic patients and suggested that excess release of TNF may play a role in the pathology of pre-eclampsia. TNF was not consistently detected in the plasma from patients with pre-eclampsia in Vince's study. However, differences in the circulating receptors were more obvious. Measuring TNF receptors may therefore be a better marker of excessive TNF release *in vivo* rather than measuring TNF itself. The strong correlation between IL-6 levels and levels of TNF receptors suggest a complex interaction among different cytokines and their receptors in the development of pre-eclampsia. Although we found increased concentrations of IL-6 in our pre-eclamptic patients we found no significant increase in TNFα production. However we did not measure the TNF receptor concentrations in our patients. TNFα may not have been detected in a single blood sample from our patients since it is released locally in small quantities and the half life of TNFα is only a few minutes, thus it may be difficult to measure in the circulation. Measuring the TNF-R concentrations may give more consistent results. Our results showing increased concentrations of IL-6 and IL-1ra in patients with pre-eclampsia agree with previous reports that increased cytokine concentrations play a role in the mediation of inflammatory and immune responses, in this case contributing to the endothelial damage and dysfunction in pre-eclampsia.

4.3.3 GM-CSF

The granulocyte-macrophage-stimulating factors are specific glycoproteins that control the production and function of granulocytes and macrophages (Metcalf et al, 1986). GM-CSF can stimulate mature neutrophils. Since neutrophils are activated in pre-eclampsia perhaps GM-CSF could play a role in this activation. However, we found no significant increase in the concentration of GM-CSF in our pre-eclamptic patients.

4.3.4 IL-8

The circulating concentrations of IL-8 were not increased in our patients with pre-eclampsia. Tanaka et al (1993) found that IL-8 was captured by receptors on the endothelial surface, providing an adhesion-inducing signal to leucocyte subsets, and therefore IL-8 may not be detected in the maternal circulation in pre-eclampsia.

4.4 General discussion

We have shown that the cytokines IL-6 and IL-1ra and the cell adhesion molecules VCAM-1 and E-Selectin are increased in the maternal circulation in pre-eclampsia. The increased expression of the cell adhesion molecules could be partly responsible for the neutrophil activation which occurs in pre-eclampsia. Cytokines could also trigger neutrophil activation and cell adhesion on the endothelium. They are emerging as candidates for physiological adhesion triggers (Harlan and Liu, 1992) and could be partly responsible for the increased expression of cell adhesion molecules seen in pre-eclampsia. Both HUVECS in culture and endothelial cells in the vessels of normal placenta express IL-6 receptors. Our results have shown, for the first time, that IL-6 can induce the expression of both VCAM-2 and E-Selectin mRNA on endothelial cells *in vitro* which was both time and dose dependent (Lyall et al, 1997). We have also shown that E-Selectin and VCAM-1 protein were induced on HUVECS following treatment with IL-6. It is only possible to assess the immunocytochemical staining semi-quantitatively. The endothelial cells did not express these adhesion molecules before treatment with IL-6 so semi-quantitative analysis was straightforward. We used a VCAM-1 antibody when carrying out immunocytochemistry and a VCAM-2 cDNA. The VCAM-2 cDNA recognises the same sequences as VCAM-1.

Hession et al (1991) cloned a new form of human VCAM-1 which contained an additional immunoglobulin homologous domain. The long and short forms of VCAM-1 could not be discriminated by Northern analysis but could be discriminated by reverse transcriptase polymerase chain reaction (RT-PCR). This explains why we only had one

transcript on the autoradiograph. Both the six and seven-domain forms of VCAM-1 support the adhesion of several VLA-4 expressing cell lines indicating that domain 4 (which is missing in the six-domain form of VCAM-1) is not essential to the binding of leucocytes to VCAM-1.

The expression of cell adhesion molecules is partly regulated by changes in cytokine concentrations. The expression of E-Selectin is regulated, in part, by changes in the concentration of the cytokines IL-1 or TNFα whilst the expression of VCAM-1 is partly regulated by the cytokines IL-1β, IL-4, TNFα and IFNγ (Masinovsky et al, 1990; Thornhill and Haskard, 1990). Our results suggest that the expression of VCAM-1 and E-Selectin is mediated partly by IL-6, levels of which increase significantly as gestation progresses in normal pregnancy (Opsjon et al, 1993). There may be an abnormal or "over rigourous" response to the increasing levels of IL-6 occurring in pre-eclampsia which in turn allows the cytokine to exert a pathological effect on the endothelium, increasing the expression of cell adhesion molecules so resulting in damage.

There are numerous studies which indicate that neutrophil activation occurs in pre-eclampsia but the mechanism underlying this activation remains to be elucidated. The change in cell adhesion molecules and cytokines described in this thesis may be important components of the neutrophil activation process. In addition to this work we recently examined whether neutrophil activation, occurring in the maternal circulation of women with pre-eclampsia, was due to a factor in the plasma or serum which increased neutrophil locomotion (Clark et al, 1996a). The early stages of neutrophil recruitment require, not only changes in cell adhesion, but also motor capacity. The late stages of neutrophil recruitment are marked by dramatic activation of the cell metabolism. This study used a microcomputer-based system for real-time analysis of neutrophil behaviour *in vitro*. The results found no evidence of a humoral factor in the plasma/serum of women with pre-eclampsia which could alter the locomotion of human neutrophils. The results suggested that although IL-6 is increased in plasma from women with pre-eclampsia it did not stimulate neutrophil motility directly. We also examined whether neutrophil activation in pre-eclampsia was due to a factor in the maternal serum which increased neutrophil adhesion to endothelial cells (Clark et al, 1997). Significantly increased adhesion of neutrophils to endothelial cells in response to IL-1β and TNFα was shown, which confirmed a report by Bochner et al (1991). IL-6 did not stimulate neutrophil-endothelial cell adhesion. We have shown a dose and time dependent increase in E-Selectin and VCAM protein and mRNA on HUVECS in repsonse to IL-6. We would hypothesise that neutrophil adhesion increases the response to IL-6 due to the recruitment of E-Selectin onto the endothelium however this was not shown. IL-6 may be inactive at some stages of neutrophil activation. IL-6 has been reported to "prime" neutrophils, enhancing superoxide secretion by formyl-methionyl-leucyl-phenylalanine (FMLP)-treated neutrophils by over 50% (Borish et al, 1989). Bass et al (1986) termed

the ability of a substance to potentiate the stimulated oxidative burst of a cell without directly stimulating a respiratory burst itself as "priming". IL-6 may mediate a respiratory burst which releases oxygen radicals and inflammatory mediators at the later stages of neutrophil activation but does not have a direct effect on neutrophil activation. Tsukimori et al (1993) found that FMLP-induced superoxide production of neutrophils was significantly higher in pre-eclamptics compared to normal pregnant women. When neutrophils prepared from normal non-pregnant women were incubated with sera from normal pregnant and pre-eclamptic women, the sera from the pre-eclamptics enhanced the FMLP-induced superoxide production to nearly twice that of the other subjects. Barden et al (1997) measured basal neutrophil CD11b (Mac-1) to assess neutrophil activation and found that neutrophil CD11b was positively correlated with plasma uric acid levels suggesting that the extent of neutrophil activation correlates with disease severity in pre-eclampsia. Perhaps plasma or serum from women with severe pre-eclampsia alters neutrophil locomotion or adhesion. From these results we can see that neutrophil activation occurs in pre-eclampsia but plays a small role in a complex disease process.

We have shown that the cell adhesion molecules VCAM-1 and E-Selectin and the cytokines IL-6 and IL-1ra are increased in the maternal circulation in patients with pre-eclampsia compared to normal pregnant controls. Furthermore we have shown that IL-6 induces VCAM-2 and E-Selectin mRNA and protein expression although this is not the only factor necessary for increased neutrophil activation. Pre-eclampsia is a multisystem disorder characterized by vascular damage brought about by endothelial dysfunction and activation of platelets, neutrophils and the coagulation system. The presence of increased concentrations of VCAM-1, E-Selectin, IL-6 or IL-1ra may all contribute to endothelial damage and dysfunction seen in this disorder but this is only one small part of a complex disease process. Effective diagnosis and treatment will only come as a result of a clearer understanding of the pathogenesis of the disease.

4.5 Future Work

In retrospect it would have been ideal to measure the soluble cell adhesion molecule and the soluble cytokine concentrations in both plasma and serum from women with pre-eclampsia and determine why plasma and serum concentrations differ. In addition it could be useful to measure the soluble IL-6 concentration in maternal plasma throughout gestation to elucidate whether there was a specific time when IL-6 was significantly increased and whether this related to any changes in features in the pre-eclamptic patient or perhaps even trigger the onset of clinical symptoms. It would be potentially valuable to relate the increase in IL-6 to other specific factors. Are there any other clinical markers that relate to this increase in IL-6? We could measure soluble VCAM-1 and E-Selectin concentrations throughout gestation and again try to find out when the expression of

these cell adhesion molecules became significant. Is there a specific time when neutrophil activation occurs in pre-eclampsia? We have found a relationship between the cytokine IL-6 and the expression of VCAM-2 and E-Selectin. Does TNFα have the same effect on cell adhesion molecule expression or are there any other cytokines which stimulate cell adhesion molecule expression? We recently examined neutrophil locomotion and adhesion *in vitro* but found no evidence of a factor in the plasma or serum from pre-eclamptic women which stimulated neutrophil activation. The HUVECS in our study were from control patients and the neutrophils from healthy non-pregnant women. We could examine the effects of neutrophils from pre-eclamptic women on adhesion to endothelial cells that have been incubated with pre-eclamptic serum or plasma.

Recent studies have suggested that a dysfunction in lipid and lipoprotein metabolism may contribute to endothelial cell dysfunction in pre-eclampsia (Hubel et al 1996; Lorentzen et al 1994, 1995). The increased expression of cell adhesion molecules on the endothelium also contributes to endothelial dysfunction. We could study the effect of varying doses of lipoprotein particles and determine their potential for stimulating the expression of the adhesion molecules E-Selectin, ICAM, VCAM and PECAM.

NF-κB is a polypeptide which controls the transcription of genes. It is rapidly translocated from the cytoplasm to the nucleus in response to extracellular signals (Siebenlist et al, 1994). NF-kB has been shown to be involved in controlling a variety of genes involved in inflammation. It may therefore play a central role in the inflammatory response to infection and tissue injury in diseases such as toxic/septic shock, acute inflammatory conditions, atherosclerosis and cancer. It has been reported that cell adhesion molecules are regulated through NF-κB expression. Neish et al (1992) suggested that NF-κB-mediated activation of VCAM-1 gene expression may lead to endothelial expression of a mononuclear leucocyte adhesion molecule associated with initial events in the development of an atherosclerotic lesion. Manning et al (1995) found that NF-κB activation preceded the transcriptional activation of P-Selectin, E-Selectin, VCAM-1 and ICAM-1 genes in rat lung and heart tissues, when exposed to endotoxin. They suggested that NF-κB activation is an early event in the initiation of acute inflammation *in vivo*. A study by Libermann and Baltimore (1990) suggested a role for NF-κB in the expression of the cytokine IL-6. They found that the promotor region of the IL-6 gene had a NF-κB binding site and mutations in the NF-κB binding site abolished inducibility of IL-6 in U-937 cells by lipopolysaccharides and TNFα. Satriano and Schlodorff (1994) found increased nuclear proteins bound to a NF-κB specific DNA oligonucleotide after mesangial cells were activated with TNFα. We have shown that increased expression of cytokines and cell adhesion molecules by the endothelium could be responsible for neutrophil activation which occurs in pre-eclampsia. NF-kB regulates these adhesion molecules and therefore may play a role in pre-eclampsia.

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PUBLICATIONS

Whilst working for this MSc, the following papers have been published;

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