EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR-A AND ITS RECEPTORS IN UTEROPLACENTAL TISSUES OF THE NORMOTENSIVE AND HYPERTENSIVE PREGNANT RAT

A Thesis Submitted
for the Degree of

MASTER OF SCIENCE

by

HUILING HE

Centre for Bioprocessing and Food Technology
Victoria University of Technology
Melbourne
AUSTRALIA

1999

PREFACE

I hereby certify that this thesis contains no material which has been accepted for the award of any other degree in any university and, that to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

The study presented in this thesis was completed by the author at Victoria University of Technology under the supervision of Dr. Kerry Dickson and Dr. Sarah Fraser.

Huiling He
Centre for Bioprocessing and Food Technology,
Victoria University of Technology,
Melbourne, Australia.
1999

ACKNOWLEDGEMENTS

Although my name goes on the cover, this thesis would not be a reality without the hard work and dedication of countless people.

First and foremost, I am very grateful to my principal supervisor, Dr Kerry Dickson, who has offered an opportunity for me to study at Victoria University and provided financial support during my second year of study. Many thanks for her support, guidance, encouragement, constructive criticism throughout the course of my study, and critical reading of the manuscript.

I am sincerely indebted to my co-supervisor, Dr Sarah Fraser, who has provided invaluable supervision through her wealth of knowledge in molecular biology and development of new techniques. She has made a major contribution to the success of this thesis. The time that we have shared in the laboratory including evenings and weekends will be forever imprinted in my memory as some of the best of my professional career.

I would like to express my thanks to Professor Bob Fairclough, for the invaluable encouragement and emotional support, enabling me to complete my study.

I would like to convey my thanks to Professor Margaret Britz, the former Director of the Centre for Bioprocessing and Food Technology (CBFT), for supporting this work.

I would like to thank Dr David Finkelstein, Department of Medicine, Monash University, for allowing me to use his laboratory and equipment to collect the rat tissues. This project would not have proceeded without his help.

I would like to thank Professor Graham Jenkin, Department of Physiology, Monash University, for the initial stimulus to work on VEGF-A, and for providing me with the initial VEGF-A primers.

I would like to thank Ms Usula Manuelpillai for kindly providing the human G3PDH primers and for sequencing the resultant RT-PCR product.

I would like to acknowledge Dr Mrinal Bhave, for her guidance and help during the initial stages of this project, and for her kind donation of a clone G3PDH plasmid.

I would like to thank Dr Tseng Lau, who has kindly provided assistance for the immunohistochemistry and *in situ* hybridisation experiments.

I am particularly grateful to my best friend, Helen Chunying Feng, for her enthusiastic help and technical advise, particularly for her assistance in the initial RNA work, and for her mental stimulus that has led me to complete this study.

My sincere thanks go to Ms Hong Wang from the Institute for Reproduction and Development, Monash University, who has provided enthusiastic assistance and excellent technical advice in immunohistochemistry and *in situ* hybridiazation.

Many thanks to the lovely friends and colleagues at the CBFT and the School of Life Sciences. I would like to thank Karoline Tellbach, for her help with the administration of anaesthesia, the holding of the rats, the preparation of surgical equipment; and for her warm help and sharing in the laboratory.

I would like to thank Matthew Knight, for his warm advise and wonderful friendship. I would also like to thank Hemalatha Jegasothy; Aslam Khan and Diana Emslie for their kindness and for sincerely caring.

Finally, I would like to dedicate this thesis to my husband John, for his understanding and full support especially as we experienced extreme financial difficulty and the project took away all my attention and time. Even though he suffered from severe illness in hospital, he still encouraged me to concentrate on my study. I also want to dedicate this project to my family members, especially my Dad, from him came all care and blessings.

CONFERENCE PRESENTATIONS

Part of this work has been presented at the following conferences

H. L. He, S. M. Fraser, K. A. Dickson. mRNA Expression of Vascular Endothelial Growth Factor (VEGF) and its Receptor (Flt-1) in Uteroplacental Tissues of the Pregnant Spontaneously Hypertensive Rat (SHR). *Fetal and Neonatal Physiological Society 25th Annual Meeting*. California, U.S.A, pp 42, 1998.

H. L. He, S. M. Fraser, K. A. Dickson. mRNA Expression of Vascular Endothelial Growth Factor (VEGF) and its Receptor (Flt-1) in Uteroplacental Tissues of the Pregnant Spontaneously Hypertensive Rat (SHR). *Fertility Society Association Conference.* Hobart, Australia, pp 72, 1998.

ABBREVIATIONS

aa	Amino acid
A_2	Thromboxane
H_2O_2	Hydrogen peroxide
ANGIS	Australian National Genomic Information Service
Ang-1	Angiopoietin 1
Ang-2	Angiopoietin 2
β-FGF	β fibroblast growth factor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CT	Cytotrophoblast
DDT	Dithiothreitol
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Equimolar mixture of dATP, dCTP, dGTP, dTTP
EB	Ethidium bromide
ECM	Extracellular matrix
EDTA	Ethylenediaminotetra-acetic acid
EGF	Epidermal growth factor
EP	Epithelial cell

Flk-1	Fetal liver kinase (rat)
Flt-1	c-fms-like tyrosine kinase
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
HCG	Human chorionic gonadotrophin
HGF	Hepatocyte growth factor
IFNα	Interferon alpha
IL	Interleukin
IUGR	Intrauterine growth retardation
KDR	Kinase insert domain-containing receptor (human)
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
NO	Nitric oxide
PA	Plasminogen activator inhibitors
PAI	Plasminogen activator inhibitors
PBS	Phosphate buffered saline
PDGF	Platelet derived endothelial cell growth factor
PGI_2	Prostacyclin
PIGF	Placental growth factor
RNase	Ribonuclease
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SHR	Spontaneously hypertensive rat
TGF-α	Transforming growth factor alpha
TIMP	Tissue inhibitor of metalloproteinase

TNFα	Tumour necrosis factor alpha
TSP1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor 1
VEGFR-2	Vascular endothelial growth factor receptor 2
VEGF-A	Vascular endothelial growth factor A
VEGF-B	Vascular endothelial growth factor B
VEGF-C	Vascular endothelial growth factor C
VEGF-D	Vascular endothelial growth factor D
VPF	Vascular permeability factor
UV	Ultraviolet
WKY	Wistar Kyoto rat

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

Hypertension, is a common clinical complication of pregnancy, often leading to pre-eclampsia and fetal intrauterine growth retardation. Many studies have shown that vascular endothelial growth factor A (VEGF-A) is a potent angiogenic factor, that is, it is responsible for the formation of new blood vessels from existing vessels. VEGF-A has been shown to be expressed in uteroplacental tissues, particularly during implantation. Levels of VEGF-A mRNA have also been shown to be significantly lower in placental tissue from pre-eclamptic women compared with control women (Cooper *et al*, 1996). The first aim of this project, was to establish the use of RT-PCR (reverse transcriptase-polymerase chain reaction), Northern blot analysis, immunohistochemistry and *in situ* hybridization, to measure the expression and localization of VEGF-A, vascular endothelial growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor receptor 2 (VEGFR-2) in normal rat uteroplacental tissues at early, mid and late gestation. The second aim of this project was to measure expression of VEGF-A, VEGFR-1 and VEGFR-2 in uteroplacental tissues of the normotensive (WKY) and the spontaneously hypertensive rat (SHR) at 7, 11 and 19 days of gestation. Spontaneously hypertensive rats have been previously established as an animal model for pre-eclampsia.

The uterus containing the placenta and fetus was removed from normal Sprague Dawley, WKY and SHR rats over a range of gestational ages. After 11 days of gestation, the uterus and placenta were separated. Tissues were frozen immediately in liquid nitrogen and stored at -80^{o}C, or fixed in 10% formaldehyde for immunohistochemistry. Total RNA was extracted using TRIZOL reagent and mRNA levels of VEGF-A and its receptors were examined using RT-PCR and Northern blot analysis. mRNA levels of VEGF-A and its receptors were compared with those of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH).

We have successfully used RT-PCR to measure mRNA expression of VEGF-A, VEGFR-1 and VEGFR-2; Northern blot analysis to measure expression of VEGF-A, and immunohistochemistry to localize VEGF-A protein in rat uteroplacental tissues. We have shown that VEGF-A and its receptors were expressed in uteroplacental

tissues of the normal rat. We found no major differences in the expression levels of VEGF-A and VEGFR-1 in uteroplacental tissues at early, mid and late gestation. We showed that VEGF-A_{164} was the predominant isoform found in uteroplacental tissues. Finally, we found no major differences in the expression levels of VEGF-A, VEGFR-1 and VEGFR-2 in uteroplacental tissues in normotensive rats (WKY) compared with hypertensive rats (SHR).

We conclude that VEGF-A and its receptors, VEGFR-1 and VEGFR-2, were expressed in uteroplacental tissues of pregnant rats. Furthermore, mRNA levels of VEGF-A and its receptors were comparable in normotensive and hypertensive rats. These results suggest that VEGF-A and its receptors may have an important role in the development of the fetal and maternal portions of the placenta, but that they are not the primary factors involved in the aetiology of pre-eclampsia.

CHAPTER 1 LITERATURE REVIEW

1.0.0 INTRODUCTION

One of the most common clinical complications of pregnancy is hypertension, which often leads to pre-eclampsia. Although the exact aetiology of hypertension is not known, it is associated with constriction of the blood vessels and endothelial cell injury. Serious complications can arise in pregnant women suffering pre-eclampsia. Furthermore, the resultant decrease in uteroplacental blood flow leads to placental insufficiency and growth retarded babies.

Pre-eclampsia, and other uteroplacental vascular disorders, are the most common causes of fetal intrauterine growth retardation (IUGR). Over 60% of prenatal morbidity and mortality are related to IUGR. Many of the surviving small, fragile babies suffer long-term medical problems, do less well than their peers at school and have a significantly increased risk of developing late onset adult cardiovascular diseases, such as hypertension and diabetes (Barker *et al*, 1992). These problems result in substantial financial costs for parents, the community and the health care system.

During *in utero* life, the placenta must develop a complex capillary network to enable sufficient gas exchange and nutrient absorption. Angiogenesis is the formation of new blood vessels from existing vessels, and is induced by specific angiogenic growth factors. To date, the most potent angiogenic factor is vascular endothelial growth factor A (VEGF-A) which has been found in uteroplacental tissues during implantation and development. VEGF-A has two receptors vascular endothelial growth factor receptor 1 (VEGFR-1) also known as c-fms-like tyrosine kinase (Flt-1) and vascular endothelial growth factor receptor 2 (VEGFR-2) also known as rat fetal liver kinase 1 (Flk-1). The presence, role and cellular site of production of VEGF-A, VEGFR-1 and VEGFR-2 in uterine and placental tissues throughout gestation are yet to be fully elucidated. Very interestingly, VEGF-A expression by the placenta has been found to be significantly lower in pre-eclamptic women compared with normal pregnant women (Cooper *et al*, 1996). This suggests that VEGF-A may be an important factor in the aetiology of pre-eclampsia.

1.1.0 NORMAL PLACENTAL DEVELOPMENT

1.1.1 Structure of the Uteroplacental Circulation

During *inutero* life, "the placenta is not only an endocrine organ, it also acts as the lungs, gut, liver and kidneys of the developing fetus" (Cross *et al*, 1994, page 112). Consequently, establishing and maintaining the growth of vascular beds within the maternal and fetal portions of the placenta are essential for subsequent embryological and fetal development (Cross *et al*, 1994).

In humans and in a variety of smaller animals, most notably rats, the placenta develops as a discoid, invasive organ; that is, it is limited to one region of the uterus with the fetal tissues actively invading the maternal tissues (Figures 1.1 and 1.2, Moore, 1988). After conception, the zygote undergoes cleavage to form a blastocyst consisting of an inner cell mass, which ultimately forms the embryo, and an outer cell mass or trophoblast, which ultimately forms the placenta and fetal membranes. The trophoblast differentiates into the cytotrophoblast and the syncytiotrophoblast. Cytotrophoblasts, which contribute cells to the syncytiotrophoblast, decrease in number as the placenta approaches term. Syncytiotrophoblasts, which increase in number with advancing gestation, actively invade the maternal tissue forming numerous chorionic villi. These villi ultimately develop into the fetal portion of the placenta. The maternal portion of the placenta is formed by the decidua basalis. As the chorionic villi enlarge they form spaces in the decidua called intervillious spaces. These spaces are filled with maternal blood via spiral arteries, which branch from the uterine artery. At the same time the circulatory system of the embryo is developing. Two umbilical arteries, from the fetal descending aorta, branch to form a network of arteries at the chorionic plate. These subsequently divide into a network of capillaries, which are bathed in maternal blood. After exchange of gases, nutrients and wastes the fetal blood is transported to the fetus via a single umbilical vein (Figures 1.1 and 1.2).

During pregnancy there is extensive angiogenesis occurring, both within the fetal villi and in the maternal decidua. This vascular growth is crucial for successful implantation and development as it allows for the increased blood flow requirements of the fetoplacental unit throughout pregnancy. Although there is close proximity

between the fetal and maternal circulations, there is no direct communication between fetal and maternal blood as the placental membrane separates them. This membrane, which thins during the second half of pregnancy, is a barrier to blood cells and large molecules but allows most compounds endogenous and exogenous, to cross. Thus enabling the fetus to readily obtain oxygen and nutrients and discharge carbon dioxide and waste products. Hofbauer cells, which are phagocytic cells, are found in the fetal circulation.

1.1.2 Uteroplacental Blood Flow

To ensure an adequate exchange of gases and nutrients, uteroplacental blood flow and umbilical placental blood flow are both very high. Although there are some differences in the response of various vasodilator and vasoconstrictor agents between the mother and fetus, in general, both vasculatures remain dilated. This is primarily due to 1) a decreased sensitivity to vasoconstrictors and 2) an increased release of vasodilators. During pregnancy, the vasculature is less sensitive to vasoconstrictors such as angiotensin II, endothelial and atrial natriuretic peptide. Several agents appear to play a role in placental vasodilatation including corticotrophin-releasing hormone, oestrogen, nitric oxide, prostacyclin and Na^+/K^+ATPase (Boura *et al*, 1994).

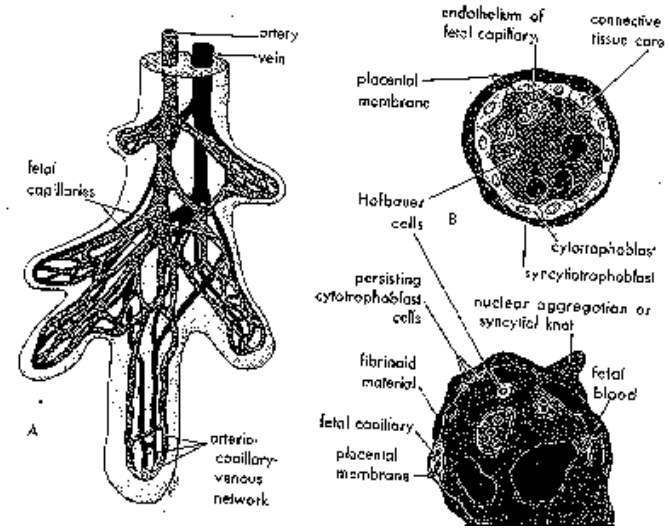
1.2.0 IMPAIRED PLACENTAL DEVELOPMENT WITH PRE-ECLAMPSIA

Hypertension during pregnancy is an important clinical problem in obstetrics as it can lead to maternal and fetal death. There are several hypertensive disorders in pregnancy When hypertension is present prior to conception it is called essential hypertension. Sometimes, women with essential hypertension subsequently develop pre-eclampsia. Women who are normotensive prior to conception may develop hypertension during pregnancy, that is, pregnancy induced hypertension. When a pregnant woman's blood pressure rises substantially (>140-160/90-110 mmHg), it causes widespread oedema and protein to be excreted in the urine (proteinuria), characteristically called pre-eclampsia. When the hypertension worsens resulting in convulsions and coma it is called eclampsia. It has been reported that uteroplacental "haemorrhage occurs in 10%

Figure 1.1. Relationship between the fetal and maternal portions of the full- term placenta. This schematic diagram shows "(1) the relationship of the villous chorion (fetal part of placenta) to the decidua basalis (maternal part of placenta), (2) the fetal

placental circulation, and (3) the maternal placental circulation. Maternal blood flows into the intervillous spaces in funnel-shaped spurts, and exchanges occur with the fetal blood as the maternal blood flows around the villi. The inflowing arterial blood pushes venous blood out into the endometrial veins, which are scattered over the entire surface of the decidua basalis. Note that the umbilical arteries carry deoxygenated fetal blood to the placenta and that the umbilical vein carries oxygenated blood to the fetus. Note that the cotyledons are separated from each other by decidual septa of the maternal portion of the placenta. Each cotyledon consists of two or more main stem villi and their many branches. In this drawing, only one main stem villus is shown in each cotyledon, but the stumps of those that have been removed are indicated"(Moore, 1988, Figure 8-5, page 92).

Figure 1.2. Structure of a chorionic villus "A) Drawing of a chorionic villus showing its arterio-capillary-venous system carrying fetal blood. The artery carries deoxygenated blood and waste products from the fetus, whereas the vein carries oxygenated blood and nutrient to the fetus. B) and C) Drawings of sections through a chorionic villus at ten weeks and at full term, respectively. The vessels are bathed externally in maternal blood. The placental membrane, composed of fetal tissues, separates the maternal blood from the fetal blood. Note that this membrane becomes very thin toward the end of pregnancy. Hofbauer cells are phagocytic cells" (Moore, 1988, Figure 8-6, page 93).



of women and cerebral haemorrhage in 5 % of women suffering from eclampsia. Fetal intrauterine death (due to placental insufficiency) occurs in about 10% of cases, with the maternal mortality rate for all cases of eclampsia being 5.8%. Among those women with antepartum eclampsia the death rate was 17% with the most common causes being cardiac failure, pulmonary oedema, cerebral haemorrhage and acute renal failure" (Daphne and Lee, 1974, page 122).

The exact aetiology of hypertension during pregnancy remains a mystery. It is associated with decreased uteroplacental perfusion, which can lead to intrauterine growth retardation, increased incidence of fetal distress and prenatal death. Many studies have shown that pathological changes in pre-eclamptic women are not secondary to increased blood pressure, rather that they are correlated with organ hypoperfusion. Furthermore, other pathophysiological changes such as activation of the coagulation cascade, increased sensitivity to vasoconstrictors and decreased response to vasodilators all precede elevated blood pressure levels (Roberts *et al*, 1989).

1.2.1 Structure of the Uteroplacental Circulation with Pre-eclampsia

In pregnancies, which are complicated by hypertension and pre-eclampsia, trophoblast invasion is shallow and maternal vascular proliferation is poor. Scanning electron microscopy and corrosion casts of capillaries have shown that, as pregnancy progresses, the tertiary stem villi fail to develop correctly with the capillaries remaining long, poorly and inappropriately branched and highly coiled. Placenta from pre-eclamptic women also show other pathological features including syncytial necrosis, distortion, increased shedding of syncytial membranes and signs of endothelial cell damage (McCarthy *et al*, 1993).

1.2.2 Uteroplacental Blood Flow with Pre-eclampsia

With pre-eclampsia, normal vasodilator stimuli are inhibited, the vasculature is hypersensitive to vasoconstrictor stimulation and endothelial cells are injured. Consequently, uteroplacental vasculature resistance is substantially higher than in normotensive pregnant women. This increased vascular resistance associated with a decreased uteroplacental perfusion leads to placental insufficiency and IUGR. Several investigators have proposed a number of mechanisms responsible for the hypertension seen during pre-eclampsia. The concentration of angiotensin II, a potent vasoconstrictor, has been shown to be higher in hypertensive pregnant women than in normotensive pregnant women. The activity of Na^+/K^+ATPase, which is partly responsible for the normal vasodilatation of the vasculature during pregnancy, has been shown to be lowered with pre-eclampsia. The balance of products of aracadonic acid metabolism has been shown to be disrupted during pre-eclampsia such that "synthesis of the vasodilator PGI_2 is decreased and synthesis of the potent vasoconstrictor thromboxane A_2 is increased" (Boura *et al*, 1994, page 743). "Synthesis of nitric oxide, a potent vasodilator, has been shown to be reduced from control levels in hypertensive rats" (Mochizuki *et al*, 1991, page 141).

There is considerable evidence that endothelial cell injury is present in women with pre-eclampsia (Roberts *et al*, 1989). Blood levels of fibronectin, factor VIII antigen, and platelet counts have been shown to be higher in pre-eclamptic women than normotensive women. A feature of endothelial cell injury that makes it appealing as a

primary event in pre-eclampsia is its tendency to auto accelerate. That is, endothelial cell damage leads to increased vasospasm and intravascular coagulation, which reduces organ perfusion which, in turn; leads to endothelial cell damage (Roberts *et al*, 1989).

Although the exact mechanisms responsible for pre-eclampsia are still under investigation the outcome is decreased uteroplacental blood flow leading to IUGR.

1.3.0 ANIMAL MODEL FOR HYPERTENSION DURING PREGNANCY

Most animal studies aimed at mimicking pre-eclampsia, have used either the spontaneously hypertensive rat (SHR) or administration of a hypertensive agent to the pregnant animal. The SHR has been used extensively as an animal model for essential hypertension, because its haemodynamic and endocrine characteristics parallel those observed in the hypertensive human (Tipton, 1984) and are significantly different to the normal Wistar Kyoto rat (WKY). "Blood pressure is substantially higher in the non pregnant SHR compared with the non pregnant WKY rat (174 ± 3 vs 114 ± 2 mmHg).

Although pregnancy, especially in the last week of gestation, decreases blood pressure in both species, it still remains higher in the SHR than in the WKY rat (136 ± 5 vs 98 ± 1 mmHg)" (Coelho *et al*, 1997, page 586). The concentration of maternal blood glucose has been shown to be higher, and the concentration of plasma insulin lower, in SHR compared with WKY rats (Johnston, 1995). Placental blood volume has been shown to be lower in SHR compared with WKY rats (Fuchi *et al*, 1995). Similarly, "placental blood flow, unlike other organ blood flows, has been shown to be 26% lower in the SHR compared with the WKY rat" (Ahokas *et al*, 1987, page 1010). However, other workers have not observed a decline, from control values, in placental blood flow in the SHR (Lindheimer *et al*, 1983).

Using light microscopy, Scott *et al*, (1985) found no major lesions, typical in human pre-eclamptic placentas, in placentas from SHR. However, they did observe more extensive haemorrhage at the lateral edges of decidua basalis in SHR compared with

the WKY rats at 15 days of gestation. By measuring cross sectional area and wall thickness, evidence of narrowing and spasms in the central blood vessels of the spiral arteries was observed in SHR but not WKY rats (Fuchi *et al*, 1995). The higher blood pressure, the possible lower placental blood flow and the damage to the placenta suggest that the SHR may be a good animal model for essential hypertension during pregnancy.

Epidemiological studies have linked low birth weight and increased placental weight with increased risk of hypertension in adult life. It has been proposed that the cardiovascular changes, which lead to hypertension, are initiated *inutero* by processes associated with IUGR (Barker *et al*, 1992). A number of workers have shown that fetal body weight, at least until a few days before term, is 10-20% lower whereas placental weight is 15-30% higher in SHR compared with WKY rats (Lewis *et al*, 1997; Johnston, 1995; Erkadius *et al*, 1995a).

Some authors, however, have shown no differences in fetal body weight between SHR and WKY at term (Lewis *et al*, 1998; Lindheimer *et al*, 1983), but these authors thought that placental hypertrophy may account for this catch up in fetal growth (Lewis *et al*, 1998). It has also been shown that the duration of gestation was 20 h longer in SHR (23d) than in WKY rats, and that maternal weight was lower in SHR than in WKY rats. When these differences were accounted for, the reduction in fetal body weight and the increase in placental weight in SHR have been shown to be negated.

It has been stated that the altered fetal and placental weight of SHR may be due to the overall reduced growth rate of the SHR strain (Erkadius *et al*, 1995b). Amniotic fluid volume has been shown to be lower at 15 days but higher at 20 days in SHR compared with WKY rats (Erkadius *et al*, 1995a). "Compared with control, SHR fetuses showed hypertrophy of the heart and kidney and lowered blood glucose concentrations. These findings suggest that under nutrition and placental insufficiency occurs in SHR" (Lewis *et al*, 1997, page 758). Although not all authors agree (Johnston, 1995), "the rate of successful pregnancies (69% verses 86%) and the litter size (7.83 $\pm$ 0.5 verses

10.41 ± 0.5) has been shown to be lower in SHR compared with WKY rats" (Pinilla *et al*, 1992, page 99). These results suggest that changes in fertilisation and/or implantation account for the reduced pregnancy rate.

Although there are discrepancies in the literature, the reduced pregnancy rate, the decreased fetal body weight and the increased placental weight support the data from human population studies, and suggest that the SHR may be a good animal model for IUGR associated with hypertension.

1.4.0 ANGIOGENESIS

Vasculogenesis is the initial formation of blood vessels during embryological development. Vasculogenesis involves the differentiation of angioblasts from mesoderm and then the formation of angioblasts into primitive blood vessels. During gastrulation, growth factors induce the mesoderm to differentiate into angioblasts and haemopoietic cells. Together, these cells constitute the blood islands of the yolk sac. The vascular endothelial growth factor family has a crucial role in the subsequent differentiation of the endothelial cells. "Endothelial cells then form primary, capillary plexuses, which undergo angiogenesis"(Flamme *et al*, 1995, page 70).

Angiogenesis is the formation of new blood vessels from the pre-existing or old blood vessels. This proliferation of blood vessels is a process necessary for the normal growth and development of tissue. In a healthy male there is little call for angiogenesis. However, it has been shown to play an important role in the female reproductive system, particularly during follicle development, implantation, embryogenesis, placental growth and fetal development. Angiogenesis also occurs as part of the bodies natural repair process, that is during wound healing. Uncontrolled angiogenesis, however, occurs in a number of pathological conditions including arthritis, diabetic retinopathy, rheumatoid diseases and tumour growth. Recently, tumour growth and the associated uncontrolled angiogenesis have been studied extensively and this has increased our knowledge of the normal process of blood

vessel growth and regression (Houck *et al*, 1991; Folkman and Klagsburn, 1987; Folkman, *et al*, 1989).

1.4.1 Mechanisms of Angiogenesis

Angiogenesis is highly regulated and dependent on controlled endothelial cell differentiation (diversification to other cell types) and proliferation (multiplication of cells) induced by specific growth factors. New blood vessels grow from pre-existing capillaries and venules. The endothelial cells and their adjacent cells carry the necessary genetic information to form tubes, branches, and whole capillary networks (Folkman *et al*, 1992). There are three primary angiogenic mechanisms: intussusception, vessel elongation and sprouting. It is likely that all three processes are involved in the physiological process of angiogenesis associated with placenta development (Caduff *et al*, 1986; Hanahan, 1997).

Intussusception involves the endothelial cells invaginating and sprouting into the lumen of the vessel, forming a septum, which divides the capillary. As the vessel remains patent and the basement membrane remains intact, there is no increase in vessel fragility or permeability with intussusception. Similarly, with capillary elongation there is no loss of vessel function. It is probable that the intussusception and elongation processes go hand in hand as intussusception increases the number of capillary branches and elongation increases capillary loop length.

To date, most research has focussed on the process of capillary sprouting. Capillary sprouting is intimately involved with the breakdown and subsequent remodelling of the extracellular matrix (ECM). The ECM encompasses components of the basement membrane and the interstitial connective tissue. Although the complete structure of the ECM remains to be defined it consists of structural components (eg. collagen fibbers, elastic fibbers, laminin, entactin, fibronectin, tenascin and proteoglycans) growth factors (eg. β-FGF), proteinases (eg. collagenase, plasminogen activator) proteinase inhibitors (eg. plasminogen activator inhibitor) and other enzymes (Dunsmore *et al*, 1996).

With capillary sprouting, the basement membrane of the pre-existing vessel breaks down. This allows degradation of the interstitial connective tissue and migration of the surrounding peryocytes. Elongation and formation of new vessels involves two processes of endothelial movement and growth that is migration and proliferation. Endothelial cells at the tip undergo migration, whereas endothelial cells just proximal to the tip undergo proliferation. Consequently, capillary sprouts baring long filopodia at their tips extend from the endothelial lining of pre-existing capillaries. The filopodia meet each other and form a long strand, which splits the intervascular space. A lumen for blood flow is thus formed. These new blood vessels are highly permeable until the basement membrane is laid down. This stabilises the vessel and allows for subsequent tissue remodelling (Risau and Flamme, 1995). Angiogenesis is stimulated via a number of factors including angiogenic factors, physical stress and blood flow, monocytes, hormones and hypoxia.

1.4.2 Role of Angiogenic and Anti-Angiogenic Factors

The process of angiogenesis has become a topic of extensive investigation, particularly because of its relevance to inflammation and tumour growth. New biotechnology techniques have allowed scientists to identify numerous angiogenic factors and with each passing year new factors or isoforms of known factors are identified and characterised. As there is a wide array of angiogenic factors, and growth factors with some angiogenic or related properties this discussion only focuses on the main factors.

Table 1.1 shows the main angiogenic factors involved in capillary sprouting. The list is not exhaustive and new factors are continually being added. The growth and, at times, the regression of blood vessels in the placental circulations are under complex endocrine, paracrine and autocrine regulation. Most importantly the process of angiogenesis is dependent on a finally tuned balance between factors which stimulate angiogenesis and factors which inhibit it.

The breakdown of the basement membrane is thought to involve enzymes, primarily matrix metalloproteinases (MMP) and plasminogen activators (PA). Tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitors (PAI) inhibit the actions of these enzymes.

Several angiogenic factors have been shown to be associated with endothelial cell migration and proliferation. The most dominant stimulatory factors are those of the VEGF family (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF). The processes of endothelial migration and proliferation have been shown to be inhibited by several factors including thrombospondin-1 (TSP1), angiostatin, endostatin, interferon alpha (IFNα) and tumour necrosis factor alpha (TNFα). Elongation of the sprout and lumen formation are stimulated by a number of factors including VEGF-A and angiopoietin 1 (Ang-1). Although, angiopoietin 2 (Ang-2) appears to antagonise the effects of Ang-1, it has a stimulatory effect on angiogenesis via cell wall breakdown (Hanahan, 1997). There also appears to be a role for cell adhesion molecules such as vascular endothelial cadherin, platelet-endothelial cell adhesion molecule-1 and cd34 in lumen formation, cell polarity and vascular permeability. Remodelling of the ECM, which

surrounds the vessels, is stimulated by several growth factors including transforming growth factor alpha (TGF-α) epidermal growth factor (EGF), hepatocyte growth factor (HGF) and angiogenin. Fibronectin, laminin, vitronectin and some integrins also appear to have important roles in the remodelling of the ECM (Risau and Flamme, 1995; Lampugnani *et al*, 1992; Bauknecht *et al*, 1986).

STAGE	ACTIVITY	ACTIVATORS	INHIBITORS
1	Basement membrane breakdown	MMP, PA	TIMP, PAI
2	Endothelial cell migration and proliferation	VEGF-A, VEGF-B, VEGF-C, VEGF-D, PlGF	TSP1, angiostatin, endostatin, IFN-α, TNF-α
3	Sprout and lumen formation and stabilization	VEGF-A, Ang-1	Ang-2
4	Remodelling	TGF-α, EGF, HGF, angiogenin	

Table 1.1 Summary of the main factors involved in capillary sprouting.

The interrelationships between various angiogenic factors are complex and remain to be fully elucidated. Although the angiogenic factors sited above appear to act directly on vascular endothelial cells, other factors may act indirectly via paracrine mediators. Examples include basic fibroblast growth factor (bFGF), which increases mitogenic activity and vascular permeability, and transforming growth factor-β, which enhances angiogenesis via its stimulatory actions on components of the ECM and activation of macrophages. Other factors which have effects on surrounding tissues as well as endothelial cells, include platelet derived growth factor (PDGF), platelet derived endothelial cell growth factor (PDEGF), interleukin-8, activin and follistatin. Furthermore, heparin, heparin sulfate and related polysaccharides, which have been shown to bind to endothelial cell growth factors, enhance the activity of several angiogenic growth factors (Franklin *et al*, 1991; Sporn and Roberts, 1990; Kiefer *et al*, 1990; Keck *et al*, 1989; Leung *et al*, 1989; Bonthron *et al*, 1988; Neufeld *et al*, 1999).

1.4.3 Role of Physical Stress and Blood Flow

Shear stress places a major mechanical force on endothelial walls. Shear stress response elements have been characterised in the promoters of various genes. It is also thought that vasoconstrictors and vasodilators, via their physiological regulation of vascular tone may influence shear stress response elements and, in turn, angiogenic growth factors (Risau and Flamme, 1995).

1.4.4 Role of Monocytes

Recruitment of monocytes (mononuclear phagocytes) from the blood compartment out into the tissue compartment is a crucial process in inflammatory reactions, immune response and angiogenesis. Chemotaxis involves the movement of these monocytes in response to a chemical concentration gradient. It has been shown that angiogenic factors (eg. PlGF, VEGF-A) induce monocyte activation via the induction of tissue factor and monocyte chemotaxis (Clauss *et al*, 1990; Koch *et al*, 1986) and that this may stimulate further expression of angiogenic factors.

1.4.5 Role of Hormones

The female reproductive tract offers an excellent model to study the effects of various hormones on the process of angiogenesis. Development of ovarian follicles and corpora lutea, proliferation and degradation of the uterine endometrium and decidualisation in response to embryo implantation all exhibit angiogenesis. Several hormones including, human chorionic gonadotrophin, luteinizing hormone, oestrogen (Cullinan-Bove *et al*, 1993), and progesterone have been implicated in the process of angiogenesis (Charnock-Jones *et al*, 1993; Dickson and Lippman, 1987; Tamura and Greenwald, 1987; Sato *et al*, 1982).

1.4.6 Role of Hypoxia

It is thought that hypoxia-mediated angiogenesis has an important role in placental development. The trophoblast forms a shell around the embryo during very early development and the maternal spiral arteries, which will ultimately perfuse the placenta, are not patent. Throughout this time, the oxygen tension of the trophoblast has been shown to be far lower than that which prevails when the spiral arteries open and perfusion of the intervillious space is established suggesting a role for hypoxia in

early decidualisation and angiogenesis. Studies examining oxygen related expression of mRNAs for VEGF, PDGF, PlGF and TGF implicate hypoxia as an important stimulator of these angiogenic factors (Levy *et al*, 1995; Shweikl *et al*, 1992; Rodesch *et al*, 1992).

1.5.0 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) FAMILY

To date, the VEGF family of growth factors has five members (Table 1.2). Vascular endothelial growth factor also known as vascular permeability factor (VPF), was discovered around 10 years ago (Gospodarowicz *et al*, 1989; Keck *et al*, 1989). As several other members of the family have now been isolated VEGF is now more correctly named VEGF-A. Very recently, 3 new members of the VEGF family of growth factors have been identified VEGF-B (Joukov *et al*, 1997; Olofsson *et al*, 1996), VEGF-C (Joukov *et al*, 1996; Lee *et al*, 1996) and VEGF-D (Achen *et al*, 1998; Yumada *et al*, 1997). The last member of the VEGF family of growth factors is placental growth factor (PlGF) (Vuorela *et al*, 1997).

	VEGF-A_{165}	**VEGF-B_{167}**	**VEGF-B_{186}**	**VEGF-C**	**VEGF-D**	**PlGF-1**	**PlGF-2**
Total length (aa)	191	188	207	419	354	149	170
Molecular weight (kDa)	22.3	21.3	21.6	46.9	40.9	16.7	19.3
Homology with VEGF-A_{165} (%)	100	42.7	33	31.4	26.7	40.4	40.1
Receptors	VEGFR-1 VEGFR-2	VEGFR-1	VEGFR-1	VEGFR-2 VEGFR-3	VEGFR-2 VEGFR-3	VEGFR-1	VEGFR-1

Table 1.2 Summary of the various properties of the VEGF family members (adapted from Joukov *et al*, 1997 with additions from Achen *et al*, 1998 and Neufeld *et al*, 1999). Note: VEGFR-1 is also named Flt-1, VEGFR-2 is named Flk-1 or KDR and VEGFR-3 is named Flt-4.

1.5.1 Vascular Endothelial Growth Factor A (VEGF-A)

VEGF-A, more commonly referred to as VEGF, has been shown to be associated with endothelial cell growth and proliferation, breakdown of the endothelial basement membrane, increasing vascular permeability and protein leakage, monocyte chemotaxis and changes in vascular tone. The importance of VEGF-A for

angiogenesis is highlighted by the finding that homozygous and heterozygous VEGF-A deficient mouse embryos have impaired and abnormal vessel formation and embryonic death occurring at mid gestation (Carmelliet *et al*, 1996; Ferrara *et al*, 1996). Two VEGF-A receptors, VEGFR-1 (flt-1) (DeVries *et al*, 1992) and VEGFR-2 (flk-1/KDR) (Terman *et al*, 1992) have been identified. To date, six VEGF-A isoforms 111, 121, 145, 165, 189, and 206 have been identified in human tissues (Neufeld *et al*, 1999; Charnock-Jones *et al*, 1993; Houck *et al*, 1991 Tisher *et al*, 1991). As VEGF-A forms the basis of this thesis it is discussed in more detail later in this chapter.

1.5.2 Vascular Endothelial Growth Factor B (VEGF-B)

VEGF-B is a nonglycolyslated, highly basic growth factor with close structural similarities to VEGF-A_{165} (Joukov *et al*, 1996). There are two, known isoforms of VEGF-B: VEGF-B_{167} and VEGF-B_{186} (Olofsson *et al*, 1996). "VEGF-B_{186} is generated by using an alternative splice acceptor site in exon 6, resulting in an insertion of 101 bp between nucleotides 410 and 411 in the coding sequence of VEGF-B_{167}. The two isoforms have an identical NH_2-terminal domain of 115 amino acids and different COOH-terminal domains"(Vuorela *et al*, 1997, page 211). VEGF-B is thought to be angiogenic. This is based on the evidence that it stimulated thymidine incorporation into DNA, which gives an indication of cell multiplication, in human umbilical vein endothelium. Using Northern blot analysis VEGF-B has been found in the term human placenta although its precise location is yet to be studied (Vuorela *et al*, 1997).

1.5.3 Vascular Endothelial Growth Factor C (VEGF-C)

VEGF-C also known as vascular endothelial growth factor-related protein (VRF) is a growth factor with close structural similarities to VEGF-A_{165} (31% homologous); VEGF-B_{167} (27% homologous), PlGF-1 (25% homologous), and PDGF-A and PDGF-B (22 - 24% homologous). "VEGF-C encodes a protein of 419 amino acid residues, with a predicted molecular mass of 46.9 kDa"(Vuorela *et al*, 1997, page 212). Although not fully characterised, VEGF-C probably has several isoforms. Two VEGF-C mRNA of 2.4 and 2.0 kb have been detected in Northern blots from many embryonic and adult tissues (Joukov *et al*, 1997).

In addition, the mouse VEGF-C has been cloned and encodes a protein of 415 amino acid residues (Kukk *et al*, 1997). Two VEGF-C receptors VEGFR-2 and VEGFR-3 have been identified (Joukov *et al*, 1996). Northern blot analysis of mRNA expression of VEGF, from human placenta showed that the 1.4 kb VEGF-B was the most highly expressed, followed by the 2.4 kb VEGF-C, and finally the 4.5 and 3.7 kb VEGF-A (Vuorela *et al*, 1997). From *insitu* hybridisation studies, VEGF-C mRNA was particularly prevalent around lymphatic vessels under going sprouting from embryonic veins. These observations suggest that VEGF-C may regulate angiogenesis of lymphatic vasculature and has a paracrine mode of action (Achen *et al*, 1998; Kukk *et al*, 1996).

1.5.4 Vascular Endothelial Growth Factor D (VEGF-D)

VEGF-D, a mitogen for endothelial cells, is a ligand for the tyrosine kinase receptors VEGFR-2 and VEGFR-3 (Achen *et al*, 1998; Yamada *et al*, 1997). VEGF-D is most closely related to VEGF-C by virtue of the presence of N-and C-terminal extensions that are not found in the other VEGF family members. In adult human tissues, VEGF-D mRNA is most abundant in heart, lung, skeletal muscle, colon, and small intestine (Achen *et al*, 1998).

1.5.5 Placental Growth Factor (PlGF)

PlGF, with around 40% homology to VEGF-A_{165}, is a glycosylated dimeric protein. It has two known isoforms PlGF-1, which encodes for a 149 amino acid protein and PIGF-2 which encodes for a 170 amino acid protein (Hauser and Weich, 1993; Maglione *et al*, 1993). *In vitro*, PlGF-1 and PlGF-2 are both mitogenic and have been shown to be expressed by human choriocarcinoma cell lines. Using *insitu* hybridisation techniques, Vuorela *et al,* (1997) has shown the PlGF mRNA was observed in the villous trophoblasts, implying that it is produced in trophoblasts. Using immunohistochemical techniques, PlGF protein has been detected in vascular endothelium. Although PlGF antibody may cross react with VEGF-A, their results suggest that PlGF has a paracrine mode of action on endothelial proliferation (Vuorela *et al*, 1997).

1.6.0 STRUCTURE OF VEGF-A

In humans, six molecular species of VEGF-A have been identified (Figure 1.4), VEGF-A_{206} (Houck *et al*, 1991), VEGF-A_{189}, VEGF-A_{165} (Tisher *et al*, 1991), VEGF-A_{145} (Charnock-Jones *et al*, 1993), VEGF-A_{121} (Tisher *et al*, 1991) and VEGF-A_{111} (Burchardt *et al*, 1999). In rats and mice, VEGF-A comprises transcripts that are one amino acid shorter than their human counterparts, namely VEGF-A_{205}, VEGF-A_{188}, VEGF-A_{164}, VEGF-A_{144}, VEGF-A_{120} and VEGF-A_{110} (Burchardt *et al*, 1999; Jakeman *et al*, 1993; Charnock-Jones *et al*, 1993; Breier *et al*, 1992; Houck *et al*, 1991; Conn *et al*, 1990). VEGF-A_{165} is a heparin-binding dimeric glycoprotein with a molecular mass of approximately 22.3 kDa. Comparisons between VEGF-A_{165} in the human and VEGF-A_{164} in the mouse and rat show a high degree of homology (Breier *et al*, 1992).

Figure 1.3 shows a schematic representation of the VEGF-A splicing variants in humans. The number, for example 165, corresponds to the actual length of the fragment, that is 165 amino acid residues, excluding the 26 amino acid leader sequence of exon 1. Isoform 206 includes, exons 1-5, 6 A and B, 7 A and 8. To date, VEGF-A_{206} has only been found in liver cells from human fetuses (Houck *et al*, 1991) and human placenta cells (Anthony *et al*, 1994). Isoform 189 includes exons 1 to 5 plus 6A, 7A and 8. The 165 form lacks the 24 amino acid residue encoded by exon 6A. The 145 form lacks the 44 amino acid residue encoded by exon 7A. The 121 form lacks both the 24 amino acid residues encoded by exon 6 A, and the 44 amino acid residues encoded by exon 7A. Exons 1 to 5 are encoded in VEGF-A isoforms 206, 189, 165, 145, 121, whereas 111 only encodes exons 1 to 3 plus 7B and 8. Isoforms 121, 165, 189 (Torry *et al*, 1996) and 145 (Charnock-Jones *et al,* 1993) have been found in human endometrium and placenta, with VEGF-A_{121} and VEGF-A_{165} being the most predominate (Torry *et al*, 1996; Charnock-Jones *et al*, 1993). Although, exon 8 is encoded in all known VEGF-A transcripts, each transcript differs in the inclusion and arrangement of the sixth and seventh exons. Exon 1 contains a 26 amino acid hydrophobic leader sequence and exons 2 and 3 encode the two VEGF-A receptor binding sites. As exon 7A specifies binding to heparin sulfate, isoforms 206, 189 and 165 have heparin-binding activity. VEGF-A_{121} lacks the amino acids encoded

by exons 6 and 7 of the VEGF-A gene and thus does not bind to heparin or the ECM. VEGF-A_{145} has some amino acids encoded by exon 6A which contains a second independent heparin binding domain and some amino acids which enable the binding of VEGF-A_{145} to the ECM. VEGF-A_{189} and VEGF-A_{206} display a higher affinity to heparin and heparin-sulfates than VEGF-A_{165} or VEGF-A_{145}. The three secreted VEGF-A splice forms, 121, 145, and 165 induce proliferation of endothelial cells and in vivo angiogenesis. Figure 1.4 shows the sequence of human VEGF-A cDNA and comparison of amino acid sequence of multiple VEGF-A molecular species (Houck *et al*, 1991). Figure 1.5 shows the DNA sequence and predicted translation products of murine VEGF-A (Breier *et al*, 1992).

When expression of the splice variants was examined, it was found that most cell types produce several VEGF-A variants simultaneously. Usually the 121 and 165 forms are the predominant forms, but expression of the 189 form could also be seen in most VEGF-A producing cell types. In contrast, expression of isoforms 206, 145 and 111 seems to be more restricted, in particular to cells derived from reproductive organs (Neufeld *et al,* 1999; Burchardt *et al,* 1999).

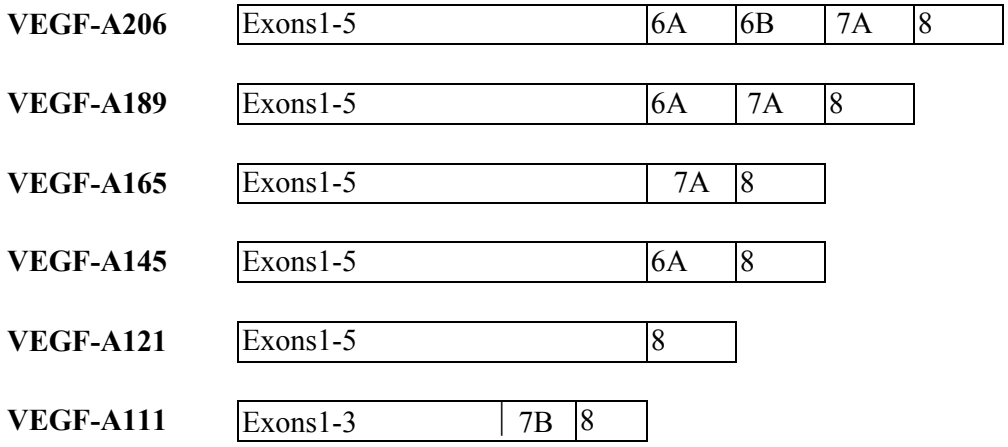
A number of workers have examined VEGF-A expression in a range of tissues using Northern blot analysis. Table 1.3 summarises their findings in terms of the size (kb) of the mRNA transcripts. The predominate expression of VEGF mRNA in the uterus from rats and mice was between 3.7 and 4.7 kb. It is interesting to note that the size of some of the transcripts is approximately the same as rRNA (4.7 kb and 1.3 kb).

Author	**Year**	**Target cells**	**Size (kb)**			
Vuorela *et al*	1997	human placenta	4.5	3.7	2.4	1.4
Pierce *et al*	1995	mouse retina	4.5	3.7		
Chakraborty *et al*	1995	mouse uterus	4.2	3.7	2.5	
Monacci *et al*	1993	various rat tissues	4.7	3.9		
Claffey *et al*	1992	mouse myogenic cell line	4.2			
Tischer *et al*	1991	human renal cells	5.5	4.4	3.7	
Atsushi *et al*	1994	human renal cells	7.5	4.4	2.4	1.4

Table 1.3 Size of VEGF-A mRNA transcripts from the literature

Figure 1.3. VEGF-A splice variants in the human (Note: human VEGF-A 206, 189, 165, 145, 121, 111: Murine VEGF-A 205, 188, 164, 144, 120, 110), (Houck *et al*, 1991; Torry *et al*, 1996; Breier *et al*, 1992; Conn *et al*, 1990; Charnock-Jones *et al*, 1993., Burchardt *et al*, 1999).

No of exons	1	2	3	4	5	6A	6B	7A	7B	8
No of amino acid	26	13	66	26	10	24	17	44	26	6



.

Figure 1.4. The sequence of human VEGF-A_{206} cDNA and comparison of amino acid sequence of multiple VEGF-A molecular species. "A). The complete sequence of VEGF-A_{206}"(Houck *et al*, 1991, Figure 2, page 1808). B). A comparison of the predicated primary amino acid sequence of six of the VEGF-A cDNA clones, illustrating the common insertion/detection site at codon 115 of VEGF-A_{165}.

B

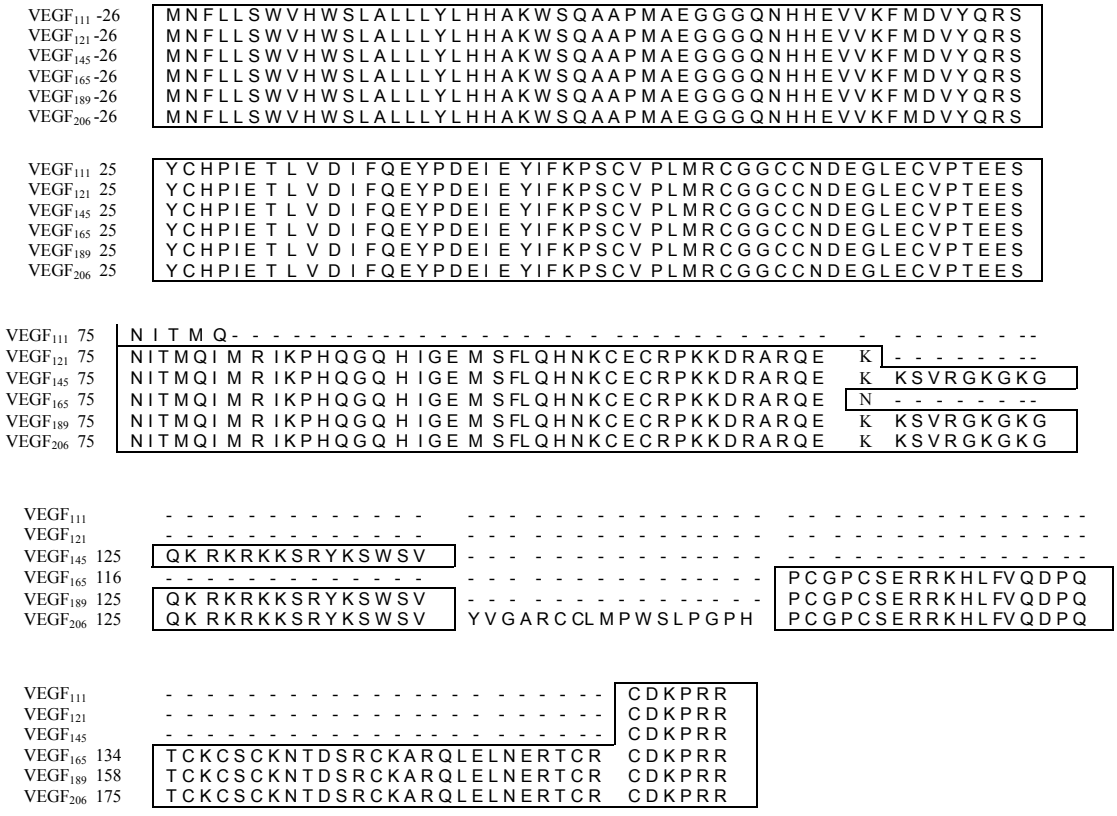


Figure 1.5. DNA sequence and predicted translation products of murine VEGF-A_{188} (Breier *et al,* 1992), exon 1 (grey) exons 2 and 3 (blue), exons 4 and 5 (yellow) exon 6 A (pink), exon 7 A (green) and exon 8 (red). VEGF-A_{164} lacks a stretch of 24 amino acid residues from position 115 to 138 in exon 6 A (pink). VEGF-A_{144} lacks a stretch of 44 amino acid residues from position 139-183 in exon 7 A (green). VEGF-A_{120} lacks a stretch of 68 (24 + 44) amino acid residues from position 115 to 183 (pink plus green). Finally, VEGF-A_{110} includes exon 1 (grey) exons 2 and 3 (blue) and exon 8 (red). The sequence of murine $VEGF_{205}$ has not been published.

```
13   ATG AAC TTT CTG CTG TCT TGG  GTG CAC  TGG ACC CTG GCT TTA CTG  CTG TAC
-26  met asn  phe leu leu ser  trp  val  his  trp  thr leu ala  leu  leu  leu tyr

64   CTC CAC CAT GCC AAG TGG  TCC CAG GCTGGA CCC ACG ACA GAA GGA GAG  CAG
-9   leu his  his  ala lys  trp  ser  gln ala  ala  pro thr thr glu gly  glu  gln

115  AAG TCC CAT GAA GTG ATC AAG TTC ATG GAC GTC TAC CAG CGA  AGC TAC TGC
9    lys ser  his  glu val  ile lys  phe met asp  val tyr  gln  arg  ser tyr cys

166  CGT CCA ATT GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCC GAC GAG  ATA
26   arg pro  ile  glu thr  leu val  asp  ile  phe gln  glu tyr  pro asp  glu  ile

217  GAG TAC ATC TTC AAG CCG TCC TGT GTG CCG  CTG  ATG CGC TGT GCA GGC TGC
43   glu tyr  ile  phe lys  pro ser cys  val  pro  leu met arg cys  ala  gly cys

268  TGT AAC GAT GAA GCC CTG GAG TGC GTG CCC  ACG TCA GAG AGC  AAC ATC ACC
60   cys asn  asp  glu ala  leu glu cys val  pro  thr ser glu  ser  asn ile thr

319  ATG CAG ATC ATG CGG ATC AAA CCT CAC CAA AGC CAG CAC  ATA GGA GAG ATG
77   met gln  ile  met arg  ile lys  pro  his  gln ser gln  his  ile gly  glu met

370  AGC TTC CTA CAG CAC AGC GGA TGT GAA TGC  AGA CCA AAG AAA GAC AGG ACA
94   ser phe  leu  gln his  ser  arg cys  glu  cys arg pro  lys  lys asp  arg  thr

421AAG CCA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGT CAA AAA CGA AAG
111 lys pro glu  lys lys  ser val arg gly lys gly lys gly gln  lys arg  lys

472  CGC AAG AAA TCC CGG TTT AAA TCC  TGG AGC  GTT  CAC TGT GAG CCT TGT TCA
128  arg lys  lys  ser arg  phe lys  ser  trp  ser  val  his cys glu  pro cys ser

523  GAG CGG AGA AAG CAT TTG TTT GTC  CAA  GAT  CCG CAG ACG TGT AAA TGT TCC
145  glu arg  arg  lys his  leu phe val gln  asp  pro gln thr cys  lys cys ser

574  TGC AAA AAC ACA GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC  GAA
162  cys lys  asn  thr  asp  ser arg  cys lys  ala arg gln  leu glu  leu asn  glu

625  CGT ACT TGC AGA TGT GAC AAG CCG AGG CGG TGA ATT C
179  arg thr  cys arg cys asp lys  pro arg arg
```

1.7.0 VEGFR-1 AND VEGFR-2

Receptor tyrosine kinases (RTK) constitute a class of signal transduction molecules, which play a pivotal role in development. RTKs are composed of an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase catalytic domain (Ullrich and Schlessinger, 1990). Ligand binding to these receptors activates the tyrosine kinases domain, which in association with intracellular target proteins transduces a signal to the nucleus of the target cell (Figure 1.6, Yarden and Ullrich, 1988).

Two tyrosine kinases, c-fms-like tyrosine kinase (Flt-1) (Shibuya *et al,* 1990; deVries *et al,* 1992) and rat fetal liver kinase 1 (flk-1) (Mathews *et al,* 1991) also known as kinase insert domain-containing receptor (KDR) in the human (Terman *et al,* 1992) have been identified as high affinity VEGF-A receptors. Flt-1 is now called VEGFR-1 and flk-1 is called VEGFR-2. Both receptors are type III RTKs, with kinase inserts domains of similar length; each contains seven extracellular immunoglobulin-like domains with a high degree of amino acid homology (Figure 1.7, Kendall & Thomas, 1993).

VEGFR-1 not only binds VEGF-A, but also VEGF-B and PlGF-1. VEGFR-2 not only binds VEGF-A, but also VEGF-C and VEGF-D. (Neufeld *et al,* 1999). Figure 1.8 shows the partial amino acid sequence of VEGFR-1 (Shibuya *et al,* 1990) and Figure 1.9 shows the partial amino acid sequence of VEGFR-2 (Millauer *et al*, 1993).

A number of workers have examined VEGFR-1 and VEGFR-2 expression in a range of tissues using Northern blot analysis. Tables 1.4 and 1.5 summarise their findings in terms of the size (kb) of the mRNA transcripts.

The importance of both VEGF-A receptors is indicated by the lethality *in utero* of either gene via knockout models. Embryos lacking the VEGFR-2 gene die before birth because differentiation of endothelial cells does not take place and blood vessels do not form (Shalaby *et al,* 1995).

VEGFR-1					
Authors	**Year**	**Targets cells**	**Size (kb)**		
Shibuya *et al*	1990	human placenta	8.0	3.0	2.2
Yamane *et al*	1994	rat liver	8.5		
Chakraborty *et al*	1995	mouse uterus	7.5	6.5	
Barleon *et al*	1996	human monocytes	7.5	3.4	2.7
Das *et al*	1997	rabbit uterus	7.5	6.5	
Barks *et al*	1998	human placenta	7.5	3.4	2.6

Table 1.4. Size of theVEGFR-1 mRNA transcripts from the literature.

VEGFR-2				
Authors	**Year**	**Targets cells**	**Size(kb)**	
Millauer *et al*	1993	mouse embryo		5.5
Yamane *et al*	1994	rat liver	7.0	
Chakraborty *et al*	1995	mouse uterus	7.5	
Barleon *et al*	1996	human monocytes	7.0	
Das *et al*	1997	rabbit uterus	7.5	5.8

Table 1.5. Size of VEGFR-2 mRNA transcripts from the literature.

A recent study, has indicated that VEGFR-2 is required for the differentiation of endothelial cells and for the movement of primitive precursors of endothelial cells from the posterior primitive streak to the yolk sac, a precondition for the subsequent formation of blood vessels (Shalaby *et al,* 1997).

Disruption of the gene encoding the VEGFR-1 receptor "did not prevent the differentiation of endothelial cells in homozygous animals, but the development of functional blood vessels from these endothelial cells was severely impaired" (Fong *et al,* 1995; page 68). Activation of the VEGFR-1 receptor promotes cell migration but does not induce cell proliferation efficiently (Barleon *et al,* 1996; Seetharam *et al,* 1995). It is possible that decreased cell migration or defects in endothelial cell to cell or cell to matrix interactions leads to defective organisation of blood vessels in mice lacking functional VEGFR-1 (Neufeld *et al,* 1999).

In 1996, Keyt *et al,* using mutational analysis, showed that VEGF-A displayed different receptor binding sites for VEGFR-1 and VEGFR-2 as shown in Figure 1.10. "One site on the VEGF-A gene, composed of basic residues (Arg, Lys and His), mediates binding to VEGFR-2. Whereas another site, composed of acidic residues (Asp, Glu, Glu), mediates binding with VEGFR-1. The binding domains are located at opposite ends of the VEGF-A monomer. In the mature VEGF-A dimer, the monomers are linked roughly head to tail, with a large overlap region, by disulfide bridges such that the binding domains are at opposite ends of the VEGF-A molecule" (Neufeld *et al,* 1999; page 14).

Both receptors are found predominantly, but not exclusively, on endothelial cells. VEGFR-1 and VEGFR-2 expression has been localised to endothelial cells, both during embryological development and in some adult tissues such as sinusoidal endothelial cells in normal rat liver (Yamane *et al*, 1994; Shweiki *et al*, 1993; Millauer *et al*, 1993; Quinn *et al*, 1993; Jakeman *et al*, 1993). Localisation of these receptors on endothelial cells indicates the important role of VEGF-A in stimulating endothelial proliferation and migration associated with angiogenesis and in regulating vascular permeability.

VEGFR-1 and/or VEGFR-2 have also been localised to nonendothelial cells including trophoblast cells (Ahmed *et al,* 1995; Charnock Jones *et al,* 1994), monocytes (Barleon *et al,* 1996; Clauss *et al,* 1996), renal mesangial cells (Takahashi *et al,* 1995), haematopoietic cells (Katoh *et al,* 1995), retinal progenitor cells (Yang and Cepko, 1996), uterine smooth muscle cells (Brown *et al,* 1997) and tumour cell lines (Cohen *et al,* 1995; Boocock *et al,* 1995; Gitay-Goren *et al,* 1993).

Four soluble forms of VEGFR-1 have been identified. These all lack the transmembrane and intracellular domains. One of these, a soluble 110 kDa form of the receptor has been identified in the supernatant of cultured human umbilical vein endothelial cells (Kednall *et al,* 1996; Kendall and Thomas, 1993). These authors showed that this soluble variant was able to inhibit VEGF-A induced endothelial cell proliferation *in vitro* and hence they suggest it may be a naturally occurring antagonist

Figure 1.6. Structure of receptor tyrosine kinases. "Indicated are amino (N) and carboxy (C) terminal ends of the receptor polypeptide chain, potential binding sites for the ligand, ATP, and endogenous substrates. Cysteine residues (open circles) that may

play a role in forming the ligand-binding pocket and tyrosine residues (filled circles) involved in receptor function are emphasized" (Yarden and Ullrich, 1988; Figure 1, page 445).

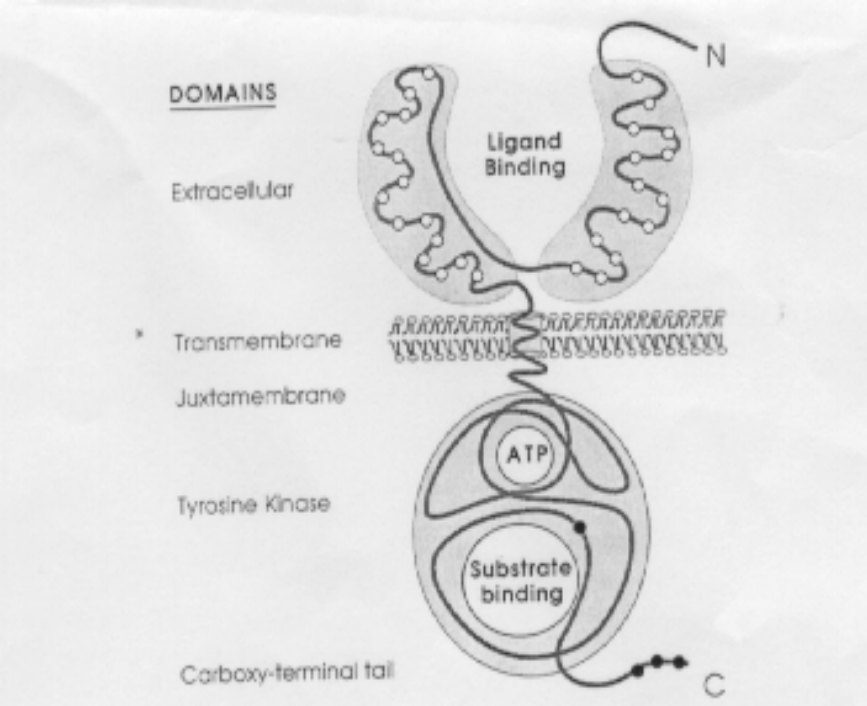


Figure 1.7. Structure of VEGFR-1 (or Flt-1) and VEGFR-2 (or KDR). Each receptor has seven extracellular immunoglobulin-like domains, "a single plasma membrane spanning sequence and an intracellular tyrosine kinase domain. The numbers 2 to 7 indicate the immunoglobulin-like domains in the extracellular ligand-binding regions from the **N** termini with the characteristic immunoglobulin-fold disulfide-bonded cysteine-reside sulfur atoms (**S**) marked. Soluble VEGFR-1 (sFlt-1) and which lacks a membrane-spanning domain is also schematically illustrated" (Kendall and Thomas, 1993; Figure 1, page 10,706).

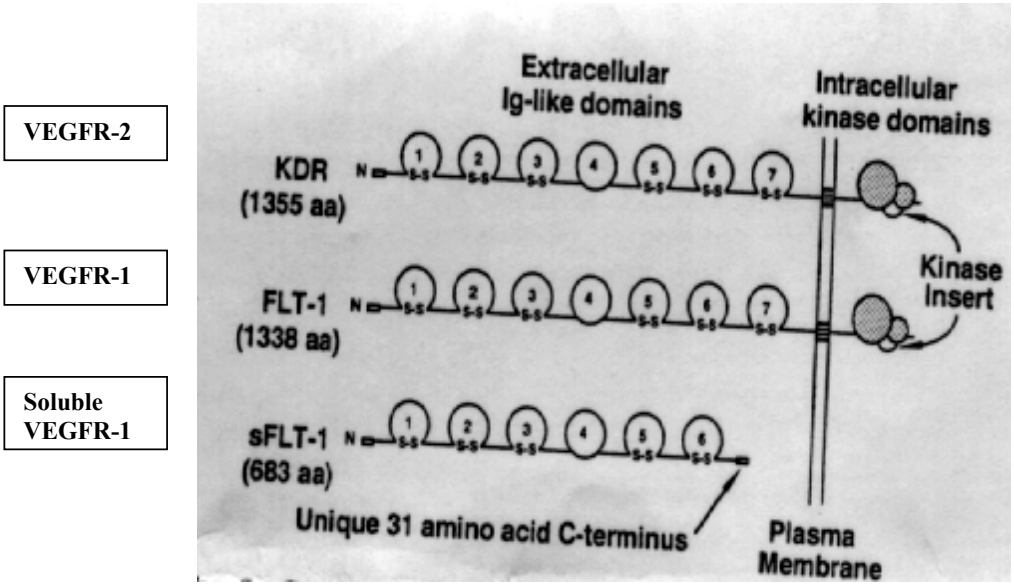


Figure 1.8 The partial VEGFR-1 amino acid sequence (286) from human placentae (Shibuya *et al*, 1990).

```
                1                                                  40
VEGFR-1     HSEAPCDFCSNNEESFILDADSNMGNRIESITQRMAIIEG
               41                                                  80
VEGFR-1   KNKMASTLVVADSRISGIYHCIASNKUGTVGRNISFYITD
               81                                                 120
VEGFR-1   VPNGFHVNLEKMPTEGEDLKISCTVNKFLYRDVTWILLR
              121                                                 160
VEGFR-1  TVNNRTMHYSISKQKMAITKEHSITLNLTIMNVSLQDSGT
              161                                                 200
VEFDR-1  YACRARNVYTGEEILQKKEITIRDQEAPYLLRNLSDHTVA
              201                                                 240
VEGFR-1  ISSSTTLDCHANGVPEPQ I TWFKNNHKIQQEPG I I LGPGS
              241                                                 280
VEGFR-1  STLFI ERVTEEDEGVYHCKATNQKGSVESSAYLTVQGTSD
              281
VEGFR-1  KSNLEL
```

Figure 1.9 VEGFR-2 amino acid sequence from mouse. "Comparison of the VEGFR-2 (Flk-1) amino acid sequence with human KDR, only difference are shown at positions 25 (The instead of Pro), 783 (val instead of Leu), 784 (Leu instead of Val), and 917 (Cys instead of Ser)" (Millauer *et al,* 1993; Figure 1, page 836).

Figure 1.10. Interaction of VEGF-A with its receptors VEGFR-1 and VEGFR-2. The interaction of VEGF-A with the binding sites of VEGFR-1 and VEGFR-2 are shown in A and B, as viewed below. "The two VEGF monomers are shown in a head to tail orientation, indicated by arrows, and held together by disulfide bonds shown in orange. The main cluster of VEGF-A amino acids that bind VEGFR-1 is located at one end of the VEGF monomer as shown in Figure A; the main cluster of VEGF-A amino acids that bind VEGFR-2 is located at the opposite pole of the VEGF-A monomer as shown in Figure B. Nevertheless, the VEGF-A domains that bind VEGFR-1 and VEGFR-2 overlap as shown. The main VEGF binding domain of the VEGFR-1 and VEGFR-2 receptors is located in immunoglobulin-like loop 2, but loop 3 also participates in the binding. Two VEGFR-1 receptors from a dimer that undergoes autophosphorylation on tyrosine residues located in the cytoplasmic part of the VEGFR-1 receptor (P), leading to the initiation of signal transduction. The dimer is held together by the interaction of each VEGFR-1 with a common VEGF-A dimer and is further stabilised by interactions between amino acids located at the loop 4 dimerization domain as shown in Figure C" (Neufeld *et al,* 1999; Figure 2, page 14).

of VEGF-A. Boocock *et al,* (1995) found two soluble forms of VEGFR-1 in ovarian carcinoma cell lines, primary ascitic cells and cultured human umbilical vein cells. Recently, Banks *et al,* (1998) have found a fourth soluble 150 kDa form of VEGFR-1 to be present in placenta, amnion, chorion, umbilical vein, maternal blood and fetal blood.

1.8.0 FUNCTIONS OF VEGF-A

Although, the functions of VEGF-A are not fully understood, it has been shown to have a role in 1) endothelial cell growth and proliferation, 2) breakdown of the endothelial basement membrane, 3) increasing vascular permeability and protein leakage, 4) monocyte chemotaxis and 5) changes in vascular tone. Perhaps its most important role, and certainly the most investigated to date, is as the most potent known mitogen for vascular endothelial cells derived from arteries, veins and lymphatics. Thus, it has a pivotal role in vasculogenesis and angiogenesis.

1.8.1 Endothelial Cell Growth and Proliferation

VEGF-A has been shown to be expressed by endothelial cells and by non-endothelial cells such as epithelial cells, monocytes, smooth muscle cells, macrophages and trophoblast cells. Therefore, VEGF-A acts in an autocrine and a paracrine fashion (Brown *et al,* 1997; Gitay-Goren *et al,* 1996; Pierce *et al,* 1995; Berse *et al,* 1992; Plate *et al,* 1992; Connolly *et al,* 1989). It binds to its receptors which are found predominantly on endothelial cells but also on non-endothelial cells including trophoblast cells (Ahmed *et al,* 1995; Charnock-Jones *et al,* 1994), monocytes (Barleon *et al,* 1996; Clauss *et al,* 1990) and tumour cell lines (Cohen *et al,* 1995; Boocock *et al,* 1995). Although some non endothelial cells, such as human myometrial smooth muscle cells (Brown *et al,* 1997), have been shown to respond to VEGF-A, it remains the most selective vascular endothelial cell mitogen known (Thomas, 1996).

As a potent endothelial mitogen, VEGF-A has been shown to elicit an angiogenic response in a range of *in vivo* models including the chick chorioallantoic membrane (Leung *et al,* 1989; Connolly *et al,* 1989) the rabbit cornea (Phillips *et al,* 1995), the primate iris (Tolentino *et al,* 1996), and rabbit bone (Connolly *et al,* 1989). The mitogenic activity of VEGF-A has been shown to be important in tissue repair from injury (Thomas, 1996). Exogenous VEGF-A induces angiogenesis and thus increases perfusion in ischaemic rabbit limbs (Bauters *et al,* 1995), in response to decreased blood flow in porcine coronary arteries (Harada *et al,* 1996) and in promoting repair of damaged carotid arteries in the rat (Asahara *et al,* 1995). Elevated expression of VEGF-A has also be shown to be correlated with the progression of several diseases,

such as tumours (Shweiki *et al,* 1992), diabetic retinopathy (Aiello *et al,* 1994), rheumatoid arthritis (Koch *et al,* 1994; Fava *et al,* 1994) and psoriasis (Detmar *et al,* 1994).

1.8.2 Breakdown of the Endothelial Basement Membrane

Angiogenesis by capillary sprouting involves the breakdown of the basement membrane by enzymes, "primarily MMP and PA, while TIMP and PAI inhibit the actions of these enzymes. VEGF-A has been shown to induce expression of the MMP collagenase and PA suggesting that VEGF-A stimulates factors responsible for the breakdown of the endothelial basement membrane" (Pepper *et al*, 1991; page 903). VEGF-A has also been shown to induce expression of PAI, and it is thought that this provides a negative regulatory step that serves to balance the proteolytic process (Pepper *et al*, 1991). It certainly appears that a fine balance in VEGF-A expression is required for appropriate rather than inappropriate angiogenesis (Ferrara and Davis-Smyth, 1997).

1.8.3 Increasing Vascular Permeability and Protein Leakage

VEGF-A increases vascular permeability, in fact, it was originally called vascular permeability factor (VPF). VEGF-A induces vascular leakage *in vivo* (Keck *et al,* 1989: Jakeman *et al,* 1992; Senger *et al,* 1983). When it was injected intradermally, (Connolly *et al,* 1989) or administered topically, it was shown to induce fenestrations or openings in endothelial cells lining small venules and capillaries consequently increasing their permeability (Roberts *et al,* 1995). The observation that blood vessels undergoing angiogenesis, for example in and around tumours, display increased permeability toward plasma fluid and proteins has led to the hypothesis that tumour cells secrete VEGF-A which acts to increase vessel permeability and the induction of plasma protein leakage (Dvorak *et al,* 1995). This results in the formation of an extravascular fibrin gel, a substrate for endothelial and tumour cell growth (Ferrara and Davis-Smyth, 1997).

Ferrara *et al*, (1997) have forwarded a model (Figure 1.11) for the complex series of effects of VEGF-A on the vascular endothelium, "including cell sprouting; induction of interstitial collagenase" (Ferrara *et al*, 1997; page 6), PA and PAI-1, increased

vascular permeability and the associated leakage of plasma proteins. Plasminogen activation results in the production of plasmin, "which may cleave extracellular matrix bound VEGF-A_{189} or VEGF-A_{206} to release the diffusible VEGF-A_{110}" (Ferrara *et al*, 1997; page 6). Plasmin may also result in an increase in collagenase expression. To ensure a balanced approach rather than rampant angiogenesis PAI may play a regulatory role by inhibiting PA (Ferrara *et al*, 1997).

1.8.4 Monocyte Chemotaxis

VEGF-A promotes monocyte chemotaxis (Baeleon *et al,* 1996; Clauss *et al,* 1990). Following stimulation, monocytes move from the blood compartment in to the tissues. "Movement of these mononuclear phagocytes is a crucial process in inflammatory reactions and immune response. It has been postulated that VEGF-A may even stimulate tumour growth by allowing the tumour to avoid the body's normal immune response" (Gabrilovich *et al,* 1996; page 1098). "Fetal macrophages, which are located in Hofbauer cells within the fetal blood compartment"(Clark *et al,* 1996; page 1092), express VEGFR-1 and thus have a role to play in monocyte chemotaxis *in utero* (Clark *et al,* 1996; Banks *et al,* 1998).

1.8.5 Changes in Vascular Tone

VEGF-A induces vasodilatation *in vitro* in a dose dependent fashion (Ku *et al,* 1993) and produces hypotension *in vivo* (Yang *et al,* 1996). These effects appear to be mediated primarily by endothelial cell derived nitric oxide. VEGF-A_{165} has been shown to stimulate nitric oxide release (Ahmed *et al,* 1997). It has been postulated that enhanced in nitric oxide release, by VEGF-A, may aid syncytiotrophoblast invasion and prevent platelet aggregation within the placenta (Ahmed *et al,* 1997).

VEGF-A has also been shown to have additional actions. VEGF-A stimulates transport of hexose, a monosaccharide, across endothelial plasma membranes (Pekala *et al,* 1990). This may satisfy the increased energy demands of endothelial cells during the process of growth and proliferation (Ferrara and Davis-Smyth, 1997). Melder *et al,* (1996) have shown that VEGF-A promotes adhesion of natural killer cells to endothelial cells.

1.9.0 REGULATION OF VEGF-A

A number of factors have been shown to regulate the expression of VEGF-A and its receptors VEGFR-1 and VEGFR-1, these include 1) a large array of growth factors, 2) physical stress and blood flow, 3) hormones, 4) hypoxia, 5) erythropoietin and 6) cell differentiation. Due to the high potential for therapeutic use, a substantial amount of work is currently being conducted to understand the factors, which regulate expression of VEGF-A and its receptors. Knowledge of the factors responsible for stimulating VEGF-A expression, would be of considerable therapeutic value for disorders characterised by inadequate tissue perfusion, such as, obstructive atherosclerosis or coronary insufficiency. On the other hand, knowledge of the factors responsible for inhibiting VEGF-A expression, would be of considerable therapeutic value for disorders characterised by uncontrolled tissue growth, such as cancer, rheumatoid arthritis, psoriasis and endometriosis.

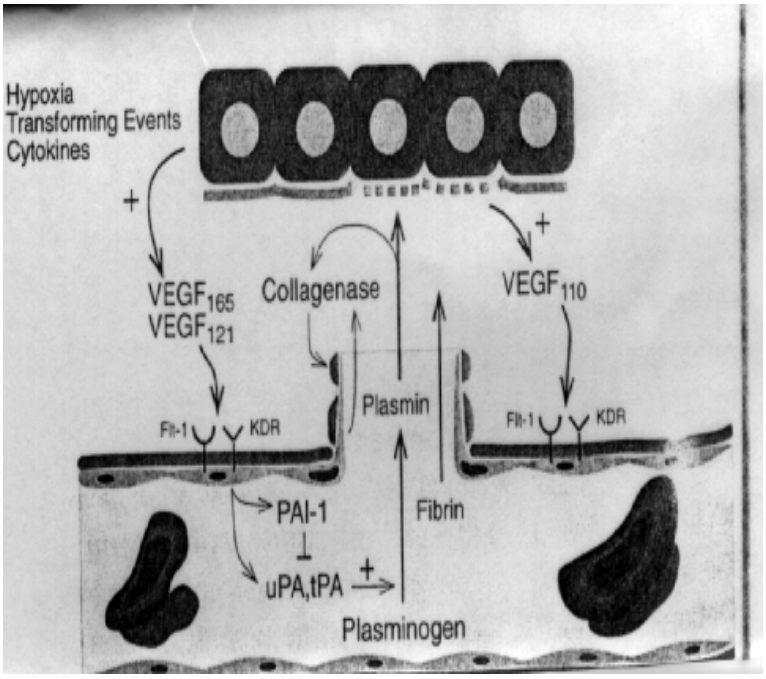
We would make inroads into the clinical repair of tissues exposed to injury or hypoxia and if we knew how to limit VEGF-A expression. Thus, we would be able to control tumour growth. Consequently, the availability of special monoclonal antibodies capable of inhibiting VEGF-A induced angiogenesis by 70 - 95% *invivo* and *invitro* (Kim *et al,* 1992) has engendered much excitement in this research area (Ferrara and Davis-Smyth, 1997).

1.9.1 Growth Factors

Several growth factors up-regulate VEGF-A mRNA expression or enhance the release of VEGF-A protein. From a variety of cultured cells, VEGF-A expression has been shown to be enhanced by platelet derived growth factor (Brogi *et al,* 1994)

Figure 1.11. Schematic model for the complex series of effects of VEGF-A on the vascular endothelium. "Several stimuli may result in the release of the diffusible alternatively spliced VEGF-A isoforms (VEGF-A_{165}, VEGF-A_{121}) from a variety of cell types. These proteins may induce a complex series of effects on the vascular endothelium, including cell sprouting, induction of interstitial collagenase, plasminogen activators (PA), and plasminogen activator inhibitor-1 (PAI-1), as well as extravasation of plasma proteins. Plasminogen activation results in generation of

plasmin, which may cleave extracellular matrix-bound VEGF-A (VEGF-A_{189} or VEGF-A_{206}) to release a diffusible proteolytic fragment (VEGF-A_{110}). Plasmin may also activate procollagenase. Activation of PAI-1 may constitute a negative regulatory step, by inhibiting the action of PA" (Ferrara and Davis-Smyth, 1997; Figure 1, page 6).



keratinocyte growth factor (Frank *et al,* 1995), tumour necrosis factor α (Frank *et al,* 1995), epidermal growth factor (Frank *et al,* 1995; Goldman *et al,* 1993), insulin like growth factor 1 (Warren *et al,* 1996), transforming growth factor-β (Frank *et al,* 1995; Petovara *et al,* 1994; Brogi *et al,* 1994), interleukin-1α (Ben-Av *et al,* 1995), interleukin-1β (Li *et al,* 1995) and interleukin-6 (Cohen *et al,* 1996). On the other hand, interleukin 10 and interleukin 13 inhibit the release of VEGF-A (Matsumoto *et al,* 1997).

1.9.2 Physical Stress and Blood Flow

Pulsatile stretch has been shown to stimulate VEGF-A expression by cultured rat cardiac myocytes (Seko *et al*, 1999).

1.9.3 Hormones

VEGF-A expression has been shown to be up regulated by prostaglandin E_2 (PGE_2) (Ben-Av *et al,* 1995; Harada *et al,* 1994), estradiol E_2 and E_3 (Hyder *et al,* 1996 Cullinan-Bove *et al,* 1993) and possibly progesterone (Charkraborty *et al,* 1995).

1.9.4 Hypoxia

Evidence has accumulated that hypoxia is a strong inducer of VEGF-A mRNA *invitro* and *invivo* (Minchenko *et al,* 1994; Brogi *et al,* 1994; Ladoux and Frelin, 1993; Shweiki *et al* 1992). It is thought that hypoxia may be the stimulus for increased VEGF-A expression in tumour growth, in placental development and in tissue repair from injury (Ferrara and Davis-Smyth, 1997).

Evidence suggests that the hypoxia-inducible factor 1 (HIF-1) is responsible for activation of the VEGF-A gene transcription during hypoxia (Forsythe *et al,* 1996; Levy *et al,* 1995; Wang and Semenza, 1993). Although the exact mechanisms responsible for hypoxia induced VEGF-A expression are unclear, adenosine, (Takagi *et al,* 1996) and a hypoxia-induced protein (Levy *et al,* 1996) have been implicated (Ferrara and Davis -Smyth, 1997).

Similarly, hypoxia has been shown to play an important role in the regulation of the VEGF-A receptors. Exposing rats to acute or chronic hypoxia has been shown to induce expression of VEGFR-1 and VEGFR-2 in the lung vasculature (Tuder *et al,* 1995). Myocardia infarction in the rat and the resultant hypoxia has been shown to up regulate both receptors (Li *et al,* 1996).

1.9.5 Erythropoietin

Erythropoietin enhances VEGF-A expression and the mechanisms responsible appear to be similar to those of hypoxia (Goldberg and Schneider, 1994).

1.9.6 Cell Differentiation

Cell differentiation has been shown to play an important role in VEGF-A expression (Claffey *et al,* 1992). VEGF-A expression is up-regulated during the differentiation of preadipocytes into adipocytes. Activators of adenylate cyclase, namely phorbol esters, forskolin and luteotrophic hormone has been shown to induce VEGF-A expression. Similarly, tumour suppressor genes and other oncogenic mutations induce VEGF-A expression. These findings suggest that alterations in some pathways which regulate cellular activities up-regulate VEGF-A expression leading to uncontrolled proliferation (Neufeld et al, 1999; Ferrara and Davis -Smyth, 1997).

1.10.0 VEGF-A IN UTEROPLACENTAL TISSUES

To date, very little is known about the functional role of VEGF-A in normal and abnormal placental development. Several workers have examined the distribution of proteins and mRNA for VEGF-A and its receptors, within the human and animal placenta and decidua. Except for VEGF-A_{110}, all other isoforms of VEGF-A have been reported to be expressed in uteroplacental tissues and embryonic tissues of the human, particularly during implantation. The predominate isoforms in placental cells were VEGF-A_{165} and VEGF-A_{121} (Athanassiades *et al,* 1998; Vuorela *et al,* 1997; Wheeler *et al,* 1995; Anthony *et al,* 1994; Houck *et al,* 1991).

VEGF-A_{188}, VEGF-A_{164}, and VEGF-A_{120} have been found in uteroplacental tissues of rabbits and rats (Cullinan-Bove *et al,* 1992; Leung *et al,* 1989; Keck *et al,* 1989). Charnock Jones *et al,* (1993) has detected VEGF-A_{145} in the human uterus. Cullinan-Bove *et al*, (1992) has reported the expression of VEGF-A_{164} and VEGF-A_{120} by the rat uterus.

Srivastava *et al,* (1998) using RT-PCR analysis, detected only one major PCR product corresponding to VEGF-A_{164} in antimesometrial and mesometrial decidual tissues of a pseudopregnant rat. Using Northern blot analysis and *insitu* hybridisation, Das *et al,* (1997) examined expression and localisation of VEGF-A and its receptors in the uteroplacental tissues of the rabbit from oestrus (day 0) to implantation (day 8). Expression of VEGF-A and its receptors was shown to be highest at days 0 and 6-8, with the syncytiotrophoblast as the predominate localisation site. A similar pattern of expression was found in the mouse uteroplacental tissues during implantation (Chakraborty *et al,* 1995).

Using PCR and *insitu* hybridisation on human placental tissues collected in the first trimester and at term, Sharkey *et al,* (1993) showed that VEGF-A was expressed in cytotrophoblast and syncytiophoblast, but was primarily localised in fetal and maternal macrophages and in glandular epithelium in the maternal decidua.

Later studies (Clark *et al,* 1996; Charnock-Jones *et al,* 1994) showed strongest localisation of VEGF-A mRNA within the maternal macrophages, whereas immunoreactive VEGF-A protein was also localised in cytotrophoblast, syncytiophoblast and Hofbauer cells. Similarly, other workers (Vuorola *et al,* 1997; Cooper *et al,* 1995; Ahmed *et al,* 1995) have also detected VEGF-A protein in Hofbauer cells, decidua and maternal macrophages. Taken together, these studies indicate that VEGF-A is widely distributed in the placenta and the decidua.

VEGFR-1 and VEGFR-2 have also been shown to be expressed by human placenta early in gestation and at term (Banks *et al,* 1998; Clark *et al,* 1996). Although found in extravillous trophoblast throughout pregnancy, expression of VEGFR-1 was "temporally and spatially regulated with patchy expression in villous trophoblast being down-regulated in mid-gestation and increasing at term" (Clark *et al,* 1996; page 1195).

Immunohistochemical and *in situ* hybridisation studies revealed the presence of VEGFR-2 protein and mRNA in endothelial cells of the placenta and decidua but not

in the placental trophoblast cells (Clark *et al,* 1996; Vuckovic *et al,* 1996). Subsequent work by Athanassiades *et al,* (1998) showed that cultured extravillious trophoblast cells expressed both VEGFR-1 and VEGFR-2. Taken together, these findings suggest that both the endothelial and trophoblast cells are potential target sites for VEGF-A but that each may have a different role in regulating growth and proliferation.

Perhaps some of the most interesting, but conflicting, clinical findings to date are those relating to the association between VEGF-A and pre-eclampsia. Serum concentrations of VEGF-A have been shown to be higher, or on the other hand lower, in patients with pre-eclampsia compared to values from normotensive women (Baker *et al,* 1995). Additionally, mRNA expression levels of VEGF-A in the placenta have been shown to be reduced, below control values, in women with pre-eclampsia (Cooper *et al,* 1996).

1.11.0 AIMS

From the literature review, we can see that VEGF-A has a vital role in vasculogenesis and angiogenesis in uteroplacental issues. However, there is a paucity of work examining the role of VEGF-A following experimental manipulations known to inhibit placental growth and development, including pre-eclampsia.

This master's project forms part of a larger program investigating the role of growth factors in the development of the placenta, fetal membranes and fetal lungs. We have focused on VEGF-A, as it is the most potent angiogenic factor found to date. Although many laboratories throughout the world are working on VEGF-A, our laboratory is new to this growth factor. Consequently, the first aim of this project was to establish techniques in our laboratory, for measuring expression and localisation of VEGF-A and its receptors in rat uteroplacental tissues at early, mid and late gestation.

VEGF-A mRNA expression has been shown to be lower in placentas from women suffering from pre-eclampsia than from women with normal pregnancies. To further investigate these findings, one of the aims of this study, was to examine whether

expression of VEGF-A and its receptors differs in the normotensive and the spontaneously hypertensive rat, as this is a previously developed animal model of pre-eclampsia.

1.11.1 Specific Aims

1. To establish the use of RT-PCR, Northern blot analysis, immunohistochemistry and *insitu* hybridisation, to measure expression and localisation of VEGF-A, VEGFR-1 and VEGFR-2 in normal rat uteroplacental tissues at early, mid and late gestation.

2. To examine, using RT-PCR and Northern blot analysis, the expression of VEGF-A, VEGFR-1 and VEGFR-2 in the uteroplacental tissues of the normotensive (WKY) and the spontaneously hypertensive rat (SHR) at early, mid and late gestation.

CHAPTER 2 MATERIALS AND METHODS

2.1.0 ANIMALS

2.1.1. Animal Husbandry

Animals were obtained either from Monash University Central Animal House (Sprague Dawley rats) or from the Alfred Hospital Animal House (spontaneously hypertensive rats: SHR and Wistar Kyoto rats: WKY). Animals were given water and food pellets as required. Their cages were cleaned twice weekly and they were housed in a room with a 12-hour light/dark cycle. At least 3 days prior to post mortem the animals were transported to either the Department of Physiology or the Department of Anatomy at Monash University and allowed to acclimatise to their new environment.

2.1.2 Ethical Approval

All methods conformed to the Australian National Health and Medical Research Council published code of practice for the use and care of animals in research. Ethical approval was received from the Monash University Physiology Animal Ethics Committee (AEETH 95/112) and the Victoria University Animal Ethics Committee (AEETH 97/11).

2.1.3 Collection of Uteroplacental Tissues

Rats were anaesthetised by over exposure to CO_2 or by over dose of sodium pentobarbitone (100mg/kg i.p.). The pleural cavity was incised to ensure that the animals did not recover from anaesthesia. A midline abdominal incision was made to expose the uterus (Figure 2.1). In most experiments, the number of fetuses in each uterine horn was counted. In animals with a gestation greater than 11 days, an incision was made into each conceptus and the placenta easily removed from the uterus. In these older animals the placenta (fetal portion) and the uterus were frozen separately. In animals with a gestation less than 11 days, it was difficult to successfully separate the placenta and uterus; hence in these animals the uterus and placenta were frozen together. At 11 days, the uterus was collected either with or without the conceptus, depending on the size of the mother. When available, a dissecting microscope was used to remove the tissues. Consequently, samples taken at 5, 7, 9 and sometimes 11 days of gestation, consisted of uterus, placenta and fetus combined. At 13, 15, 17, 19

Figure 2.1 Collection of uteroplacental tissues. A) Dissection of uteroplacental tissues using a dissecting microscope. B) Rat with both uterine horns exposed.

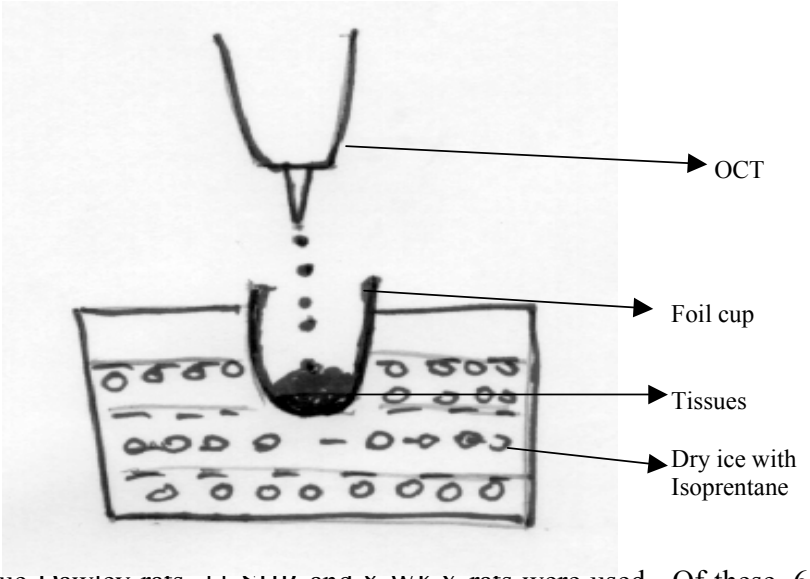
A

B

and 21 days of gestation the uterus and placenta were always collected separately. Once removed, the samples were washed twice with phosphate buffered saline (0.1M, pH 7.2).

For measurement of mRNA expression by RT-PCR or Northern blot analysis, the samples were snap frozen in liquid nitrogen, placed in pre labelled tubes and stored at -80^{0}C until required. In addition, tissues were either frozen or fixed for detection of VEGF-A protein and mRNA using immunohistochemistry and *in situ* hybridization techniques, respectively. Tissues were frozen in OCT (Tissue-TEK, MILES). The tissue was placed with OCT in a foil cup, which was floating in a slurry of dry ice and Isopentane (Figure 2.2). Once frozen, the tissues were stored at -80^{0}C until required. Alternatively, tissues were fixed in 10% formaldehyde buffer at room temperature for 3-4 hours. Following a PBS rinse, the tissues were stored in PBS at 4^{0}C until required.

Figure 2.2 Schematic diagram showing the technique for freezing tissues in OCT



A total of 30 Sprague Dawley rats, 11 SHR and 8 WKY rats were used. Of these, 6 Sprague Dawley rats, 5 SHR and 2 WKY rats were unsuccessfully plug mated. We used 4 Sprague Dawley rats (2 at 19 and 2 at 11 days) to establish some of our techniques.

The experimental design for Aim 1, which was to measure the expression of VEGF-A, VEGFR-1 and VEGFR-2 uteroplacental tissues from normal Sprague Dawley rats over a range of gestational ages is summarised in Table 2.1.

Gestation (days)	0	5	7	9	11	13	15	17	19	21
Sprague Dawley rats (no.)	2	2	2	2	2	2	2	2	2	2

Table 2.1. Number of normal Sprague Dawley rats obtained at each gestational age.

The experimental design for Aim 2, which was to compare the expression of VEGF-A, VEGFR-1 and VEGFR-2 the uteroplacental tissues from normotensive (WKY) and the spontaneously hypertensive rat (SHR) at early, mid and late gestation, is summarised in the Table 2.2.

Gestation (days)	7	11	19
SHR (no.)	2	2	2
WKY (no.)	2	2	2

Table 2.2 Number of normotensive (WKY) and spontaneously hypertensive (SHR) rats obtained at each gestational age.

2.2.0 RNA EXTRACTION

Total RNA was extracted from uteroplacental tissues using either the guanidinium (single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction, Chomczynski and Sacchi, 1987) technique (24 pieces of tissue) or the commercially available Trizol (GIBCOBRL) technique (65 pieces of tissue).
All reagents used for RNA extraction were RNase free. Solutions were treated with DEPC (0.1%) overnight at room temperature. Following the incubation period, the treated solutions were autoclaved at 121^{o}C for 20 minutes to remove any trace amounts of DEPC. All solutions not DEPC treated, were made in oven (160 ^{o}C)

baked glassware or appropriately sterilised equipment. These solutions were then autoclaved at 121^{o}C for 15 minutes on a wet cycle.

2.2.1 Total RNA Extraction by the Guanidinium Technique

The technique of Ausubel *et al,* (1994) was used to extract total RNA. Tissues were homogenised with an appropriate volume of denaturing solution and a mild detergent (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1 M 2-Mercaptoethanol; in a 20 ml glass-teflon homogeniser). The homogenate was then transferred to a 5 ml poly-propylene tube (~ 4 ml/0.2g tissue). Sequentially, 2 M sodium acetate, pH 4.0; 0.4 ml of water-saturated phenol and chloroform isoamyl alcohol (49:1) was added to the homogenate and mixed thoroughly by inversion. Aqueous and organic phases were separated by centrifugation at 10,000 rpm for 20 minutes at 4^{o}C. The upper aqueous phase was transferred to a fresh tube and RNA precipitated by the addition of an equal volume of 100% isopropanol at -20^{o}C for 1 hour to overnight. Following centrifugation at 10,000 rpm for 20 minutes at 4^{o}C, the supernatant was discarded, and the pellet resuspended in denaturing solution. This was then transferred to a 1.5 ml eppendorf tube and the RNA precipitated with 100% isopropanol for 30 min at -20^{o}C, followed by centrifugation at 10,000 rpm for 10 minutes at 4^{o}C. The RNA pellets were washed twice with 75% ethanol followed by centrifugation at 7,500 rpm for 5 minutes at room temperature. Finally, the pellets were air dried in a vacuum desiccator (about 15 minutes) and dissolved in 100 - 200 µl TE buffer or DEPC H_2O for storage at -80^{o}C.

2.2.2 Total RNA Extraction by the Trizol Technique

Total RNA was extracted using Trizol Reagent, (GIBCO BRL, Live Technologies, Cat No. 15596-018), according to the manufacturer's instructions. Tissues were homogenised (3 ml of Trizol reagent/50 mg of tissue) using the Polytron homogeniser (Kinematica, PTMR-2100, Cat No: 285567). The sample volume did not exceed 10% of the volume of Trizol reagent used for homogenisation. The sample was incubated for 5 minutes, at room temperature, to permit the complete dissociation of nucleoprotein complexes. Following addition of 0.6 ml of chloroform (0.2ml/ml of Trizol), the sample was mixed thoroughly by vigorous shaking for 15 seconds and then incubated at room temperature for a further 2-3 minutes. The samples were then

centrifuged at 12,000 rpm for 15 minutes at 4°C. The upper aqueous phase, containing the RNA was transferred to a new tube. RNA was precipitated by the addition of Isopropanol (0.5ml/ml of Trizol). The sample was centrifuged again at 12,000 rpm for 10 minutes at 4°C. The RNA pellet was re-dissolved with 3 ml of 75% ethanol (1ml/1ml of Trizol). The mixture was vortexed well and centrifuged at 7,500 rpm for 5 minutes at 4°C. The pellet was dried and resuspended in DEPC H_2O or TE buffer by passing the solution a few times through a pipette. Samples were then stored at -80°C until required.

2.2.3 mRNA Extraction by the Poly-AT Tract Isolation Technique

mRNA was extracted from total RNA using the Poly-AT tract mRNA Isolation Kit (Promega, Cat No. Part TM021), according to the manufacturer's specifications. Total RNA (0.1-1 mg) was resuspended in 250 µl DEPC H_2O, and denatured at 65°C for 10 minutes. The annealing reaction was prepared by adding 1.5 µl of Biotinylated-Oligo (dT) probe and 6.5 µl of 20 x SSC to the sample. It was gently mixed and then incubated at room temperature for 15 minutes.

The streptavidin-paramagnetic particles (SA-PMP) were prepared for use by gently flicking the bottom of the tube until they were completely dispersed. Subsequently the particles were captured by placing the tube in the magnetic stand until the SA-PMPs had collected at the side of the tube (approximately 30 minutes). The particles were then washed with 150 µl of 0.5 X SSC. This procedure was then repeated 3 times and particles were finally resuspended in a total volume of 50 µl of 0.5 X SSC ready for use.

Capture of oligo (dT) –mRNA hybrids was performed by mixing the entire contents of the annealing reaction with the SA-PMPs, followed by incubation at room temperature for 10 minutes. Following magnetic capture of the particles and 4 washes with 150µl 0.1 X SSC, as much of the aqueous phase as possible was removed, without disturbing the SA-PMPs particles. Finally, mRNA was eluted by adding 40 µl DEPC H_2O. This elution was repeated and the mRNA was ethanol precipitated following addition of

1/10 volume 3M sodium acetate (pH 4.6) for 40 minutes at -70^{o}C. The concentration of mRNA was assessed using a spectrophotometer.

2.3.0 QUALITY AND QUANTIFICATION OF RNA

2.3.1 Quality of RNA by Electrophoresis

2.3.1.1 Denaturing gel electrophoresis

The quality of the isolated total RNA was assessed by denaturing formaldehyde gel electrophoresis. Formaldehyde gel electrophoresis was performed according to the technique of Ausubel *et al*, (1994). A 6% formaldehyde gel was prepared with 1g agarose, 20 ml 37% formaldehyde, 11 ml 10 x MOPs buffer, and 81 ml DEPC H_2O. The sample buffer was prepared by mixing 250 μl formamide (deionised), 83 μl formaldehyde, 50 μl 10 x MOPs buffer and 17 μl H_2O; total volume 400 μl. The loading buffer contained 0.1 mg/ml bromophenol blue in 50% glycerol. After mixing 10 μg total RNA or 0.1 μg mRNA (16 μl) with the sample buffer (4 μl) and the loading buffer (4 μl), the total volume (24 μl) was denatured for 10 minutes at 65°C. Electrophoresis was carried out overnight at 15 volts or for 4-5 hours at 50 volts. Standard RNA molecular weight markers (Promega, Cat No. G3191), were loaded in each gel. Gels and a fluorescent ruler were routinely photographed under UV light.

2.3.1.2 Non-denaturing gel electrophoresis

Non-denatured gels were sometimes used to confirm concentrations assessed by the spectrophotometer and to analyse RNA quality when only small amounts of total RNA were available. Electrophoresis was carried out in 2% agarose gels in 1 X TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH7.4) or 1 X TBE and run for 45 minutes at 80 volts. RNA samples (5 μl) (usually containing 0.1 μg mRNA or 1-5 μg total RNA) were loaded after addition of 1 ul 6 X loading buffer which contained 10% Ficoll, 10 mM Tris-HCL, 50 mM EDTA, 0.01% bromophenol blue, 0.25% Xylene Cyanol.

2.3.2 Concentration and Purity of RNA

The concentration and purity of total and mRNA were assessed using a spectrophotometer. To measure the concentration of RNA, the absorbance (A) of the sample was measured at 260nm. As an absorbance of 1 unit at 260nm corresponds to 40 μg of RNA the concentration of each sample can be calculated using the equation: concentration of sample (μg/ml) = A260 X dilution factor X 40 (μg /ml). Purity levels of RNA can be estimated by calculating the ratio between the absorbance value at 260 nm and that at 280 nm.

2.4.0 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Expression of VEGF-A and its receptors, VEGFR-1 and VEGFR-2, was investigated by reverse transcriptase polymerase chain reaction (RT-PCR). The design of the primers, DNase treatment and RT-PCR methods and specifications are outlined below.

2.4.1 Design of Primers

2.4.1.1 G3PDH primers

G3PDH was used as an internal control during semi quantitative RT-PCR to ensure comparison between equal amounts of mRNA. RT-PCR primers for detection of G3PDH were designed based on the published human liver mRNA G3PDH sequence (Arcali *et al*, 1984). These were kindly provided by Ms Usula Manuelpillai (School of Life Sciences and Technology, Victoria University). The G3PDH sequence was confirmed by Ms Usula Manuelpillai. These primers only have one mismatch with the published rat sequence for G3PDH. The estimated (Tm) for G3PDH sense and antisense was 58°C and 66°C, respectively. The theoretical size of the RT-PCR product was 525 base pairs.

Human G3PDH Forward primer: 5'-TCC TGC ACC ACC AAC TGC TT-3'
Human G3PDH Reverse primer:5'-AGG TCC ACC ACC CTG TTG CTG TA-3'

2.4.1.2 VEGF-A primers

Set 1:

The design of our first set of primers was based on primers kindly donated by Professor Graham Jenkin, Department of Physiology, Monash University. For these *set 1* VEGF-A primers, the forward primer was identical to the residues 342-364, inclusive, of the rat VEGF-A cDNA sequence and VEGF-A reverse primer was designed complementary to the residues 577-556, inclusive, of the rat VEGF-A cDNA sequence (Conn *et al*, 1990, Acc No: M32167) except for position 568 where a Guanosine residue was introduced. These primers have the ability to detect isoforms 205, 188, 164, 144 and 120. The estimated (T_m) for VEGF-A forward and reverse primers were 72°C and 66°C, respectively. The theoretical size of the RT-PCR product for VEGF-A_{205} was 359 base pairs; VEGF-A_{188} was 308 base pairs; VEGF-A_{164} was 236 base pairs; VEGF-A_{144} was 176 base pairs and VEGF-A_{120} was104 base pairs (Table 2.3). Both primers were synthesised by Operon Technologies company.

VEGF-A Forward primer: 5'- GCC AGC ACA TAG GAG AGA TGA GC-3'
VEGF-A Reverse primer: 5'- TCA CCG CCT GGG CTT GTC ACA T-3'

Set 2:

Further VEGF-A primers (*set 2*) were designed and kindly provided by Dr. Sarah Fraser from the School of Life Sciences at Victoria University. These primers were located at residues 88-108, inclusive, (forward) and 577-556, inclusive, (reverse) of the rat cDNA sequence (Conn *et al*, 1990; Acc No: M32167) and have the ability to detect isoforms 205, 188, 164, 144, 120 and 110. The estimated (T_m) for VEGF-A forward and reverse primers were 72°C and 66°C, respectively. Theoretical size of RT-PCR products for VEGF-A_{205} was 619 base pairs, VEGF-A_{188} was 568 base pairs, VEGF-A_{164} was 496 base pairs; VEGF-A_{144} was 436 base pairs, VEGF-A_{120} was 364 base pairs and $VEGF_{110}$ was 334 base pairs. Both primers were synthesised by Pacific Oligos Pty.Ltd.

VEGF-A Forward primer: 5'-CAC GAC AGA AGG GGA GCA GAA-3'
VEGF-A Reverse primer: 5'-TCA CCG CCT TGG CTT GTC ACA T-3'

VEGF-A isoforms	*Set 1* primers (bp)	*Set 2* primers (bp)
VEGF-A_{205}	**359**	**619**
VEGF-A_{188}	**308**	**568**
VEGF-A_{164}	**236**	**496**
VEGF-A_{144}	**176**	**436**
VEGF-A_{120}	**104**	**364**
VEGF-A_{110}	**----**	**334**

Table 2.3 Theoretical sizes of the RT-PCR products using *set 1* and *set 2* VEGF-A primers.

2.4.1.3 VEGFR-1 primers

The design of the VEGFR-1 (Flt-1) forward and reverse primers was based on the published rat cDNA sequence for tyrosine kinase receptor (Flt-1) (Yamane, 1994, Acc No: RATFLT1, ANGIS). The forward primer was located at the residues 1771 to 1794; the reverse primer was located at the residues 2050 to 2028 of the rat Flt-1 cDNA sequence. The estimated ($T_{m)}$ for VEGFR-1 forward and reverse primers were 72°C and 68°C, respectively. The theoretical size of the RT-PCR product was 280 base pairs. Both primers were synthesised by Operon Technologies Company. Sequence analysis by BLASTN and FASTA indicated that this region of VEGFR-1 showed no significant homology with other receptor tyrosine kinases.

VEGFR-1 Forward primer: 5'-CTG CTA CGG ACA GTT AAC AAC AGG-3'

VEGFR-1: Reverse primer: 5'-CTT GAC AGT CTA AGG TCG TGG AG-3'

2.4.1.4 VEGFR-2 primers

The design of the VEGFR-2 (Flk-1) primers was based on the published rat cDNA sequence for Flk-1 (Wen *et al*, 1997, Acc No: RNU93306, ANGIS). The forward primer was located at the residues 1371-1394; the reverse primer was located at the residues 1754- 1731 of the rat Flk-1 cDNA sequence. The estimated (Tm) for VEGFR-2 forward and reverse primers were 68°C and 70°C, respectively. The theoretical size of the RT-PCR products equals 383 base pairs. Both primers were synthesised by Operon Technologies Company. Sequence analysis by BLASTN and FASTA indicated that this region of VEGFR-2 showed no significant homology with other receptor tyrosine kinases.

VEGFR-2 Forward primer: 5'-ACC ACA TCC AAT GGT ACT GGC AG-3'
VEGFR-2 Reverse primer: 5'-TGT GAG CCA AGC TTG TAC CAC GT-3'

2.4.2 DNase Treatment of RNA

When using RT-PCR it was essential to establish that PCR product formation was a measure of mRNA expression. Therefore, all extracted total RNA was routinely treated with deoxyribonuclease (DNase, GIBCOBRL). The method for DNase treatment was as follows: 10 x DNase buffer containing 200 mM Tris-HCl (pH) 8.4, 20 mM $MgCl_2$, 500 mM KCl (1 µl), DNase 1 µl (1U/µl), total RNA sample 2 µl (0.98 µg/µl), and d.d. H_2O (6 µl) incubated for 15 minutes at 37 °C, the reaction was then stopped by adding 25 mM EDTA (1 µl), denatured for 10 minutes at 65°C, cooled on ice and used immediately for 1st strand synthesis. This RNA sample was divided equally (4µl per reaction) for first strand synthesis in the presence or absence of reverse transcriptase. Success at DNase treatment was assessed by the inability to obtain PCR products in the absence of reverse transcriptase.

2.4.3 First Strand cDNA Synthesis Using SUPERSCRIPT™ II RT

First strand cDNA synthesis was performed in a reaction volume of 20 µl. All reaction mixtures were prepared on ice. For each RNA template, a negative control reaction omitting addition of reverse transcriptase was performed. The contents of the tube were gently mixed and incubated at 42°C for 2 minutes prior to the addition of 1 µl (200 units) of Superscript II RNase H-Reverse Transcriptive (GIBCOBRL). Following an incubation period of 50 minutes at 42°C the reaction was terminated by heating at 70°C for 15 minutes. The cDNA was stored at -20°C and used as a template for PCR amplification of G3PDH, VEGF-A and VEGFR-1. This system was found not to be optimal for VEGFR-2 amplification.

	RT+		RT-
Component	**Volume**	**Final Concentration**	
Oligo(dT)12-18	1 μl	0.5 ng/μl	1 μl
Total RNA	4 μl	2.5 ng/μl	4 μl
5 x 1st strand buffer	4 μl	50 mM Tris,75 mM KCl, 3 mM $MgCl_2$	4 μl
0.1 M DTT(0.1M)	2 μl	10 mM	2 μl
10 mM dNTP mix	1 μl	500 μM	1 μl
RNAsin (40U/μl)	0.5 μl	1U/μl	0.5 μl
Sterile distilled water	6.5 μl		7.5 μl
Total volume	19 μl		20 μl

Table 2.4 First strand cDNA synthesis using SUPERSCRIPT^{TM} IIRT

2.4.4 PCR Amplification

Factors which affect the quality of the RT-PCR product, including the concentrations of primer, template and magnesium, the annealing temperature and the cycle number, were optimised for G3PDH, VEGF-A, VEGFR-1 and VEGFR-2. A reaction volume of 50 μl was used. Thermocycling was performed using the DNA Engine PTC-220 (MJ Research, Bresatec). The standard optimal RT-PCR programs for G3PDH, VEGF-A, VEGFR-1 and VEGFR-2 are outlined in the following tables. Analysis of the PCR products was performed on a 2% agarose gel in 1 X TBE buffer containing 0.1% Ethidium Bromide.

2.4.4.1 G3PDH

Component	Volume	Final Concentration
10 x PCR buffer	5 µl	20 mM Tris, 50 mM KCl
25 mM $MgCl_2$	3 µl	1.5 mM
10 mM dNTP mix	1 µl	200 µM
10 µM upstream primer	1.25 µl	0.25 µM
10 µM downstream primer	1.25 µl	0.25 µM
First strand DNA	4 µl	
Taq DNA polymerase	0.5 µl	0.05U/µl
Sterile water	34 µl	
total volume	50 µl	

Table 2.5 The composition of the PCR reaction for the amplification of G3PDH

Cycle	Denaturation	Annealing	Polymerisation
First cycle	3 min, at 94°C		
Subsequent	30 seconds	30 seconds	1 minute
Cycles (n=35)	at 94°C	at 58°C	at 72°C
Last cycle			5 minutes at 72°C
Hold			at 4°C

Table 2.6 PCR cycling parameters for G3PDH

2.4.4.2 VEGF-A (primer *set 1*)

Component	Volume	Final Concentration
10 x PCR buffer	5 µl	20 mM Tris,50 mM KCl
25mM $MgCl_2$	3 µl	1.5 mM
10 mM dNTP mix	1 µl	200 µM
10 µM upstream primer	1.25 µl	0.25 µM
10 µM downstream primer	1.25 µl	0.25 µM
First strand DNA	4 µl	
Taq DNA polymerase	0.5 µl	0.05 U/µl
Sterile water	34 µl	
Total volume	50 µl	

Table 2.7 The composition of the PCR reaction for the amplification of VEGF-A (primer *set 1*).

Cycle	Denaturation	Annealing	Polymerisation
First cycle	3 min, at 94°C		
Subsequent	30 seconds	30 seconds	1 minute
Cycles (n=35)	at 94°C	at 58°C	at 72°C
Last cycle			5 minutes at 72°C
Hold			at 4°C

Table 2.8 PCR cycling parameters for VEGF-A (primer *set 1*).

2.4.4.3 VEGF-A (primer *set 2)*

Component	Volume	Final Concentration
10 x PCR buffer	5 µl	20 mM Tris,50 mM KCl
25mM $MgCl_2$	3 µl	1.5 mM
10 mM dNTP mix	1 µl	200 µM
10 µM upstream primer	1.25 µl	0.25 µM
10 µM downstream primer	1.25 µl	0.25 µM
First strand DNA	4 µl	
Taq DNA polymerase	0.5 µl	0.05U/µl
Sterile water	34 µl	
Total volume	50 µl	

Table 2.9 The composition of the PCR reaction for the amplification of VEGF-A (primer *set 2*).

.

Cycle	Denaturation	Annealing	Polymerisation
First cycle	3 min, at 94°C		
Subsequent	30 seconds	30 seconds	1 minute
Cycles (n=35)	at 94°C	at 63°C	at 72°C
Last cycle			5 minutes at 72°C
Hold			at 4°C

Table 2.10 PCR cycling parameters for VEGF-A (primer *set 2*).

2.4.4.4 VEGFR-1

Component	Volume	Final Concentration
10 x PCR buffer	5 µl	20 mM Tris,50 mM KCl
25mM $MgCl_2$	3 µl	1.5 mM
10 mM dNTP mix	1 µl	200 µM
10 µM upstream primer	1.25 µl	0.25 µM
10 µM downstream primer	1.25 µl	0.25 µM
First strand DNA	4 µl	
Taq DNA polymerase	0.5 µl	0.05 U/µl
Sterile water	34 µl	
Total volume	50 µl	

Table 2.11 The composition of the PCR reaction for the amplification of VEGFR-1

Cycle	Denaturation	Annealing	Polymerisation
First cycle	3 minutes, at 94°C		
Subsequent	30 seconds	30 seconds	1 minute
Cycles(n=35)	at 94°C	at 65°C	at 72°C
Last cycle			5 minutes at 72°C
Hold			at 4°C

Table 2.12 PCR cycling parameters for VEGFR-1

2.4.4.5 VEGFR-2

An alternative RT-PCR system was found to be more successful to investigate VEGFR-2 expression. The Access RT-PCR kit (Promega) was used according to the manufacturers specifications. All components of first strand synthesis and the PCR reaction were combined, on ice, as described in the table below.

Component	**Volume**	**Final Concentration**
Reverse transcriptase (5U/µl)	1 µl	0.1 U/µl
25 mM MgCl2	4 µl	2 mM
10 mM dNTP mix	1 µl	200 µM
10 pM upstream primer	5 µl (50 pM)	1 pM
10 pM downstreamprimer	5 µl (50 pM)	1 pM
5 x AMV/Tf1 Buffer	10 µl	1 X
Tf1DNA polymerase (5U/µl)	1 µl	0.1 U/µl
Total RNA (2 µl) +d.d.H_2O	10 µl	40 ng/µl
Sterile water	13 µl	
Total volume	50 µl	

Table 2.13: The composition of the PCR reaction for the amplification of VEGFR-2.

The mixture was incubated concurrently at 48 °C for first strand and 94 °C for subsequent cycles as described below.

First strand cDNA synthesis			
65°C for 10 minutes ----->	48°C for 45 minutes -->	94°C for 2	minutes
Seconds strand synthesis			
Cycle	Denaturation	Annealing	Polymerisation
Subsequent cycles(n=35)	94°C for 30 seconds	65°C for 1 minute	72°C for 2 minutes
Last cycle			72°C for 5 minutes

Table 2.14: PCR cycling parameters for VEGFR-2.

2.5.0 SEQUENCE ANALYSIS

Automated sequence analysis was performed at Monash University using an ABI prism sequencer (model 373). Sequence analysis of PCR products was performed in a 16 μl reaction volume. This mixture contained product (30 ng), 3.2 pmol sense or antisense primer and 6ul Big dye. Cycling was performed using a thermocycler (PTC-220). Cycling parameters are shown in the table below.

Cycle	Denaturation	Annealing	Polymerization
First cycle	4 minutes at 96°C		
Subsequent	30 seconds	15 seconds	4 minutes
cycles(n=25)	at 96°C	at 50°C	at 60°C
Last cycle			4 minutes at 60°C

Table 2.15. PCR cycling parameters for sequencing

Extension products were purified by ethanol precipitation after the addition of 3 μl 3M sodium acetate (pH 4.6). The precipitate was recovered by centrifugation (14,000 rpm) at room temperature for 30 minutes and the pellet was then rinsed briefly with 250 μl 70% ethanol. The ethanol was removed and the pellet was air dried.

Sequence alignments were analysed using Blast, Fasta and Pile up programs on ANGIS.

2.6.0 DENSITOMETRY

When a qualitative difference was observed in the levels of expression of VEGF-A and its receptors, either at different gestational ages or between normotensive (WKY) and hypertensive (SHR) rats, the data were analysed using semi quantitative analysis. Data were calculated by scanning the RT-PCR negative film using a GXL Ultrasound laser scanner. The ratio between specific bands of interest and G3PDH was calculated.

2.7.0 STATISTICAL ANALYSIS

Data were presented as mean ± standard error of the mean (SEM). Unpaired t test was conducted using Excel software.

2.8.0 PROBES FOR NORTHERN BLOT ANALYSIS

2.8.1 G3PDH

A plasmid DNA with rat G3PDH cDNA insert (Piechaezyk *et al*, 1984) was kindly provided by Dr. Mrinal Bhave from the School of Life Sciences at Victoria University. The 2 kb Pst I fragment, blunt-ended, was ligated into the Hind III site in the Gemini 3E vector. A 550 bp G3PDH fragment was cut out by Apa I and Hind III. This fragment was purified and labelled with α-^{32}P dCTP for hybridisation.

2.8.2 VEGF-A

The 236 base pair RT-PCR product corresponding to VEGF-A_{164} (primer *set 1*) was used directly as a probe for analysis of VEGF-A mRNA expression by Northern blot analysis. This probe was ideal for studying expression of VEGF-A_{164}. Theoretically, this probe will also hybridise with isoforms 205, 188, 144 and 120, but with different levels of nucleotide sequence homology. This PCR fragment was also cloned (see section 2.10.0) and the cloned DNA used for probe preparation in one of the following ways:

1. The plasmid (see section 2.10.0), containing the 236 bp VEGF-A_{164}, was digested using EcoR1. The reaction (50 µl) which included 2 µl (1.32 µg/µl) plasmid DNA, 5 µl (10 U/µl) EcoR1, 5 µl EcoR1 buffer and 38 µl d.d. H_2O was incubated for 2 hours at 37°C. The insert was purified as described in section 2.9.0. The purified clone product is shown in Figure 2.3.
2. A single colony (see section 2.10.2) was removed from the plate and mixed with 50 µl H_2O. Following this, 2 µl of this mixture was used directly for amplification of the VEGF insert by PCR. Alternatively a 1 µl aliquot from an overnight culture of the clone in Luria-Bertani (LB) medium with ampicillin (50 µg/ml) was used as a template for PCR, as shown in the table below.

Component	Volume	Final Concentration
10 x buffer	2 μl	20 mM Tris, 50 mM KCl
10 mM dNTP mix	1 μl	0.5 mM
10 uM upstream primer	1 μl	0.5 μM
10uM downstream primer	1 μl	0.5 μM
Template	1μl aliquot	
Advantage	0.5 μl	0.025 U/μl
Sterile water	13.5 μl	
Total volume	20 μl	

Table 2.16 The composition of the PCR reaction for the amplification of the VEGF-A probe.

Cycle	Denaturation	Annealing	Polymerisation
First cycle	3 minutes at 95°C		
Subsequent	1 minute	1 minute	1 minute
Cycles (n=10)	at 94°C	at 54°C	at 72°C
Last cycle			5 minutes at 72°C
Hold			at 4°C

Table 2.17 PCR cycling parameters for the VEGF-A probe.

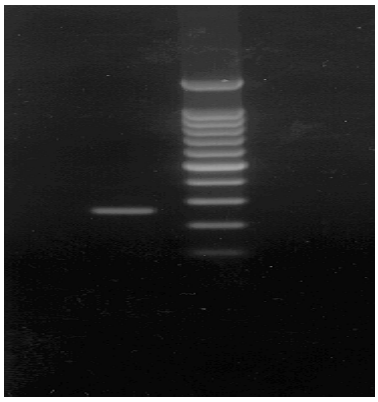
The PCR product was subjected to electrophoresis on a 2% agarose gel and purified as described in the Methods section 2.9.0. Figure 2.3 shows the purified 236 bp band which was confirmed by sequence analysis to be the partial sequence of VEGF-$A_{164.}$

2.8.3 VEGFR-1

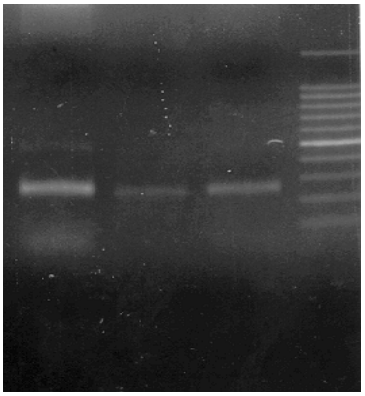
The 280 bp VEGFR-1 cDNA fragment generated by RT-PCR was used directly as a probe for Northern blot analysis. Analysis of the VEGFR-1 sequence by Blast program revealed no DNA sequence homology with other tyrosine kinase receptors.

Figure 2.3. Gels showing purified VEGF-A DNA fragments. A) The purified RT-PCR product (5 μl) on a 2% agarose gel with 1 x TAE buffer stained with ethidium bromide (1%). B) The 236 bp VEGF-A cDNA fragment excised from the plasmid cloned into the PCR vector (3.9 kb) by EcoR1 digested as described in the Methods 2.10.0.

A


1
M
1500 bp
1000 bp
500 bp
200 bp
VEGF-A
236 bp

B


1
2
3
M
1500 bp
1000 bp
500 bp
200 bp
VEGF-A
236 bp

2.8.4 VEGFR-2

The 383 bp VEGFR-2 cDNA fragment generated by RT-PCR was used directly as a probe for Northern blot analysis. Analysis of the VEGFR-2 sequence indicated that this sequence was specific for VEGFR-2.

2.9.0 DNA PURIFICATION

The cDNA fragments (PCR products and restriction enzyme digests) were purified by the BANDPURE™ Purification Kit (Progen, Cat No: 600-0000). The bands of interest were excised from the gel under UV light and weighed. A volume of sodium iodide (NaI) binding solution, 2.5 times that of the weight of the band of interest, was added into a pre-weighed 1.5 ml eppendorf tube. The tube was then incubated at 55°C for 10 minutes with frequent gentle mixing. The silica matrix (Bandpure, 5 µl) was added, mixed and incubated at room temperature for 5 minutes. The tube was then centrifuged for 30 seconds so that the pellet containing the silica matrix and bound DNA was formed. The supernatant was discarded and the pellet washed in 500 µl of cold ethanol. The pellet was re-suspended, centrifuged and the supernatant discarded. This washing procedure was repeated twice. After this 10 µl of d.d. H_2O was added, the pellet was resuspended and incubated at 55 °C for 2-5 minutes to elute the purified DNA from the silica matrix. Finally, the tube was centrifuged briefly and the supernatant that contained the eluted DNA was transferred to another tube.

2.10.0 CLONING OF PROBES

The VEGF-A_{164} probe was cloned using the TA cloning kit (Invitrogen). Initial attempts to clone this insert into pBluescript (Stratogene) using EcoR1/Sal1 restriction sites generated at the termini of the RT-PCR product were unsuccessful. Therefore, the VEGF-A_{164} probe was cloned using the TA cloning kit (Invitrogen).

2.10.1 Ligation

The VEGF-A fragment generated by RT-PCR was purified. Ligation was performed in a total volume of 13 μl containing 1 μl ligase, 2 μl vector (PCR 2.1 vector, 3.9 kb, TA cloning Kit, Invitrogen) and 1 μl 10 x ligation buffer, VEGF-A cDNA 3 μl (3 ng/μl). The reaction was mixed gently on ice with a pipette tip and incubated at 18°C overnight.

2.10.2 Transformation

For transformation a 50 μl vial of One-Short Top 10F' competent cells was thawed on ice for 30 minutes. To this was added 2 μl of 0.5M β-mercaptoethanol (β-ME) and 2 μl of ligation mixture. Cells were heat shocked for exactly 30 seconds at 42°C without mixing and returned to the ice for 2 minutes. 250 μl of SOC media was added to each tube. Cells were plated on LB plates containing Ampicillin (50 μg/ml) and X-gal (40 μl) and IPTG (40 μl). Plates were incubated overnight at 37° C. Plates were then trasferred to 4°C for 3-4 h to allow proper colour development. Single colonies were screened for the insert using alkaline lysis miniprep.

2.10.3 Alkaline Lysis Miniprep

The single colonies containing the DNA insert, were grow in LB broth, containing ampicillin (5 ml), overnight at 37°C in a shaking incubator. The culture was then transferred to several 1.5 ml tubes and centrifuged, at maximum speed, for 1 minute at room temperature. The supernatant was aspirated and the pellet was resuspended in an ice-cold solution (50 mM Glucose, 25 mM Tris-HCl pH8, 10 mM EDTA pH 8). Then, 10 mg/ml Lysozyme (20 μl) was added to each tube and mixed by inversion. To this, 0.2 N NaOH :1% SDS (200 μl) was added, mixed and stored on ice for 5 minutes. Finally, 3M NaOAC pH 5.2 (50 μl) was added and the reaction centrifuged for 5 minutes. The supernatant was transferred to a new microfuge tube, whereupon 10 mg/ml DNase free RNase (5 μl) was added and incubated at 37°C for 15 minutes. The supernatant was extracted with equal volume Phenol: Chloroform: Isoamylalchol (25:24:1). After centrifuging for 15 minutes at maximum speed, the aqueous phase was re-extracted by adding 100% ethanol (2:1). The sample was stored at -20°C overnight. Then, the sample was centrifuged at 14,000 rpm for 15 minutes. The

supernatant was decanted, the pellet was washed with 70% ethanol and dried. The pellet was then dissolved in d.d. H_2O (50 μl). When an insert of the correct size was detected, Glycerol (30%) was added for longer storage.

2.11.0 NORTHERN BLOT ANALYSIS

2.11.1 Preparation of Blots

Northern blot analysis was also used to analyse expression of VEGF-A and its receptors in uteroplacental tissues. Electrophoresis of total or mRNA was carried out on 6% formaldehyde containing gels as described in the Methods section 2.3.1.1.

The gel was checked and photographed under UV light with a fluorescent ruler. The gel was soaked in a glass dish with 300 ml 10 X SSC buffer for 30 minutes to remove excess formaldehyde. Pieces of Whatman filter paper (3 mm) were placed over the platform to act as a wick. The wick was moistened with 10 X SSC and all air bubbles were removed with the aid of a 5 ml pipette. The gel was placed face down, taking care to avoid air bubbles the edges of the gel were surrounded by clingfilm. A Nylon membrane (Boehringer Mannheim) was cut to the same size as the gel and soaked in 10 X SSC for 2 minutes. This was placed on top of the gel followed by three pieces of 3 mm Whatman filter paper towels presoaked in 10 x SSC. One of the corners from the gel and membrane was excised to help in identification later. A large pile of Kleenex paper towel was placed over the Whatman paper to absorb the buffer. Finally, a weight (about 500g) was placed on the top of the paper towels, and the transfer was allowed to proceed overnight. Once completed, the blot was disassembled and the wells were marked with a pencil. Successful transfer of RNA was detected by visualisation of ethidium bromide stained rRNAs on the membrane under UV light.
The Nylon membrane was rinsed in 2 x SSC, then air dried for 30 minutes, placed on the UV box RNA side down for 5 minutes to crosslink the mRNA to the membrane. The membrane was stored on a flat surface at 4°C or used directly for hybridisation.

2.11.2 Radioactive Labelling of cDNA Probes

cDNA probes were radioactivity labelled by High Prime (random oligonucletides Boehringer Mannheim, Cat No: 1585592) with radioactive α-^{32}P dCTP (ADC-2 Bresatec, Cat No: H2907). Double stranded cDNA (25-100 ng) in a final volume of 11 µl was denatured by heating in a boiling water bath for 10 minutes and chilling quickly in ice. After the addition of 4 µl of high primer solution (containing 1U/µl Klenow polymerase, labelling grade, 0.25 mM dATP, 0.25 mM dGTP, 0.25 mM dTTP and 5 x reaction buffer in 50% glycerol) and 5 µl α-^{32}P dCTP (50 µCi, 3000 Ci/mMol) the reaction was incubated at 37°C for one hour. The reaction was stoped by adding 2 µl 0.2 M EDTA (pH 8). In early experiments, the Spin™ Columns, Sephadex G-50 (fine) was used to remove non-incorporated deoxyribonucleoside-triphosphates prior to hybridisation. However, this procedure was found to be unnecessary and omitted from later experiments.

2.11.3 Non-radioactive Labelling of cDNA Probes

Double strand cDNA probes were labelling with digoxigenin (DIG) by the random primed method according to the supplied protocol supplied by Boehringer Mannheim. cDNA template (80 ng -1 µg) in a final volume of 16 µl was denatured by heating in a boiling water bath for 10 minutes and cooling on ice. After addition of 4 µl DIG-High prime mix, the reaction was centrifuged briefly. Following incubation for 16-20 hours at 37°C (usually in PCR machine), the reaction was stopped by adding 2 µl 0.2 M EDTA (pH 8) or heating at 65°C for 10 minutes.

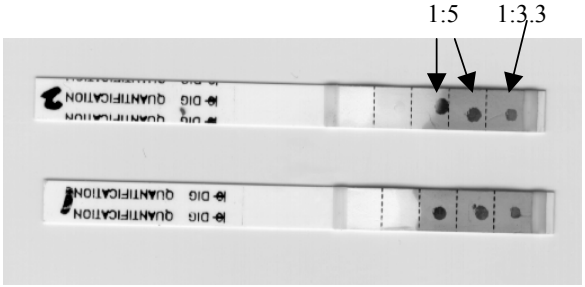
DIG Quantification Teststrips from Boehringer Mannheim (Cat No 1669958) were used to test the success of the labelling. Labelled cDNA was diluted to a concentration of 1:3.3 in buffer (20 X SSC: DEPC H_2O: Formaldehyde, 5:3:1) and a 1ul dot placed on the test strip. The signal was compared with a control strip loaded with 5 defined dilutions of a control DNA. Test strips were subjected to immunological detection with Anti-Digoxigen-AP and the colour substrate NBT/BCIP. Strips were held back to back and immersed in prepared solutions as described in the Table 2.18.

Procedure	Time
Blocking	2 minutes
Antibody binding	3 minutes
Blocking	1 minute
Washing	1 minute
Equilibration	1 minute
Colour reaction (in the dark)	30 minutes

Table 2.18 DIG quantification test strip procedure.

A 1 µl (1 - 40 µg/ml) spot of each dilution was applied onto the marked squares of a DIG quantification teststrip and air dried for approximalely 2 minutes. The above procedure was used to quantify the yield of probe labelled. The blocking solution contained blocking reagent stock solution 1:10 with Maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5). The antibody solution was prepared by diluting 1 µl of Anti-Digoxigenin-alkaline phosphatase in 2 ml blocking solution. The colour reaction contained 9 µl NBT solution (4.5 µl/ml) and 7 µl BCIP solution (3.5 µl/ml) and 2 ml detection buffer. The detection buffer containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ (pH 9.5). The washing solution contained 0.3% (w/v) Tween 20 in Maleic acid buffer.

Figure 2.4 Photograph showing DIG quantification test strip. For example, the concentration at a dilution of 1:3.3 was approximal 300 pg per micro litre as estimated by comparison with the control strip loaded with known amounts of DIG-labelled DNA.



2.11.4 Northern Hybridisation Using Radioactive cDNA Probes

2.11.4.1 pre-hybridisation

When using radioactive cDNA probes pre-hybridisation was carried out in an RNase free sealed dish, in a hybridisation oven at 42°C with gentle agitation. Pre-hybridisation was performed in pre-warmed (42°C) buffer (2 x Denhart's, 5 mM EDTA, 10 mM Tris-HCL, 5% SDS, 0.5 M Na_2PO4 pH 7.0; 0.5 M NaH_2PO4 pH 7.0; 1.5ml/10cm2 membrane). Salmon Sperm DNA (ssDNA: 100 ng/ml) was denatured at 100°C for 10 minutes and added to the prehybridisation solution. The membrane was placed RNA side up in a baking dish. Pre-hybridisation was performed for a minimum of 2 hours, usually 3-4 hours with gentle shaking.

2.11.4.2 hybridisation

The pre-hybridisation solution was discarded and replaced with 15 ml buffer containing the freshly denatured probe. Probes were then denatured at 100°C for 10 minutes and quickly cooled on ice immediately prior to use. Hybridisation was performed at 42°C for a minium of 8 hours, usually overnight.

2.11.4.3 post-hybridisation

Following hybridisation, the following basic washing procedure was used to remove unbound probe and reduced non- specific binding.

Solution	Time (min)	Temperature (°C)
4 x SSC	10 min x 1	Room temperature
2 x SSC + 0.1% SDS	10 min x 2	Room temperature
1 x SSC + 0.1% SDS	10 min x 1	37°C
1 x SSC + 0.1% SDS	10 min x 1	45°C

Table 2.19 Post-hybridisation washing protocol for radioactive cDNA probes.

2.11.4.4 autoradiography

Autoradiography was performed with a single intensifying screen at -80°C for 16-72 hours or one week depending on the strength of the signal. We used Kodak film (X-OMAT XAR5). The cassette was removed from the freezer and allowed to warm to room temperature prior to development of the film. The film was developed manually, allowing 5 minutes in the developer (1: 4 H_20), 1 minute in the acetic acid (3%) and 2 minutes in the fixer. Finally the film was washed extensively under running water and air dried.

2.11.5.0 Northern Hybridisation Using Non-Radioactive cDNA Probes

2.11.5.1 pre-hybridisation

Pre-hybridisation was performed in DIG Easy buffer (5 x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent). The blot was sealed in a pre-hybridisation bag containing 20 ml DIG-easy buffer per 100 cm^2 of membrane surface area. Prehybridisation was at 50°C for at least 1 hour, usually 4 hours or overnight.

2.11.5.2 hybridisation

The buffer was discarded and replaced with DIG- Easy buffer (100 cm^2/20 ml) containing the freshly denatured probe (25 ng/ml). Hybridisation was performed with gentle shaking for 8 hours or overnight at 50°C.

2.11.5.3 post-hybridisation

Following hybridisation the membrane was washed, according to the protocol below, to remove unbound probe and to reduce non-specific binding.

Solution	Time(min)	Temperature (°C)
2 x SSC+ 0.1% SDS	5 min x 1	Room temperature
2 x SSC+ 0.1% SDS	15 min x 2	Room temperature
1 x SSC + 0.1% SDS	15 min x 2	68°C
0.5 x SSC + 0.1% SDS	10 min x 1	68°C
0.1 Maleic acid + 3% Twee 20	5 min x 1	Room temperature

Table 2.20 Post–hybridisation washing protocol for non-radioactive cDNA probes.

2.11.5.4 immunological and chemiluminescence detection

Membranes were subjected to immunological detection with anti-Digoxigen –AP and the chemiluminescence substrate CSPD according to the manufacturers instructions. The membrane was incubated in 1 ml/per cm^2 blocking solution (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5) for 30 minutes. Incubation was carried out for 30 minutes in diluted anti-Digoxigen–AP (1:10,000 in blocking buffer). The membrane was washed twice for 15 minutes in 100 ml washing buffer (0.1 M Maleic acid, and equilibrated for 5 minutes in 20 ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). Final detection was performed by placing the membrane with RNA side facing up on a hybridisation bag and covering with about 1 ml CSPD (1:100 dilution). After 5 minutes incubation at room temperature, excess liquid was removed and the hybridisation bag sealed. The membrane was incubated for 30 minutes at 37°C to activate the luminescent reaction. Finally, the blot was exposed to X-ray film (Kodak BIOMAX™MR) for 1-20 or 30 minutes depending on the strength of the signal. Films

were processed as follows: 3-5 minutes in developer, 1 minute in acetate acid and 1 minute in fixative.

2.12.0 IMMUNOHISTOCHEMISTRY

Localisation of VEGF-A protein was investigated using immunohistochemistry. Uteroplacental tissues were either frozen in isopentane or fixed in 10% formaldehyde buffer.

2.12.1 Frozen Tissues

Sections (6 μm thick) were cut on a cryostat (ZEISS HM325 MICROM), fixed in acetone or 10% formalin buffer for 10 minutes at room temperature, air dried for 2-3 hours and stored in ethanol vapour at -20°C or -70°C.

2.12.2 Fixed Tissues

Fixed uteroplacental tissues were processed (Grale Scientific Company) by dehydrating (graded ethanol: 30%, 50% 70%, 90% 100% X 2), clearing (Histolene X 2) and embedding the tissue with paraffin wax (59°C). Each step took 2 hours. Tissues were fixed in the middle of the paraffin block (SIMPORT, M475-2, and Lot: 303985769), quickly chilled (ZEISS AP 280-1) to 4°C and then stored at 4°C. Sections (6 μm thick) were cut on a microtome, dried at 37°C over night, and returned to room temperature.

2.12.3 VEGF-A Immunohistochemistry

Fixed tissue was used for the immunohistochemical study. The sections were dewaxed (histolene X 2 for 4 minutes) and rehydrated (100% ethanol X 2 for 4 minutes, 70% ethanol X for 4 minutes) and washed (d.d H_2O X 2 for 5 minutes, PBS buffer X 1 for 3 minutes). Antigen retrieval was enhanced with 10 mM Citrate (pH 6) buffer or 2 x SSC buffer for 10-20 minutes at 100°C. After which the slides were returned to 30°C and washed with d.d H_2O. Endogenous peroxidase was blocked by 3% H_2O_2 in 50% Methanol for 10 minutes at room temperature, followed by a wash (d.d H_2O). Non-specific binding was reduced by the addition of the blocking agent,

10% Normal Rabbit Serum in PBS buffer (ZYMED, Lot No: 80943701) for 10 minutes at room temperature.

A monoclonal VEGF-A antibody (SANTA CRUZ, Cat: Sc-7269) which is a mouse antibody was used in this experiment. Titration curves were conducted to established the correct concentration of antibody, the best concentration was 1 in 50. Each slide was incubated with 80 - 100 µl of primary antibody for 2 hours at 37°C or overnight at 4°C. Then, the slides were washed twice in 1 x PBS buffer for 2 minutes at room temperature.

Negative controls, which excluded the primary antibody were similarly treated. The slides were then incubated for 20 minutes at room temperature with the secondary antibody, a rabbit anti mouse IgG antibody (ZYMED. Lot No: 80140011R4) at a dilution of 1:100. Then, the slides were washed twice in PBS for 2 minutes at room temperature. The slides were then incubated for 20 minutes at room temperature with Horseradish Peroxidase-Streptavidin (ZYMED, Lot No: 80441388), at a dilution of 1:400. Then, the slides were washed twice in PBS for 2 minutes at room temperature.

The slides were then incubated for 10 minutes in 3,3'-Diaminobenzidine tetrahydrochloride (DAB) using a substrate A.B.C. kit (ZYMED, Lot No: 80743042). Following the manufacturers protocol, two drops of each reagent (A: 20 x buffer; B: 20 x DAB solution; C: 20 x hydrogen peroxide) were diluted in 2 ml d.d.H_2O. The slides were then washed and clear mount applied. Positive staining, as indicated by a yellow to brown colour, was observed under a light microscope (Olympus, OM System, CH-2).

2.13.0 *IN SITU* HYBRIDIZATION

Localisation of mRNA VEGF-A in uteroplacental tissues was investigated using *insitu* hybridization as described in section 2.13.4.

2.13.1 Preparation of Riboprobe

A novel system from Ambion (LigNScribe) was used to add T7 promotor directly to the VEGF-A_{164} RT-PCR product. This product was purified from an agarose gel prior to use as described in section 2.9.0.

A ligation volume of 10 µl containing 10 x ligation buffer 1 µl, T7 promoter 1µl, PCR fragment 2 µl (10 ng/µl), T4 DNA ligase 1µl (4.0 units/µl) and d.d water 5 µl was set up. The reaction was mixed and incubated at room temperature for 15 minutes. Of this reaction mixture, 2 µl was used for PCR. In order to generate the DNA template for transcription of the antisense riboprobe, PCR was performed with an Adapter primer and VEGF-A_{164} upstream primer. In order to generate the DNA template for transcription of the sense riboprobe PCR was performed with an Adapter primer and VEGF-A_{164} downstream primer. The composition of the PCR is shown in Table 2.21.

Component	**Volume For Sense Riboprobe**	**Volume For Antisense Riboprobe**	**Final Concentration**
10 x PCR amplitaq buffer	5 µl	5 µl	20 mM Tris, 50 mM KCl
25 mM $MgCl_2$	3 µl	3 µl	15 mM
2.5 mM dNTP mix	4 µl	4 µl	200 µM
10 µM upstream primer	------	1.25 µl	2.5 µM
10 µM downstream primer	1.25 µl	-------	2.5 µM
Ligation reaction	2 µl	2 µl	
Amplitaq	0.5 µl	0.5 µl	0.05 U/µl
Sterile water	33 µl	33 µl	
Total volume	50 µl	50µl	

Table 2.21 The composition of the PCR reaction for amplification of the VEGF-A riboprobe

Cycle	Denaturation	Annealing	Polymerisation
First cycle	3 min, at 94°C		
Subsequent	30 seconds	30 seconds	3 minutes
Cycles (n=30)	at 94°C	at 58°C	at 72°C
Last cycle			3 minutes at 72°C
Hold			at 4°C

Table 2.22 PCR cycling parameters for the VEGF-A riboprobe.

2.13.2 Non-radioactive Labelling of Riboprobes for *In situ* Hybridisation

The reaction mix contained 10 x transcription buffer containing DTT 2 μl (0.1 M), T7 RNA polymerase 2 μl (40 units /20 μl), RNAsin 1 μl (40 U/μl), dNTP mix 2 μl (10 mM), and d.d water (8 μl). Total volume was 20 μl in the final concentration of ligation PCR products (as described in 2.13.1) 2.5 ng/μl and T7 RNA Polymerase 2 μl (40 units/20 μl). The reaction was incubated at 37°C for 2 hours, terminated by adding 2 μl 0.2 M EDTA pH 8.0 2.5 μl LiCl (4 M) was added and 75 μl 100% ethanol to final reaction mix and the mixture was incubated at -70°C for overnight. The riboprobe was centrifuged at 12,000 rpm (14g) for 15 min at 4°C, pellets were washed in 80% ethanol, dried and resuspended in 50-100 μl DEPC water. The riboprobes was stored at -80°C prior to use.

2.13.3. Northern Blot Analysis using Riboprobes

Two antisense riboprobes were prepared complementary to VEGF-A nucleotides 342-577, inclusive, and 88-282, inclusive. Sense probes were also prepared. A concentration of 12.9 ng (0.3 ng/μl) of antisense riboprobe and 15.6 ng (0.52 ng/μl) of sense riboprobe was denatured in a 65°C water bath for 10 minutes. After cooling on ice, the reaction was mixed with 5 ml DIG easy buffer (Boehringer Mannheim), then pre-hybridised at 68°C for 4 hours and hybridisation overnight at 68°C with shaking. The post-hybridisation washes were performed as described in section 2.11.5.3. An anti-Digoxigen-AP and chemiluminescence (CSPD) detection was used in this experiment.

2.13.4 *In situ* Hybridisation

Paraffin embedded tissues were used to detect mRNA VEGF-A using the technique of *insitu* hybridisation. The sections were dewaxed (histolene X 2 for 4 minutes) and rehydrated (100% ethanol X 2 for 4 minutes, 70% ethanol X 2 for 4 minutes). HCl digestion (0.2 M HCl) occurred for 20 minutes and the slides were washed twice with DEPC water. The protein digestion was carried out by adding 50 µl of proteinase K (1 - 50 µg/ml), washed in 0.2% glycine at 4°C for 10 minutes to deactivate proteinase K. Salmon sperm DNA (ss DNA) and tRNA was denatured by heating to 95°C for 10 minutes, coolling on ice and adding 50-1000 µl of each to dextran sulphate solution. Slides were washed in 0.1 M triethanolamine for 2 minutes to reduce non-specific binding. After washing again with DEPC water, pre-hybridisation was carried out by adding 80 µl of dextran sulphate solution and incubating at 37 - 42°C for 2 hours. Hybridisation was carried out by adding 80 µl diluted riboprobe (20 - 1000 ng/µl) and incubating overnight at 42°C. The post-hybridisation washing consisted of 2 x SSC (300 ml) at room temperature for 15 minutes and 2 x SSC with RNase at 42 °C for 30 minutes, followed by two further washes in 1 x SSC at 42°C for 15 minutes.

Immuno-detection was carried out in blocking solution at room temperature for 30 minutes. To this, 80 µl of anti-DIG antibody (1:1000 in blocking solution) was added followed by incubation either a room temperature for 1 hour or overnight at 4°C. After washing in buffer, 80 µl of NBT (4.5 µl/ml) and BCIP (3.5 µl/ml) was added to the slides. The slides were incubated in the dark for 15 minutes or overnight. The slides were then washed and mounted in GVA mounting solution (ZYMED).

CHAPTER 3: ESTABLISHMENT OF RT-PCR, NORTHERN BLOT ANALYSIS, IMMUNOHISTOCHEMISTRY AND *IN SITU* HYBRIDISATION TECHNIQUES TO MEASURE EXPRESSION AND LOCALISATION OF VEGF-A, VEGFR-1 AND VEGFR-2 IN NORMAL UTEROPLACENTAL RAT TISSUES AT EARLY, MID AND LATE GESTATION.

3.1.0 INTRODUCTION

This project forms part of a larger project at Victoria University investigating the role of various growth factors in the development of the placenta, fetal membranes and fetal lungs. The development of the circulatory system, in particular vessel growth and development in the placenta and the fetal lungs, is vital for adequate gas exchange in utero and postnatally. Our reproductive unit, at Victoria University, is interested in understanding angiogenic processes in the development of the placenta and the fetal lungs. As VEGF-A is the most potent angiogenic factor known, we wished to, firstly, establish techniques to measure expression and localisation of VEGF-A and its two receptors. These techniques include RT-PCR, Northern blot analysis, immunohistochemistry and *insitu* hybridisation.

All isoforms of VEGF-A, except VEGF-A_{110} have been reported to be expressed in uteroplacental and embryonic tissues of the human, particularly during implantation. The predominant isoforms expressed by placental cells were VEGF-A_{165} and VEGF-A_{121}. (Athanassiades *et al*, 1998; Vuorela *et al,* 1997; Wheeler *et al,* 1995; Anthony *et al,* 1994; Houck *et al,* 1991). VEGFR-1 and VEGFR-2 have also been shown to be expressed by human placenta early in gestation and at term (Banks *et al,* 1998; Clark *et al,* 1996). VEGF-A_{188}, VEGF-A_{164}, and VEGF-A_{120} have been found in uteroplacental tissues of pregnant rabbits and rats, with VEGF-A_{120} and VEGF-A_{164} being the dominant isoforms expressed (Cullinan-Bove *et al,* 1992; Leung *et al,* 1989; Keck *et al,* 1989). Srivastava *et al,* (1998) using RT-PCR analysis, detected only one major PCR product corresponding to VEGF-A_{164} in antimesometrial and mesometrial decidual tissues of a pseudopregnant rat.

Using Northern blot analysis and *insitu* hybridisation, Das *et al,* (1997) examined expression and localisation of VEGF-A and its receptors in the uteroplacental tissues of the rabbit from oestrus (day 0) to implantation (~day 8). Expression of VEGF-A and its receptors was shown to be highest at days 0 and 6-8, with the syncytiotrophoblast as the predominate localisation site. A similar pattern of

expression was found in the mouse uteroplacental tissues during implantation (Chakraborty *et al,* 1995).

The first aim of this thesis was to establish the use of RT-PCR, Northern blot analysis, immunohistochemistry and *insitu* hybridisation, to measure expression and localization of VEGF-A, VEGFR-1 and VEGFR-2 in normal rat uteroplacental tissues at early, mid and late gestation.

3.2.0 METHODS

The methods are outlined in Chapter 2.

3.3.0 RESULTS

3.3.1 Quality and Quantification of RNA Extraction

The quality of total RNA extraction was assessed by gel electrophoresis (Figure 3.1), and the yield and purity of total RNA extraction was assessed by spectrophotometry (Table 3.1). Extraction of mRNA from total RNA, using the Poly A tract mRNA Promega Isolation System Kit, was successful as shown in Figure 3.2.

Sample		Guanidinium thiocyanate			Trizol reagent			
No.	OD 260	OD 280	Yield (μg/mg)	Purity	OD260	OD 280	Yield (μg/mg)	Purity
1	0.095	0.054	0.30	1.7	0.225	0.14	0.36	1.6
2	0.085	0.051	0.26	1.6	0.372	0.205	0.59	1.7
3	0.069	0.029	0.22	2.3	0.271	0.132	0.43	2.0
4	0.188	0.109	0.60	1.7	0.456	0.281	0.72	1.5
5	0.038	0.027	0.12	1.4	0.432	0.242	0.68	1.8
6	0.183	0.115	0,58	1.5	0.589	0.343	0.94	1.7
7	0.227	0.165	0.72	1.3	0.401	0.225	0.64	1.7
8	0.011	0.008	0.03	1.3	0.113	0.08	0.18	1.4
Mean	0.112	0.069	0.353	1.60	0.315	0.206	0.567	1.68
SEM	0.072	0.050	0.233	0.30	0.174	0.080	0.221	0.17
n =	8	8	8	8	8	8	8	8

Table 3.1 Comparison of yield (μg RNA/mg of tissue) and purity, from a representative selection of total RNA extractions, using either the Guanidinium thiocyanate or Trizol technique.

Figure 3.1. Electrophoresis gels of total RNA extraction using the A) Guanidinium thiocyanate technique and B) Trizol technique. Samples of total RNA (10 μg/well) from normal uteroplacental tissues were loaded in 6% Formaldehyde gel (1 X MOPs buffer) at 15 volts for 19 hours. The gels were stained with Ethidium Bromide (0.1%). A) Lanes 1 to 7 were uterine tissue from day 7 to 19 of gestation, in 2 day intervals, respectively. B) Lanes 1 to 7 were uterine tissue from day 5 to 15 of gestation, in 2 day intervals, respectively.

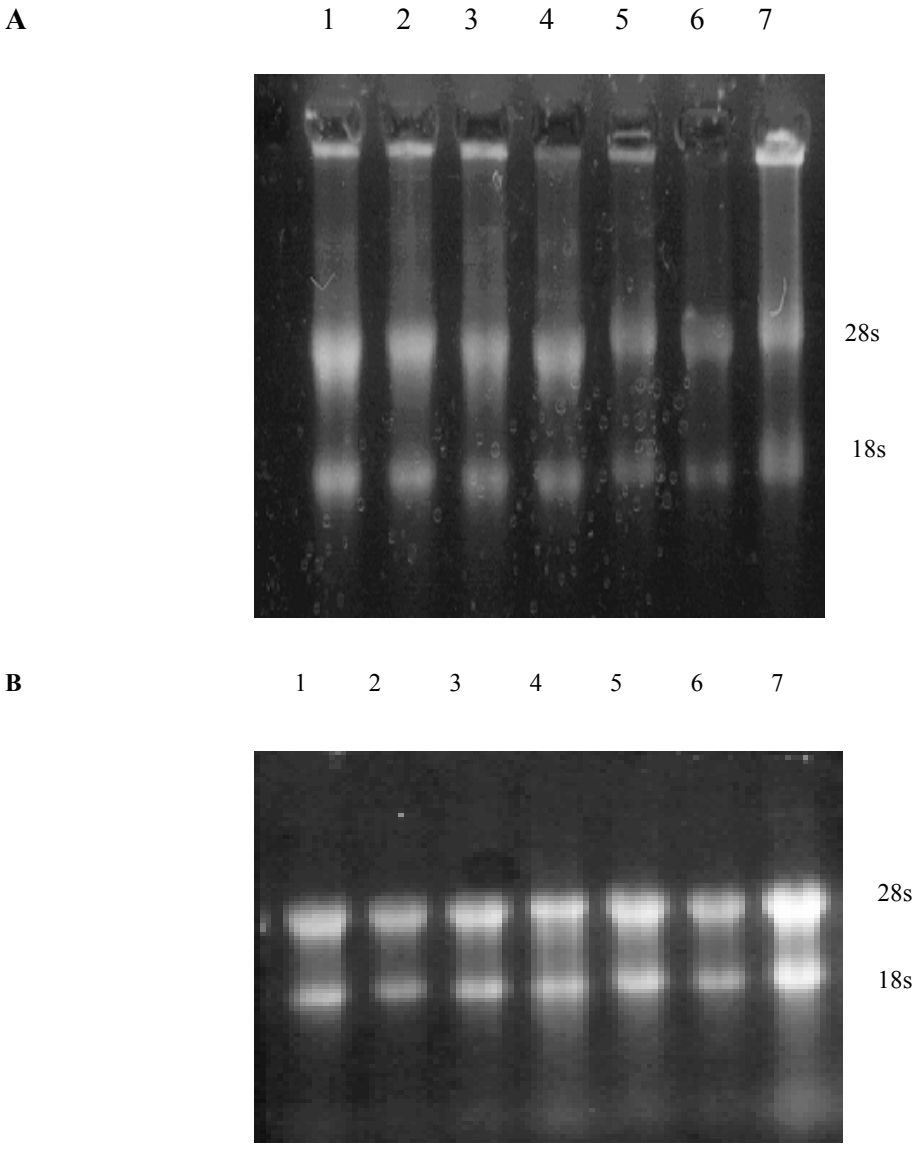
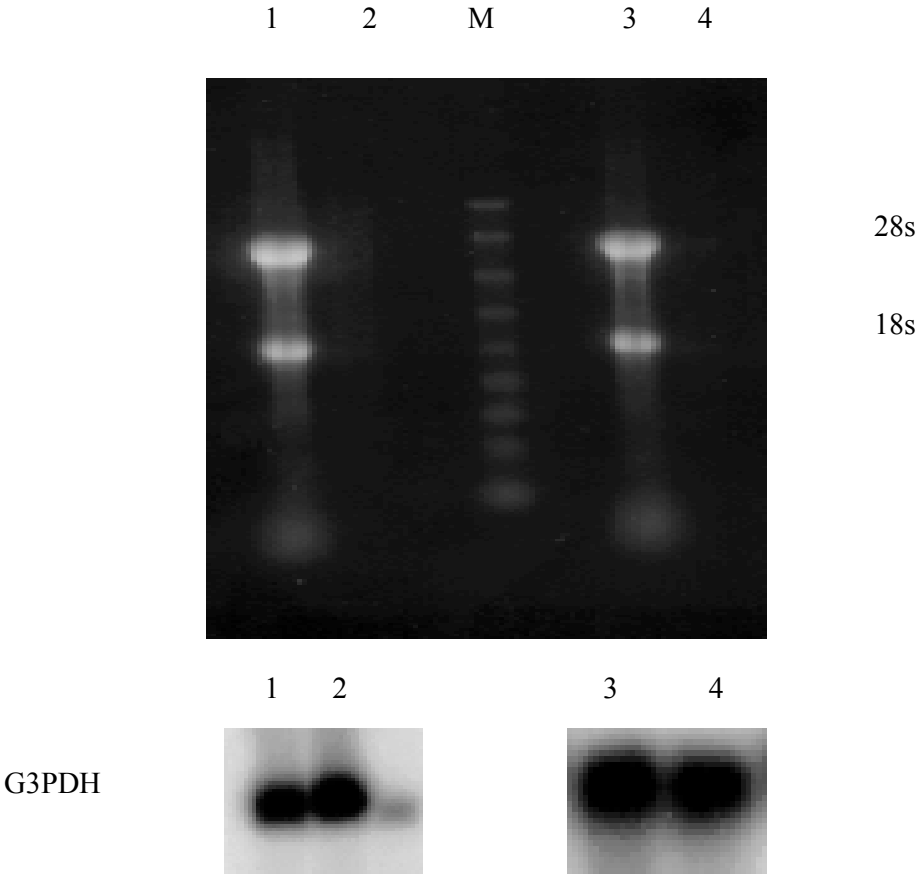


Figure 3.2. Electrophoresis gel of mRNA extracted from total RNA. Samples, 1.6 μg of mRNA and 10 μg of total RNA (per well), were loaded in a 6% formaldehyde gel

(1 X MOPs buffer) at 20 volts for 14 hours. The gel was stained with ethidium bromide. Although total RNA was visible under UV light, mRNA was not. The integrity of the mRNA was demonstrated by the corresponding Northern blot showing hybridisation to G3PDH. Lanes 1 and 3 were total RNA, lanes 2 and 4 were mRNA. RNA marker (M) was used to show molecular weights.



By visual observation, the quality of total RNA extracted using the Guanidium thiocyanate technique was comparable to that using the Trizol technique. Similarly, the yield and purity of total RNA were comparable in the two techniques. However, extraction of total RNA was simpler, easier and faster using the Trizol technique than

the Guanidinium thiocyanate technique. The Trizol reagent was stable for a much longer period of time than the phenol alone which is required for the Guanidium thiocyanate technique. Consequently, the Trizol technique was used to extract total RNA from the majority of samples used in this project.

3.3.2 Reverse Transcriptase Polymerase Chain Reaction

RT-PCR was used as a technique to investigate levels of G3PDH, VEGF-A, VEGFR-1 and VEGFR-2 mRNA.

3.3.2.1 DNase treatment

When conducting RT-PCR to measure mRNA expression it is important to ensure that the resultant products are dependant on reverse transcript activity and not derived from genomic DNA. Therefore, all extracted total RNA was routinely treated with DNase. Successful DNase treatment was judged by the inability to obtain PCR products in the absence of reverse transcriptase. A representative analysis of RT-PCR products formed in the presence and absence of reverse transcriptase is shown in Figure 3.3.

3.3.2.2 G3PDH RT-PCR

Throughout these studies, G3PDH gene was used as an internal standard to establish equal mRNA template levels for RT-PCR analysis. Optimal conditions for PCR cycling have been described in the Methods section 2.4.4.1.

RT-PCR analysis showed the presence of G3PDH mRNA in uteroplacental tissues from normal rats as shown in Figure 3.4. One band of approximately 525 bp was consistently observed. The size of this RT-PCR product was as expected, size and identity were confirmed by sequence analysis at the onset of these experiments by Ms. Usula Manuelpillai.

Figure 3.3 Dependence of PCR product formation on the presence of reverse transcriptase. PCR was performed with reverse transcriptase (lanes 1, 2, 4, 5, 7, 8) and without reverse transcriptase (lanes 3, 6, 9). DNA 100 bp marker (M) is shown.

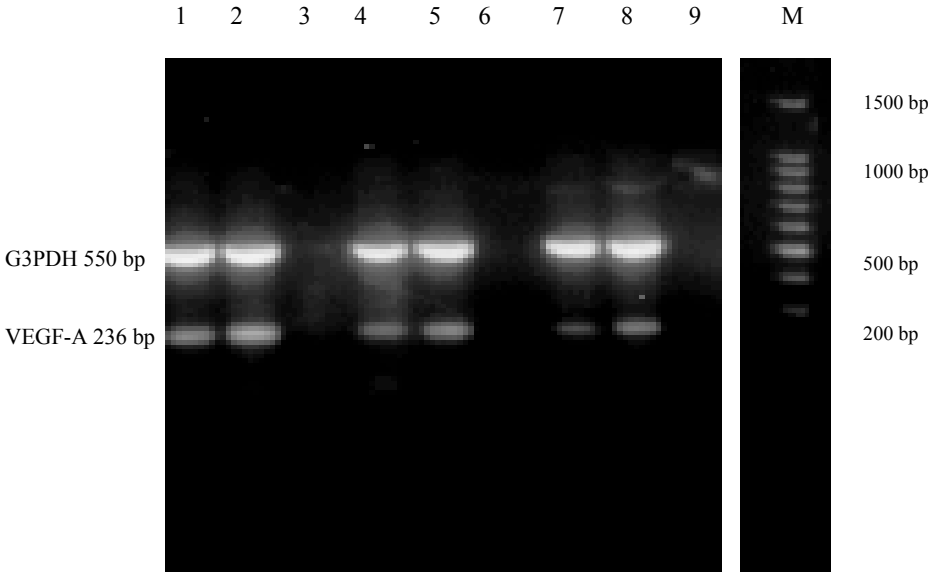
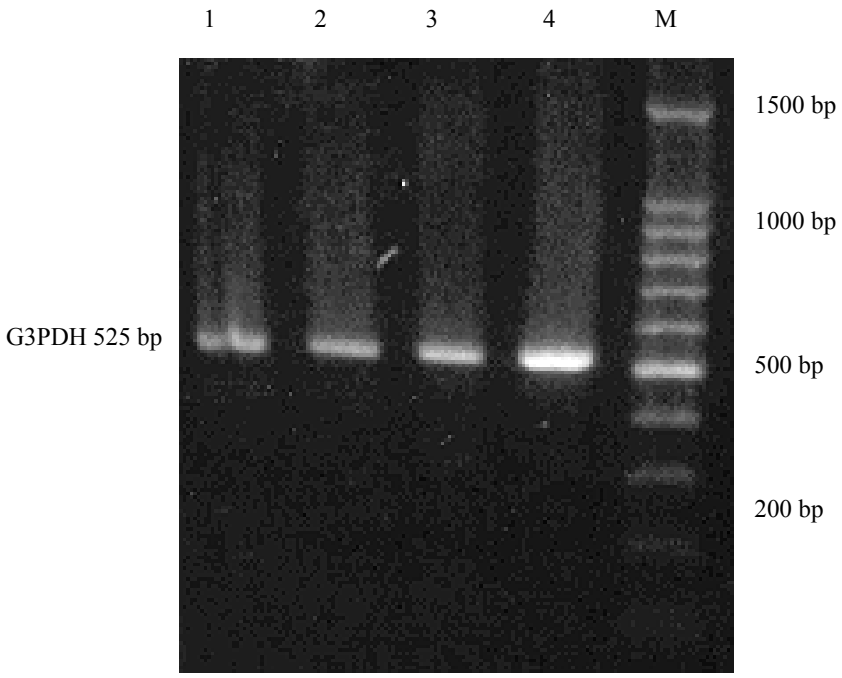


Figure 3.4. RT-PCR analysis of G3PDH expression in uteroplacental tissues. A single product of ~525 bp was observed (lanes 1 - 4), and it corresponded to the theoretical size of the G3PDH RT-PCR product. The DNA 100 bp marker (M) is shown.



3.3.2.3 VEGF-A RT-PCR

Two sets of primers were used to detected VEGF-A isoforms. *Set 1* primers were used for the vast majority of the work in this thesis. Theoretically, these VEGF-A primers have the ability to detect isoforms 205, 188, 164, 144 and 120. Using RT-PCR, we confirmed that these primers do detect various isoforms in a range of rat tissues. Figure 3.5 shows three bands of approximately 308, 236 and 104 bp derived from placental tissues of Sprague Dawley rats at 19 days of gestation and one band at approximately 236 bp derived from uterine tissues at 19 days of gestation. The 308 bp and 236 bp bands were sequenced and they corresponded to VEGF-A_{188} and VEGF-A_{164}, respectively (Figure 3.6 and Figure 3.7). Although several attempts were made, the band at approximately 104 was not successfully sequenced, therefore we were unable to confirm that band represented VEGF-A_{120} as predicted.

We found *set 1* primers adequate for separating three of the VEGF-A isoforms using RT-PCR and adequate for detection and qualitative analysis. In some cases, results obtained with those primers were confirmed via another set of primers (*set 2*) which were designed and provided by Dr Sarah Fraser at Victoria University. Theoretically, these primers can detect all known isoforms of VEGF-A as the primers are in exon 1 and exon 8. Four bands were found in uteroplacental tissues using the VEGF-A primer *set 2* (Figure 3.8). Two major PCR products from placental tissues, at approximately 496 bp and 364 bp were sequenced and found to correspond to VEGF-A_{164} and VEGF-A_{120} (Figure 3.9), respectively. The other two bands (approximately 568 bp and 436 bp) that were observed have the correct theoretical size for VEGF-A_{188} and VEGF-A_{144}, however, identity was not confirmed by sequence analysis.

3.3.2.4 VEGFR1 RT-PCR

RT-PCR analysis produced a band at 280 bp in uterine tissue at 19 days of gestation (Figure 3.10). This 280 bp band was sequenced and it corresponded to VEGFR-1 (Figure 3.11).

Figure 3.5. RT-PCR analysis of VEGF-A (primer *set 1*) expression in A) placental and B) uterine tissues at day 19 days of gestation. Three bands (308, 236 and 104 bp) were evident in some placental tissues (lane 1), whereas one band was evident in uterine tissues (lane 2). The 308 and 236 bp bands were sequenced and corresponded to VEGF-$A_{188,}$ and VEGF-$A_{164,}$ respectively. The 104 bp band could not be sequenced. DNA 100 bp marker (M) is shown.

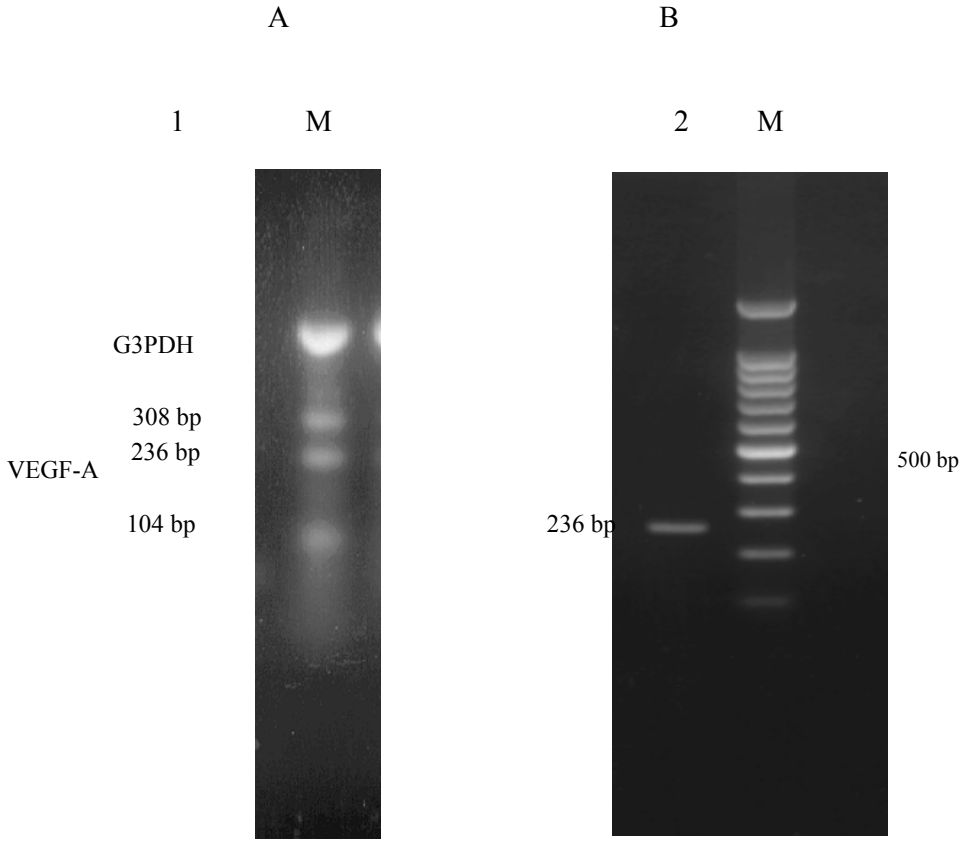


Figure 3.6. Partial sequence alignment of the VEGF-A_{188} (308 bp) RT-PCR product (primer *set 1*). The sequence of the RT-PCR product, shown as the upper sequence, had 94% homology with the published VEGF-A_{188} sequence (Breier *et al,* 1992, Acc No: AF0626), shown as the lower sequence. The sequences used to design the primers are underlined and the unconfirmed sequences are highlighted.

```
                10        20        30        40        50
         GCCAGCACATAGGAGAGATGAGCTTCCTGCAGCACAGCAGATGTGAATGCAGACCAAA
         :::::::::::::::::::::::::::::::::: :::::::::::::::::::::::
AF0626 AAGCCAGCACATAGGAGAGATGAGCTTCCTGCAGCATAGCAGATGTGAATGCAGACCAAA
              10        20        30        40        50        60

       60        70        80        90       100       110
       GAAAGATAGAACAAAGCCAGAAAAANAATCAGTTCGAGTAAAGGAGCAGGGACAATATCG
       ::::::::::::::::::::::::: :::::::::::: :::::   :::: ::: : ::
AF0626 GAAAGATAGAACAAAGCCAGAAAAAAAATCAGTTCGAGGAAAGGGAAAGGGTCAAAAACG
              70        80        90       100       110       120

      120       130       140       150       160       170
       AAAGCGCCCGAAATCCCGGTTTAAATCCTGGAGCGTTCACTGTGAGCCTTGTTCAGAGCG
       :::::::  ::::::::::::::::::::::::::::::::::::::::::::::::::::
AF0626 AAAGCGCAAGAAATCCCGGTTTAAATCCTGGAGCGTTCACTGTGAGCCTTGTTCAGAGCG
             130       140       150       160       170       180

      180       190       200       210       220       230
       GAGAAAGCATTTGTTTGTCCAAGATCCGCAGACGTGTAAATGTTCCTGCAAAAACACAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF0626 GAGAAAGCATTTGTTTGTCCAAGATCCGCAGACGTGTAAATGTTCCTGCAAAAACACAGA
             190       200       210       220       230       240

      240       250       260       270       280       290
       CTCGCGTTGCAAGGCGAGGCAGCTTGAGTTTACGATACGAACCGGCAGATGTGACAAGCC
       ::::::::::::::::::::::::::::::::    ::: :: ::..............
AF0626 CTCGCGTTGCAAGGCGAGGCAGCTTGAGTTAAACGAACGTACTTGCAGATGTGACAAGCC
             250       260       270       280       290       300

       AAGGCGGTGA
       ..........
AF0626 AAGGCGGTGA
```

Figure 3.7. Partial sequence alignment of the VEGF-A_{164} (236 bp) RT-PCR product (primer *set 1*). The sequence of the RT-PCR product, shown as the upper sequence, was identical with the published VEGF-A_{164} sequence (Conn *et al*, 1990; Acc No: M32167), shown as the lower sequence. The sequences used to design the primers are underlined.

```
                                                  10        20        30
                                                GCCAGCACATAGGAGAGATGAGCTTCCTGC
                                                ::::::::::::::::::::::::::::::
 M32167TGCAGATCATGCGGATCAAACCTCACCAAAGCCAGCACATAGGAGAGATGAGCTTCCTGC
             320       330       340       350       360       370

               40        50        60        70        80        90
       AGCATAGCAGATGTGAATGCAGACCAAAGAAAGATAGAACAAAGCCAGAAAATCACTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
M32167 AGCATAGCAGATGTGAATGCAGACCAAAGAAAGATAGAACAAAGCCAGAAAATCACTGTG
             380       390       400       410       420       430

              100       110       120       130       140       150
       AGCCTTGTTCAGAGCGGAGAAAGCATTTGTTTGTCCAAGATCCGCAGACGTGTAAATGTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
M32167 AGCCTTGTTCAGAGCGGAGAAAGCATTTGTTTGTCCAAGATCCGCAGACGTGTAAATGTT
             440       450       460       470       480       490

              160       170       180       190       200       210
       CCTGCAAAAACACAGACTCGCGTTGCAAGGCGAGGCAGCTTGAGTTAAACGAACGTACTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
M32167 CCTGCAAAAACACAGACTCGCGTTGCAAGGCGAGGCAGCTTGAGTTAAACGAACGTACTT
             500       510       520       530       540       550

              220       230
       GCAGATGTGACAAGCCAAGGCGGTGA
       :::::::::::::::::::::::::
M32167 GCAGATGTGACAAGCCAAGGCGGTGA
              560       570
```

Figure 3.8 RT-PCR analysis of VEGF-A expression (primer *set 2*) in uteroplacental tissues. Four bands (~ 568 bp, 496 bp, 436 bp and 364 bp) were evident. The most highly expressed band was at 496 bp. When sequenced, the 496 bp band corresponded VEGF-A_{164} and the 364 bp band corresponded to VEGF-A_{120}. Lane 1 (uteroplacentae day 7), lane 2 (uteroplacentae day 11), lane 3 (placentae day 19). DNA 100 bp marker (M) is shown.

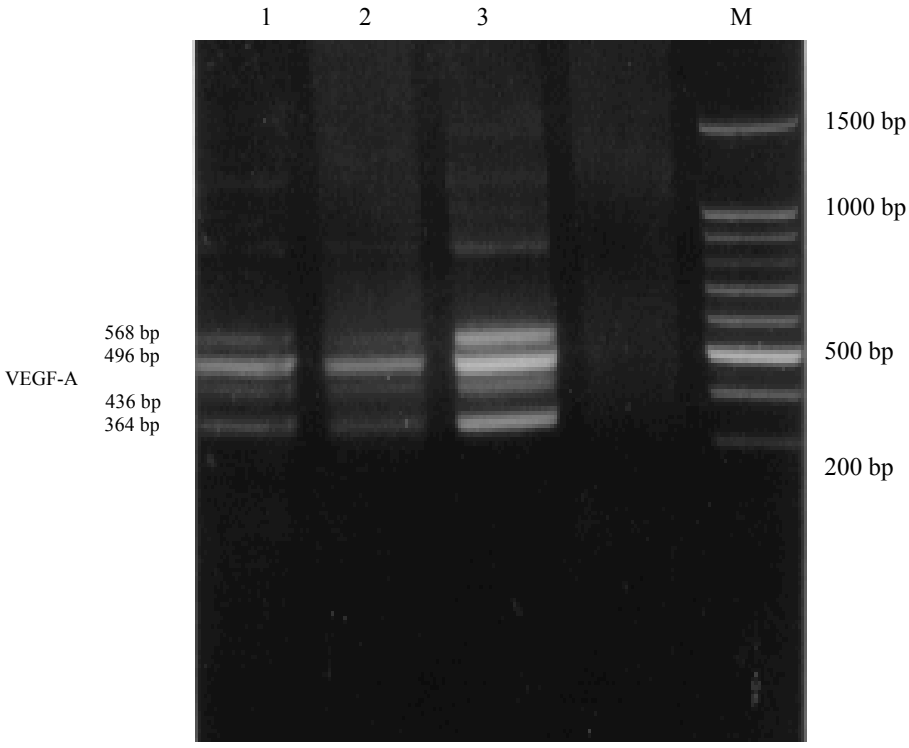


Figure 3.9. Partial sequence alignment of the VEGF-A_{120} (364 bp) RT-PCR product (primer *set 2*). The sequence of the RT-PCR product, shown as the upper sequence, was identical with the published VEGF-A_{120} sequence (Acc No: S38100), shown as the lower sequence. The sequences used to design the primers are underlined.

```
                                                10        20
                                              CACCCACGACAGAAGGAGAGCAGAAGTC-C
                                              :::::::::::::::::::::::::::: :
S38100 ACCTCCACCATGCCAAGTGGTCCCAGGCTGCACCCACGACAGAAGGAGAGCAGAAGTCCC
               70        80        90       100       110       120

      30        40        50        60        70        80
       ATGAAGTGATCAAGTTGCATGGACGTCTACCAGTGAACCTACTGCCGTCCGATTGAGACC
       ::::::::::::::: ::::::::::::::: ::: :::::::::::: :::::::::
S38100 ATGAAGTGATCAAGTT-CATGGACGTCTACCAGCGAAGCTACTGCCGTCCAATTGAGACC
             130       140       150       160       170       180

      90       100       110       120       130       140
       CTGGTGGACATCTTCCAGGAGTACCCCGACGAGATAGAGTACATCTTCAAGCCGTNNTGT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::  :::
S38100 CTGGTGGACATCTTCCAGGAGTACCCCGACGAGATAGAGTACATCTTCAAGCCGTCCTGT
              190       200       210       220       230       240

     150       160       170       180       190       200
       GTGCCGCTGATGCGCTGTGCAGGCTGCTGTAACGATGAAGCCCTGGAGTGCGTGCCCACG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
S38100 GTGCCGCTGATGCGCTGTGCAGGCTGCTGTAACGATGAAGCCCTGGAGTGCGTGCCCACG
              250       260       270       280       290       300

     210       220       230       240       250       260
       TCAGAGAGCAACATCACCATGCAGATCATGCGGATCAAACCTCACCAAAGCCAGCACATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
S38100 TCAGAGAGCAACATCACCATGCAGATCATGCGGATCAAACCTCACCAAAGCCAGCACATA
              310       320       330       340       350       360

     270       280       290       300       310       320
       GGAGAGATGAGCTTCCTACAGCACAGCAGATGTGAATGCAGACCAAAGAAAGACAGAACA
       :::::::::::::::::::::::::: :::::::::::::::::::::::::::: :::
S38100 GGAGAGATGAGCTTCCTACAGCACAGCCGATGTGAATGCAGACCAAAGAAAGACAGGACA
              370       380       390       400       410       420

     330       340       350       360
       AAGCCNGAAAAATGTGACAAGCCAAGGCGGTGA
       ::::: ::::::::::::::::: :::::::::
S38100 AAGCCAGAAAAATGTGACAAGCCGAGGCGGTGA
              430       440       450
```

Figure 3.10 RT-PCR analysis of VEGFR-1 expression in uterine tissue at 19 days of gestation. The 280 bp product (lane 1) was sequenced and shown to correspond to VEGFR-1. DNA 100 bp marker (M) is shown.

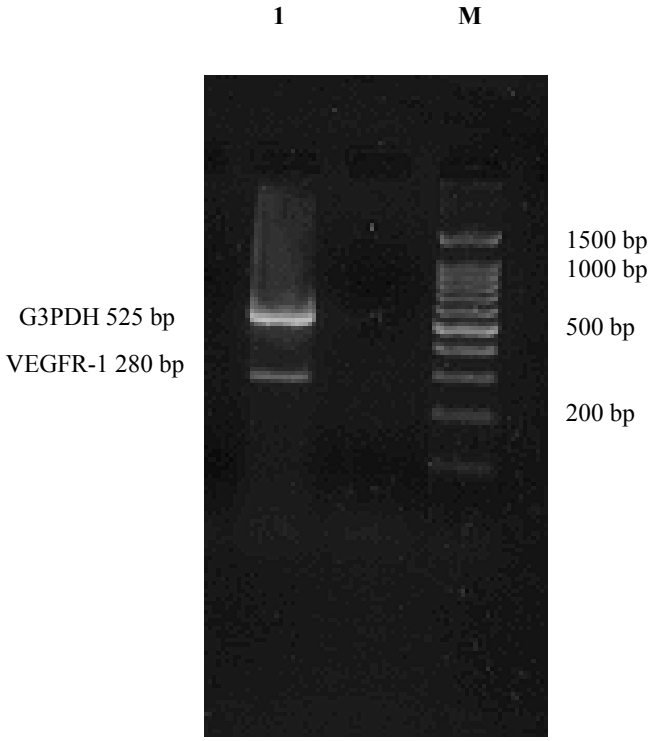


Figure 3.11 Partial sequence alignment of the VEGFR-1 (280 bp) RT-PCR product. The sequence of the RT-PCR product shown as the upper sequence, was 99.2% homologous with the published VEGFR-1 sequence (RNFLT1 Acc No: 4734), shown as the lower sequence. The sequences used to design the primers are underlined.

```
                                                  10        20        30

                                        CTGCTACGGACAGTTAACAACAGGACCATG
                                        ::::::::::::::::::::::::::::::
RNFLT1 AAATTCCTGTACAGAGACATTACCTGGATCCTGCTACGGACAGTTAACAACAGGACCATG
       1980      1990      2000      2010      2020      2030

               40        50        60        70        80        90
       CACCATAGCATCAGTAAGCAAAAAATGGCCACCACTCAGGACTACTCCATCACTCTGAAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNFLT1 CACCATAGCATCAGTAAGCAAAAAATGGCCACCACTCAGGACTACTCCATCACTCTGAAC
       2040      2050      2060      2070      2080      2090

              100       110       120       130       140
       CTTGTCATCAAGAATGTGTCTC-GGAAGANTCGGGCACCTATGCCTGCAGAGCCAGGAAC
       :::::::::::::::::::::: :::::: ::::::::::::::::::::::::::::::
RNFLT1 CTTGTCATCAAGAATGTGTCTCTGGAAGACTCGGGCACCTATGCCTGCAGAGCCAGGAAC
       2100      2110      2120      2130      2140      2150

     150       160       170       180       190       200
       ATATACACAGGGGAAGAGATCCTTCGGAAGACAGAAGTTCTCGTTAGAGATTTGGAAGCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNFLT1 ATATACACAGGGGAAGAGATCCTTCGGAAGACAGAAGTTCTCGTTAGAGATTTGGAAGCG
       2160      2170      2180      2190      2200      2210

     210       220       230       240       250       260
       CCACTCCTGCTTCAAAACCTCAGTGACCACGAGGTGTCCATCAGTGGCTCCACGACCTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNFLT1 CCACTCCTGCTTCAAAACCTCAGTGACCACGAGGTGTCCATCAGTGGCTCCACGACCTTA
       2220      2230      2240      2250      2260      2270

     270       280
       GACTGTCAAGA
       :::::::::: ...
RNFLT1 GACTGTCAAGCTAG
       2280      2290
```

Figure 3.12 RT-PCR analysis of VEGFR-2 expression in uteroplacental tissues. The 383 bp product was sequenced and it corresponded to VEGFR-2. Lane 1 (uteroplacentae, day 7), lane 2 (uteroplacentae, day 11), lane 3 (uterus, day 19). DNA 100 bp marker (M) is shown.

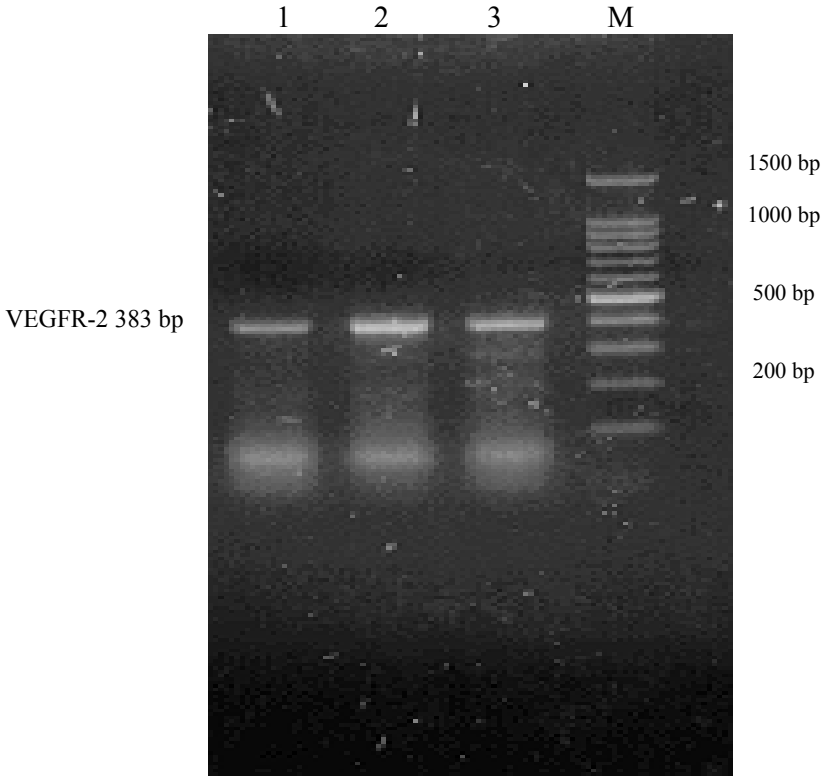


Figure 3.13 Partial sequence alignment of the VEGFR-2 (383 bp) RT-PCR product. The sequence of the RT-PCR product, shown as the upper sequence, was 98% homologous with the published VEGFR-2 sequence (RNU 93306), shown as the lower sequence. The sequences used to design the primers are underlined and unconfirmed sequences are highlighted.

```
                                                  10        20        30
                                          ATCCAATGGTACTGGCAGCTAGAAGAAGCA
                                          ::::::::::::::::::::::::::::::
RNU933 ACAGTCTATGCCAACCCTCCCCTGCACCACATCCAATGGTACTGGCAGCTAGAAGAAGCA
          1590      1600      1610      1620      1630      1640

       TGNTCCTACAGGCCCAGCCAAACAAACCCATATACTTGTAAAGAATGGAGACACGTGAAG
       :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNU933 TGCTCCTACAGGCCCAGCCAAACAAACCCATATACTTGTAAAGAATGGAGACACGTGAAG
          1650      1660      1670      1680      1690      1700

              100       110       120       130       140       150
       GATTTCCAGGGGGGNAAATAAGATCGAAGTCACCAANAACCAATATGCCCTAATTGAAGG
       :::::::::::::: :::::::::::::::::::: ::::::::::::::::::::::::
RNU933 GATTTCCAGGGGGG-AAATAAGATCGAAGTCACCAAAAACCAATATGCCCTAATTGAAGG
          1710       1720      1730      1740      1750      1760

              160       170       180       190       200       210
       AAAAAACAAAACTGTAAGTACTCTGGTCATCCAGGCTGCCAACGTGTCCGCATTATACAA
       :::::::::::::::::::::::::::::::::::::::: ::::::::::::::::::
RNU933 AAAAAACAAAACTGTAAGTACTCTGGTCATCCAGGCTGCCTACGTGTCCGCATTATACAA
           1770      1780      1790      1800      1810      1820

              220       230       240       250       260       270
       ATGTGAAGCCATCAACAAAGCAGGACGAGGAGAGAGGGTCATCTCCTTCCATGTGATCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNU933 ATGTGAAGCCATCAACAAAGCAGGACGAGGAGAGAGGGTCATCTCCTTCCATGTGATCAG
           1830      1840      1850      1860      1870      1880

              280       290       300       310       320       330
       GGGTCCTGAAATTACTGTCCAGCCTGCTACCCAGCCAACCGAGCAGGAGAGTGGGACGTG
       ::::::::::::::::::::::::::::::::::::::::: ::::::: .......
RNU933 GGGTCCTGAAATTACTGTCCAGCCTGCTACCCAGCCAACCGAGCGGGAGAGTATGACGTG
           1890      1900      1910      1920      1930      1940

              340       350       360       370       380

       GTACAAGCTTGGCTCACA
       ..................
RNU933 GTACAAGCTTGGCTCACA
```

3.3.2.5 VEGFR-2 RT-PCR

RT-PCR analysis produced a band at 383 bp in uteroplacental tissues at day 7, 11 and 19 of gestation (Figure 3.12). This 383 bp band was sequenced and it corresponded to VEGFR-2 (Figure 3.13).

3.3.3 VEGF-A and VEGFR-1 Expression, Using RT-PCR, at Early, Mid and Late Gestation

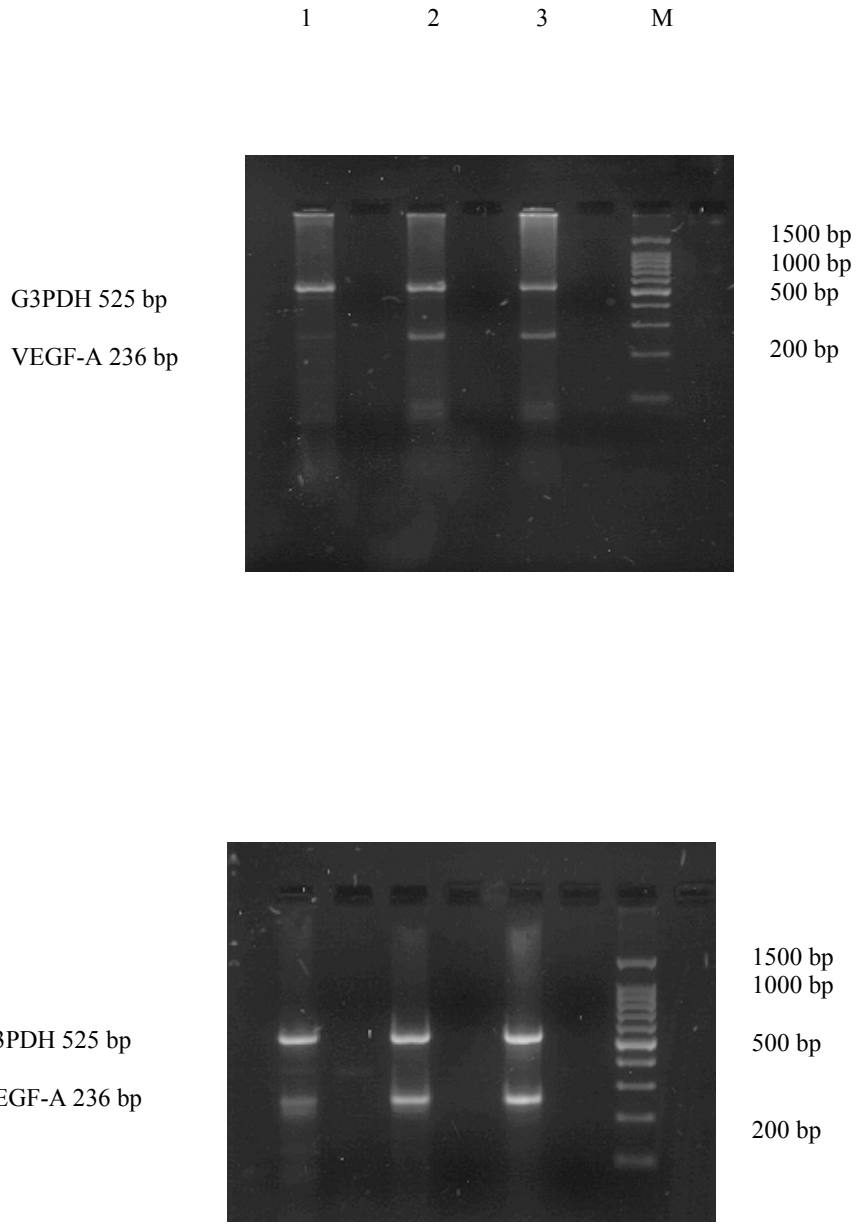
As part of method development for investigating expression of VEGF-A and its receptors, it was considered important to assess the suitability, reliability and limitations of RT-PCR and Northern blot for qualitative and quantitative analysis. We chose to examine expression patterns throughout gestation. This initial work was carried out on the cheaper and more readily available Sprague Dawley rats.

We examined VEGF-A expression in uteroplacental tissues of Sprague Dawley rats, at early (day 5), mid (day 11) and late (day 21) gestation and compared the RT-PCR products with those of G3PDH (Figure 3.14). At 5 and 11 days of gestation it was technically too difficult to divide the uteroplacental tissues into uterus and placenta, thus values for these different tissue were only available at 21 days.

Using VEGF-A primers (*set 1*), one band was found in uteroplacental tissues at 5 and 11 days and in both uterine and placental tissues at 19 days of gestation in one experiment (see Figure 3.5). The 236 bp band corresponded to VEGF-A_{164}. We were able to detect 3 bands in placental tissue at 19 days of gestation. However we were unable to detect all these bands reproducibly. The reasons for this are unknown.

There were no major differences observed in the ratio of VEGF-A_{164} to G3PDH throughout gestation. However, it was observed that this ratio was slightly lower at day 5 of gestation than at day 11 or day 21 of gestation. We used semi-quantification analysis to investigate these observations. The results, shown in Table 3.2, suggest that the ratio of VEGF-A_{164} expression to G3PDH expression was lower at 5 days of gestation than at 11 days and 21 days of gestation. However, further investigation into the kinetics of the RT-PCR was warranted (see section 3.3.4) and a larger sample size required to confirm these results.

Figure 3.14 RT-PCR analysis of VEGF-A and G3PDH expression in uteroplacental tissues at day 5 (lane 1, same sample in upper and lower gels), day 11 (lane 2, same sample in upper and lower gels) and in uterine (lane 3, upper gel) and placental tissues (lane 3, lower gel) at day 21 of gestation. DNA 100 bp marker (M) is shown.



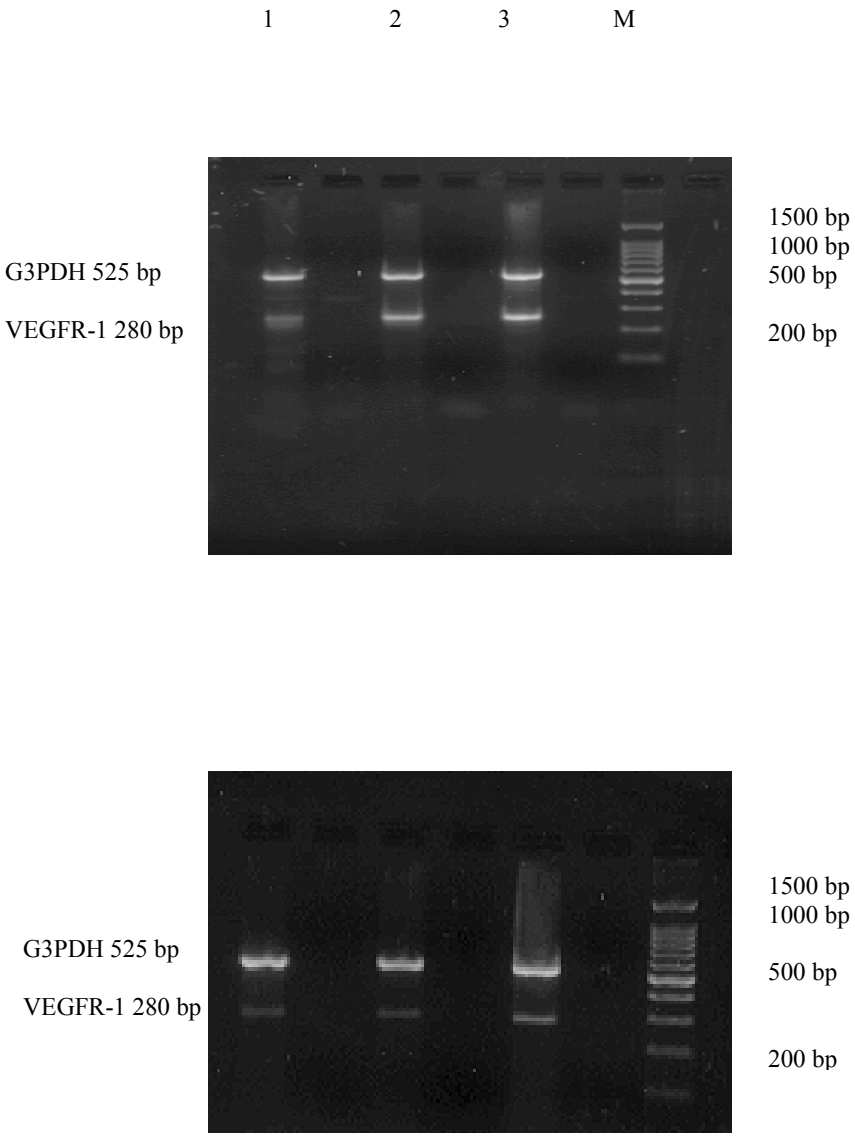
Gestation (days)	**5**	**5**	**11**	**11**	**21**	**21**
Tissue	**Utero-placental**	**Utero-placental**	**Utero-placental**	**Utero-placental**	**Uterus**	**Placenta**
VEGF-A_{164}	0.344	0.347	0.788	1.029	0.827	1.417
G3PDH	1.417	0.945	1.464	1.432	1.476	1.822
Ratio	0.24	0.36	0.52	0.71	0.56	0.77

Table 3.2 Ratio of VEGF-A_{164} to G3PDH in uteroplacental tissues at 5, 11 and 21 days of gestation, assessed by laser densitometry of RT-PCR products.

We examined VEGFR-1 expression in uteroplacental tissues, at early (day 5), mid (day 11) and late (day 21) gestation and compared the RT-PCR products with those of G3PDH (Figure 3.15). There were no major differences observed in the ratio of VEGFR-1 expression to G3PDH expression at 5, 11 and 21 days of gestation.

VEGFR-2 expression was confirmed in uteroplacental tissues (day 7 and 11), and uterus day 19 (see Figure 3.12). Semi-quantitative analysis was not performed.

Figure 3.15 RT-PCR analysis of VEGFR-1 and G3PDH expression in uteroplacental tissues at day 5 (lane 1, same sample in upper and lower gels) and day11 (lane 2, same sample in upper and lower gels) and in uterine tissues (lane 3, upper gel) and placental tissues (lane 3, lower gel) at 21 days of gestation. DNA 100 bp marker (M) is shown.



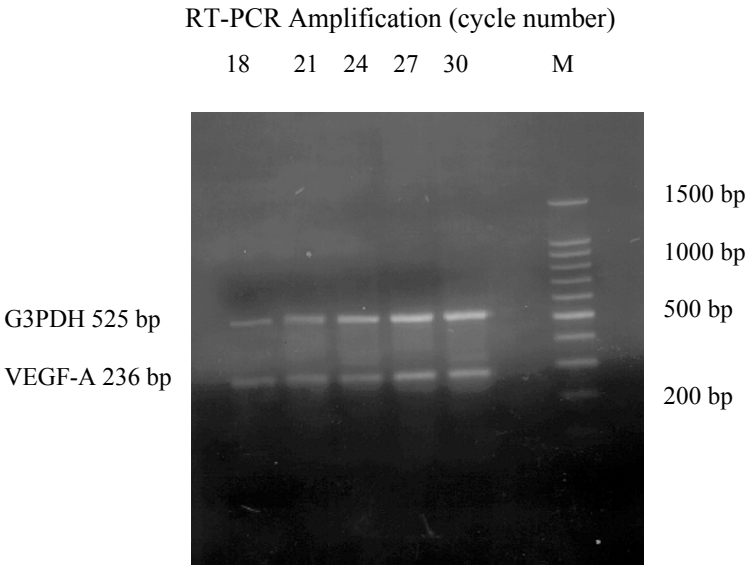
3.3.4 Semi-quantitative Analysis of VEGF-A and VEGFR-1 Expression

Our results demonstrated that the primers used in this study were suitable for qualitative investigation of VEGF-A, VEGFR-1 and VEGFR-2 expression. The G3PDH gene was included to demonstrate comparisons made between equal amounts of mRNA. As such, this system is also used for semi-quantitative analysis. As we observed a small difference in VEGF-A expression between early gestation and mid to late gestation, we wished to investigate this difference further. The precision of semi-quantitative analysis, using our RT-PCR conditions, was subsequently tested by examining the kinetics of the RT-PCR. Using uterine tissues at 19 days of gestation, we performed RT-PCR at 18, 21, 24, 27 and 30 cycles. The products were run on agarose gels. The amount of PCR product from G3PDH and VEGF-A primers (*set 1*) increased with increasing cycle number (Figure 3.16). The gel was scanned in an Y-axis direction and the intensity of each band calculated. It was clear that the expected doubling of intensity level with each cycle did not occur. Furthermore, the relationship between cycle number and intensity of the resultant band for VEGF-A_{164} differed from that of G3PDH, that is, the slopes of the regression lines differed (Figure 3.16). These results showed that the PCR amplification rate of VEGF-A_{164} differed from that of G3PDH. Consequently, we were unable to use semi-quantitative analysis with any degree of certainty.

We also endeavoured to quantify the levels of VEGFR-1 using semi-quantitative analysis. The amount of PCR product from G3PDH and VEGFR-1 primers increased with increasing cycle number (Figure 3.17). The gel was scanned in an Y-axis direction and the intensity of each band calculated. Again it was clear that the intensity did not double with each cycle. However, the relationship between cycle number and intensity of the resultant band for VEGFR-1 and G3PDH, showed no major difference between the slopes of the regression lines (Figure 3.17). This indicates that the PCR amplification rate of VEGFR-1 was comparable with that of G3PDH. Consequently, we were able to use semi-quantification of VEGFR-1 using G3PDH as the relative control. VEGFR-2 RT-PCR did not attempt semi-quantitative analysis with VEGFR-2.
Figure 3.16 Kinetics of RT-PCR for VEGF-A and G3PDH. A) Electrophoresis gel showing the RT-PCR products from G3PDH and VEGF-A primers (*set 1*) at 18, 21,

24, 27, and 30 cycles. A 100 bp DNA marker (M) is shown. The amount of PCR product increased with increasing cycle number. B) Relationship between RT-PCR amplification (cycle number) and intensity of the RT-PCR band (AU.mm) for VEGF-A_{164} and G3PDH. The slopes of the regression lines differed, indicating that PCR amplification rate of VEGF-A_{164} differed from that of G3PDH.

A



B

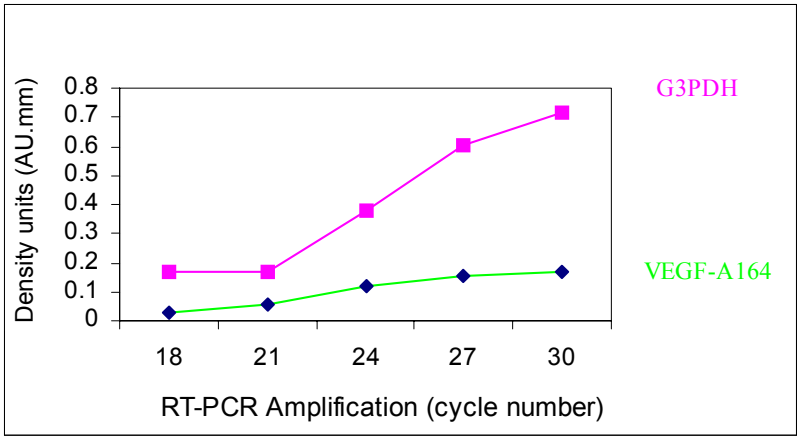
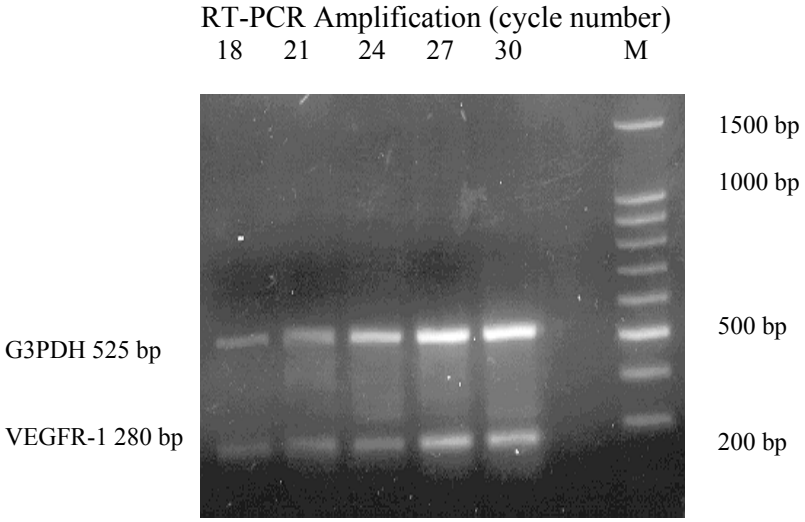


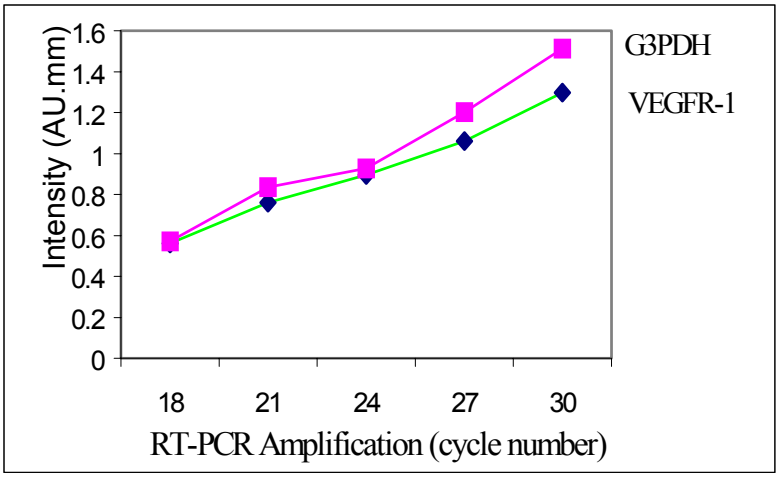
Figure 3.17 Kinetics of RT-PCR for VEGFR-1 and G3PDH. A) Electrophoresis gel showing the RT-PCR products from G3PDH and VEGFR-1 primers at 18, 21, 24, 27, and 30 cycles. A 100 bp DNA marker (M) is shown. The amount of PCR product increased with increasing cycle number. B) Relationship between RT-PCR amplification (cycle number) and intensity of the RT-PCR band (AU.mm) for

VEGFR-1 and G3PDH. There were no major differences between the slopes of the regression lines, indicating that PCR amplification rate of VEGFR-1 was comparable to that of G3PDH.

A



B



3.3.5 NORTHERN BLOT ANALYSIS

3.3.5.1 VEGF-A

VEGF-A expression was assessed, by Northern blot analysis, using a cDNA probe comprising nucleotides 342 to 577 as described in the Methods. As discussed, this probe corresponds exactly to the sequence of VEGF-A_{164} but exhibits variable sequence homology with the remaining isoforms 205, 188, 144 and 120. Hybridisations were initially performed using total RNA and digoxigenin labelled cDNA probes. Bands in the region of 3.5 to 4.7 kb and 1.4 to 2.4 kb were located in uteroplacental samples hybridized to a non-radioactive labelling (Figure 3.18) and a radioactive labelling (Figure 3.19). No major differences were seen in total RNA expression of VEGF-A over gestation (Figures 3.18 and 3.19) or between uterus and placenta (Figure 3.20 and 3.21). These bands were comparable to previously published results. However, it was observed that these bands were also very close in molecular weight to the ribosomal RNA species. Due to our initial uncertainty regarding the specficity of this probe we chose to carry out all further studies using mRNA.

Using mRNA, Northern blot analysis of VEGF-A expression in uteroplacental tissues detected 4 bands at approximately 4.5 kb, 3.5 kb, 2.4 kb and 1.4 kb (Figure 3.22). Due to the quality of the scanner used to import the autoradiograph, the lower two bands are not clearly seen in this figure. The bands correspond to published VEGF-A mRNA transcript sizes. Northern blot analysis was also conducted using a riboprobe, made from primer *set 1*, (nucleotides from 342 to 577) which was homologous to VEGF-A_{164} mRNA. This ribobrobe was also found to detect 4 bands of approximately the following sizes 4.5 kb, 3.5 kb, 2.4 kb and 1.4 kb. These results are shown in Figure 3.23 and discussed further in section 3.3.7.

3.3.5.2 VEGFR-1

VEGFR-1 expression was assessed, by Northern blot analysis, using a cDNA probe comprising nucleotides 1771 to 2050 as described in the Methods. Northern Blot analysis of VEGFR-1 expression in uteroplacental mRNA detected very faint bands in the region of 5.5 to 7.5 kb, less faint bands in the region of 3.5 to 4.5 kb with more intense bands around 2.0 kb and 1.0 kb (Figure 3.24). This blot shows VEGFR-1

mRNA expression in normotensive (WKY) rats and hypertensive (SHR) rats. However, for technical reasons the blot was not hybridised to G3PDH. Therefore, it was not possible to conclude whether there were differences in VEGFR-1 mRNA expression between WKY and SHR rats. Due to the inconclusive nature of the blot, these results are presented in this chapter rather than Chapter 4.

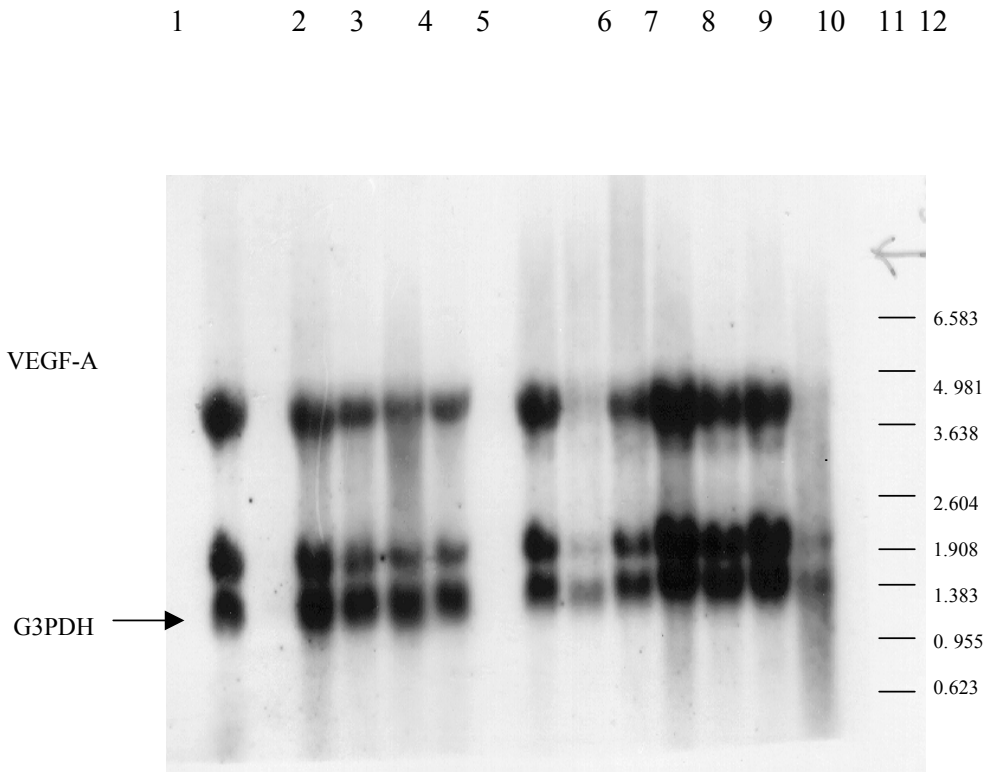
3.3.6 VEGF-A IMMUNOHISTOCHEMISTRY

Using immunohistochemistry, VEGF-A protein was localised in the glands and stoma of the maternal decidua as shown in Figures 3.25 and 3.26. A negative control slide, which was not incubated with VEGF-A antibody shows no specific binding (Figure 3.27)

3.3.7 VEGF-A *IN SITU* HYBRIDISATION

During the later stages of this project, both sense and antisense riboprobes were developed for use in *insitu* hybridisation. Two sets were prepared by direct ligation of a T7 adaptor promote to a RT-PCR fragment, as described in the Methods, section 2.13.1. A riboprobe, which corresponded to nucleotides 342 to 577 of the published VEGF-A sequence, was used for Northern blot analysis (Figure 3.23). The hybridisation was carried out at 68°C overnight with Digoxigenin labelling. The antisense riboprobe produced 4 bands. However, the sense riboprobe hybridised with an unknown mRNA (Figure 3.23) under the same experimental conditions. Consequently, a second riboprobe complementary to nucleotides 88-282 provided by Dr. Sarah Fraser was used for *in situ* hybridisation. Our efforts to establish the technique of *insitu* hybridisation, to localise VEGF-A mRNA expression in uteroplacental tissues, were unsuccessful and require further optimisation.

Figure 3.18. Northern blot analysis of VEGF-A and G3PDH expression in uteroplacental tissues over a range of gestational ages. Total RNA was hybridised to a DIG labelled VEGF-A_{164} cDNA probe. Lanes 1-5 (day 11, uteroplacentae, replicates from rat 1), lane 6 (day 11, uteroplacentae, rat 2), lane 7 (day 15, uterus, rat 1), lane 8 (day 15, uterus, rat 2), lane 9 (day 17 uterus, rat 1), lane 10 (day 17, uterus, rat 2), lane 11 (day 19, uterus, rat 1), and lane 12 (day 19, uterus, rat 2). RNA marker (M) was used to show molecular weights.



Figures 3.19. Northern blot analysis of VEGF-A and G3PDH expression in uteroplacental tissues over a range of gestational ages. Total RNA was hybridised to α-^{32}P dCTP labelled VEGF-A_{164} cDNA probe. Lane 1 (spleen, negative control), lane 2 (day 5, uteroplacentae), lane 3 (day 7, uteroplacentae), lane 4 (day 9, uteroplacentae), lane 5 (day 11, uterus), lane 6 (day 13, uterus), lane 7 (day 15, uterus), lane 8 (day 17, uterus), lane 9 (day 19, uterus), lane 10 (day 21, uterus). RNA marker (M) was used to show molecular weights.

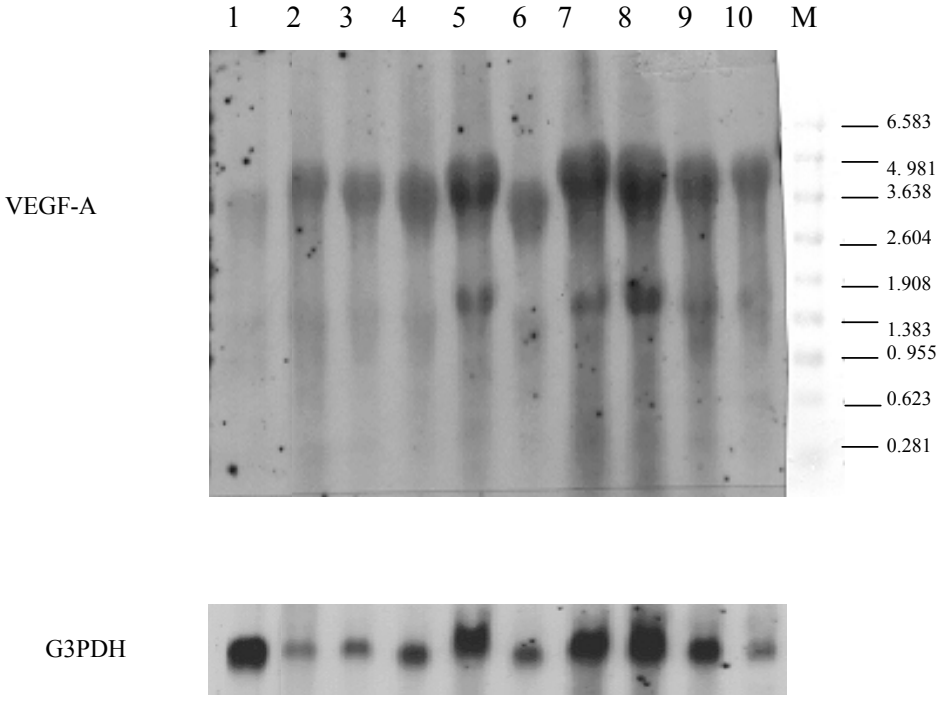


Figure 3.20. Northern blot analysis of VEGF-A and G3PDH expression in uterine tissues from day 13 to day 21 of gestation. A) Total RNA was hybridised to a DIG labelled cDNA probe, lane 1 (day 13), lane 2 (day 15), lane 3 (day 17), lane 4 (day 19) and lane 5 (day 21). B) Corresponding formaldehyde gel. RNA marker (M) is shown.

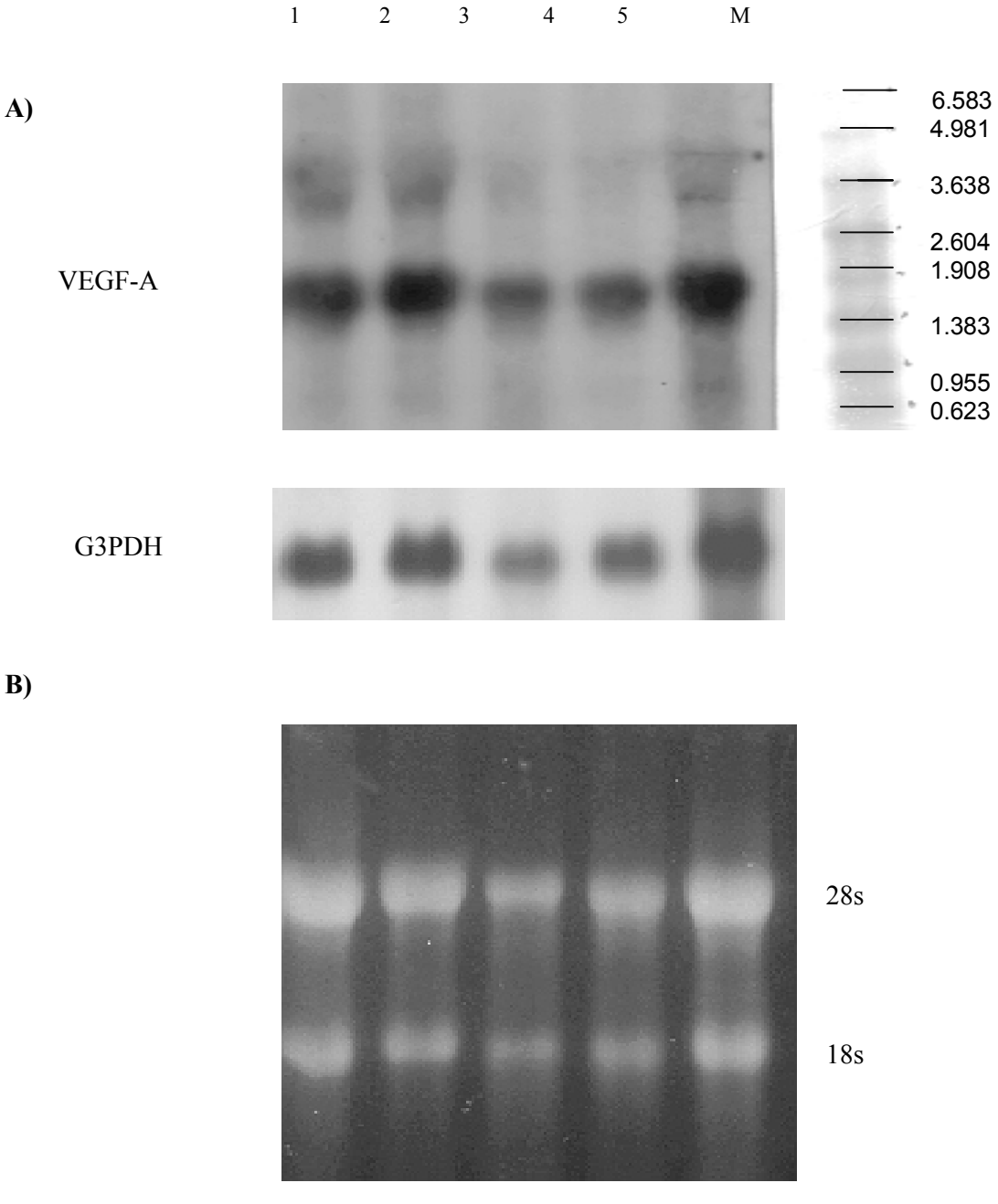


Figure 3.21. Northern blot analysis of VEGF-A and G3PDH in placental tissues from day 13 to day 21 of gestation. Total RNA was hybridised to DIG labelled cDNA probe. Samples from left to right: A) lane 1 (day 13), lane 2 (day 15), lane 3 (day 17), lane 4 (day 19) and lane 5 (day 21). RNA marker (M) is shown. B) shows the formaldehyde gel for this Northern blot.

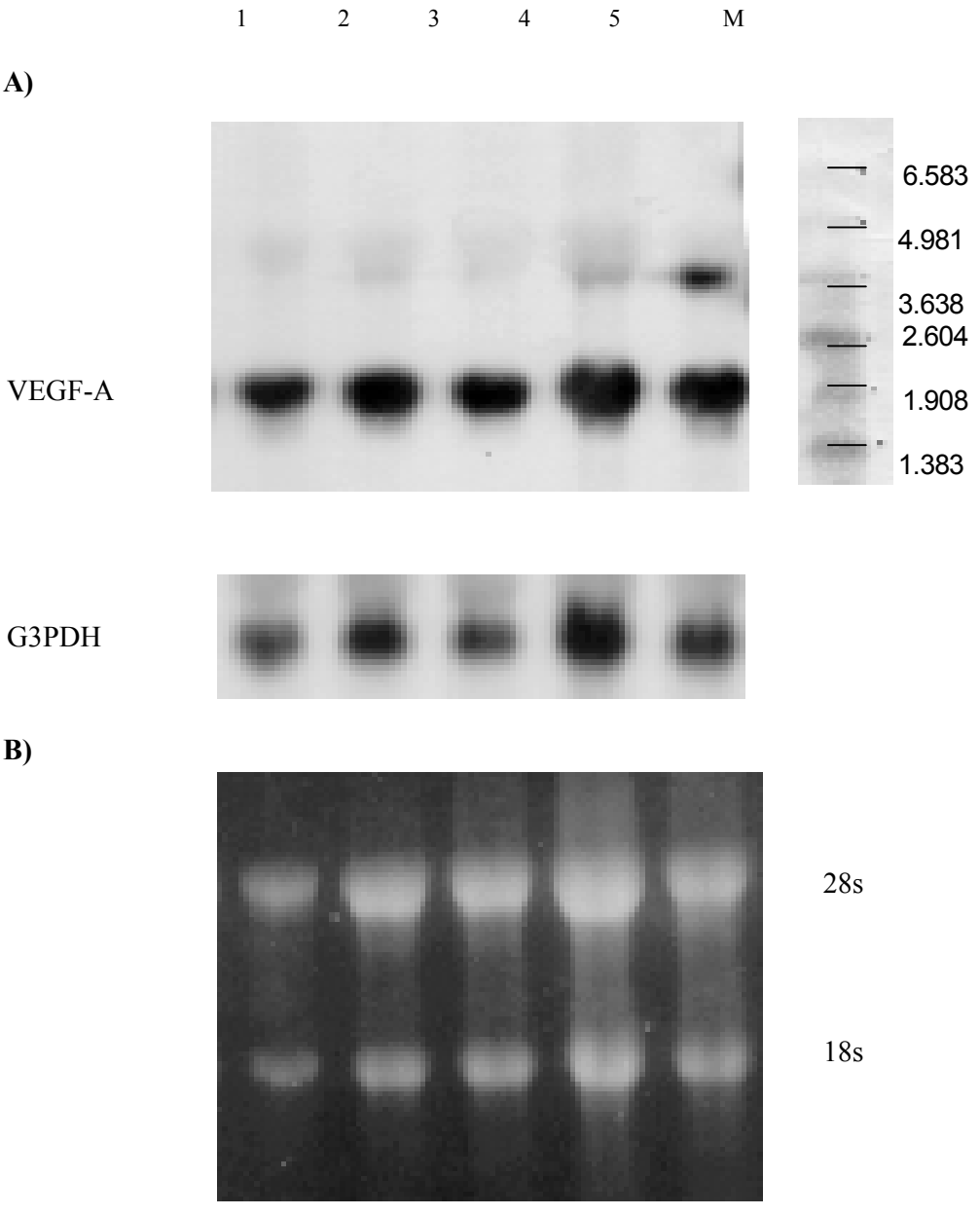
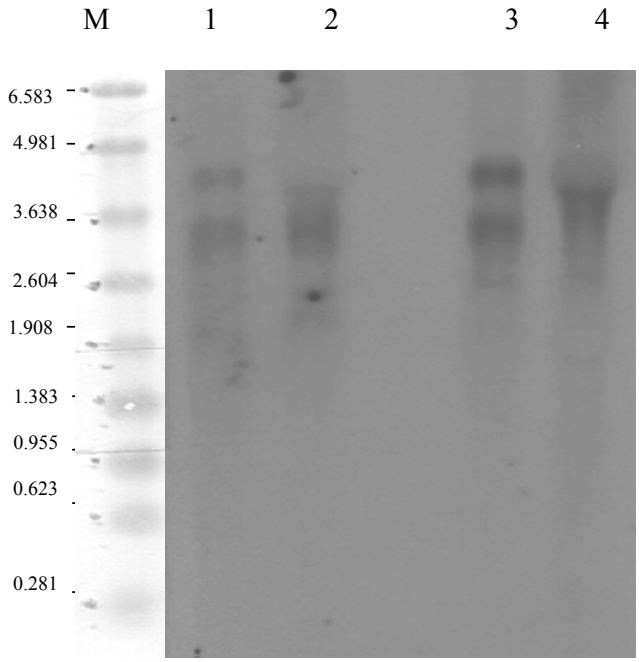


Figure 3.22. Northern blot analysis of VEGF-A expression in uterine and placental tissues at day 19 of gestation. Total RNA and mRNA were hybridised to a DIG labelled cDNA probe. Four bands were detected in the mRNA samples, lane 1 (mRNA, uterus), lane 2 (total RNA, uterus), lane 3 (mRNA, placentae), lane 4 (total RNA, placentae). RNA marker (M) is shown.



3.4.0 DISCUSSION

This project forms part of a larger program investigating the role of growth factors in the development of the placenta, fetal membranes and fetal lungs. We have focused on VEGF-A as it is the most potent angiogenic factor found to date. Although many laboratories throughout the world are working on VEGF-A, our laboratory was new to this growth factor. Consequently, the first aim of this project was to establish techniques, in our laboratory, for measuring expression and localization of VEGF-A and its receptors in rat uteroplacental tissues at early, mid and late gestation. We wished to establish the use of RT-PCR, Northern blot analysis, immunohistochemistry and *insitu* hybridization, to measure expression and localization of VEGF-A, VEGFR-1 and VEGFR-2.

We have successfully established the use of RT-PCR to measure expression of VEGF-A, VEGFR-1 and VEGFR-2; Northern blot analysis to measure expression of VEGF-A; and immunohistochemistry to localize VEGF-A protein in rat uteroplacental tissues. For a variety of reasons, primarily technical and a lack of time, we were unable to optimise the measurement of VEGFR-1 and VEGFR-2 expression by Northern blot analysis, to localize VEGFR-1 and VEGFR-2 proteins using immunohistochemistry, nor to optimize our *insitu* hybridization technique.

In this study, we have shown that VEGF-A and its receptors were expressed in uteroplacetal tissues of the normal rat. The level of VEGF-A and VEGFR-1 expression was not significantly different between early, mid and late gestation.

VEGF-A is one of the potent angiogenic factors known to date. It plays an important role in the induction of vascular growth in the decidua, placentae, vascular membranes, and fetal development. Our finding that expression levels of VEGF-A and VEGFR-1 were comparable throughout gestation suggests that other angiogenic factors may be more highly expressed in early gestation to account for the more rapid vasculogenesis and angiogenesis which occurs at the time of implantation. Some of these factors may include hormones, such as progesterone, human chorionic

gonadotropin, or growth factors, such as placental growth factor (Evain-Brion *et al,* 1994; Hauser, 1993).

3.4.1 Extraction of Total and mRNA

We have successfully extracted total RNA, using both the Guanidinium thiocyanate and Trizol techniques, and mRNA using the Poly A tract mRNA Promega Isolation System Kit. We have also shown that our DNase treatment removed any possible DNA contamination.

3.4.2 RT-PCR

Using primer set 1, we detected 3 bands and, using primer *set 2*, we detected 4 bands which corresponded to the theoretical sizes VEGF-A isoforms 188, 164, 145 and 120. We were able to sequence 2 of the bands, using primer *set 1* products, and they corresponded to VEGF-A_{188} (308 bp) and VEGF-A_{164} (236 bp). We were able to sequence another band, using primer *set 2* products, and it corresponded to VEGF-A_{120} (364 bp). The partial sequence alignments had high homology to published Gene Bank Acc No: AF0626 (94% homology) M32167; (99.6% homology) S38100 (99.2% homology) and agreed with published studies (Connet *et* al, 1990, Houck *et al*, 1991, Berier *et al*, 1992). Although numerous efforts were made, we were unable to sequence the ~ 104 bp band, using primer *set 1*, or the ~ 436 bp band, using primer *set 2*. This may have been due to the small amount of RT-PCR product available or to technical errors encounter during the sequencing procedure at Monash University.

We have detected VEGF-A in normal uteroplacental tissues at day 7, 11 and 21 by RT-PCR. VEGF-A_{164}, appeared to be the predominant isoform expressed in uteroplacental and uterine tissues. Although our findings were not always consistent, both VEGF-A_{164} and VEGF-A_{121} appeared to be the predominant isoforms in placental tissues. In general these findings support those of other workers who showed that VEGF-A_{164} and VEGF-A_{121} appeared to be the predominant isoforms in a range of reproductive tissues (Srivastava *et al*, 1998; Athanassiades *et al*, 1998; Vuorela *et al*, 1997; Wheeler *et al*, 1995; Anthony *et al*, 1994; Houck *et al*, 1991; Cullinan-Bove *et al,* 1992; Leung *et al,* 1989; Keck *et al,* 1989).

Srivastava *et al,* 1998, detected only one major PCR product, corresponding to VEGF-A_{164}, in antimesometrial and mesometrial decidual tissues of the pseudopregnant rat. Their tissues were from Sprague Dawley rats at 8 to 14 days of pseudopregnancy. Similar to our study, they used guanidinium thiocyanate to extract total RNA. They used 1-5 μg total RNA as the template and reverse transcribed at 42°C in the presence of random hexamer primers (100 pmol) and Moloney murine leukema virus reverse transcriptase in a total 20 μl reaction mixture. Their annealing temperature was 60 °C and they amplified for 30 cycles. They used rat ribosomal protein L19 as an internal standard, whereas we used G3PDH. Finally, they analysed their RT-PCR products using 8% polyacrylamide non-denaturing gels, autoradiography and Image Quant version software; whereas we used 2% agarose gel and a very old fashioned densitomiter. Although there were some differences in our protocol compared with that of Srivastava *et al,* (1998), both studies found that VEGF-A_{164}, was the predominant isoform expressed in uterine tissues. It is possible that $VEGF_{164}$ is the most highly expressed isoform because it has the restoring ability to bind VEGFR-2 cell-surface and heparan-surface proteoglycans (Weksberg *et al,* 1996; Siemeister *et al*, 1996).

Cullinan-Bove *et al*, (1992) reported that the predominate isoforms expressed in the rat uterus, at term, were VEGF-A_{164} and VEGF-A_{120}. They measured expression levels in control rats and rats administered Estriol (E_3). Using RT-PCR, they detected three bands (VEGF-$A_{188,}$ VEGF-A_{164} and VEGF-A_{120}) in the uterus, with the strongest signals corresponding to VEGF-A_{164} and VEGF-A_{120}. VEGF-A_{164} and VEGF-A_{120} are secreted in freely dissolvable form, while VEGF-A_{188} is associated with the cell or extracellular matrix (Ferrara *et al*, 1992; Houch *et al*, 1991). Although physiological roles of the various forms have not been fully defined, VEGF-A_{164} and VEGF-A_{120} have been shown to increase vascular permeability and to be mitogenic for endothelial cells.

We examined VEGF-A expression, in uteroplacental tissues, at early (day 5), mid (day 11) and late (day 21) gestation and compared the RT-PCR product with those of

G3PDH. At 5 and 11 days of gestation, it was technically too difficult to divide the uteroplacental tissues into uterus and placenta, and as a consequence expression levels in uterus and in placenta could only be measured at 21 days. The level of VEGF-A_{164} expression was not significantly different between these three gestational ages. However, we observed that there was a tendency for expression levels to be slightly lower in the day 5 rat than those in the day 11 and day 21 rats. We used semi-quantification analysis to investigate these observations further and the results showed that the ratio of VEGF-A_{164} expression to G3PDH expression was lower in the day 5 rat than in the day 11 rat and the day 21 rat. Due to technical difficulties, we were unable to repeat these experiments in more rats. To investigate whether we could use semi-quantitative analysis on our results with any degree of certainty, we examined the kinetics of VEGF-A and G3PDH RT-PCR. Our results, which showed that the amplification rate of VEGF-A_{164} and G3PDH differed considerably, were not encouraging. Therefore, we concluded that the error in our RT-PCR technique prevented us from quantitating the results with any degree of certainty. Consequently, the small differences in expression levels of VEGF-A with gestational age that we observed were within the experimental error of our technique.

We examined VEGFR-1 expression in uteroplacental tissues, at early (day 5), mid (day 11) and late (day 21) gestation and compared the RT-PCR products with those of G3PDH. There were no major differences observed in the ratio of VEGFR-1 expression to G3PDH expression between the day 5 rat, the day 11 rat and the day 21 rat. We have shown that uteroplacental tissues at day 7 and 11 and uterine tissue at day 19 expressed VEGFR-2. As we did not measure G3PDH expression in the same samples we were unable to comment on the relative distribution of VEGFR-2 throughout gestation.

Our VEGF-A, VEGFR-1 and VEGFR-2 results, were in partial agreement with previous workers. In 1998, Shivastaua *et al,* found that VEGF-A mRNA expression was similar in both antimesometrial and mesometrial decidual tissues and remained constant throughout pseudo-pregnancy. Das *et al,* (1997) examined expression and localisation of VEGF-A and its receptors in the uteroplacental tissues of the rabbit

from oestrus (day 0) to implantation (day 8) and found VEGF-A expression was highest at days 0 and 6-8, with the syncytiotrophoblast being predominate localisation site.

3.4.3 Northern Blot Analysis

We have successfully established the use of Northern blot analysis to measure expression of VEGF-A in rat uteroplacental tissues. For a variety of reasons, primarily technical and a lack of time, we were unable to optimise the measurement of VEGFR-1 and VEGFR-2 expression by Northern blot analysis.

We have shown, by Northern blot that uteroplacental tissues express VEGF-A. Using mRNA, we detected four bands and their molecular weights were relatively similar to those published by other workers (Vuorela *et al,* 1997, Pierce *et al*, 1995, Charkraborty *et al*, 1995, Monacci *et al*, 1993, Claffey *et al*, 1992, Tiscger *et al*, 1991, Atsushi *et al*, 1994). We also hybridised total RNA to our VEGF-A riboprobe and obtained relatively similar banding pattern to that obtained with our cDNA probe.

Northern blot analysis of VEGF-A expression over a range of gestational ages, confirmed our RT-PCR finding that VEGF-A expression was not significantly different at early, mid and late gestation.

Despite several attempts, we were unable to optimise the Northern blots for VEGFR-1 and VEGFR-2. Previous workers have found bands around 7.5, 3-5 and 2.5 kb for VEGFR-1 and 7.5 kb and 5.5 kb for VEGFR-2 (Millauer *et al*, 1993, Yamane *et al*, 1994, Chakraborty *et al*, 1995, Barleon *et al*, 1996, Das *et al,* 1997). However, until we develop specific probes and optimise our procedures, we are unable to confirm previous work.

Due to technical problems and a lack time, we were unable to conduct RT-PCR and Northern blot analysis on more than one rat at each gestational age. Our results would have been more conclusive, if we were able to increase the number of rats used.

3.4.4 Immunohistochemistry

We have successfully localised VEGF-A protein in maternal decidua as revealed by immunohistochemistry. Glandular epithelial cells within the maternal decidua, and stroma cells were positively labelled by anti-mouse VEGF-A monoclonal antibodies. These findings agree with the work of Sharkey *et al*, (1993). They reported that VEGF-A protein was present in both stroma and glandular cells during the proliferate phase of the cycle, with greatly increased expression in the glands of secretory endometrium. Using immunohistochemistry, Ferrara *et al*, (1997) showed that VEGF-A protein was present in epithelial cells, but not vascular endothelial cells, in human uterus at 22 weeks of gestation (Shifren *et al*, 1994). Due to technical problems, photographs were only taken at 40 times magnification. Our results would have been more satisfactory at 100 times magnification.

CHAPTER 4: EXPRESSION OF VEGF-A AND VEGFR-1 IN UTEROPLACENTAL TISSUES FROM NORMOTENSIVE (WKY) AND SPONTANEOUSLY HYPERTENSIVE (SHR) RATS.

4.1.0 INTRODUCTION

Perhaps some of the most interesting, but conflicting, clinical findings to date are those relating to the association between VEGF-A and pre-eclampsia. Serum concentrations of VEGF-A have been shown to be higher (Baker *et al*, 1995), or on the other hand lower (Lyall *et al*, 1997), in patients with pre-eclampsia compared to values from normotensive women. Additionally, mRNA expression levels of VEGF-A in the placenta have been shown to be reduced, below control values, in women with pre-eclampsia (Cooper *et al*, 1996). The second aim of this thesis was to examine, using RT-PCR and Northern blot analysis, the expression of VEGF-A, VEGFR-1 and VEGFR-2 in the uteroplacental of the normotensive (WKY) and the spontaneously hypertensive rat (SHR) at 7, 11 and 19 days of gestation.

4.2.0 METHODS

The methods are outlined in Chapter 2.

4.3.0 RESULTS

4.3.1 Animals

We found that pregnancy rate was lower in SHR (55%) than in WKY (75%) rats. Litter size was found to be comparable in SHR (7.2 ± 2.0 fetuses from 6 litters) and WKY (9.0 ± 1.7 fetuses from 6 litters) rats (Table 4.1 and Figure 4.1).

	day 7	day 11	day 19
WKY	10.50 ± 1.50	6.50 ± 5.50	10.0 ± 0.00
SHR	10.50 ± 0.50	1.50 ± 0.50	9.50 ± 3.50

Table 4.1. Litter size for WKY and SHR rats at 7, 11 and 19 days of gestation.

4.3.2 VEGF-A RT-PCR

Using set 1 primers, RT-PCR analysis of mRNA confirmed the presence of VEGF-A in uteroplacental tissues from both WKY and SHR at 7, 11, and 19 days of gestation as shown in Figure 4.2. The 236 bp band was sequenced and it corresponded to VEGF-A $_{164}$. Three bands (308 bp, 236 bp and 104 bp) were detected in placental tissues at 19 days.

Figure 4.1 Litter size for WKY and SHR rats at day 7, 11 and 19 of gestation.

There was no major difference observed in the ratio of VEGF-A_{164} to G3PDH between WKY and SHR rats. However, this ratio was observed to be slightly lower in SHR rats than in WKY rats.

Although the kinetics of our RT-PCR limited the reliability of semi-quantitative analysis of VEGF-A expression, Table 4.2 shows that there was no significant difference (unpaired t test) in the ratio of VEGF-A_{164} to G3PDH between SHR (n = 3, i.e day 7, 11 and 19 days of gestation) and WKY (n = 3) rats (Table 4.2).

Tissue	**U+P**	**U+P**	**U+P**	**U+P**	**Uterus**	**Uterus**	**Placenta**	**Placenta**
Gestation (days)	**Day 7**	**Day 7**	**Day 11**	**Day 11**	**Day 19**	**Day 19**	**Day 19**	**Day 19**
Rat	**SHR**	**WKY**	**SHR**	**WKY**	**SHR**	**WKY**	**SHR**	**WKY**
VEGF-A_{164}	1.08324	1.37064	0.98064	1.09344	0.74524	1.12924	1.60236	0.85036
G3PDH	1.75644	2.54884	1.79112	1.78076	2.27832	2.55376	2.49628	1.71796
Ratio	0.61	0.76	0.55	0.61	0.32	0.44	0.64	0.49

Table 4.2 Ratio of VEGF-A_{164} to G3PDH for one WKY and one SHR rat at early (7 days), mid (11 days) and late (19 days) gestation; uteroplacental (U+P), uterine and placental tissues.

Using the primer *set 2*, RT-PCR analysis of mRNA confirmed the presence of VEGF-A in uteroplacental tissues from both control and spontaneously hypertensive rats at 7, 11, and 19 days of gestation as shown in Figure 4.3. Four bands were detected. The 496 bp and 364 bp bands were sequenced and they corresponded to VEGF-A_{164} and VEGF-A_{120} (Figures 3.8 and 3.9). There was no major difference observed in the ratio of VEGF-A to G3PDH between WKY and SHR rats.

Figure 4.2 RT-PCR analysis of VEGF-A (primer *set 1*) and G3PDH expression in uteroplacental tissues from WKY and SHR rats at 7, 11 and 19 days of gestation. The 236 bp band was sequenced and it corresponded to VEGF-A_{164}. Three bands were detected in placental tissue at 19 days. There were no major differences, in the ratio of VEGF-A_{164} to G3PDH, over gestation or between WKY and hypertensive SHR rats.

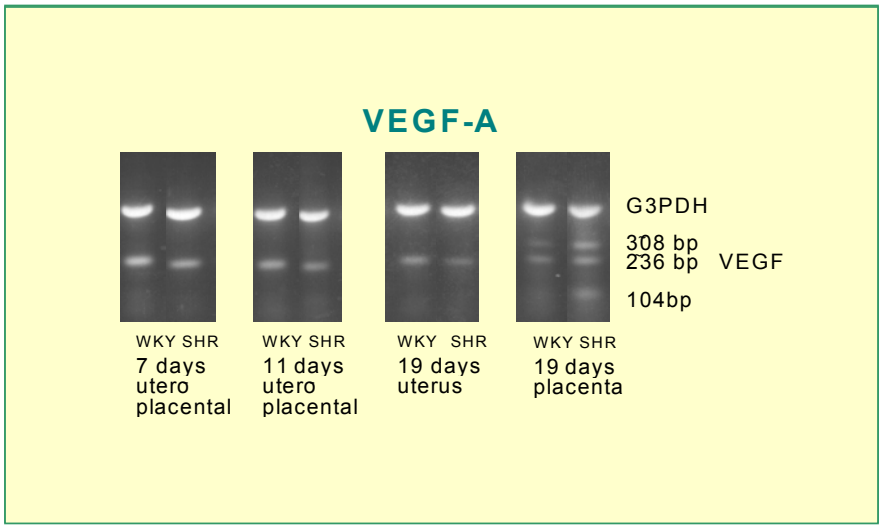
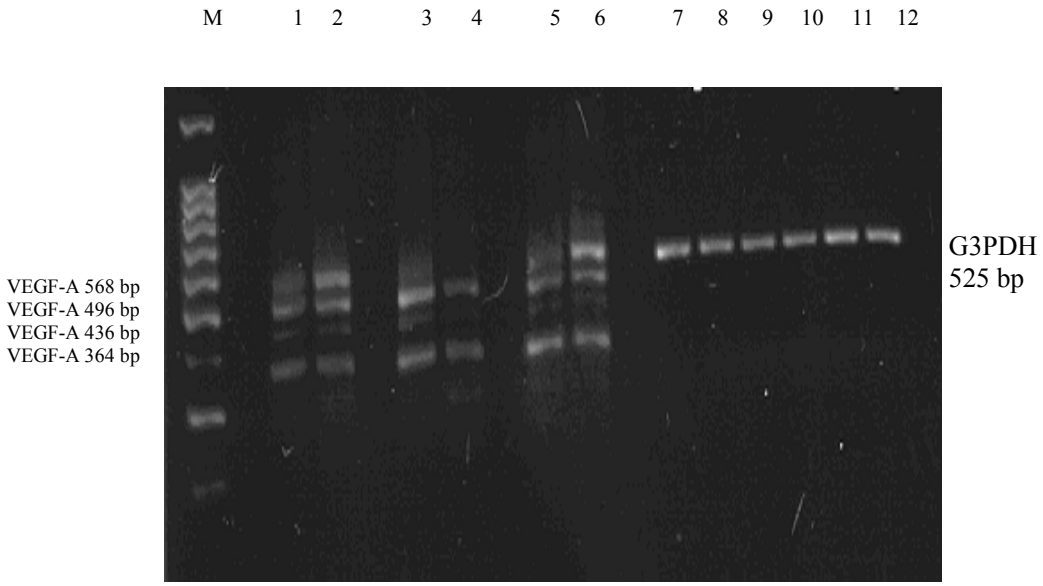


Figure 4.3. RT-PCR analysis of VEGF-A (primer *set 2*) and G3PDH expression in uteroplacental tissues from WKY and SHR rats at 11 and 19 days of gestation. Four bands were detected in each tissue sample. The 496 bp and 364 bp bands were sequenced and they corresponded to VEGF-A_{164} and VEGF-A_{120}. Lane 1 (day 11, WKY), lane 2 (day 11, SHR), lane 3 (day 19, uterus, WKY), lane 4 (day 19, uterus, SHR), lane 5 (day 19, placentae, WKY), lane 6 (day 19, placentae, SHR). Lanes 7 to 12 show the corresponding G3PDH expression for each of the previous samples, respectively. DNA marker (M) was used to show molecular weights.



4.4.0 DISCUSSION

In the present study, we have shown that VEGF-A, VEGFR-1 and VEGFR-2 were expressed in both the normotensive and the spontaneously hypertensive rat, throughout gestation. There were no major differences in the mRNA levels of VEGF-A, VEGFR-1 and VEGFR-2 between the normotensive and the spontaneously hypertensive rat. These findings suggest that VEGF-A is not a major factor involved in the aetiology of essential hypertension during pregnancy.

4.4.1 SHR as a Model for Pre-eclampsia

Most animal studies aimed at mimicking pre-eclampsia have used either the spontaneously hypertensive rat or administration of a hypertensive agent to the pregnant animal. The SHR has been used extensively as an animal model for essential hypertension because its haemodynamic and endocrine characteristics parallel those observed in the hypertensive human (Tipton, 1984) and are significantly different from the normal Wistar Kyoto rat. Blood pressure has been shown to be higher in the non-pregnant and the pregnant SHR compared with that in the WKY rat. Although it would have been prefereable, in the present study, we were unable to measure blood pressure in our rats, as we did not have a tail cuff manometer.

Light microscopy studies (Scott *et al,* 1985) have shown that although there were no major lesions, typical in human pre-eclamptic placentas, in placentas from SHR, extensive haemorrhage at the lateral edges of decidua basalis and narrowing and spasms in the central blood vessels of the spiral arteries was observed in SHR. Due to a lack of time, we did not investigate the histological structure of the uterus and placenta in our animals. A number of workers have shown that fetal body weight, at term, was 10-20% lower in SHR compared with WKY rats (Lewis *et al*, 1997; Johnston, 1995; Erkadius *et al*, 1995a). However, other workers have shown that differences in fetal body weight can be accounted for by differences in placental weight and differences in the length of gestation between SHR and WKY (Lewis *et al*, 1998; Lindheimer *et al*, 1983). We did not measure fetal body weight in our animals because we were endeavouring to freeze the tissues as rapidly as possible.

In the present study we found that pregnancy rate was lower in SHR (55%) compared with WKY (75%) rats. These results, which confirm earlier work (Pinilla *et al*, 1992), suggest that changes in fertilisation and/or implantation in SHR may account for the reduced pregnancy rate. We found that litter size was comparable in SHR and WKY rats. This finding confirms work by Johnston, (1995), but disagrees with the findings of Pinilla *et al*, (1992).

As we can see, there is disagreement in the literature regarding the appropriateness of the SHR as a model for pre-eclampsia. However, based on the strong evidence that SHR exhibit hypertension prior to and during pregnancy, it is generally agreed that the SHR is a good model for essential hypertension during pregnancy. It is certainly less well established that the SHR is a good animal model for pregnancy induced hypertension which is the most common clinical precursor to pre-eclampsia.

This is the first study to use an animal model to investigate the relationship between hypertension during pregnancy and expression levels of VEGF-A, VEGFR-1 and VEGFR-2. Our results would have been stronger if we had used a larger sample size. Furthermore, as the SHR models essential hypertension rather than pregnancy induced hypertension, our results would be strengthened by eliciting a stress induced rise in blood pressure within our SHR group. Possible ways that blood pressure could be further elevated in our SHR, would be by shaking the animal or by administering angiotensin. This could only be done with ethical approval.

4.4.2 VEGF-A, VEGFR-1 and VEGFR-2 in SHR and WKY Rats.

Using RT-PCR, we found no major differences in the mRNA levels of VEGF-A, between SHR and WKY rats. We observed a tendency for VEGF-A expression to be slightly lower in uteroplacental and uterine tissues from SHR compared with values in the WKY rats. However, due to a limited number of animals and our levels of experimental error, semi-quantitative analysis could not be conducted with any degree of certainty. Results from our Northern blot analysis were consistent with the RT-PCR result, that is, the level of VEGF-A mRNA was not significantly different

between SHR and WKY rats. These findings, which suggest that VEGF-A is not a major factor involved in the aetiology of pre-eclampsia, are in contrast to earlier work.

In a cross-sectional study, Baker *et al* (1995), measured VEGF-A levels in maternal serum from pre-eclampsic women (n = 27), nonproteinuric pregnancy induced hypertensive women (n = 15) and control normotensive pregnant women (n = 36). The average age of the women in the groups was 28 weeks for the pre-eclampsic women, 29 weeks for the pregnancy induced hypertensive women, and 26 weeks for control pregnant women. At term, these women had average blood pressures of 153/92 mmHg (pre-eclampsia), 139/89 mmHg (pregnancy induced hypertension) and 120/75 mmHg (normal pregnancy). Barker *et al* (1995) used an immunofluorometric assay technique to detect levels of VEGF-A in the blood. They showed that, VEGF-A levels, in maternal serum, were above the detection level of the assay in 7 of the 27 pre-eclampsic women, in 2 of the 15 pregnancy induced hypertension women and only 1 of the 36 normal pregnant women. They also used longitudinal data, and showed that VEGF-A levels rose during pregnancy and then fell postpartum in 3 to 4 of the 12 women who developed pre-eclampsia. They also found levels were consistently higher, that is prior to, during and after pregnancy, in one woman with pregnancy induced hypertension and 2 control women. Furthermore, when Barker *et al,* (1995) measured VEGF-A levels in umbilical cord blood they found they were not significantly different between newborns from normal and from pre-eclampsic women. Although the authors concluded that VEGF-A has a pathogenic role in the endothelial cell activation that occurs with pre-eclampsia, their results were not entirely consistent.

In contrast to Baker *et al,* (1996), Lyall *et al,* (1997) has shown that VEGF-A levels in human peripheral venous blood were significantly lower, not higher, in women with pre-eclampsia (2.34 pg/ml) than in control pregnant women (12.89 pg/ml). They concluded that discrepancies in the findings of the two papers may be due to cross-reactivity between VEGF-A antibodies and PDGF or PlGF antibodies. Lyall *et al,* (1997) also conclude that VEGF-A may be an important factor in the aetiology of pre-

eclampsia, however, the mechanisms of its actions and thus is concentration in maternal blood vary considerably to those forwarded by Baker *et al* (1995).

Using an RNase protection assay, Cooper *et al*, (1996) measured VEGF-A and VEGFR-1 in placentas from normal pregnant (n = 20) and pre-eclampsic (n = 23) women. They showed that, unlike VEGFR-1, VEGF-A levels were lower (10% at 25 weeks, 40% at 35 weeks) in placentas from pre-eclampsic women compared with those from control women. These findings were, however, inconsistent with their results obtained from *insitu* hybridisation and immunohistochemical studies where they found no detectable difference in the levels of VEGF-A mRNA and immunoreactivity between normal and pre-eclamptic placentas. Although the authors state that the RNase protection assay is the most sensitive of the 3 techniques, the inconsistencies in there findings perhaps do not fully support their conclusion that VEGF-A may be an important factor in the aetiology of pre-eclampsia.

To date, the relationship between VEGF-A and pre-eclampsia has only been investigated by Baker *et al* (1995), Cooper *et al*, (1996) and Lyall *et al,* (1997) and their results are conflicting and inconsistent suggesting either an increase or a decrease in VEGF-A expression with pre-eclampsia. Our findings of no difference in VEGF-A expression levels between SHR and WKY do not agree with any of these earlier studies. There are some differences in the experimental design and methodology between our work and the other three reports. They all used samples taken from pregnant women, whereas we used rats. They used larger sample sizes than we did. They predominantly were interested in pregnancy induced hypertension leading to pre-eclampsia, whereas we were interested in essential hypertension leading to pre-eclampsia. Two of these previous studies inferred the importance of VEGF-A, from concentrations in blood rather than measuring the expression level directly in uteroplacental tissues. Although, both Cooper et al (1996) and the present study measured VEGF-A mRNA expression levels in the placenta the techniques used varied. They used RNase protection assay, *insitu* hybridisation and immunohistochemistry and obtained inconsistent results. We used RT-PCR and

Northern blot analysis, and obtained a consistent finding of no difference between SHR and WKY rats.

Using RT-PCR, we found no major differences in the mRNA levels of VEGFR-1, between SHR and WKY rats. These findings agree with earlier work by Cooper *et al*, (1996) who found no difference in the expression level of VEGFR-1 in placentas from pre-eclamptic women and control women. Our study is the first to investigate the relationship between VEGFR-2 and pre-eclampsia. We found no major differences in the mRNA levels of VEGFR-2, between SHR and WKY rats.

4.4.3 Relationship between VEGF-A and Pre-eclampsia

Pre-eclampsia, first described more than 100 years ago, may result in maternal and newborn death. The relationship between pre-eclampsia and VEGF–A is still undefined. VEGF-A was first identified, in human tumour cells, by Senger *et al,* (1983). This work was followed by Gospodarowicz *et al* (1989) who measured VEGF-A in pituitary-derived follicle stellate cells. VEGF-A was then identified in the rat (Conn *et al*, 1990; Hock *et al*, 1991). In 1993, Charmock-Jones *et al*, identified VEGF-A in the endometrium with Jackson *et al*, (1994), identifing it in the placentae, particularly in cytotrophoblasts in the first trimester and in syncytiotrophoblasts throughout gestation.

Since 1983, more and more work has been published examining the role of VEGF-A and its receptors. However, its physiological significance during pregnancy, particularly in pathological states such as pre-eclampsia, remains to be fully understood. In pre-eclampsia, endothelial cell activities change, such that anticoagulants and vasodepressor substances are reduced, there are disturbances in endothelial tight junctional complexes, fluid and proteins leak from vessels and there is a loss in transport function. Injured endothelial cells produce mitogens, such as plate-derived growth factor, which is a vasoconstrictor. Additionally, injured endothelial cells increase membrane permeability, clot formation, vasospasm and blood vessel remodelling, resulting in physiological disturbances. It is thought that injured endothelial cells indirectly induced VEGF-A (Robert *et al*, 1989a; Clauss *et al*,

1990). However, our results suggest that endocrine, paracrine or autocrine factors, other than VEGF-A, may be of principal importance in the aetiology of pre-eclampsia. These factors may include the other members of the VEGF family, VEGF-B, VEFD-C, VEGF-D and PIGF.

Evidence has accumulated the VEGF-A mRNA expression is stimulated by a range of endocrine and paracrine factors. Estrogen (Reynods *et al*, 1998; Hyder *et al*, 1996; Cullinan-Bove *et al*, 1993), progesterone (Charkraborty *et al*, 1995), follicle stimulating hormone (Chun *et al,* 1995), leutinising hormone (Shaw *et al*, personal communication), and prostaglandin E_2 (Ben-AV *et al*, 1995; Harada *et al,* 1994), have all been shown to stimulate VEGF-A expression. On the other hand, prostacyclin (PGI_2), has been shown to inhibit VEGF-A expression. It is possible that endothelial cell damaged associated with pre-eclampsia, leads to the release of prostacyclin and thus an inhibition of VEGF-A expression. PGI_2 has been used for the treatment of pre-eclampsia and in 1992, Furuhashi *et al*, injected PGI_2 (1mg/kg) into SHR and WKY rats, and they found that placental blood flow was reduced in SHR but increased in WKY rats.

Studies examining oxygen related expression of mRNAs VEGF-A have shown that hypoxia is a potent stimulator of VEGF-A (Levy *et al*, 1995; Shweiki, *et al*, 1992; Rodesch *et al*, 1992). Lower partial pressure of carbon dioxide may also cause VEGF-A expression to increase (Lyall *et al*, 1997). For a long time, it has been thought that in pregnancies complicated by pre-eclampsia, the placenta and the fetus are hypoxic. Consequently, as hypoxia is a potent stimulator of VEGF-A, it was expected that VEGF-A expression would be higher during pre-eclampsia than during normal pregnancy. The results of the present study, which showed no change in VEGF-A expression, and those of Cooper *et al* (1996) and Lyall *et al* (1997), which showed lower VEGF-A levels in pre-eclamptic women, support the recent contention that the fetus and placenta are not hypoxic with pre-eclampsia (Lyall *et al,* 1997).

Carbon monoxide and nitric oxide have been shown to indirectly cause VEGF-A expression to be decreased by inhibition of hypoxia induced factor activation (Huang

et al, 1998). With pre-eclampsia, blood flows are reduced due to vessel constriction and placental dysfunction, thus reducing oxygen levels and increasing carbon monoxide and carbon dioxide levels. Nitric oxide is then released to act as a potent vasodilator. Our results, support the contention that factors such as nitric oxide may be released during pre-eclampsia which inhibit hypoxia induced factor activation and thus do not lead to an increase in VEGF-A expression.

A soluble form of Flt-1 has been shown to inhibit VEGF-A expression. Lyall *et al*, (1997) has suggested that there may be an excess of this soluble receptor produced during pre-eclampsia and thus as it binds with high affinity to VEGF-A, the levels of this growth factor may be reduced with pre-eclampsia. Conversely, other workers have shown that other factors, such as interleukin 1 and epidermal growth factor are altered during pre-elampsia possibly leading to an indirect increase in VEGF-A expression (Sheppard *et al*, 1981).

Our results suggest that factors, other than VEGF-A, play the most important role in the aetiology of pre-eclampsia. Any future studies of ours examining the relationship between VEGF-A and pre-eclampsia, need to optimise our techniques, such as RT-PCR kinetics and Northern blot analysis; to improve our animal model to include pregnancy induced hypertension; and to increase our sample size.

CHAPTER 5: CONCLUSIONS AND FURTHER STUDIES

The first aim of this project was to establish techniques, in our laboratory, to measure expression and localisation of VEGF-A and its receptors in uteroplacental tissues at early, mid and late gestation. The second aim was to examine whether expression of VEGF-A and its receptors differs in normotensive and spontaneously hypertensive rats, as this previously developed animal model mimics pre-eclampsia.

We have successfully established the use of RT-PCR to measure expression of VEGF-A, VEGFR-1 and VEGFR-2; Northern blot analysis to measure expression of VEGF-A; and immunohistochemistry to localize VEGF-A protein in rat uteroplacental tissues. For a variety of reasons, primarily technical and a lack of time, we were unable to optimise the measurement of VEGFR-1 and VEGFR-2 expression by Northern blot analysis, to localize VEGFR-1 and VEGFR-2 proteins using immunohistochemistry, nor to optimize our *insitu* hybridization technique.

In this study, we have shown that VEGF-A and its receptors were expressed in uteroplacetal tissues of the normal rat. The level of VEGF-A and VEGFR-1 expression was not significantly different between early, mid and late gestation.

In the present study, we have shown that VEGF-A, VEGFR-1 and VEGFR-2 were expressed in both the normotensive and the spontaneously hypertensive rat, throughout gestation. There were no major differences in the mRNA levels of VEGF-A, VEGFR-1 and VEGFR-2 between the normotensive and the spontaneously hypertensive rat.

Our preliminary results suggest that VEGF-A and its receptors may have an important role in the development of the fetal and maternal portions of the placenta, but not the primary aetiology of essential hypertension during pregnancy or pre-eclampsia.

Further studies are required to fully understand the relationship between VEGF-A and pre-eclampsia. We need to optimise our techniques, such as the RT-PCR kinetics, Northern blot analysis and *insitu* hybridisation. We also need to improve our animal model to include pregnancy induced hypertension; and to increase our sample size.

REFERENCES

Achen, M. G., Stacker, S. A. The vascular endothelial growth factor family; proteins which guide the development of the vasculature. *Int J Exp Pathol*. 79: 255-65, 1998.

Ahmed, A., Li, X. F., Dunk, C., Whittle, M. J., Ruchton, D. I., Rollason, T. Colocalisation of vascular endothelial growth factor and its Flt-1 receptor in human placenta. *Growth Factors* 12: 235-243, 1995.

Ahmed, A., Dunk, C., Kniss, D., Wilkes, M. Role of VEGF receptor-1 (Flt-1) in mediating calcium-dependent nitricoxide release and limiting DNA synthesis in human trophoblast cells. *Lab Invest*. 76: 779-791, 1997.

Ahokas, R. A., Reynolds, S. L., Anderson, G. D., Lipshitz, J. Uteroplacental blood flow in the hypertensive, term pregnant, spontaneously hypertensive rat. *Am J Obstet Gynecol.* 156: 1010-5, 1987.

Aiello, L. P., Avery, R., Arrigg, R., Keyt, B., Jampel, H., Shah, S., Pasquale, L., Thieme, H., Iwamoto, M., Park, J. E., Nguyen, H., Aiello, L. M., Ferrara, N., King, G. l. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *Engl J Med.* 331: 1480-1487, 1994.

Anthony, F. W., Wheeler, T., Elcock, C. L., Pickett, M., Thomas, E. J. Short report: Indentification of a specific pattern of vascular endothelial growth factor mRNA expression in human placental and cultured placental fbroblasts. *Placenta.* 15: 557-561, 1994.

Asahara, T., Bauters, C., Pastore, C., Bunting, S., Ferrara, N., Symes, J. F., Isner, J. M. Local delivery of vascular endothelial growth factor accelerates re-endothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation.* 91: 2802-2809, 1995.

Athanassiades, A., Hamiliton, G. S., Lala, P. K. Vascular endothelial growth factor stimulates poliferation but not migration or invasiveness in humam extravillous trophoblast. *Biol. Reprod.* 59: 643-654, 1998.

Ausubel, F. M., Brent, R., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Albright, L. M., Coen, D. M., Varki, A., Chanda, V. B. *Current protocols in molecular biology*. 4.9: Supplement 27, 1994.

Banks, R. E., Forbes, M. A., Searles, J., Pappin, D., Canas, B., Rahman, D., Kaudmannm, S., Walters, C. E., Jackson, A., Eves, P., Linton, G., Keen, J., Walker, J. J., Sellby, P. J. Evidence for the existence of a novel pregnancy-associated soluble variant of the vascular endothelial growth factor, Flt-1. *Mol Hum Reprod*. 4: 377-388, 1998.

Barker, D. J. P. Fetal and infant origins of adult disease. *Br Med J London.* 1-343, 1992.

Baker, P. N., Krasnow, J., Roberts, J. M., Yeo, K. T. Elevated serum levels of vascular endothelial growth factor in patients with preeclampsia. *Obstet Gynecol*. 86: 815-21, 1995.

Barker, D. J. In *utero* programming of chronic disease. *Clin Sci*. 95: 115-28, 1998.

Barleon, B., Sozzani, S., Zhou, D., Weich, H. A., Mantovani, A., Marme, D. Migration of human monocytes in responese to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Am Soc Hematol*. 3336-3343, 1996.

Bauknecht, T., Kiechle, M., Bauer, G., Siebers, J. W. Characterization of growth factor in human ovarian carcinoma. *Cancer Res*. 46: 2614-2618, 1986.

Bauters, C., Asahara, T., Zheng, L. P., Takeshita, S., Buntring, S., Ferrara, N., Symes, J. F., Isner, J. M. Recovering of disturbed endothelium dependent flow in collateral perfused rabbit ischemic limits following administration of VEGF. *Circulation.* 91: 2793-2801, 1995.

Ben-Av, P., Crofford, L. J., Wilder, R. L., Hla, T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett.* 372:83-87, 1995.

Berse, B., Brown, L.F., Van, L., De, Water., Dvorak, H. F., Senger, D. R. Vascular permeability factor (vascular endotheilail growth factor) gene is expressed differentiation in normal tissues, macrophages, and tumours. *Mol Biol Cell.* 3:211-220, 1992.

Bonthron, D. T., Morton, C. C., Orkin, S., Collins, T. Platelet-derived growth factor A chain: gene structure, chromosomal location, and basis for alternative mRNA splicing. *Proc Natl Acad Sci. U S A.* 85: 1492-1496, 1988.

Boura, A. L., Walters, W. A., Read, M. A., Leitch, I. M. Autacoids and control of human placental blood flow. *Clin Exp Phamacol Physiol*. 21: 734-748, 1994.

Breier, G., Albrecht, U., Sterrer, S., Risau, W. Expression of vascular endothelial growth factor during embryonic angiogensis and endothelial cells differentiation. *Development.* 114: 541-532, 1992.

Brogi, E., Wu, T., Namiki, A., Isner, J. M. Indirect angiogenic cytokines upregulate VEGF and bFGF expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. *Circulation.* 90: 649-652, 1994.

Boocock,C. A., Charnock-Jones, D. S., Sharkey, A. M., Mclaren, J., Barker, P. J., Wright, K. A., Twentym, P. R., Smith, S. K. Expression of vascular endothelial growth factor and its rexeptors Flt and KDR in ovarian carcinoma. J Natl Cancer Inst. 87: 506-516, 1995.

Brown, L F., Detmar, M., Tognazzi, K., Abu-Jawden, G., Iruela-Arispe, M. L. Uterine smooth muscle cells express functional receptors (Flt-1 and KDR) for vascular permeability factor/ vascular endothelial growth factor. *Lab Invest*. 76: 245-254, 1997.

Burchardt, M., Burchardt, T., Chen, M. W., Shabshi, A., dela-Taille, A., Buttyan, R., Shabsigh, R. Expression of messenger ribounucleic acid splice variants for vascular endothelial growth factor in the penis of adult rats and humans. *Biol. Reprod*. 60: 398-404, 1999.

Caduffffischer, L. C., Burri-ph, Anat-rec. Scaning electro microscope study of the developing incrovasculature in the postnatal rat lung. 216: 154-64, 1986.

Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., Van-Vlaenderen, I., Demunck, H., Kasper, M., Breier, G., Evrard, P., Muller, M., Risau, W., Edgington, T., Collen, D. Role of tissue factor in embryonic blood vessel development. *Nature.* 383: 73-5, 1996.

Chakraborty, C., Katsumata, N., Myal, Y., Schroedter, I. C., Brazeau, P., Murphy, L. J., Shiu, R. P., Friesen, H. G. Age-related changes in peptide-23/pancreatitis-associated protein and pancreatic stone protein/reg gene expression in the rat and regulation by growth hormone-releasing hormone. *Endocrinology.* 136: 1843-1849, 1995.

Chakraborty, S. K. Das., Dey, S. K. Differential expression of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation. *Journal of Endocrinology.* 147: 339-352, 1995.

Charnock-Jones, D. S., Sharkey, A. M., Boocock, C. A., Ahmed, A., Ferrara, R. Plevin., Smith, S. K. Vascular endothelial growth Factor receptor localiztion and activation in human trophoblast and choriocarcinoma Cells. *Biology of Reproduction.* 51: 524-530, 1994.

Charnock-Jones, D. S., Sharkey, A. M., Rajput-williams, D. J., Burch, J., Paul, S., Shaun, A. F., Boocock, C. A., Strphen, K. Smith. Identification and Localisation of Alternately Spliced mRNAs for Vascular Endothelial Growth Factor in Human Uterus and Estrogen Regulation in Endometrial Carcinoma Cell Line. *Biol Reprod.* 48: 1120-1128 1993.

Chun, S. Y., Eisenhauer, K. M., Kubo, M., Hsueh, A. J. Interleukin-1 beta suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. *Endocrinology*. 136: 3120-3127, 1995.

Claffey, K. P., Wilkison, W.O., Spiegelman, B. M. Vascular endothelial growth factor: regulation by cell differentiates and activated second messenger pathways. *J Biol Chem.* 267: 16317-16322, 1992.

Clark, D. E., Smith, S. K., Starkey, A. M., Charnock-Jones, D. S. Localisation of VEGF and expression of its receptor Flt and KDR in human placenta throughout pregnancy. *Hum Reprod*. 11: 1091-1196, 1996.

Clauss, M., Gerlach, M., Gerlach, H., Brett, J., Wang, F., Familletti, P. C., Pan, Y. C., Olander, J. V., Connolly, D. T., Stern, D. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J Exp Med*. 172: 1535-45, 1990.

Clauss, M., Weich, H., Breier, G., Knies, U., Rockl, W., Waltenberge, J., Risau. W. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. *J Biol Chem*. 271: 17629-17634, 1996.

Coelho, E. B., Ballejo, G., Salgado, M. C. Nitric oxide blunts sympathetic of pregnant normotensive and hypertensive rat arteries. *Hypertension.* 3: 585-8, 1997.

Cohen, T., Gitay-Goren, H., Sharon, R., Shibuya, M., Halaban, R., Levi, B. Z., Neufeld, G. $VEGF_{121}$, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparin sulfates for efficient binding to the VEGF receptors of human melanoma cells. *J Biol Chem.* 270: 11322-11326, 1995.

Cohen, R. F., Contrino, J., Spiro, J. D., Mann, E. A., Chen, L. L., Kreutzer, D. L. Interleukin-8 expression by head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg.* 121: 202-209, 1995.

Conn, G., Bayne, M. L., Soderman, D. D., Kwok, P. W., Sullivan, K. A., Palist, T. M., Hone, D. A., Thomas, K.A. Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor, *Proc Natl Acad Sci USA*. 87: 2628-2632,1990.

Connolly, D. T., Heuvelman, D. M., Nelson, R., Olander, J. V., Eppley, B. L., Delfino, J. J., Siegel, N. R., Leimgruber, R. M., Feder, J. Tumor vascular permeability factor stimulators endothelial cell growth and angiogenesis. *J Clin Invest*. 84: 1470-1478, 1989.

Cooper, J. C., Sharkey, D. M., Charnock-jones, S. D., Palmer, C. R., Stephen, K. Smith. VEGF mRNA levels in placentae from pregnancies complicated by pre-eclampsia. *British Journal of Obstetrics and Gynaecology.* 103: 1191-1196, 1996.

Cooper, J. C., Sharkey, A. M., Mcharen, L., Charnack-Jones, D. S., Smith, S. K. Localisation of vascular endothelial growth factor and its receptor, Flt, in human placenta and decidua by immunohistochemistry. *J Reprod Fertil*. 105: 205-213, 1995.

Cooper, J. S., Sharkey, A.M., Charnock-Jones, D. S., Palmer, C. R., Smith, S. K. VEGF mRNA levels in placentae from pregnancies complicated by pre-eclampsia. *Brit. J. Obstet. Gynaecol*. 103: 1191-1196, 1996.

Cross, J. C., Werb, Z., Fisher, S. J. Implantation and the placenta: key pieces of the development puzzle. *Science* 266:1509-1518, 1994.

Culliana-Bove, K., Koos, R. D. Vascular endothelial growth factor/ vascular permeability factor expression in the rat uterus: rapid stimulation by Estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. *Endocrinology.* 133: 829-836, 1992.

Daphne, W. C., Lee, K. H. Toxaemia of pregnancy. *Practical Obstetrics.* 115-122, 1974.

Das, S. K., Chakraboty, I., Wang, J., Dey, S. L., Horffman, L. N. Expression of vascular endothelial growth factor (VEGF) and VEGF receptor messenger ribonucleic acids in the per-implantation rabbits uterus. *Biol Reprod*. 56: 1390-1399, 1997.

De Vries, Carllie., Jaime, E. A., Hikaru, Ueno., Houck, Kith., Ferrara, Naooleone., Lewis, T. Willams. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science.* 989-991, 1992.

Detmar, M., Brown, L. F., Claffy, K. P., Yeo, K.T., Kocher, O., Jackman, R.W., Berse, B., Dvorak, H. F. Over expression of vascular permeability factor/ vascular endothelial growth factor and its receptors in psoriasis. *J. Exp. Med*. 180:1141-1146, 1994.

Dickson, R. B., Lippman, M. E. Estrogenic regulation of growth and polypeptide growth factor. *Science*. 255: 989-991, 1987.

Dunsmore, S. E., Lee, Y. C., Aratinez, Williams, C., Rannels, D. E. Synthesis of fibromection and laminin by type II pulmonary epithelial cells. *Am. J. Physiol*. 270: L215-223, 1996.

Dvorak, H. F., Brown, L. F., Detmar, M., Dvorak. A. M. Vascular permeability factor/ vascular endothelial growth factor, microvascular permeability and angiogenesis. *Am J Pathol.* 146: 1029-1039, 1995.

Erkadius, E., Morgan, T. O., Di-Nicolantonio, R. Altered uterine environment in the spontaneously hypertensive rat. *Clin Exp Pharmacol*. 1: S281-2, 1995a.

Erkadius, E., Morgan, T. O., Di Nicolantonio, R. Amniotic fluid composition and fetal and placental growth rates in genetically hypertensive and normotensive rats. *Reprod Fertil Dev*. 7: 1563-7, 1995b.

Etsuo, Hashimoto., Teruhiko, Ogita., takashi, Nakaoka., Rumiko, Matsuoka., Atsuyoshi, Takao., Yuji, Kira. Rapid induction of vascular endothelial growth factor expression by transient ischemia in rat heart. *The American Physiological Society*. H1948-1954, 1994.

Evain-Brion, D. Hormonal regulation of fetal growth. *Horm Res.* 42: 207-214, 1994.

Fava, R.A., Olsem, N. J., Spencer-Green, G., Yeao, T. K., Yeo, K.T. B., Jackman, R.W., Senger, D.R., Dvorak, H. F., Brown, L. F. Vascular permeability factor/ vascular endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids in rheumatoid arthritis. *J Exp Med.* 180: 340-346, 1994.

Ferrara, Napoleone., Davis-Smyth, Terri. The biology of vascular endothelial growth factor. *Endocrine Reviews*. 18: 4-25, 1997.

Ferrara, N., Houck, K., Jakeman, L., Leung, D. W. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev.* 13: 18-22, 1992.

Flamme, Ingo., Breier, Georg., Risau, Werner. Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (Flk-1) are expression during vasculogenesis and vascular differentiation in the Quail embryo. *Developmental Biology*. 169: 699-712, 1995.

Folkman, J., Shing, Y. Angiogenesis. *J Biol Chem*. 267: 10931-4, 1992.

Folkman, J., Watson, K., Ingber, D., Hanahan, D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature*. 339:58-61,1989.

Folkman, J., Klagsburn, M. Angiogenic factors. *Science*. 235: 442-447, 1987.

Fong, G.H., Rossant, J., Gertsenstein, M., Brietman, M. L. Role of Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature.* 376: 66-70, 1995.

Forsythe, J. A., Jiang, B. H., Lyer, N. A., Agai, F., Leing, S. W., Koos, R. D., Semenza, G. L. Activation of Vascular endothelial growth factor gene transcription by hypoxia-induced factor-1. *Mol Cell Biol.* 16: 4604-4613, 1996.

Frank, S., Haber, G., Breier, G., Longaker, M. T., Greenhalgh, D. G., Werner, S. Regulation of VEGF expression in cultured keratinocytes. Implications for normal and impaired wound healing. *J. Biol. Chem*. 270: 12607-12613, 1995.

Franklin, G. C., Donovan, M., Adam, G. l., Holngren, L., Ohlsson, Pfeifer. S., Ohlsson, R. Expression of the human PDGF-B gene, is regulated by both positively and negatively acting cell type-specific regulatory elements located in the first intron. *EMBO J.* 10: 1365-1373, 1991.

Fuchi, I., Higashino, H., Noda, K., Suzuki, A., Matsubara, Y. Placental Na^+, K^+ activated ATP-ase activity in SHRSP in connextion with pregnancy induced hypertension and intra-uterine growth retardation. *Clin Exp Pharmacol Physiol*. 22: S283-285, 1995.

Gabrilovich, D. I., Chen, H. l., Girgis, K. R., Cunningham, H. T, Meny, G. M., Nadaf, S., Kavanaugh, D., Carbone, D. P. Production of vascular endothelial growth factor by tumours inhibits the functional maturation of dendritic cells. *Nature Med.* 2: 1096-1103, 1996.

Gitay-Goren, H., Cohen, T., Tessler, S., Soker, S., Gengrinovitch, S., Rockwell, P., Klagsburn, M., Levi, B. Z., Neufeld, G. Selective binding of $VEGF^{121}$ to one of three vascular endothelial growth factor receptors on vascular endothelial cells. *J Biol Chem*. 271: 5519-5523, 1996.

Goldberg, M. A., Schneider, T. J., Khan, S., Petersen, J. R. Clinical validation of an RIA for natural and recombinant erythropoietin in serum and plasma. *Clin Biochem*. 26: 183-189, 1993.

Goldberg, M. A and Schneider, T. J. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *Biol Chem.* 269: 4355-4359, 1994.

Goldman, C. K., Kim, J., Wong, W. L., King, V., Brock, T., Gillespie, G.Y. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiform pathophysiology. *Mol. Biol. Cell.* 4: 121-133, 1993.

Gospodarowicz, D., Abraham, J. A., Schlling, J. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Pro Natl Acad Sci*. 86: 7311-7315, 1989.

Hanahan, Douglas. Signalling vascular morphogenesis and maitenace. *Science.* 277: 48-50. 1997.

Harada, S., Nagy, J. A., Sullivan, K. A., Thomas, K. A., Endo, N., Rodan, G. A., Rodan, S. B. Induction of vascular endothelial growth factor expression by prostaglandin E2 and E1 in osteoblasts. *J Clin Invest.* 93: 2490-2496, 1994.

Harada, K., Friedman, M., Lopez, J., Wang, S., Li, J., Prasad, P. V., Pearlman, S. D., Edelmam, E., Selke, F. W., Simons, M. Vascular endothelial growth factor in chronic myocardial ischaemia. *Am J Physiolo*. 270: H1791-1802, 1996.

Hauser, S., Weich, H. A. A heparin-binding form of placenta growth factor (PIGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors.* 9: 259-268, 1993.

Houck, Keith. A., Ferrara, Napoleone., Winer, Cachianes., Jane, Geoge., Li, Bing., Leung, David. W. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Molecular Endocrinology.* 1806-1814, 1991.

Huang, L. E., William, G., Willmore, Jie. Gu., Mark, A. Goldberg., Bunn, H. F. Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. *Journal of Biological Chemistry*. 274: 9038-9044, 1998.

Hyder, S. M., Stancel, G. M., Chiappetta, C., Murthy, L., Boettger-Tong, H. L., Makela, S. Uterine expression of vascular endothelial growth factor is increased by Estradiol and Tamoxifen. *Cancer Res*. 56: 3954-3960, 1996.

Jakeman, L. B., Winer, J., Bennett, G. L., Altar, C. A., Ferrara, N. Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *J Clin Invest.* 89: 244-253, 1992.

Jakeman, L. B., Armanini, M., Phillips, H. S., Ferrara, N. Developmental expression of binding sites and messanger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. *Endocrin*. 133: 848-859, 1993.

Johnston, B. M. Fetal growth retardation and increased placental weight in the spontaneously hypertensive rat. *Reprod Fertil Dev*. 7: 639-45, 1995.

Joukov, V., Kaipainen, A., Jeltsch, M., Pajusola, K., Olofsson, B., Kumar, V., Eriksson, U., Alitalo, K. Vascular endothelial growth factor VEGF-B and VEGF-C. *J Cell Physiol* 173: 211-215, 1997.

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., Alitalo, K. A novel vascular endothelial growth factor is a ligand for the Flt4 (VEGF-3) and KDR (VEGF-2) receptor tyrosine kinases. *EMBO J*. 15: 290-298, 1996.

Kendal, R. L., Thomas, K. A. Inhibition of vascular endothelial growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci.* 90: 10705-10709, 1993.

Kendall, R. L., Wang, G., Thomas, K. A. Identification of a natural soluble of the vascular endothelial growth factor receptor, Flt-1, and its heterodimerization with KDR. *Biochem Biophys Res Comm.* 226: 324-328, 1996.

Katoh, O., Tauchi, H., Kawaishi, K., Kimura, A., Satow, Y. Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation. *Cancer Res.* 55: 5687-5692, 1995.

Keck, P. J., Masbuki, S., Hauser, D., Krivi, G., Sanzo, K., Warren, T., Connolly, D. T. Vascular permeability factor, an endothelial growth mitogrn related to PDGF. *Science.* 246: 1309-1312, 1989.

Kiefer, M., Stefihens, J. C., Crawford, K., Okino, K., Barr, P. J. Ligand-affinity cloning and structure of a cell surface heparan sulface heparan salfate proteoglycan that binds basic fibroblast growth factor. *Proc Natl Acad Sci USA*. 87: 6985-6989, 1990.

Kim, K. S., Li, B., Houck, K., Winer, J., Ferrara, N. The vascular endothelial growth factor proteins identification of biological relevant regions by neutralising monoclonal antibodies growth factors. 7: 53-54, 1992.

Koch, A. E., Harlow, L. A., Haines, G. K., Amento, E. P., Unemori, E. N., Wong, W. L., Pope, R. M., Ferrara, N. Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol*. 152: 4149-56, 1994.

Koch, A.E., Polverini, P. J., Leibovich, S. J. Induction of neovascularization by activated human monocytes. *J Leukov Biol*. 39: 233-238, 1986.

Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V., Alitalo, K. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development.* 122: 3829-37, 1996.

Ku, D. D., Zaleski, J. K., Liu, S., Brock, T. Vascular endothelial growth factor induces EDRF-dependent relaxation of coronary arteries. Am J Physiol. 265: 586-592, 1993.

Ladoux, A., Frelin, C. Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart. *Biochem Biophys Res Commun.* 195: 1005-10, 1993.

Lampugnani, M. G., Resnati, M., Ratiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco,L. P., Dejana, E. A novel endothelial-specific membrane protein is a marker of cell-cell contracts. *J Cell Biol*. 118: 1511-22, 1992.

Lee, D., Stefano, G., Mandriota, J., Monteano, R., Orci, L., Peter, M. S. Vascular endothelial growth factor is increased in devascularized rat islets in vitro. *Proc Natl Acard Sci U.S.A*. 93: 1988-1992, 1996.

Leung, D. W., Cachianes, G. W., Kuand, J., Goeddel, D. V., Ferrara, N. Vascular endothelial growth factor is a secreted napoleonic mitogen. *Factors.* 2: 9-19, 1989.

Leung, D. W., Cachianes, G. W., Kuang, J., Wun, J., Goeddel, D. V., Ferrara, N. Vascular endothelial growth factor is a secrected angiogenic mitogen. *Science*. 246: 1306-1309, 1983.

Levy, A, P., Levy, N. S., Goldberg, M. A. Hypoxia-inducible protein binding to vascular endothelial growth factor mRNA and its modulation by the von hippel-lindau protein. *J Biol Chem*. 271: 25492-25497, 1996.

Levy, A. P., Levy, N. S., Wegner, S., Goldber, M. A. Transcriptional regulation of the rat vascular endothelial factor gene by hypoxia. *J Biol Chem.* 270: 13333-13340, 1995.

Lewis, R. M., Bassett, N. S., Johnston, B.M., Skinner, S. J. Fetal and placental glucose and amino acid uptake in the spontaneously hypertensive rat. *Placenta.* 19: 403-8, 1998.

Lewis, R. M., Batchwlor, D. C., Bassett, N. S., Johnston, B. M., Napier, J., Skinner, S. J. Perinatal growth disturbance in the spontaneously hypertensive rat. *Pediatr Res*. 42: 758-64, 1997.

Li, H. T., Honbo, N. Y., Karliner, J. S. Chronic hypoxia increases beta 1-adrenergic receptor mRNA and density but not signalling in neonatal rat cardiac myocytes. *Circulation.* 94: 3303-3310, 1996.

Li, J., Perrella, M.A., Tsai, J. C., Yet, S. F., Hsieh, C. M., Yoshizumi, M., Patterson, C., Endege, W. O., Zhou, F., Lee, M. E. Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. *J Biol Chem.* 270: 308-12, 1995.

Lindheimer, M. D., Katz, A. I., Koeppen, B. M., Ordonez, N. G., Oparil, S. *Hypertension.* 5: 498-506, 1983.

Lyall, F., Greer, I. A., Boswell, F., Fleming, R. Suppression of serum vascular endothelial growth factor immunoreactivity in normal pregnancy and in pre-eclampsia. *Br J Obstet Gynaecol.* 104: 223-228, 1997.

Magilone, D., Guerriero, V., Viglietto, G., Ferraro, M. G., Aprelikova, O., Alotalo, K., Del Vecchios, S., Lei, K. J., Chou, J. Y., Persico, M. G. Two alternative mRNA coding for the angiogenic factor, placenta growth factor (PLGF), are transcribed from a single gene of chromosome 14. *Oncogene.* 8: 935-931, 1993.

Matsumoto, Y., Kato, M., Tamada, Y., Mori, H., Ohashi, M. Enhancement of interleukin-1 alpha mediated autocrine growth of cultured human keratinocytes by sho-saiko-to. *Jpn J Pharmacol*. 73: 333-6, 1997.

Matthews, W., Jordan, C. T., Gravin, M., Jemkins, N. A., Copeland, N. G., Lemischka, I. R. A receptor tyrosine kinase cDNA isolated from a population of enriched mprimitive haematopoietic cells and exhibiting close genetic linkage to c-kit. *Proc Natl Acad Sci USA*. 90: 10705-10709, 1991.

McCarthy, P. W. Sparse substance P-like immunoreactivity in intervertebral discs. nerve fibers and endings in the rat. *Acta Orthop Scand.* 64: 664-8, 1993.

Melder, R. J., Koenig, G. C., Witwer, B. P., Safabakhsh, N., Munn, L. L., Jain, R, K., During angiogenesis, vascular endothelial growth factor and basic fibroblast growth factor regulate natural killer cell adhesion to tumour endothelium. *Nat Med*. 2: 992-7, 1996.

Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W., Ulrch, A. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell.* 72: 835-846, 1993.

Minchenko, A., Bauer, T., Salceda, S., Caro, J. Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. *Lab Invest*. 71: 374-379, 1994.

Mochizuki, N., Morikawa, H., Yamasaki, M., Maruo, T. Vascular reactivity in normal and abnormal gestation. *Am J Kiney Dis.* 17: 139-143, 1991.

Monacci, W. T., Marsha, J. Merrill., Edwaud, H. Oldfiels. Expression of vascular Endothelial Growth Factor/ Vascular Endothelial Growth Factor in rat tissues. *Am J Physiol.* 264: 995-1002, 1993.

Moore, K. L. Before we are born basic embryology and birth defects. Third edition. W. B. Saundels Co. *Philadelphia U. S. A*. 92-93, 1989.

Neufeld, G., Cohen, T., Gengrinovitch, S., Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *The FASEB Journal*. 13: 9-21, 1999.

Olofson, B., Paiusola, K., Kaipainen, A., Von-Euler, G., Joukov, V., Sakela, O., Orpana, A., Petterson, R. F., Alitalo, K., Eriksson, U. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci*. 93: 2576-2581, 1996.

Pekala, P., Marlow, M., Henvelman, D., Connolly, D. Regulation of hexose transport in aortic endothelial cells by V P F and tumor necrosis factor-alpha, but not be insulin. *J Biol Chem*. 265: 18051-18054, 1990.

Pepper, M. S., Ferrara, N., Orci, L., Montesano, R. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res Commun*. 181: 902-906, 1991.

Pierce, E. A., Avery, R. L., Llord, E. D., Lloyd, P., Aiello, L., Smith, E. H. Vascular endothelial growth factor/ vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc Natl Acad Sci USA*. 92: 905-909, 1995.

Pinilla, L., Rodriguez-Criado, J., Gaaytan, F., Aguilar, E. Mechanism of reproductive deficiency in spontaneously hypertensive rats. *Physiol Behav*. 51: 99-104, 1992.

Pertovarra, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O., Alitalo, K. Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and endothelial cells. *J Biol Chem*. 269: 6271-6274, 1994.

Phillips, G. D., Stone, A. M., Schultz, J. C., Jones, B. D., Lisowski, M. J., Goodkin, M. L., Knighton, D. R. Tumor necrosis factor alpha (rhTNF) fails to stimulate angiogenesis in the rabbit cornea. *Anat Rec.* 245: 53-56, 1996.

Plate, K. H., Breier, Georg., Herbert, A. Weich., Werner, Risau. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature.* 359: 845-8, 1992.

Quinn, T., Peter, K. G., deVries, C., Ferrara, N., William, S. L. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expression in vascular endothelium. *Proc Natl Acad Sci USA* 90: 7533-7537.

Reynolds, L. P., Kirsch, J. D., Kraft, K.C., Redmer, D. A. Time-course of the uterine response to estradiol-17 beta in ovariectomized ewes: expression of angiogenic factors. *Biol Reprod*. 59: 613-20, 1998.

Risau, W., Flamme, I. Vasculogenesis. *Annu Rev Cell Dev Biol.* 11: 73-91, 1995.

Roberts, J. M., Robert, N., Taylor, M. D., Thomas, J., Musci, M. D., Gerge, M., Rodgers, M. D., Carl, A. Hubel., Margeret, K. Mclaughlin. Preeclampsia: An ecdothelial cell disorder. *Am J Obstet Gynecol*. 161: 1200-4, 1989b.

Roberts, W. G., Palade, G. E. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *Cell Sci.* 108: 2369-79, 1995.

Rodesch, F., Simon, P., Donner, C., Jaunaux, E. Oxygen measurements in endothmetrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol*. 80: 383-285, 1992.

Sato, E., Ishibashi, T., Koide, S. S. Inducment of blood vessel formation by ovarian extracts from mice injected with gonadotropins. *Experientia.* 38: 1248-1249, 1982.

Scott, J. N., Goecke, J. C., Ream, L. J. Placentas from spontaneously hypertensive rats and control strain Wistar-Kyoto rats. *Lab Anim Sci.* 35: 146-9, 1985.

Seetharam, L., Gotoh, N., Maru, Y., Neufeld, G., Yamaguchi, S., Shibuya, M. A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Oncogene.* 10: 135-47, 1995.

Seko, Y., Yuko, Seko., Naoyuki, Takahashi., Masabumi, Shibuya., Yoshio, Yazaki. Pulatile stretch stimulates vascular endothelial growth factor (VEGF) secretion by cultured rat cardiac myocytes. *Biochemical Biophysical communications.* 254: 462-465, 1999.

Senger, D. R., Golli, S. J., Dvorak, A. M., Perruzzi, C. A., Ttarveg, V. S., Dvorak, H. F. Tumour cells secrete vascular permeability factor that promotes accumulator of ascites fluid. *Science.* 219: 983-985, 1983.

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A., Rossant, J. A requirement for Flk1 in primitive and definitive haematopoiesis and vasculogenesis. *Cell.* 89: 981-90, 1997.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., Schuh, A. C. Failure of blood-island formation and vasculogenesis in Flk-1 deficient mice. *Nature.* 376: 62-66, 1995.

Sharkey, A. M., Chaunick-Jones, D. S., Boocock, C. A., Brown, K. D., Smith, S. K. Expression of mRNA for vascular endothelial growth factor in human placenta. *J Reprod Ferti.* 99: 609-615, 1993.

Sharkey, A. M., Cooper, J. C., Balmforth, J. R, McLaren, J., Clark, D. E., Charnock-Jones, D. S., Morris, N. H, Smith, S. K. Maternal plasma levels of vascular endothelial growth factor in normotensive pregnancies and in pregnancies complicated by pre-eclampsia. *Eur J Clin Invest*. 26: 1182-5, 1996.

Sheppard, B. L., Bonnar, J. An ultrastructural study of utero-placental spiral arteries in hypertensive and normotensive pregnancy and fetal growth retardation. *Br J Obstet Gynaecol*. 88: 695-705, 1981.

Siemester, G., Weinner, K., Monhrs, K., Barleon, B., Martiny-Baron, G., Marme, D. Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor suppressor protein. *Cancer Res.* 56: 2299-2303, 1996.

Shibuya, M., Yamaguchi, S., Yamane, A., Ikada, T., Tojo, T., Matsu-shune, H., Sato, M. Nuckeotide sequence and expression of a novel human receptor-tyrosine kinase (flt) closely relared to the fms family. *Oncogene* 8: 519-527, 1990.

Shifren, J. L, Doldi, N., Ferrara, N., Mesiano, S., Jaffe, R. B. In the human fetus, vascular endothelial growth factor (VEGF) is expressed in epithelial cells and myocytes, but not vascular endothelium: implications for mode of action. *J Clin Endocrinol Metab*. 79: 316-322, 1994.

Shweiki, D., Itin, A., Soffer, D., Keshet, E. Vascular edothelial growth factor induced by hypoxia may mediatc hypoxia-induced angiogenesis. *Nature*. 359: 843-845, 1992.

Shweiki, D., Itin, A., Neufeld, G. Gitay., Goren, H., Keshet, E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest*. 91: 2235-2243, 1993.

Sporn, M. B., Robert, A. B. TGF-beta: Problems and prospects. *Cell Regul*. 1: 875-82, 1990.

Srivastava, R, K., Gu, Y., Ayloo, S., Ziberstein, M., Gibori, G. Developmental expression and regulation of basic fibroblast growth factor and vascular endothelial growth factor in rat decidua and in a decidual cell line. *Journal of Endocrinogy.* 21: 355-362, 1998.

Takagi, H., King, G. L., Robinson, G. S., Ferrara, N., Aiello, L. P. Adenosine mediates hypoxic induction of vascular endothelial growth factor in retinal pericytes and endothelial cells. *Invest Ophthalmol Vis Sci.* 37: 2165-76, 1996.

Takahashi, T., Shirasawa, T., Miyake, K., Yahagi, Y., Maruyama, N., Kasahara, N., Kawamura, T., Matsumura, O., Mitarai, T., Sakai, O. Protein tyrosine kinases expressed in glomeruli and cultured glomerular cells: Flt-1 and VEGF expression in renal mesangial cells. *Biochem Biophys Res Commun*. 209: 218-26, 1995.

Tamura, H., Greenwald, G. S. Angiogenesis and its hormone control in the corpus luteum of the pregnant rat. *Biol Reprod*. 33: 1149-1154, 1987.

Terman, B., Khandke, L., Dougher-Vermazan, M., Maglione, D., Lassam, N. J., Gospodarowicz, D., Persico, M. G., Bohlen, P., Eisinger, M. VEGF receptor subtypes KDR and FLT1 show different sensitivities to heparin and placenta growth factor. *Growth Factors*. 11: 187-95, 1994.

Terman, B.I., Dougher, Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., Bohlen, P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Bioche Bioph Res Commun.* 187: 1579-1586, 1992.

Thomas, K. A. Vascular endothelial growth factor, a potent and selective angiogenic agent. *J Biol Chem*. 271: 603-606, 1996.

Tipton, C. M., Sturk, M. S., Oppliger, R. A., Matthes, R. D., Overton, J. M., Edwards, J. G. Responses of SHR to combinnation of chemical sympathecomy, adrenal demedullation, and training. *Am J Physiol Jul.* 247: 109-118, 1984.

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., Abraham, J. A. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J biol Chem*. 266: 11947-54, 1991.

Tisher, C. C., Madsen, K. M., Verlander, J. W. Structural adaptation of the collecting to acid-base disturbances. *Kiney Int Suppl.* 33: 57-63, 1991.

Tolentino. M. J., Miller, J. W., Gragondas, E. S., Chatzistefanon, K., Ferrara, N., Adamis, A. P. VEGF is sufficient to produce iris neovascularorization and neovascular glanloma in a nor-human primate. Arch Ophthalmol. 114: 964-970, 1996.

Torry, D. S., Holt, V. J., Jeffrey, B. S., Keenan, A., G., Harris, M. R., Caudie, T. R. Vascular endithelial growth factor expression in cycling human endometrium. *Fertility and Sterility*. 66: 72-80, 1996.

Tuder, R. M., Flook, B. E., Voelkel, N. F. Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia. Modulation of gene expression by nitric oxide. *J Clin Invest*. 95: 1798-807, 1995.

Ullrich, A., Schlessinger, J. Signal transduction by receptors with tyrosine kinase activity. *Cell*. 61: 203-212, 1990.

Vuckovic, M., Ponting, J., Ferrara, B. I., Niketic, V., Seif, M. W., Kimar, S. Expression of the vescular endothelial growth factor receptor, KDR, in human placentae. *J Anat.* 188: 361-366, 1996.

Vuorela, P., Hatva, E., Lymboussaki, A., Kaipainen, A., Joukov, V., Persico, M. G., Alitalo, K., Halmesmaki, E. Expression of vascular endothelial growth factor and placenta growth Factor in human placenta. *Biol Reprod.* 56: 489-494, 1997.

Wang, G. L., Semenza, G. L. Purification and charactorization of hypoxia-inducible factor. *J Biol Chem.* 270: 1230-1237, 1995.

Warren, R. S., Yuan, H., Matli, M. R., Ferrara, N., Donner, D. B. Induction of vascular endothelial growth factor by insulin-like growth factor-1 in colorectal carcinoma. *J Biol Chem.* 271: 29483-29488, 1996.

Weksberg, R., Squire, J. A., Templeton, D. M. Glypicans: a growth trend. *Nat.Genet*. 12: 225-227, 1996.

Wheeler, T., Elcock, C. L., Anthony, F. W. Angiogenesis and the placental enviroment. *Placenta.* 16: 289-296, 1995.

Yamane, A., Seetharam, L., Yamaguchi, S., Gotoh, N., Takahashi, T., Neufeld, G., Shibuya, M. A new communication system between hepatocytes and sinusoidal endothelial cells in liver through vascular endothelial growth factor and Flt tyrosine kinase receptor family (Flt-1 and KDR/Flk-1). *Oncogene.* 9: 2683-2690, 1994.

Yang, X. J., Cepko, C. L. Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. *J Neurosci*. 16: 6089-6099, 1996.

Yarden, Y., Ullrich, A. Growth factor receptor tyrosine kinases. *Ann Rev Biochem*. 57: 443-478, 1988.

Yamada, Yoshiki., Neu, Jun-ichi., Shimane, Miyuki., Yuichi, Hirata. Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics.* 42: 483-488, 1997.

SOLUTION AND BUFFERS

SOLUTIONS AND BUFFERS

Acetone-APES buffer (2%)

The buffer containing 6 ml 3-Amihopropyl-Triethoxysilane and 300 ml Acetone was mixed at room temperature immediately prior to use.

Denhardt's reagent X 50

5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone and 5 g of bovine serum albumin was dissolved and made up to 500 mL with DEPC water, filter sterilised (0.2 μm filter) and stored at -20°C.

DEPC water

Deionised water containing 0.1% of DEPC was shaken vigorously, incubated overnight at 37°C and autoclaved for 30 minutes.

DNA extraction buffer

The buffer constituted of Tris (pH 8.0) 100 mM, EDTA (pH 8.0) 50 mM, NaCl 100 mM, SDS 1% and 10 mM (mercaptoethanol was added immediately before use). The solution was autoclaved.

EDTA, 0.5 M (pH 8.0)

Disodium ethylenediaminetetra-acetate.$2H_20$ (186.1 g) was dissolved in 800 mL dH_2O by continuous stirring and the pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 1 litre with dH_2O and autoclaved.

Ethidium Bromide (10 mg/mL)

Ethidium bromide (10 mg/mL) in autoclaved dH_2O was stirred for several hours to ensure that the dye has dissolved. The solution was stored in an amber bottle at 4°C.

6% Formaldehyde gel for northern blot

The buffer contained of 37% Formaldehyde 10 ml and 5.5 ml 10 x MOPs buffer, to added 40.5 ml DEPC water with 0.5 g agarose.

Formaldehyde gel-loading buffer

The buffer constituted of 50% autoclaved glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol FF.

MOPS X 10

The buffer constituted of 200 mM of 3-[N-Mopholino] propane sulfonic acid, 80 mM sodium acetate and 10 mM EDTA (The pH was adjusted to 7 with 2N NaOH). Following 0.2 μm filter sterilisation, the stock buffer was stored in an amber bottle at room temperature.

10% Normal Rat Serum (N.R.S.)

The solid was dissorved in 10 ml d.d.H2O, aliquotes of 150 ul each tube, stored in -20°C. To prepare for using, 150 ul of each tube was mixtured with 1350 ul 1 x P.B.S. buffer.

Phenol-chloroform-isoamyl alcohol (25:24:1; v / v)

Liquified phenol (melted at 65°C), to which 8-hydroxyquinoline was added at a concentration of 0.1% (w/v), was equilibrated by extracting several times until the pH was 8. One part of this phenol was mixed with one part of chloroform-isoamyl alcohol (24:1) and stored under 50 mM Tris-Cl (pH 8) buffer in an amber glass bottle at 4°C.

Prehybridisation solution

The solution constituted of 2 parts of Denhardt's reagent, 5 mM EDTA, 10 mM Tris-Cl, 0.5 M Na_3PO_4 (pH = 7), 5% SDS and treated salmon sperm DNA solution (100 ng/mL) addedimmediately before use. This was stored at -20°C.

RNAase (DNAase-free)

Ribonuclease A (from bovine pancreas) was dissolved at a concentration of 10 mg/mL in 0.01 M sodium acetate (pH 5.2) and heated at 100°C for 15 minutes. It was then cooled slowly to room temperature, dispensed into aliquots and stored at -20°C.

RNA marker stain

Methylene blue (0.04% w/v) dissolved in 0.5 M sodium acetate (pH 5.2).

Salmon sperm DNA

Salmon sperm DNA (ssDNA) solution (from Gibco-BRL) was prepared at a concentration of 10 mg/mL by dissolving in autoclaved dH_2O. The required amount for prehybridisation was denatured in a boiling water bath for 10 minutes and chilled for 2-3 minutes.

Sample buffer (for RNA)

The buffer was made up by mixing 250 µL formamide, 83 µL of 37% formaldehyde (pH > 3.5), 50 µL of 10 X MOPS buffer and the volume was adjusted to 400 µL with DEPC water and stored -20°C.

SDS 10%

Sodium dodecyl sulphate (100 g) was dissolved in 900 mL dH_2O by heating it to 68°C and the pH was adjusted to 7.2 with concentrated HCL. The volume was made up to 1 litre.

Sodium acetate 3 M (pH 5.2)

Sodium acetate.$3H_2O$ (408.1 g) was dissolved in 800 mL dH_2O, the pH was adjusted to 5.2 with glacial acetic acid and the volume made to 1 litre with dH_2O. The solution was autoclaved.

Sodium phosphate 1 M (pH 7)

To prepare 100 mL of the solution, 68.4 mL of 1 M Na_2HPO_4 was added to 31.6 mL of 1 M NaH_2PO_4.

SSC X 20

Sodium chloride (175.3 g) and sodium citrate (88.2 g) were dissolved in 800 mL of dH_2O. The pH was adjusted to 7 with 10 N NaOH and the volume was made up to 1 litre with dH_2O and autoclaved.

Tris-acetate buffer (TAE) X 50

An amount of 242 g of Tris (pH 7.4), 57.1 ml sodium acetate and 100 ml of 0.5M EDTA (pH 8.0) were mixed and the pH was adjusted to 7.4 with glacial acetic acid. The volume was made up to 1 litre with dH_2O and the solution was autoclaved. The working solution is 1 X TAE.

Tris-borate buffer 0.1 M (pH 8.3) X 5

Tris base (54 g) and boric acid (27.5 g) were made up to 1 litre. The working solution is 1 X Tris-borate.

Tris-HCl 1 M

Tris base (121.1 g) was dissolved in 800 mL of dH_2O. The pH was adjusted with concentrated HCl as follows when solution was at room temperature:

pH	HCl
7.4	70 mL
7.6	60 mL
8.0	42 mL

The volume was made up to 1 litre and the solution was autoclaved.

Tris-HCl 0.5 M (pH 6.8)

Tris base (6 g) was dissolved in ~60 mL dH_2O, the pH was adjusted to 6.8 with 1 N HCl and the volume was made up to 1 litre.

TE Buffer (pH 7.4)

The solution constituted of 10 mM Tris-Cl (pH 7.4) and 1 mM EDTA (pH 8.0). The solution was autoclaved.

Water-Saturated Phenol

100 g phenol crystals dissolved in 500 ml water with 8-Hydroquiline (0.1% hydroquiline) heating at 65°C water bath for 2-3 hours, phases were allowed to separated phenol, cool down in 4°C. The solution allowed to using for 1month and stored at 4°C.

X-ray developer

400 mL developer concentrate (AGVA GEVART)
2 litres water