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Vascular Endothelial Growth Factor Expression in Breast Cancer.

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Thesis submitted for the degree of Doctor of Medicine at the University of Glasgow.

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September 2002

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Acknowledgements

I would like to thanks to:

Dr Susan Jamieson for her advice and criticism during the conduct of this work and during the preparation of the thesis.

Dr Richard Lamb and Dr George Birnie of the Beatson Institute for Molecular Biology, for their guidance during the cloning of the VEGF sequence.

Maura Farquharson and Dr Anne-Marie McNicol, Department of Pathology, Glasgow Royal Infirmary for teaching me the techniques of *in-situ* hybridisation.

Professor R.N.M McSween and Dr Elizabeth. Mallon for supplying tissue sections for immunohistochemistry and *in situ* hybridisation.

Dr Napoleone Ferrara, Dr Kenneth Hillan and the staff of Genentec, San Francisco for making available the Genentec monoclonal and polyclonal anti-VEGF antibodies.

Dr Valerie Speirs, Department of Medicine, University of Hull, for making available the breast cDNA samples used in the preliminary trials of the RT-PCR assay.

Professor WD George for his advice and for giving me the opportunity to perform these studies.

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List of Abbreviations

cDNA	Complementary Deoxyribonucleic acid
CI	confidence interval
CMF	cyclophosphamide, methotrexate & 5-flurouracil
DEPC	diethylpyrocarbonate
Dig	digoxigenin
DTT	dithioethreitol
EDTA	ethylene diamine tetra acetate
ELISA	enzyme-linked immunoabsorbent assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IPTG	isopropyl-thiogalactoside
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PNK	polynuclotide kinase
RT	reverse transcrpition
SDS	sodium dodecyl sulphate
SSC	sodium chloride/sodium citrate buffer
TE buffer	tris-EDTA buffer
TBE	tris borate buffer
TBS	tris buffered saline
VEGF	vascular endothelial growth factor
x-gal	5-bromo-4-chloro-3-indolyl-β-galactosidase

Summary.

In order to grow, a tumour must obtain a blood supply. This process is called angiogenesis. It is likely that the better a tumour is able to obtain a blood supply, the faster it may grow and the more access the cells of the tumour may have to the circulation for haematogenous metastasis.

Several authors have reported that breast tumours that contain large numbers of blood vessels have a poor prognosis. This suggests that angiogenesis is an important mediator of the behaviour of breast tumours.

A protein called vascular endothelial growth factor (VEGF) has been described which is a potent inducer of angiogenesis. It is a specific endothelial mitogen produced by both normal tissues and tumours.

Prior to commencement of this thesis the role of VEGF in breast cancer had not been reported. Given the apparent importance of vascularity to prognosis in breast cancer it was hypothesised that VEGF may be of importance in the behaviour of these tumours.

The aim of this research was to develop and apply techniques for studying the expression of VEGF in breast cancers. It was hoped that such techniques might be of value identifying a group of patients whose prognosis was poorer than would be expected from conventional predictors such as lymph node status. This would allow the potential for intervention either with specific anti-angiogenic therapy or conventional adjuvant therapy.

A part of the cDNA sequence of VEGF was cloned into a plasmid expression vector. The vector was reproduced in bacterial cultures and the presence of the cloned sequence verified by Southern blotting, PCR and DNA sequencing.

The vector was then used to produce an RNA riboprobe with a non-radioactive digoxigenin label. Attempts were made to use this probe and a DNA oligonucleotide

probe to seek VEGF RNA both by Northern blotting and by *in situ* hybridisation. These attempts were hampered by high levels of non specific binding of the probe.

Several antibodies against VEGF protein were tested to investigate the feasibility of identifying VEGF by immunohistochemisty. Despite the use of antigen unmasking techniques, this proved unsuccessful with the tissue samples used.

A semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) based assay for studying the relative expression of VEGF in breast tissue was developed and optimised. Amplification of β-actin mRNA was also carried out to control for the quantity and quality of the mRNA in the tissue samples. A linear relationship between the amount of mRNA samples and the amount of amplified cDNA product after RT-PCR was verified. VEGF and β-actin mRNA concentrations in the tissue samples was compared to a standard RNA solution extracted from a breast cancer cell line (T47D).

It proved possible to use this assay to compare samples of breast tumours and adjacent breast. Due to its inherent molecular amplification, this RT- PCR assay has the advantage of requiring very small amounts of RNA and is therefore applicable to small samples of archival material or clinical biopsies.

Relationships of the expression of VEGF to the pathological characteristics and clinical behaviour of the tumours were examined. No clear difference was seen between VEGF expression in tumours and adjacent breast though the numbers of tissue samples studied was not sufficiently large to draw conclusions regarding any link between VEGF and tumour behaviour.

Recently several authors have found evidence that VEGF is indeed important in breast cancer. The literature regarding this has been summarised in the introduction to this thesis.

Declaration

I declare that the planning and execution of all of the work described in this thesis was carried out by me. Whilst guidance was sought from those acknowledged above, all the experimental work described was performed by me.

The work presented here has not been presented in whole or part for any other degree.

Part of the work relating to the development of the RT-PCR based has been presented by me to the British Association of Surgical Oncology, Oxford, July 1996.

Chapter 1. Introduction

1.1 Tumour Angiogenesis

Angiogenesis is the process of formation of new blood vessels. The term, derived from the Greek words angion (a vessel) and genesis (birth), was used by Judah Folkman [1] over thirty years ago to describe the mechanisms by which tumours obtain a blood supply. It has been the subject of much research since then. Blood vessel formation starts in the third week of foetal development and continues as a normal physiological event throughout life. It is a mechanism essential to growth and wound healing but also plays a part in many pathological processes including cancer.

The behaviour of tumours placed in situations where they are not able to obtain a blood supply has been studied by implanting them, floating in suspension, in the anterior chamber of the rabbit's eye [2]. In these situations, the size of the tumour is limited by the capacity of the central cells to obtain nutrients by diffusion and generally does not exceed a few millimetres in diameter. By contrast it was observed that a tumour implanted in vascularised tissue grows rapidly and that tumours usually contain a large number of blood vessels. Thus, it was assumed that blood vessels grew into a tumour from the host vasculature. The methods initially used to study this process involved implanting tumours in chambers into the ears of rabbits [3], or the cheek pouches of hamsters [4]. By enclosing tumours in chambers made from millipore filters, it could be demonstrated that the angiogenic response was mediated by small diffusible elements [5]. The rabbit cornea or the chorioallantoic membrane of the chick egg [6,7] proved convenient sites since the development of blood vessels could be continuously observed. Other substances could also be introduced to test their effect on the angiogenic process [8]. Observations made on these systems have led to a better understanding of the cellular events of the angiogenic response. The new vessels originate from small venules and existing capillaries and the neovascularisation process principally involves endothelial cells [2, 8]. The earliest event is the degradation of the capillary basement membrane under the influence of proteolytic enzymes produced by the endothelium [9]. Endothelial cells migrate out of

this breech and move towards the tumour. These cells form a column with most of the mitotic activity occurring a few millimetres behind the advancing tip. The column develops a lumen and loops of these new capillaries form with blood flowing in them.

In 1971 Folkman's group [1] found that one fraction isolated by gel filtration from tumour cell extracts was angiogenic when introduced into a dorsal pouch on a rat. They called this fraction, which consisted of carbohydrates, RNA and protein, tumour angiogenesis factor (TAF). Klagsbrun et al [10] purified an endothelial mitogen released by hepatoma cells and demonstrated that this was structurally similar to basic fibroblast growth factor (bFGF). Schweigerer et al [11] have shown that capillary endothelial cells secrete bFGF and are capable of autocrine stimulation. Some of the factors which have been purified, such as that obtained by Keegan et al [12], are only effective *in vitro* if a collagen substrate is used. Thompson et al [13] found that plasmin digests of fibrin were angiogenic in the chorioallantoic membrane assay while West et al [14] showed that fragments of hyaluronic acid between four and twenty five disaccharides long were angiogenic in this assay. The production of proteolytic enzymes by endothelial cells has been well documented [14, 15]. Gross et al [9] found that capillary endothelial cells in culture could produce low levels of plasminogen activator and collagenase. This production could be stimulated by agents such as tissue plasminogen activator. If this activity could be stimulated by tumour products, there is a possibility of a positive feedback loop of endothelial cell stimulation.

A number of growth factors have been documented which stimulate endothelial cells *in vitro* and are angiogenic *in vivo*. These include acidic and basic fibroblast growth factors, transforming growth factor-alpha, epidermal growth factor, platelet derived endothelial growth factor and the vascular endothelial growth family of angiogenic peptides that will be discussed in depth later.

Whilst cells in a pre-vascular tumour may replicate as rapidly as those in a vascularised tumour, the generation of new cells is balanced by cell death. The switch to an angiogenic phenotype results from over expression of an angiogenic protein, mobilisation of an angiogenic protein from the extracellular matrix, recruitment of

host cells such as macrophages that produce angiogenic proteins or a combination of these factors. It also appears that the angiogenic phenotype depends on the decreased production of inhibitors of angiogenesis such as thrombospondin-1, glioma derived angiogenesis inhibitor and angiostatin.

1.2 Angiogenesis in Breast Cancer.

The capacity of tissues from the human breast to induce angiogenesis was demonstrated by Brem et al [16] in 1978. They found that some samples of normal breast tissue would generate an angiogenic response in the rabbit's eye model. The response was stronger if the tissue was derived from a breast containing a tumour and stronger still if the cancer tissue, either *in situ* or invasive, was used. Jensen et al [17] subsequently confirmed that 15% of lobules from normal breasts gave rise to an angiogenic response compared to 28% of histologically apparently normal lobules from breasts containing ductal carcinomas.

Weidner [18] looked at the relationship between the number of microvessels (capillaries and venules) in histological sections from breast carcinomas. Using immunostaining for factor VIII, he counted the number of microvessels per high power field in the areas that appeared to be richest in these vessels. He found that tumours from patients with lymph node metastases had a significantly higher number of microvessels. There was a steady increase in the likelihood of lymph node metastasis with increasing vessel count. All fifteen tumours with greater than one hundred vessels per 200x field had lymph nodes involved compared to only one of seven patients with less than thirty three vessels. He also noted rings of increased vascularity in the immediate vicinity of ducts containing carcinomas *in situ.*

This work created considerable interest and many other groups performed similar studies.

In 1992, Bosari [19] confirmed that node-positive patients had tumours with a higher microvessel count. He also found that, within the node-negative group, patients who

subsequently developed distant metastases had a significantly higher microvessel count than those who did not.

The following year, To et al [20] reported microvessel counts in tumours from 125 patients with and without lymph node involvement by staining using both factor VIII and CD 31 antibodies. They found that in both node-positive and negative groups high microvessel density was an independent strong predictor of poor relapse-free survival.

In 1994, Obermair et al [21] also found that tumours with greater than ten microvessels per 1mm^2 ocular raster were associated with five year disease free survival of 43% compared with 88% for those with less than ten. Whilst lymph node metastases were more common in the tumours with higher microvessel count, the effect of microvessel density on survival appeared to be an independently significant.

Horak et al [22] found an increased number of microvessels in the majority of breast cancers compared to normal breast. This had a strong correlation with lymph node metastases. Tumours with a high vascular count (above 160 microvessels /mm^2) had an 86% incidence of lymph node metastases compared with 15% of those with a low vascular count. Indeed, vascular count appeared to be more significant than the degree of lymphatic infiltration in predicting lymph node metastases. An association was also seen between increased vascular count and poor three year survival.

In 1994, Gasparini and Weidner [23] examined a series of potential prognostic markers in node-negative breast cancer patients including size, histological characteristics, p53 and erb B expression. In a series of 254 patients, they found that high microvessel density (assessed by CD31 staining) was the best predictor of poor disease-free survival over six years of follow up.

Van Hoef et al. [24] however found no association between tumour vascularity and disease free survival in a study of 93 node-negative patients. They used a graticule giving a smaller counting field than the previous authors but otherwise very similar methods.

Hall et al in 1992 [25] also showed no association between microvessel counts and metastasis. However, although one group of thirty-seven patients in their study had been followed–up for a median 9.5 years, the remaining 50 patients had a median follow up of only 1.5 years. Furthermore, for the analysis, the authors grouped together a heterogeneous group of patients with "metastasis". These comprised those with lymph node involvement at the time of surgery and those who developed either lymph node or distant metastasis on follow-up.

In 1995, Axelsson [26] reported a large study of 220 patients with both node-positive and node-negative ductal carcinomas over a median follow up of 11.5 years. They found that obtaining consistent results for microvessel counting was difficult with considerable variation between different areas of a tumour and between different observers. They concluded that microvessel count did not predict for either relapse free or overall survival

There has been much discussion about improving the reproducibility of vascular assessment. In 1943, Chalkley [27] described a method of measuring the relative volumes of the component parts of tissues by noting which part of the tissue was superimposed on a graticule point. Hansen [28] found that Chakley counting yielded less observer variation than Weidner's microvessel counting method.

Variations of this technique have been applied to the assessment of tumour vascularity. Fox et al [29] used a technique involving superimposing a 25 point graticule on the area of apparent maximum vascularity and aligning it in such a way that the maximum number of point overlay immunostained vessels or their lumens. This removes the random nature of the point placement intrinsic to Chalkley's method. They showed a significant association between high vessel count and both poor survival and an increased likelihood of lymph node metastasis.

Vascularity can also be assessed by measurement of the cross sectional area of tumour vessels by computerised image analysis and some studies have showed that this

correlates better with prognosis breast carcinoma than microvessel counting and is less subject to observer variation [30].

1.3 Vascular Endothelial Growth Factor

In 1983 Senger [31] described a protein secreted by rodent tumour cell lines, which reversibly increased vascular permeability. He called this protein vascular permeability factor (VPF). Three years later [32], he reported that several cell lines derived from human tumours including bladder & cervical carcinomas and osteogenic sarcomas also produced this factor. He found that tumour-derived cell lines produced more of this protein than cell lines derived from non-malignant tissues.

In 1989, Napoleone Ferrara [33] described a protein called vascular endothelial growth factor (VEGF) isolated from normal pituitary follicular cells. It had selective mitogenic activity for endothelial cells. The same year, Connelly [34] discovered that VPF was also a selective endothelial mitogen, stimulating thymidine incorporation in a bovine endothelial cell line but not in a range of other connective tissue cell lines. Later in 1989, Leung, working with Ferrara, published the cDNA sequence of VEGF [35]. Almost simultaneously, Pamella Keck published the sequence of the VPF cDNA [36]. She initially commented on homologies between this sequence and the B chain of platelet derived growth factor but added during proof that the sequence of VPF was the same as the VEGF sequence newly published by Ferrara's group. It had become apparent that VPF and VEGF were products of the same gene.

VEGF is a dimeric glycoprotein composed of two disulphide bonded polypeptide subunits. Five different polypeptide variants have been discovered. These have identical N termini but showing variations in other regions. They arise by alternative splicing of the transcript from a gene with eight exons [37] to produce polypeptides with 121, 145, 165, 189 and 206 amino acids. $VEGF_{145}$ [39] has so far only been demonstrated in placenta and tumours of the female reproductive tract. The action of VEGF on endothelial cells is mediated via transmembrane tyrosine kinase receptors.

$VEGF_{121}$ does not bind to heparin. The other variants bind to heparin with an affinity that increases with molecular weight. $VEGF_{189}$ and $VEGF_{206}$ are secreted but are almost completely bound to proteoglycans containing heparan sulphate in the extracellular matrix [40].

Keyt and his colleagues at Genentec [41] discovered that plasmin cleaves a 110 amino acid fragment from $VEGF_{165}$ bound to extracellular matrix. This fragment, which is devoid of the heparin-binding domain, binds to the KDR (VEGF-II) receptor with a similar affinity to $VEGF_{121}$ and $VEGF_{165}$ in the absence of heparin. Addition of heparin however potentiates the binding of $VEGF_{165}$ to KDR by 3-4 fold. The 110 and 121 amino acid forms bind the FTL-1 (VEGF-1) receptor with up to twenty times less affinity $VEGF_{165}$ and this is not affected by heparin. The mitogenic potency of the 110 and 121 forms is over a hundred-fold less than $VEGF_{165}$.

Several growth factors and cytokines induce transcription of VEGF RNA. These include platelet-derived growth factor-BB, epidermal growth factor, tumour necrosis factor alpha, transforming growth factor $\beta 1$ and interleucin 1β [42] and it therefore appears that some agents with angiogenic activity may work via there ability to promote VEGF production.

Having observed that tumour cells in necrotic areas of gliomas appeared to express high levels of VEGF RNA, Shweiki et al [43] investigated the effect of exposing lines of benign and malignant cells to hypoxic conditions. They found that cells grown in hypoxic conditions showed upregulation of VEGF RNA, which was reversed by introducing oxygen. Subsequent studies have shown that there is both increased transcription and stabilisation of the VEGF RNA in hypoxic conditions.

Levy et al [43] showed that in rat phaeochromocytoma cells, hypoxia increased the VEGF mRNA transcription rate by three per cent but the steady state concentration of VEGF mRNA increased by twelve percent. This suggests that the most important influence on the increase is post-transcriptional stabilisation of the mRNA. They subsequently found [45] that in hypoxic conditions, the half-life of VEGF mRNA in these cells increased from 43 to 106 minutes. VEGF mRNA in common with other

unstable mRNA s has an area rich in adenine and uracil bases called A-U rich elements (AREs). In particular an AUUUA pentamer, in the 3' untranslated region appears to be important. Levy's group found that this area in VEGF formed protein-RNA complexes in hypoxia. They found [46] that in a human cell line hypoxia increased the half-life of VEGF RNA from 4.8 to 8 hours. They used transformed versions of these cells that did not express the 36KDa RNA binding protein HuR and concluded that this protein was necessary for the hypoxia-induced stabilisation of VEGF RNA. However, HuR is not overexpressed in hypoxic cells and is probably only one component of the stabilisation. Shih and Claffey [47] discovered that in hypoxic cells, heterogeneous nuclear ribonucleoprotein L (hnRNP L) formed a complex with a hypoxia stability region in the 3' untranslated region of VEGF RNA. This region was juxtaposed to the ARE region and the authors suggested that binding hnRNP L may prevent the binding proteins such as hnRNP C to this region which are believed to destabilise mRNA. They found that in hypoxic cells there is a slight increase in hnRNP L whilst cytoplasmic levels of hnRNP C fall.

Several endothelial growth factors with high levels of homology to VEGF, encoded by different genes, have recently been identified [42,48,49]. VEGF-B exists as two alternatively spliced forms with 167 and 186 amino acid subunits and can form heterodimers with VEGF. It is most abundant in heart and skeletal muscle. It appears to stimulate the release of both urokinase type plasminogen activator (uPA) and plasminogen activator inhibitor 1 by endothelial cells. Placental growth factor (PlGF) is a similar protein with angiogenic activity. Originally cloned from human placenta, it has subsequently been found in other tissues including thyroid and lung. It has two splice variants PlGF 1 (129 amino acids) and PlGF 2 (152 amino acids). PlGF also appears to be able form heterodimers with VEGF though these are less mitogenic for endothelium than VEGF homodimers. VEGF C and D show lower levels of homology (about 30%). They are found at low levels in heart and intestine and appear to be angiogenic. Cao et al [50] found that VEGF-C was a potent inducer of angiogenesis in mouse corneal micropockets and chick chorioallantoic membrane. A gene encoding a polypeptide with a 25% amino acid homology to VEGF has been found in the genome of the Orf virus. It has been called VEGF E [51]. It binds to the

Flk-1 (VEGFR-2) receptor but not to the Flt-1 (VEGFR-1) receptor. Despite having no heparin-binding region, it has similar potency in inducing endothelial proliferation and vascular permeability as $VEGF_{165}$. Salven et al [52] studied a range of human tumours including breast and skin tumours. They demonstrated VEGF B in 90% and VEGF C in about 50%

The members of the VEGF family are regulated by different stimuli [42, 49]. Growth factors such as TGF-β and cytokines increase expression of VEGF and VEGF-C mRNA but do not affect VEGF-B. Amplification of the ras oncogene, mutation of the p53 tumour suppressor gene and hypoxia both upregulate VEGF expression but not VEGF B or C

The VEGF family peptides bind to a group of three receptors called VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (FLT-4) [42, 53]. All three comprise a seven segment immunoglobulin-like domain linked to a tyrosine kinase. VEGFR 1 and 2 are found on endothelial cells whilst VEGFR-3 is found on lymphatic endothelium.

VEGFR-1 binds VEGF, VEGF-B and PlGF. VEGFR-2 binds VEGF, VEGF-C and VEGF-D whilst VEGFR-3 binds VEGF-C and VEGF-D. Cao's group [50] found that VEGF-C was capable of inducing proliferation and chemotaxis of vascular endothelial cells via both VEGFR-2 and VEGFR-3. VEGFR-3 is not normally expressed on adult vascular endothelium but has been demonstrated on a cell line derived from umbilical vein [54]. Expression of the VEGFR-3 receptor is increased in some tumours and in lymph nodes containing metastases. It has been suggested that the VEGFR-1 receptor may be important for mediating endothelial survival whilst VEGFR-2 mediates angiogenesis. A mutation that prevents VEGFR-2 expression in nude mice prevents tumour growth and this receptor appears to be essential for tumour angiogenesis. Bernatchez et al [55] studied the effect of suppressing both Flt-1 and Flk-1 by treating endothelial cells *in vitro* with antisense oligonuclotides complementary to the mRNA coding for the two receptors. They found that inhibition of Flk-1 (VEGFR-2) almost eliminated the stimulation of proliferation, migration and production of platelet activating factor (PAF) by VEGF. They had

previously demonstrated that the action of VEGF on vascular permeability was mediated through PAF synthesis. Some production of PAF was observed when endothelial cells were stimulated by PlGF, which is a specific Flt-1 (VEGFR-1) agonist. This suggested that simulation of this receptor also plays some part in the induction of vascular permeability.

Hypoxia appears to directly upregulate VEGFR-1. Increased expression of VEGF appears to lead to upregulation of both VEGFR-1 and VEGFR-2 whilst exposure of cells to TNF-α increases VEGFR-2 expression.

Yonekura et al [56] have shown that endothelial cells produce VEGF, VEGF-B, VEGF-C and PlGF creating a potential autocrine loop of endothelial stimulation.

Kaposi's sarcoma cells have been found to express both VEGFR-2 [57] and VEGFR-3 [54] and to produce both VEGF and VEGF-C. This has been shown to give rise to autocrine stimulation of tumour growth by both these members of the VEGF family. More recently Dias et al [58] showed that VEGFR-2 was present in leukaemic cells from five out of ten samples of peripheral blood from patients with acute leukaemia and two out of three leukaemic cell lines. Eight out of ten of the leukaemia cell samples also expressed VEGFR-1. All the patient samples and cell lines produced VEGF. Exogenous $VEGF_{165}$ increased leukaemic cell proliferation and migration whilst monoclonal antibody against VEGFR-2 inhibited leukaemic cells in vitro and improved survival in xenografted mice. VEFG and VEGF-C appear to act as autocrine growth factors in leukaemia.

1.4 VEGF in Cancer

Shortly after the discovery of VEGF, it was found that some tumours appeared to produce more VEGF than their tissues of origin. In 1992 two groups found that those central nervous system tumours associated with a high degree of neovascularisation and oedema such as glioblastomas, meningiomas and capillary haemangioblatomas commonly produced higher levels of VEGF than normal brain [59, 60]. Berger [61] examined VEGF expression by Northern blotting in 65 human tumours that had been xenografted into mice. Expression of VEGF varied considerably even between

tumours of the same histology with some producing high levels and others not producing any detectable VEGF. Only three samples of normal tissues were however included making valid comparisons between tumour and normal tissues difficult. Subsequently there has been great interest in the significance of VEGF expression to the behaviour and prognosis of different forms of cancer.

Following the early discovery by Brown et al [62] that VEGF was expressed by normal kidney, the same group went on to show, by *in situ* hybridisation, that 11 out of 12 renal cell carcinomas expressed high levels of VEGF [63]. They also found high levels of VEGF RNA staining in three transitional cell carcinoma of the bladder. Tominisawa et al [64] examined the significance of VEGF expression in renal cell carcinomas in more detail. They found by Northern blotting that VEGF RNA, especially the 189 splice variant, was overexpressed in the tumours compared to the surrounding tissues. Liu et al [65] examined expression of VEGF and the KDR receptor in fifteen cell lines derived from a variety of human tumour including breast, colon ovary, cervix and melanoma. All showed expression of VEGF RNA by RT PCR but the three melanoma cell lines were unusual in also showing expression of KDR (VEGFR-2) giving the potential for autocrine stimulation. They confirmed this potential by demonstrating increased proliferation of the three melanoma cell lines in response to exogenous VEGF.

It was discovered that, as well as being overexpressed in tumour tissues, VEGF could also be found in the raised levels in the serum of tumour-bearing animals and of cancer patients. In 1993, Kondo et al [66] grafted both HeLa/v5 a cell line that produces high levels of VEGF and HeLa/c, a line that does not produce VEGF into nude mice. Prior to transplantation, no VEGF could be detected in the mouse serum but after transplantation VEGF could be detected in the serum of those transplanted with HeLa/v5 by enzyme linked immunosorbent assay (ELISA). They went on to show that whilst only very low levels of VEGF were detectable in the serum of healthy subjects, significantly higher levels were found in serum derived from patients with uterine, cervical, ovarian and lung cancers.

Dirix et al [67] measured the serum levels of VEGF by ELISA in a variety of cancer patients. They compared these to the control group described by the manufacturers of the kit (R&D) but they had no local controls. Five out of fourteen patients with operable colonic cancer had serum levels above the 95th centile of the reference group. Whilst the mean serum concentration of VEGF decreased slightly with tumour resection, this was not statistically significant. They investigated patients with a variety of metastatic carcinomas, breast, colorectal, ovarian & renal, and found that 40% had serum levels above the 95th centile of the manufacturer's reference group. There most striking finding was however that 63% of patients with rapidly progressive cancer had a raised VEGF level compared to only 13% with slowly progressive disease.

The rather small differences between normal subjects and controls in this study may at least in part be explained by the discovery by Webb et al [68] that large amounts of VEGF were released into the serum when plasma clotted. The amount released varied considerably between samples. The concluded therefore that plasma rather than serum was the preferred medium for measuring circulating VEGF.

This finding was confirmed by Hyodo et al [69] who found high and variable levels of VEGF in serum from normal subjects by ELISA. They found that most patients with either gastric or colonic cancer had higher plasma levels of VEGF than their normal controls. They found that a subset of patients with metastases (38% of those with metastatic gastric cancer and 50% of those with colorectal cancer) had VEGF levels above the 95th centile of the levels in patients without metastases. This group showed shorter survival and a poorer response to chemotherapy.

Ishigami et al [70] compared the ratio of VEGF mRNA expression in colorectal cancers and adjacent bowel (T/N ratio) by Northern blotting. They found that the presence of liver or lymph node metastasis was associated with a significantly higher VEGF T/N ratio. They found that a high VEGF T/N ratio was an independent indicator of increased risk of death from tumour recurrence with a greater hazard ratio than tumour size, depth of infiltration or degree of differentiation.

Landriscina et al [71] also found that the amount of VEGF, measured by ELISA, was higher in colorectal cancers than uninvolved mucosa. The same was not true of the angiogenic factor bFGF although raised levels of both factors were found in mesenteric blood at the time of surgery.

Uchida et al [72] found that 60% of oesophageal cancers showed immunohistochemical staining for VEGF. There was a significant association of VEGF positivity with both advanced tumour stage and short survival.

1.5 Therapeutic Implications of VEGF in Cancer

Several clinical trials of anti-angiogenic drugs are in progress [73]. The main strategies currently being investigated are as follows.

- Blockage of endothelial cell proteases preventing breakdown of the surrounding matrix.
- Direct inhibition of endothelial cells
- Blockage of the activities of specific factors that stimulate angiogenesis
- Blockage of the action of integrin on the endothelial cell surface

Given the importance of VEGF in angiogenesis, it is not surprising that attention has been given to agents that may block VEGF action. Several VEGF receptor inhibitors have been developed. SU5416 [74] is a 3-substituted indolinone that binds to VEGFR-2. It is currently undergoing phase III trials for metastatic colorectal cancer and in phase I and II trials [75] for many other tumours including advanced breast cancer. A similar substance, SU6668 [74], inhibits VEGFR-2 and also the PDGF and FGF receptors. It is currently undergoing phase I clinical trials. Several other receptor inhibitors have recently been developed including a series of substituted 4-anilinoquinazolines which are potent inhibitors of both VEGFR-1 and VEGFR-2 that are active by oral administration [74]. Piossek et al [76] described a peptide derived from the third Ig-like domain of VEGFR-2 that blocked binding of $VEGF_{165}$.

Monoclonal antibody against VEGF is currently undergoing phase I trials in refractory solid tumours and has reached phase II trials in metastatic renal cell carcinoma. Antibodies against the VEGF-2 receptor have also been developed.

1.6 VEGF in Breast Cancer

In 1994, Toi and his colleagues at Tokyo Komagone Hospital [77] investigated whether there was a link between the emerging evidence of that increased microvessel density was an indicator of poor prognosis and the expression by these tumours of VEGF. They carried out immunoperoxidase staining of breast cancer sections using FVIII related antibody and an anti-VEGF monoclonal antibody developed by Genentec. They graded VEGF staining as negative, moderate or high. Their initial study of 103 primary breast cancer patients with 51 months follow up confirmed that microvessel density correlated inversely with relapse-free survival. They showed a significant link between VEGF and microvessel density and found that VEGF expression had an independent prognostic significance for disease-free survival on multivariant analysis. The following year, the same group published a larger study [78], using the same methodology, involving 328 patients with 56 months follow up. They still found that microvessel density was an independent predictor of poor relapse-free survival. Whilst there was still a significant association between VEGF expression and poor relapse-free survival on univariant analysis, this did not prove to be independent on multivariant analysis.

In 1995 Brown et al [79] reported their results of *in situ* hybridisation in breast tumours using a riboprobe to the common region of VEGF mRNA. They found that normal breast tissues from both reduction mammoplasties and breasts which had contained tumours stained weakly while 12 specimens of ductal carcinomas and 4 specimens of comedo ductal carcinoma in situ (DCIS) stained strongly. Two specimens of lobular carcinoma did not show any staining. They also found that endothelial cells adjacent to areas of strong VEGF expression in both tumours and DCIS stained strongly for both the KDR (VEGFR-1) and ftl-1 (VEGFR-2) receptors.

In 1996, Anan et al [80] found by RT- PCR that $VEGF_{121}$ and $VEGF_{165}$ were both expressed in only 17% of benign breast samples whilst $VEGF_{121}$ was expressed in 84% of breast tumours and $VEGF_{165}$ in 78%. TGF-α and bFGF were expressed in almost all of the samples of both tumour and benign breast. They also found significantly more VEGF expression in the group of tumours with a high microvessel count than in the group with a low count.

By 1996 [81] Toy and the Tokyo group had succeeded in measuring VEGF protein levels in breast tumours by ELISA. They showed a considerable variation in protein levels in the 135 breast cancers examined. These ranged from 3.3 to 2032 pg per mg of protein. There was a significant correlation between the intratumoural VEGF concentration and the microvessel density in the tumours.

The following year saw the start of a rush of publications looking at VEGF in breast cancer. Gasparini [82] in Vincenza collaborated with the Tokyo group in studying VEGF protein concentrations in cytosolic extracts from tumours obtained from 260 women with node-negative breast cancer who had a median period of follow up of 66 months. Postoperative radiotherapy was given where appropriate but they received no other adjuvant therapy. Again, there was a considerable range of VEGF levels from 5 to 6523pg per mg of protein. They found that, on multivariant analysis, there was a significant association between VEGF levels and both disease free and overall survival. This was independent of other factors such as age, menopausal status, tumour size, histological type or hormone receptor status. The same year, in Oxford, Relf [83] described expression of a range of angiogenic factors, including VEGF in 35 node-negative and 29 node-positive tumours. VEGF expression was measured using an RNAase protection assay. High VEGF expression correlated with a high risk of tumour recurrence on univariant analysis.

Obermair [84] in Vienna studied 89 patients with breast cancer (28 of which were node-positive) treated by either mastectomy or local excision. Patients undergoing local excision had postoperative radiotherapy. Oestrogen or progesterone receptor positive patients received tamoxifen whilst receptor-negative patients received CMF

chemotherapy. Three patients developed local recurrences and fourteen developed distant recurrences. They measured VEGF in tumour cytosols using the same ELISA technique used by the previous authors. Whilst confirming the previously described association between high VEGF concentrations and microvessel density, they failed to find a significant association between VEGF and relapse-free survival.

Two papers published the same year suggested that VEGF might be important in the early development of breast tumours. Guidi et al [85] found that the tumour cells in ductal carcinoma *in situ* of the breast stained more strongly than the surrounding breast epithelium by *in situ* hybridisation using a probe for VEGF mRNA. Yoshiji et al [86] at the National Cancer Institute reported a model system using subcutaneously implanted T47D tumour cells with VEGF expression controlled by a tetracycline regulated retroviral vector system. They found that suppression of VEGF production strongly inhibited tumour growth in small tumours but did not do so once the tumours were large. In the larger tumours, basic fibroblast growth factor (bFGF) and transforming growth factor alpha (TGFα) appeared to be able to take over from VEGF

Anan's group [87, 88] described the use of a semi-quantitative RT-PCR based assay to measure VEGF in breast cancers compared to actin, which was amplified in separate reactions. PCR products were measured by Southern Blotting or computer analysis of gel photographs. They found a close correlation between VEGF expression measured in fine needle aspirates from tumours with the expression measured in the tumours after resection. There was a significant correlation between vascular counts and VEGF expression in forty eight ductal carcinomas but not in the small group of non-ductal (mucinous, medullary and lobular) carcinomas.

In 1998 two groups simultaneously published studies looking at the significance of VEGF in node-negative breast cancer. Linderholm [89] studied 525 consecutive patients of whom 500 received no systemic treatment. Cytosolic $VEGF_{165}$ protein was measured by ELISA. VEGF was a significant indicator of poor overall survival both when analysed as a continuous variable or divided into groups above and below

the median value of 2.40 pg/μg of DNA. The relative risk of a cytosolic VEGF higher than the mean was greater than that of tumour size, grade, type or hormone receptor status. This was also the case when analysis was limited to the patients who did not receive any systemic treatment or to the patients who were oestrogen receptor positive. Eppenberger [90] described the analysis of VEGF, bFGF, angiogenin, urokinase type plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1) in two patient populations. One was a heterogeneous set of 305 patients, both node-negative and positive, from their own institution undergoing surgery between 1992-93 with a median follow up of 36 months. The second population consisted of a group of node-negative patients operated on in another institution between 1978 and 1988 who had received no adjuvant treatment. This group had a median follow up of 76 months. Cytosolic VEGF protein levels were measured using a chemiluminescence immunosorbent assay. They grouped the tumours into those above and below the first quartile of VEGF protein levels and found that the group with higher levels of VEGF had a significantly higher chance of disease recurrence in both populations.

Lee at Guys hospital [91] used in situ hybridisation to study the proportion of tumour cells staining for VEGF in ductal and lobular carcinomas and correlated this with the microvessel count from the three most vascular fields in the tumour. They confirmed the results reported by Brown three years earlier that VEGF was expressed more strongly in ductal than lobular cancers. Nine out of eleven ductal cancers showed moderate or strong expression whilst the remaining two showed weak expression. Eight out of nine lobular cancers showed absent or weak expression with one showing moderate expression. As in the previous study, the majority of tissue samples of ductal carcinoma in situ showed strong or moderate staining. These results were confirmed by the staining patterns for VEGF on the sections by immunohistochemistry with both monoclonal and polyclonal antibodies. Microvessel density was slightly higher in the ductal carcinomas than the lobular carcinomas. Correlation was seen between VEGF expression and microvessel density in the ductal but not in the lobular carcinomas. The authors conclude that VEGF is an important

mediator of angiogenesis in ductal carcinoma but that angiogenesis may be mediated by other factors in lobular carcinoma.

Given the emerging evidence regarding the importance of VEGF, Heer et al in Hull [92] looked at serum VEGF concentrations throughout the menstrual cycle and speculated on the implications of these for the timing of surgery. They measured serum VEGF by ELISA in 14 healthy premenopausal subjects along with FSH oestodiol and progesterone. The use of measurements in serum is questionable in the light of previously described work [68, 69]. They found that VEGF concentrations were significantly lower in the luteal phase and showed a negative correlation with progesterone. They exposed the MCF7 breast cancer cell line to luteal phase serum from one subject and found a reduction in VEGF production. This however contrasts with the work of Hyder [93] earlier the same year. He found that progesterone in physiological concentrations caused an increase in up to four fold in VEGF production in the T47D cell line, which is a strong expresser of the progesterone receptor. Progesterone did not have any effect on VEGF production in several other cell lines, including MCF7, that show lower levels of progesterone receptor expression. The significance of background VEGF production rather than tumour production is not known and the significance of varying serum levels in health subjects must be regarded as speculative A subsequent study by McIlhenny in Glasgow did not show any variation of VEGF with the menstrual cycle in health volunteers and no correlation with FSH or LH levels [121].

Linderholm et al published a further paper in 1999 [94] looking at a homogeneous group of T1-2 node-negative tumours treated by local excision with radiotherapy but no systemic treatment. They studied 302 patients with a median follow up of 56 months of whom 43 developed recurrent disease. Cytosolic VEGF was measured by the same ELISA method that they had used in their previous studies. Again, they found that patient with high VEGF levels had significantly shorter relapse free and overall survival. The relative risk of high VEGF expression was even more pronounced when analysis was confined to the smaller T1 tumours. The authors

suggest that in the tumours that would normally have a good prognosis, high VEGF expression was negating the protection normally conferred by radiotherapy. Therefore, high VEGF expression might be considered as a risk factor indicating the need for systemic therapy when this might otherwise not be deemed necessary.

The same year Gasparini's group [95] published a study of cytosolic VEGF protein concentration in two groups of node-positive patients. One group of 137 patients were treated with adjuvant CMF chemotherapy whilst the other group of 164 were treated with tamoxifen, with a median follow-up of 72 months in both groups. Cytosolic VEGF concentration proved to be a significant indicator of poor relapse-free and overall survival on univariant analysis and a significant independent adverse prognostic indicator for both recurrence and survival on multivariant analysis.

Balsari and collaborators in Milan [96] published an analysis of 36 heterogeneous patients with stage 1-3 breast cancer. They assessed VEGF production by a subjective assessment of immunohistochemical staining. Their results supported the association between VEGF production and microvessel density. They did not find a significant correlation between serum VEGF concentrations and either VEGF staining in the tumours or microvessel density. This is, however, not surprising in view of the previous studies showing the unreliable nature of serum measurements.

Two papers published in 1999 looked at hormonal influences in VEGF production. Ruohala et al [97] looked at the influence on expression of the VEGF family of growth factors of exposing breast cancer cell lines to oestrodiol and antioestrogens *in vitro.* They measured VEGF RNA by Northern blotting and VEGF protein by ELISA. They found that oestodiol promoted production & stability of VEGF mRNA and increased VEGF protein production in an oestrogen-sensitive MCF7 cell line. Whilst one oestrogen receptor blocker abolished this effect, tamoxifen and toremifene induced VEGF mRNA expression. VEGF-B levels were not affected by the addition of oestodiol whilst those of VEGF-C were reduced. Greb et al [98] attempted to look at the effect of ovarian hormones on VEGF mRNA in human breast *in vivo* by comparing tissue samples of both neoplastic and adjacent non-neoplastic breast from premenopausal and postmenopausal women. They used a quantitative RT-PCR based

assay using GAPDH primers in the same tube as the VEGF reaction. They found that in the non-neoplastic tissues VEGF mRNA expression decreased with age and was significantly lower in the postmenopausal women. There was however, no significant difference in VEGF mRNA expression in the tumour samples from the premenopausal and postmenopausal women. Indeed there was a slight, though not significant, increase in tumour VEGF mRNA levels with age. Of the nineteen women studied, 15 had oestrogen receptor positive tumours and 14 had progesterone receptor positive tumours. The assumption that the decrease of VEGF mRNA in the non-neoplastic tissues in this study is hormonally mediated is unproven. It could be hypothesised that it was due to some other non-hormonal effect of tissue ageing. The authors also admit that the results in tumours may be questionable since GAPDH may be upregulated in some breast tumours.

The effects of tamoxifen on VEGF production were explored further the following year by Adams et al. [99]. They confirmed the previous work regarding the advantage of plasma over serum measurements. Neither of these measures however correlated with an assessment of VEGF immunostaining on tumour tissue sections. Plasma levels of VEGF were higher in patients being treated with tamoxifen. Since the tumours had been removed however, the source of VEGF was unknown and the authors suggested it may arise form uterine tissue. Oestrogen receptor expression in the tumours was associated with stronger VEGF immunostaining.

Linderholm's [100] group added to their previous work on node-negative tumours with a study examining VEGF in a series of 362 node-positive tumours. They measured cytosolic VEGF protein by ELISA as in their previous study. They did not find a significant difference in VEGF concentration between these node positive patients and node negative series they had previously studied [94]. In contrast to Adams [99], they found significant inverse association between oestrogen receptor and VEGF expression. As with the node-negative patients, high VEGF expression was an independent predictor of poor overall survival and a predictor of poor relapse-free survival. High VEGF expression predicted poor overall survival in patients receiving both endocrine therapy and chemotherapy. It predicted poor relapse-free

survival in patients receiving endocrine therapy but not in those receiving chemotherapy. This group has published a further study [101] examining the relationship between mutation in the p53 tumour suppresser gene and VEGF expression in 485 node-negative and 348 node-positive patients with breast cancer. They used a commercial ELISA kit that claimed to detect mutant but not wild type p53 and measured cytosolic VEGF by ELISA as in their previous studies. They found a statistically significant association between expression of mutant p53 and high VEGF expression. Sixty four percent of patients with mutant p53 had increased VEGF expression whereas only 43 percent of patients without mutant p53 had raised VEGF concentrations. Both raised VEGF and mutant p53 expression predicted poor relapse-free and overall survival except in a small subgroup of 25 high-risk patients receiving CMF chemotherapy. It is not clear whether the failure of VEFG to be a significant predictor of outcome in this group was due to the chemotherapy or purely a reflection of the small size of this subgroup.

The role of other members of other members of the VEGF family in breast cancer has been investigated. Gunningham et al [102] found that whilst there was no significant difference in VEGF-B expression between normal breast and breast tumours, there was a significant association between expression of this protein and the number of involved lymph nodes in breast cancers.

Evidence has begun to emerge that VEGF is probably an important predictor of a group of patients at high risk of tumour relapse. The ability to identify these patients allows the possibility for intervention. This may take the form either of more aggressive use of adjuvant therapy than would normally be indicated from conventional prognostic indicators such as node status or the employment of specific anti-angiogenic agents as described. It remains to be seen whether the ability to identify this group of patients will ultimately improve their outcome.

1.7 Purpose of the Present Study

When this study was commenced, VEGF expression had been demonstrated in a small number of tissues using immunohistochemistry or in-situ hybridisation. There had

been no published accounts of VEGF expression in breast tumours. As has been described, there was evidence that the amount of neovascularisation within breast tumours may be of considerable prognostic importance. Estimation of neovascularisation by examination of histological sections is a laborious process subject to observer variation and requiring fairly large tissue samples. A molecular marker of the angiogenic potential of the tissue would therefore be valuable. The evidence which had emerged in the proceeding few years suggested that VEGF had great potential as such a marker.

A technique for measuring VEGF which was rapidly quantifiable was needed. It was intended that this technique could be used with small samples of archival breast tumours. If VEGF proved to be of prognostic significance that the technique could be applied to very small biopsy samples, and fine needle aspirates. A fundamental requirement of the assay technique was that it should be provide sensitive detection of VEGF in the smallest possible samples.

The more established techniques of *in situ* hybridisation and immunohistochemistry were explored, primarily because these had the advantage of allowing localisation of VEGF production in the case of *in situ* hybridisation and tissues localisation of the protein with immunohistochemistry. The main part of this work however consisted of the development of an assay for VEGF based on the technique of the polymerase chain reaction. Intrinsic to this technique is the amplification of the nucleic acid species being investigated making it extremely sensitive and therefore ideal for the intended purpose. The emergence in the last few years evidence of the importance of VEGF to the behaviour of breast tumours makes the existence of a sensitive assay, usable on very small amounts of tissue increasingly important.

The first part of this work describes the production of a riboprobe based on the published sequence of VEGF RNA and attempts to use it for *in situ* hybridisation. Attempts to carry out immunohistochemisty using some antibodies which had recently been produced are also described. These techniques yielded little success.

Discussions with workers in other laboratories confirmed that the available antibodies were not ideal for immunohistochemistry. Since these techniques are difficult to quantify and require larger samples of tissue, the PCR based technique had greater potential for yielding an assay suitable for the intended purposes stated above, the focus of the work moved to this assay. The development and optimisation of this assay and it use in a pilot study to determine the relationship between the amount of VEGF mRNA in archival tumours and the clinico-pathological behaviour of those tumours is described in the subsequent sections of the work.

Chapter 2. Cloning of VEGF Sequence into an Expression Vector

2.1 Introduction

The aim of this part of the work was to clone a section of DNA derived from the VEGF cDNA sequence. A section was chosen from the first exon of the VEGF gene, which is common to all splice variants. By splicing this sequence into an expression vector, it could then be used as a template to produce RNA molecules with a sequence complimentary to the VEGF RNA. These could be used as a probe to detect VEGF RNA by hybridisation.

2.2 Methods

2.2.1 Reverse Transcription Reaction.

RNA that had been purified from phorbol-activated HL60 cells was used. This was diluted in DEPC treated water and the optical density of the solution determined at 260nm on a Beckman DU-600 spectrometer. The RNA solution was then further diluted to a final concentration of 1µg/µl.

Reverse transcription was carried out using the Perkins Elmer RT-PCR kit. One microgram of HL60 RNA was mixed with PCR reaction buffer, 4µl of 25mM $MgCl_2$, 20 units of RNAase inhibitor, 8µl of 2.5mM dNTPs, either 100ng of VEGF 3' oligonucleotide or 5nmol random hexomers and 50u of MuLu reverse transcriptase in a final volume of 20µl. A negative control was set up as above with random hexomers but no reverse transcriptase.

The reaction mixes were incubated at room temperature for ten minutes then at 42°C for forty five minutes. They were then denatured at 99°C for five minutes and cooled on ice.

2.2.2 Polymerase Chain Reaction.

Primers which amplify a 330 base pair segment of cDNA complementary to part of the first exon of VEGF mRNA, common to all five splice variants, were designed with the help of Dr Richard Lamb, Beatson Institute, Glasgow and were synthesised by Cruachem, Glasgow. They include a terminal Eco R1 endonuclease restriction site to facilitate plasmid ligation.

Primers were as follows:

5' CGG GAA TTC CCT TGC TGC TCT ACC TCC ACC AT
3' CCG GAA TCC GAA GCT CAT CTC TCC TAT GTG CT

The polymerase chain reaction was carried out using the Perkins Elmer RT-PCR kit. A further 4µl of 25mM $MgCl_2$ together with PCR buffer and water to a volume of 77µl was added to the products of each of the four reverse transcription reaction. 100ng of VEGF or CD44 3' and 5'oligo was added to the random hexomer VEGF and positive control reactions respectively and to the -RT negative control. Two and a half units of Taq polymerase were then added to each reaction mix in a final volume of 80µl. The reaction mix was overlaid with 20µl of mineral oil to prevent evaporation

The mixtures were denatured at 95°C for five minutes, annealed at 55°C for five minutes and extended at 73°C for fifteen minutes. They were then subjected to 30 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute and extension at 73°C for five minutes.

2.2.3 Gel Electrophoresis

One hundred millilitres of 1.2% solution of agarose in Tris-borate buffer (TBE) (0.045M Tris-borate, 0.001M EDTA) was prepared and heated in a microwave oven to dissolve the agarose. The solution was allowed to cool to 60°C. One microlitre of

ethidium bromide at 10mg/ml was added and the solution poured into an 8x10cm gel mould. The gel was allowed to set for forty five minutes then placed in a tank containing TBE buffer with ethidium bromide added.

Ten microlitres from each PCR reaction was mixed with 2μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and loaded on the gel beside molecular weight markers. The gel was run at 100v for two hours.

2.2.4 Five Prime End Labelling of Oligo Probe.

A further oligonucleotide was created complementary to an area in the centre of the sequence that should be generated by the PCR reaction above. This was synthesised by Cruachem, Glasgow.

It had the following sequence:

GGCTGCTGCAATGACGAGGGCCTG

It was used to produce a probe to confirm the identity of the PCR product prior to cloning. End labelling of this oligonucleotide with γ^{32}P dATP was carried out using the enzyme T4 Polynucleotide Kinase (PNK). A 20μl reaction was set up containing 200ng of the internal oligonucleotide, 2μl of 10x PNK buffer, 2μl of 100mM DTT, 3μl of [γ^{32}P] dATP with an activity of 10μCi/μl and 1μl of T4PNK. This mixture was incubated at 37°C for one hour. The reaction was then stopped and the DNA precipitated by addition of 1μl of 0.25M EDTA, 1μl of 10μg/μl glycogen, 10μl of 7.5M ammonium acetate and 100μl of ethanol. This mixture was stored at –70°C for fifteen minutes and centrifuged at 14,000rpm for fifteen minutes at 4°C. The resulting pellet was washed with 70% alcohol and re-suspended in 100μl of water. The activity of the resulting labelled probe was determined by scintillation counting.

2.2.5 Southern Blotting and Hybridisation.

The gel was blotted overnight onto a Hybond N+ filter using 0.4M NaOH. The filter was then washed with 2x SSC.

Prehybridisation buffer was prepared. This was composed of 6x SSC salt solution (sodium chloride / sodium citrate), 10x Denhard's reagent (Ficoll, Polyvinyl pyrollidone and bovine serum albumin), 0.5% sodium dodecyl sulphate (SDS) and denatured salmon sperm DNA (50µg/ml). The membrane was sealed in a plastic bag with pre-hybridisation buffer and incubated for one hour in a 65°C shaking water bath. The radio-labelled oligonucleotide probe was then added to the buffer and incubated overnight in a 65°C shaking water bath.

The filter was then washed in 6x SSC, 0.1% SDS for fifteen minutes at room temperature then at 65°C for one hour. The periphery of the filter was checked with a Geiger counter to ensure that the washing was adequate (<10cpm). The membrane was resealed in a bag and placed in a radiographic cassette with X ray film overnight at -70°C. The film was then processed using an automated x-ray film processor.

2.2.6 ECO R1 Digests

The PCR product was purified by extracting twice with phenol and once with chloroform. It was then precipitated with sodium acetate and alcohol at –70°C and washed with 70% alcohol. The product was re-suspended in 30µl of water. Prior to ligation of the product into a plasmid vector, the cloning sites on the plasmid and insert must be exposed by endonuclease digestion.

An ECO R1 digest was set up with 30µl of the PCR product, React 3 buffer, 25nMol spermidine and 20 units of EcoR1 in 50µl of water. A further digest was set up with 1µg of bluescript SK plasmid in the same way. Both mixtures were incubated at 37°C overnight. Ten microlitres of each digest were then mixed with 2µl of loading buffer and run on a TBE-agarose gel containing ethidium bromide as previously described.

2.2.7 Plasmid Ligation.

Using the gel to estimate the relative concentration of PCR product, a ligation mix was set up containing an approximately three-fold excess of insert (PCR product) over plasmid. In order to prevent ligation without an insert, phosphatase-treated bluescript was used. Plasmid and insert were combined in a solution of ligation buffer, 50mMol rapt and 2U of T4 DNA ligase in a total volume of 50µl.

Three control ligation mixes were set up. The first contained bluescript that had been ECO R1 digested but not phosphatase-treated. No insert was added. This was intended to test the efficiency of the ligase enzyme. The second control contained phosphatase-treated bluescript but no insert to test the extent of self-ligation of the plasmid. The third contained phosphatase-treated plasmid and a commercial test insert with ECO R1 ends with a known high ligation rate. The mixtures were incubated at 12°C for eighteen hours.

2.2.8 Preparation of Competent E. Coil.

An overnight culture of XL-1 E. coli was prepared. One millilitre of this culture was added to 100 ml of L-Broth. This was then incubated at 37°C in a shaking incubator. The bacteria were pelleted by centrifugation at 560g for ten minutes. The bacteria were then re-suspended in 20ml of 10mM $MgSO_4$ (10ml in each of two 50ml Falcon tubes) and kept on ice for five minutes. This solution was then centrifuged as before. The supernatant was discarded and the bacteria re-suspended in 20ml of 50mM $CaCl_2$ (10ml per tube). This mixture was kept on ice for thirty minutes then centrifuged as before. The pellets from each tube were re-suspended in 1ml of 50mM $CaCl_2$ and combined.

2.2.9 Transformation of E. coli

Falcon tubes (Type2059) were cooled on ice. One hundred microlitres of either XL-1 bacteria rendered competent by the above method or DH5α competent bacteria were placed in each tube. Five microlitres of each ligation mix was added to each tube. Two further controls were also set up, one with intact, unligated bluescript plasmid added to the bacteria and one with bacteria alone. The mixtures were kept on ice for one hour. They were then heat shocked for forty five seconds (DH5α) or two minutes (XL-1) and placed back on ice. Nine hundred microlitres of L-broth was added to each tube and they were incubated at 37°C in a shaking incubator for one hour.

Nine hundred microlitres of L-Broth bacterial culture medium was added to each tube. The tubes were placed in a shaking incubator at 37°C for one hour.

The bluescript plasmid cloning vector confers ampicillin resistance on host bacteria. Therefore transfected bacteria can be selected for using ampicillin-containing media. The cloning site of the bluescript plasmid lies within the β-galactosidase gene. Therefore successful ligation of a DNA segment into this plasmid will inactivate this enzyme. If transformed bacteria are cultured in the presence of the lac operon inducer homologue, IPTG, and the chromogenic substrate, x-gal, cells transfected with plasmids which have not undergone ligation at the multiple cloning site will produce a blue colour change.

One and a half grams of bacterial grade agar (Bactagar) was mixed with 100ml of L-Broth and autoclaved. Ampicillin was added to a concentration of 50μg/ml along with IPTG and x-gal. Agar plates were poured and allowed to set. 100μl of each of the liquid cultures described above was smeared onto separate agar plates. Plates were then cultured overnight at 37°C. The blue and white colonies on each plate were counted and the white colonies harvested for characterisation.

2.2.10 Preparation of Master Clone Plates

Agar plates containing L-Broth, ampicillin, X-Gal and IPTG were prepared as above. The plates were laid on a colony identification grid and an orientation mark made. Bacterial clones were transferred to the master plate by picking up some of the original colony with the tip of an autoclaved wooden toothpick. This was stabbed several times into the agar plate overlying a single numbered square on the grid and then the square of the same number on a replicate plate. Two non-recombinant blue colonies were transferred onto each plate in this way to act as controls and the remainder of the grid squares inoculated with white colonies. A total of 100 colonies were transferred onto these plates.

2.2.11 Identification of Recombinant Clones by PCR

Colonies were screened for inserts using a technique described by Kilger and Schmid [120]. Part of each white colony believed to contain an insert was picked up with a sterile toothpick. The bacteria were then smeared into a PCR tube and the toothpick used to inoculate a new agar plate, which was placed on a colony grid so that its position could be identified. The PCR tubes were microwaved for two minutes at 600W to rupture the bacteria. PCR master mix containing the 3' and 5' PCR primers described above was added to each tube and the PCR reaction carried out as before. Products were electrophoresed on an agarose gel and the presence of product confirmed by ethidium bromide fluorescence. For further confirmation of the identity of the product, Southern blotting and hybridisation with the radio-labelled internal oligonucleotide was carried out as before.

2.2.12 Mini Plasmid Preparation.

Smears were taken with a cocktail stick from eighteen colonies that did not show any blue colouration on the master plates. Each stick was placed in 5ml of L Broth containing 50µg/ml Ampicillin and incubated overnight.

One and a half millilitres of each culture was spun in a microcentrifuge tube for fifteen seconds to pellet the bacteria. The pellet was then re-suspended in 100µl of a solution of 50mM glucose, 25mM Tris pH8 and 10mM EDTA pH7.5. 200µl of 0.2M NaOH + 1% SDS was added. The tubes were gently mixed and placed on ice for five minutes. One hundred and fifty microlitres of cold 5M potassium acetate was then added to each tube and they were placed on ice for a further five minutes to precipitate the bacterial protein. This protein was then pelleted by spinning the tubes for one minute at 14 000 rpm in an Ependorph microcentrifuge. The supernatant formed was mixed with an equal volume of 1:1 phenol/chloroform, centrifuged for five minutes at 14 000 rpm and then the top layer preserved. Boiled RNAase was added to a final concentration of 10µg/ml and incubated for thirty minutes at room temperature. A quantity of 7.5M ammonium acetate solution equal to half the residual volume was added and the tubes placed on ice for twenty minutes before being centrifuged at 14000 rpm for two minutes. The supernatant was then mixed with twice its volume of ethanol and precipitated at room temperature for ten minutes. The precipitate was pelleted by centrifugation for five minutes. The pellet was washed with 70% ethanol, dried in a lyophyliser and re-suspended in 50µl of TE buffer.

Fifteen microlitres of each sample were then digested overnight with ECO R1 as before. Ten microlitres of each digest were then run on a 1% agarose-TBE gel, blotted onto Hybond N+ membrane and hybridised with 5' end labelled VEGF internal oligonucleotide as described above.

2.2.13 Further Digest of Restriction Sites within the Bluescript Multiple Cloning Site

The remaining 35μl of each mini-plasmid preparation was mixed with 4.5μl of react 4 buffer, 2 μl of 100mmol spermidine, 12 U of KPN I endonuclease and 8 U of SMA I endonuclease. These mixtures were incubated overnight at 37°C. The solutions were then frozen on dry ice and lyophilised. The solute was re-suspended in 10μl of water, run on a 1% agarose-TBE gel, blotted onto Hybond N+ membrane and hybridised with 5' end labelled VEGF internal oligonucleotide as before.

2.2.14 Large scale plasmid preparation

Five hundred millilitre cultures of recombinant DH5α bacteria were grown by inoculating either L-Broth or terrific broth with 1ml of small-scale liquid culture in the presence of ampicillin (50μg/l) and incubating overnight in a 37°C shaking incubator. The bacteria were sedimented by centrifugation at 5000 rpm (4°C) for ten minutes. The medium was drained and the bacteria re-suspended in 25 ml of ice-cold buffer containing 50mM glucose, 25mM Tris (pH8), 10mM EDTA and 5mg/ml lysosyme. 40ml of alkaline SDS (0.2M NaOH + 1% SDS) was added and the mixture kept on ice for five minutes. Twenty millilitres of potassium acetate solution (2.5M potassium acetate, 5M acetic acid (pH=4.8)) were then added and the mixture kept on ice for fifteen minutes. The flocculate formed was sedimented by centrifugation at 8,000 rpm for five minutes. The supernatant was strained through gauze and a volume of isopropanol equal to 0.6x the collected volume added. This was left at room temperature for five minutes to precipitate nucleic acids and then centrifuged at 8,000 rpm for five minutes. The supernatant was drained and the pellet re-suspended in TE buffer (pH8) (5ml of TE per 500ml of culture). Caesium chloride (1.5g/ml) and Ethidium Bromide (0.1 ml of 10mg/ml solution per ml of TE) were added and the refractive index adjusted to1.395. After two unsuccessful attempts at density gradient separation a further centrifugation at 10,000 rpm for ten minutes was added to remove the precipitate formed after addition of ethidium bromide. The mixture was

then transferred to ultracentrifuge tubes and centrifuged for forty hours at 40 000 rpm in an ultracentrifuge (Beckman).

Under UV illumination the lowest of the fluorescent bands in the tube was aspirated. The ethidium bromide was removed by five extractions with isopropanol, the volume of the aqueous phase being restored between each extraction with TE buffer.

Caesium chloride was removed by placing the solution in collodium tubes and dialysing against TE buffer in a stirred bath at 4°C for twenty four hours with regular changes of buffer. The optical density at 260nm and 280nm was measured on a spectrophotometer.

2.2.15 Sequencing of Product

Sequencing gels were first prepared. Two glass plates were washed with soap then alcohol. One plate was siliconised on one side with Sigmacote. The plates were then assembled with 0.4 mm spacers between. The plates were then taped and clamped together.

Eight percent polyacrilamide sequencing gel was prepared. This contained 21g of urea, 21ml of water, 5ml of 10x TBE buffer, 10ml of 40% acrilamide/2% bis solution and 250μg of ammonium persulphate. Immediately before pouring, 24μl of TEMED was added to commence polymerisation. The gel was then poured and a comb inserted to create wells.

Four micrograms of the DNA prepared in the large-scale plasmid preparation was used. This was denatured by addition of 2μl 2M NaOH/ 2mM EDTA and incubation at 37°C for twenty minutes. Three microlitres of ammonium acetate (pH4.5) were then added followed by 100μl of ethanol and the mixture placed at –20°C overnight to precipitate. The DNA was pelleted by spinning for fifteen minute spin in a refrigerated microcentrifuge and the pellet washed with 70% alcohol.

Sequencing was carried out using the Sequenase version 2.0 DNA sequencing kit. (United States Biochemical). The DNA pellet was re-suspended in 7µl of water. Two microlitres of sequencing buffer and 1µl of sequencing primer were then added. The mixture was heated to 65°C for two minutes and allowed to cool slowly over thirty minutes to allow primer annealing. It was then placed on ice. Labelling reactions were then set up by adding 1µl of 0.1M dithiothreitol (DTT), 2µl of labelling mix (diluted 1:5), 0.5µl of [^{35}S]dATP and 2µl of Sequenase DNA polymerase (diluted 1:8 by mixing 1ml with 0.5µl of pyrophosphatase and 6.5 µl of enzyme dilution buffer). Four termination tubes were set up which contained all four deoxynucleotides and in different tubes one nucleotide as the dideoxynucleotide (ddNTP) analogue. This terminates the polymerase reaction at the incorporation sites of that ddNTP creating a range of different length products all terminating at the same base in each tube. After running the labelling reaction for three minutes at room temperature to allow incorporation of the radio-labelled dATP into the DNA chains, 3.5ml of labelling mix was mixed with 2.5ml of each of the four termination mixtures. These tubes were incubated at 37°C for five minutes before the addition of 4µl of sequenase stop solution (95% formamide, 20mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanol FF) to each.

These reactions were then repeated using the reverse primer that creates chains of the opposite sense reading from the other side of the multiple cloning sites.

The sequencing gel was placed in a gel tank with 1X TBE in the buffer chambers. A power supply was connected giving a constant power of 55 watts at 2000 volts. After pre running for thirty minutes, the samples were loaded onto the gel and run until the dye front approached the bottom of the gel.

The siliconised plate was then removed leaving the gel adhering to the other glass plate. The gel was then washed in 10% acetic acid for ten minutes. The gel was then removed from the glass plate onto a sheet of blotting paper, covered with cling film and dried in a vacuum gel dryer. It was then placed in an x-ray cassette overnight

with a sheet of fast and slow X ray film. These films were developed in an automatic processor. The sequence of the insert was read from the plates.

2.2.16 Preservation of Cloned Lines

Lines were preserved in two ways. Stab cultures were made by impregnating the bacteria into vials of L Agar with a needle. Glycerol stocks were prepared by freezing the bacteria to –70°C in glycerol.

2.3 Results

2.3.1 RT-PCR

Gel electrophoresis of the RT-PCR products showed a band with the sample in which 3' oligonucleotide had been used in the reverse transcription reaction. Blotting and hybridisation with ^{32}P end labelled oligonucleotide complementary to the expected sequence of the central region of the PCR product produced a strong signal in the position of the band thought to represent the VEGF PCR product.

2.3.2 Plasmid Ligation and Bacterial Transformation

An ECO R1 digest of the PCR product was carried out to produce single stranded ends. Bluescript plasmid was digested as a control. Gel electrophoresis of the digested PCR product, the bluescript control and a sample of pre-digested phosphorolased bluescript was carried out. This revealed a bands of corresponding to the expected molecular weights. Ligation of the PCR product into pre-phosphorolased bluescript plasmid and transformation of competent XL-1 E. coli was carried out as described.

Whilst a low incidence of recombinant colonies was seen with the test insert, none were seen with the PCR product.

It appeared that the ligation efficiency was lower with the PCR product than with test insert. Possible explanations were that the ECO R1 sites on the ends of the PCR product had not been fully digested by the enzyme or that the small oligonucleotides formed from a successful digestion of these ends were subsequently attaching to the ECO R1 sites on the open ends of the plasmid thus blocking ligation of the PCR product. Measures were therefore taken to overcome these two potential problems.

To improve digestion efficiency, the PCR product was subjected to a proteinase K treatment prior to ECO R1 digestion. The ECO R1 digest was then run on a 1.4% agarose gel. A single band corresponding to the expected weight of the PCR product was seen. This was cut out in a block, dissolved and phenol chloroform extracted. Ligation and transformation were carried out as before. A stock of pre-competent DH5α bacteria was available and these were used. Results are shown below.

Plate Number	Description	White Colonies	Blue Colonies
1	Unphosphorolased Plasmid	0	>1000
2	No insert	2	83
3	Test Insert	280	20
4	PCR product	180	150
5	Undigested Plasmid	0	>1000
6	Untransformed Bacteria	0	0

Table 1. Bacterial transformation with cloned plasmid

180 colonies of bacteria which appear to have been transformed by cloned plasmid were produced

2.3.3 Characterisation of Clones

Colonies from plate 4 which appeared white were transferred to master plates and cultured overnight. Some of the colonies showed a blue colour after this further growth but eighteen colonies that did not and were therefore believed to be recombinant were selected.

Products of PCR of microwaved smears were run on agarose gels. Bands of the expected size were seen from ten clones. When the gel was blotted onto nylon membrane all of these and a further four which did not yield a visible band on the gel, hybridised with ^{32}P labelled internal oligonucleotide.

The small-scale plasmid preparations were subjected to ECO R1 digestion. The gel was blotted on to nylon membrane and subsequent hybridisation with ^{32}P labelled internal oligonucleotide confirmed the presence of the VEGF insert in fourteen out of the eighteen clones. One of the colonies was selected for large-scale plasmid preparation as describe above.

2.3.4 Sequencing

A sample of the product of the large scale plasmid preparation was subjected to DNA sequencing. Sequenase sequencing reactions were carried out as described. The gel obtained is illustrated in figure 1. The base sequence of the insert and adjacent plasmid were derived from the gel and compared with the predicted sequence as shown in the appendix. The sequence was as expected.

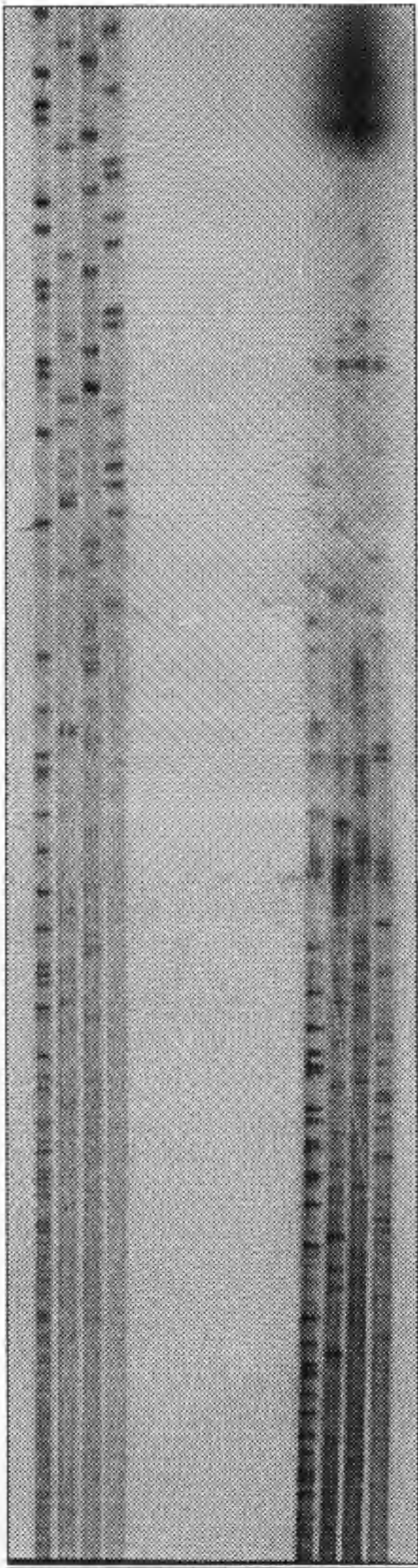


Figure 1 The sequencing gel

2.5 Discussion.

It has proved possible to generate cDNA corresponding to part of the published sequence of the first exon of VEGF. This part of the molecule is common to all splice variants. The identity of this cDNA was confirmed by verification of its size using gel electrophoresis, by hybridisation with a DNA probe based on part of its expected sequence and ultimately by chain-termination DNA sequencing. This piece of cDNA was cloned into an insertion vector to allow synthesis of RNA probes for use in further work.

In the course of this work it was found that this part of the VEGF sequence could reliably be amplified by PCR even from plasmid DNA within crude bacterial lysates. The success of these PCR primers formed the basis of the development of the semi-quantitative PCR assay of VEGF described later.

Chapter 3. Preparation and Testing of Riboprobes

3.1 Introduction

Various probes can be used for hybridisation with RNA. Whilst short oligonucleotide DNA sequences can be readily synthesised and make convenient probes, larger riboprobes produced from RNA transcripts have the advantage of a longer length of binding. . RNA-RNA binding has higher affinity compared to RNA-DNA binding. The preceding chapter describes how part of the VEGF sequence was cloned into the bluescript plasmid expression vector. This allows the production of RNA sequences complementary to cellular mRNA

To allow detection of the hybridised probe, it must carry a detectable label. Initially hybridisation techniques relied on radioactive labels that could be detected by autoradiography. This method carries with it the problems of the risks of handling of radioactive material, the limited shelf life of probes and the time required for autoradiography. More recently, non-radioactive methods such as the biotin labelling or digoxigenin system (Boeringer Mannheim) have been developed. These overcome the problems associated with radioactive methods and can yield higher resolution by avoiding scatter of radiation during autoradiography. This system was used in this work.

The aim of this part of the work was to create a VEGF riboprobe based on the cloned sequence by *in vitro* transcription and to test its ability to hybridise to mRNA.

3.2 Methods

3.2.1 Preparation of linearised plasmid templates.

Linearised plasmid construct templates for the preparation of sense and antisense riboprobes were made. This was done by cutting the construct with restriction endonucleases on the side of the insert opposite the enzyme promoter. To make a

template for the antisense probe for use with T7 transcriptase the construct was digested with PST I endonuclease. The template for the sense probe for use with T3 transcriptase was created using ECO RV endonuclease.

Digests were set up containing 40.5µl of the dialysed plasmid solution previously prepared, 5µl of 10x enzyme buffer as appropriate for the enzyme (React 2 buffer, Gibco BRL for PST I and Reaction buffer 4, NBL for ECO RV). Two and a half microlitres of 10mmol spermidine was added followed by 2µl of the appropriate enzyme (PST I, 8u/µl, Gibco BRL or ECO RV 12u/µl, NBL). This gave a concentration of plasmid construct of about 0.11µg/µl in the reaction mix. Control reactions for each enzyme were set up using an equivalent amount of bluescript plasmid. The mixtures were incubated overnight at 37°C. Fifty microlitres of DEPC treated water was then added to each mixture. They were purified by a phenol/chloroform extraction followed by a chloroform extraction as has been previously described. The resulting aqueous phase was precipitated by adding 10µl of sodium acetate and 200µl of ethanol and storing at -20°C overnight. The resulting pellet was washed with 70% ethanol then re-suspended in 10µl of DEPC treated water. Two microlitres of each solution, including the bluescript controls, were electrophoresed on a 1.2% agarose gel to ensure purity and completeness of the digestion.

3.2.2 Transcription of digoxigenin-labelled riboprobes.

Transcription reactions were set up containing 1µg of linearised plasmid template, 2µl of digoxigenin labelling mixture (10mM ATP, 10mM CTP, 10mM GTP, 6.5 mM UTP, 3.5mM DIG-11-UTP in Tris-HCl pH 7.5, Boeringer Mannheim), 2µl of 10x transcription buffer (400mM Tris-HCl pH 8, 60mM $MgCl_2$, 100mM DTT, 100mM NaCl, 20mM spermidine, RNAase inhibitor 1U/µl, Boeringer Mannheim), 40 units of T7 (for antisense) or T3 (for sense) RNA polymerase (Boeringer Mannheim) made up to a total volume of 20µl with DEPC treated water. On the first occasion that the transcription was carried out, an enzyme-free control reaction was also included. The mixture was incubated in a 37°C water bath for two hours. The reaction was then

stopped by the addition of 2µl of 200mM EDTA pH 8. The RNA was then precipitated by the addition of 2µl of 4M LiCl and 60µl of cold ethanol. This mixture was incubated at -70°C for half an hour then centrifuged at 14 000 rpm for fifteen minutes in an Ependorph microcentrifuge. The pellet was washed with 100µl of 70% ethanol in DEPC treated water and again centrifuged at 14 000 rpm for five minutes. The alcohol was removed and the pellet lyophilised in a freeze dryer before being re-suspended in 100µl of DEPC treated water.

Labelling of the probe was checked with dot blots on Hybond N+ membranes. After the first transcription duplicate 1µl samples of the T3, T7 and enzyme-free controls were dotted onto Hybond N+ membrane and allowed to air dry for one hour before being cross-linked by exposure to ultra violet for five minutes. For subsequent transcriptions, the amount of labelled probe was compared with previous reactions by preparing serial dilutions of the new and old probes, which were then dotted onto the membrane and treated as above.

3.2.3 Detection of Probe

The membrane was washed for five minutes in detection buffer 1 (100mM maleic acid, 150 mM NaCl pH 7.5). It was then blocked by incubation for thirty minutes at room temperature in 1% blocking reagent for nucleic acid hybridisation (Boeringer Mannheim) in detection buffer 1 and incubated with anti-digoxigenin-AP Fab fragments (Boeringer Mannheim) diluted 1:5000 in 1% blocking reagent solution.
The membrane was then washed twice for fifteen minutes each time in buffer 1. It was equilibrated with detection buffer 3 (100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM MgCl2.) for two minutes. Forty five microlitres of NBT solution (75mg/ml nitro-blue tetrazolium salt in 70% dimethylformamide, Boeringer Mannheim) and 35µl of BCIP solution (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100% dimethylformamide, Boeringer Mannheim) were diluted in 10ml of detection buffer 3. The membrane was incubated with this in a sealed nylon bag in the dark overnight. It was then washed in TE buffer (10mM Tris, 1mM EDTA pH 8).

3.2.4 Isolation of RNA from cultured cells.

Extraction was carried out with TRIzol (Gibco), a mono-phasic solution of phenol and guanidine isothiocyanate. Cells were grown to confluence in a tissue culture flask. Culture medium was removed and the cells re-suspended in TRIzol, 1ml per $10cm^2$ of flask). The cells were lysed by pipetting. Chloroform (0.2 ml of per ml of TRIzol) was added. The tube was shaken for fifteen seconds and left at room temperature for three minutes before being centrifuged at 11 600g in a microcentrifuge at 4°C for fifteen minutes. The aqueous phase containing the RNA was removed leaving the DNA and protein in the organic phase.

To precipitate the RNA, 0.5 ml of isopropyl alcohol per ml of TRIzol used was added and left at room temperature for ten minutes. The RNA was pelleted by centrifugation at 11 600g at 4°C for ten minutes. The supernatant was removed. The pellet washed in 1ml of 75% ethanol then centrifuged at 7250g at 4°C for five minutes. The ethanol was removed and the pellet dried in a freeze drier for 5 minutes. The pellet was re-suspended in 0.5% SDS solution in DEPC treated water and incubated for ten minutes at 55°C to ensure complete dissolution. The optical density of the solution was then measured at 260 and 280 nm.

3.2.5 Determination of optimal probe concentration for hybridisation.

Samples of RNA were precipitated as described for gel electrophoresis and re-suspended at a concentration of 10μg /μl in RNA diluting buffer (5:3:2 mixture of DEPC-treated water, 20x SSC and formaldehyde).

Six 5mm x 5mm squares of Hybond N+ membrane were cut. One microlitre of RNA solution was dotted onto three of the squares, air-dried for one hour and cross-linked by exposure to ultra-violet light for five minutes.

Hybridisation buffer as described in the Boeringer DIG system protocols was prepared containing 50% formamide, 5x SSC, 0.02% SDS, 0.1% N-lauroylsarcosine, 2% blocking reagent for nucleic acid hybridisation (Boeringer Mannheim). All six squares were incubated for one hour in this solution at 68°C

Hybridisation solutions were prepared by adding the product of the T7 transcription reaction the prehybridisation buffer in dilutions of 1ul/ml, 3 µl/ml and 5 µl/ml. These solutions were placed in 1ml screw top tubes and placed in a boiling water bath for ten minutes to denature the probe. One piece of membrane with RNA dotted on and one piece without RNA were then place in each hybridisation solution and incubated at 68°C overnight. The squares were then transferred to a small plastic box. They were washed twice at room temperature for five minutes with wash solution containing 2x SSC with 0.1% SDS and twice for twenty minutes at 68°C with wash solution containing 0.1x SSC and 0.1% SDS. Detection was then carried out as described in section 3.2.3 above, except the incubations with antibody and colour substrate were carried out in 1 ml tubes.

3.2.6 Gel Electrophoresis of RNA samples.

The volume of solution containing the required amount of RNA (10-30µg) was calculated. The RNA was precipitated from this solution by the addition of 0.1 vol of 3M Na Acetate pH 5.4 and 2.5 vol of absolute ethanol. The samples were then chilled for 5 minutes at -70°C before being centrifuged at 13530 g at 4°C for fifteen minutes. The supernatant was withdrawn and the pellet lyophilised. The pellet was then re-suspended in 10µl of RNA loading buffer. Immediately prior to being loaded onto the gel, the samples were denatured by heating to 65°C for fifteen minutes. One microlitre of 1µg/ml ethidium bromide was then added to each sample. Molecular weight markers were also prepared as above.

The electrophoresis tank, gel tray and comb were washed with detergent then soaked in 3% hydrogen peroxide for ten minutes. They were then washed with DEPC-treated water and air-dried.

Gels were prepared containing 1.2 g agarose and 20ml 10x MOPS in DEPC-treated water per 100ml. The mixture was heated in a microwave oven until the agarose was fully dissolved 10ml formaldehyde per 100ml was added to the molten gel. The gel was then poured into a gel tray with a gel comb in place and it was allowed to set for thirty minutes before use.

The gel was then placed in an electrophoresis tank containing 1x MOPS buffer and the gel comb was removed. Samples and markers were loaded into the wells. Electrophoresis was carried out at 70v.

The gel was inspected and photographed under ultra-violet light.

3.2.7 Gel Electrophoresis of Probe.

An RNA electrophoresis gel was prepared as before. A 20µl sample of the T7 RNA transcript was precipitated, lyophilised, re-suspended and denatured in the same way as the previous RNA samples used for Northern blotting. RNA molecular weight markers were also prepared in the same way.

3.2.8 Northern Blotting.

Blotting apparatus was set up as has previously been described for Southern blotting. The marker lane was excised and the gel trimmed. The RNA was blotted onto Hybond N+ membrane overnight using 20x SSC transfer buffer. The membranes were washed twice in 2x SSC for five minutes to remove adherent agarose then air-dried for two hours. The RNA was then cross-linked to the membrane by exposure to UV light for five minutes.

3.2.9 Blotting and Detection of Probe

The RNA gel on which the T7 transcript had been electrophoresed was Northern blotted onto Hybond N+ membrane. This membrane was then subjected to the detection method described above to reveal the probe

3.2.10 Probing of membranes.

Nylon membranes, which had been blotted and cross-linked as above, were placed in hybridisation bags with 20ml/100cm^2 of Northern hybridisation buffer as described above and incubated for one hour at 68°C in a shaking water bath.

DIG labelled riboprobe prepared by T7 transcription from the VEGF plasmid construct was diluted in hybridisation buffer and denatured by placing in a boiling water bath for ten minutes.

The bag containing the membrane was drained and the probe hybridisation solution (probe in hybridisation buffer) was added. This was then incubated overnight at 68°C. The membrane was then washed twice at room temperature for five minutes with wash solution containing 2x SSC with 0.1% SDS and twice for twenty minutes at 68°C with wash solution containing 0.1x SSC and 0.1% SDS. Detection was then carried out as described under Detection of Probe above.

3.2.11 Hybridisation Tests with Dot Blots

RNA from MCF7 cells, human heart and human liver were dot blotted onto two pieces of Hybond N+ membrane as before. Hybridisation buffer previously used for Southern blotting in earlier work was used. This contained 50% formamide and salmon sperm DNA.

An antisense oligonucleotide probe was prepared by end labelling the 3' oligonucleotide with digoxigenin.

Since smaller oligonucleotide probes tend to have lower binding affinity, hybridisation and washes with this probe were carried out at lower temperatures to reduce stringency.

The membranes were prehybridised by soaking in hybridisation buffer at 68°C. Hybridisation solutions containing either 1:400 riboprobe or 1:20 oligonucleotide probe were set up. One membrane was soaked overnight in the riboprobe at 68°C and the other in oligonucleotide probe at 42°C. The membrane was then washed twice at room temperature for five minutes with wash solution containing 2x SSC with 0.1% SDS. The membrane hybridised with riboprobe was washed twice for twenty minutes at 68°C with wash solution containing 0.1x SSC and 0.1% SDS. The membrane hybridised with the oligonucleotide probe was washed in the same solution at 42°C. Detection with alkaline phosphatase-conjugated anti-digoxigenin and substrate was carried out as before.

3.3 Results.

3.3.1 Preparation of Digoxigenin-Labelled Riboprobes.

Transcription reactions were set up as described with T3 & T7 transcriptase and a transcriptase-free control. Appropriate DNA templates were used for each enzyme. The probe produced in each transcription reaction was re-suspended in 100μl of water and dot-blotted onto an N+ membrane in duplicate.

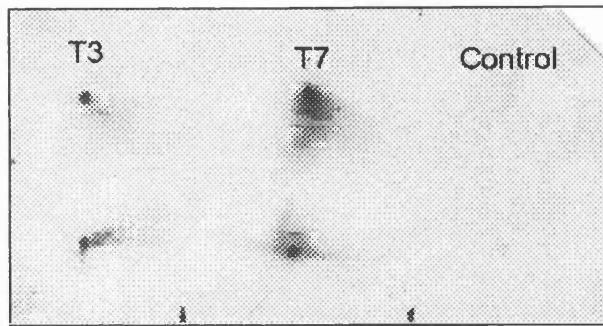


Figure 2. Blot of Probe

It can be seen that both transcriptions give strong dot blots with the T7 being strongest. Only weak staining is seen with the transcriptase-free controls, which presumably resulted from adherence of unincorporated DIG-UTP to the membrane.

3.3.2 Gel Electrophoresis and Blot of Riboprobe

Electrophoresis and blotting of the probe revealed a dense band with a tail of smaller residues representing incomplete or degenerate transcripts.

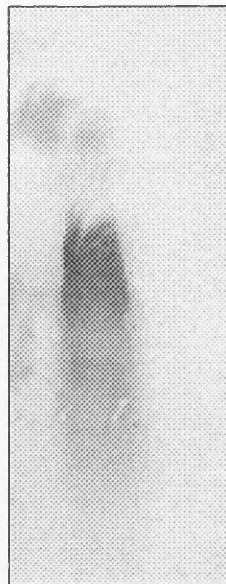


Figure 3. Blot of DIG labelled T7 transcript

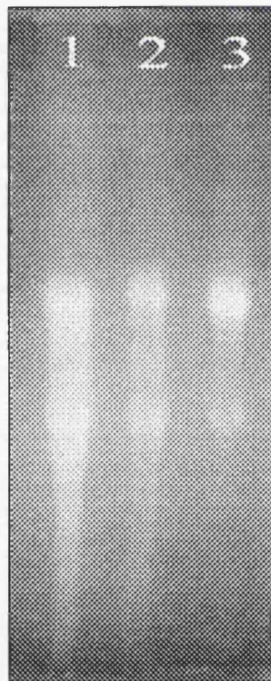
3.3.3 Estimation of Optimal Probe Concentration.

Small squares of Nylon membrane with or without MCF7 RNA dot-blotted onto them were subjected to hybridisation as described with probe dilutions of 1,3 and 5 μl of probe per ml of buffer.

No background staining of the membrane was seen at any of these concentrations. Faint discolouration of the membrane was seen in the central areas of the squares which had been blotted with MCF7 RNA and exposed to the higher two probe concentrations.

3.3.4 Northern Blots.

RNA samples were electrophoresed and blotted onto Hybond N+ nylon membrane.



Lane 1. MCF7 RNA (30μg)

Lane 2. Human Heart RNA (30μg)

Lane 3. Human Liver RNA (30μg)

Figure 4. Photograph of RNA gel

After blotting and hybridisation, the membrane showed very high levels of background staining with no distinguishable bands.

3.3.5 Dot Blots

In order to determine whether the background staining was due to the problems with the blotting or hybridisation, dot blots were carried out.

Ten microgram dots of MCF7 breast cell line derived RNA, human heart RNA and human liver RNA on Hybond N+ nylon membrane were hybridised with digoxigenin labelled riboprobe or oligonucleotide probe. The riboprobe again showed background staining of the membrane. The oligo probe caused less background staining but no visible hybridisation to the RNA samples could be demonstrated.

3.4 Discussion

Digoxigenin labelled RNA transcripts were successfully obtained from the plasmid construct containing the VEGF insert. It was demonstrated that this transcript could be detected when bonded to nylon membranes. Attempts to use the probe for Northern blotting were hampered by non-specific binding to the membrane. Discussions with Boeringer suggested that his may be due to the level of charge on the Hybond N+ membrane and that Boeringer uncharged membrane may be more suitable. A sample of this membrane was tested and did indeed result in lower levels of background. Since the PCR based assay described later appeared to offer greater sensitivity blotting with this paper was not pursued further. The probes were however used for *in-situ* hybridisation in an attempt to obtain information on the localisation of VEGF RNA expression within the tissue samples.

Chapter 4. *In Situ* Hybridisation.

4.1 Introduction

In situ hybridisation (ISH) techniques allow the detection of nucleic acid in cells or tissue sections. It relies on the binding of a nucleic acid probe to a complementary nucleic acid sequence within the cell. RNA hybridisation makes it possible to identify which cells are expressing a particular mRNA species and may therefore be producing a particular protein.

When used on archival fixed paraffin sections, the technique depends on the assumption that the fixation process has preserved the RNA species under study. These RNA molecules can then be made accessible by treatment with a protease such as proteinase K.

In order to distinguish specific hybridisation from non-specific binding of probe, it is important to include controls with a probe of similar characteristics to the test probe that is not expected to bind. A sense RNA transcript from the same construct used to generate the antisense probe makes a convenient control.

Whilst immunohistochemical methods will label cells which bind or take up a particular protein, RNA in situ hybridisation will only label cells producing the mRNA coding for that protein. However, the finding of mRNA species within a cell does not necessarily mean that that this is undergoing translation to protein. ISH and immunohistochemistry therefore yield complementary information.

4.2 Methods.

4.2.1 Tissues

Formalin fixed, paraffin embedded sections of normal human kidney, normal human breast and ductal carcinoma of breast were kindly supplied by the Department of

Pathology, Western Infirmary Glasgow. Detection of VEGF mRNA in the glomeruli of human kidneys has been reported [62] and it was therefore felt that this was a suitable test tissue for the technique

4.2.2 Preparation of Probes.

Riboprobe.

Preparation of digoxigenin-labelled riboprobe from the cloned plasmid template containing part of the VEGF cDNA sequence has been described in the preceding chapters.

Oligonucleotide probes

The antisense (3') and internal sense oligonuclotides used previously in cloning the VEGF sequence into the plasmid were used as an oligonucleotide probe and sense control respectively. These were end-labelled with digoxigenin as previously described.

The labelled oligonucleotides were passed through a sepharose column and the fractions dot blotted onto nitrocellulose membrane. The fractions containing bound digoxigenin were lyophilised.

4.2.3 *In -situ* Hybridisation

After washing in xylene, sections were rehydrated along an alcohol / DEPC treated water gradient. They were then immersed in DEPC treated 0.2 N HCl for twenty minutes then washed twice in DEPC treated PBS. The slides were then immersed in 0.3% Triton X made up in 2x SSC at 70°C for fifteen minutes. They were then washed twice with DEPC treated PBS.

Proteinase K dilutions were of 0, 10, 20, 30, 40, 50 and 100 μg/ml were prepared by diluting proteinase K in diluting buffer (0.1M Tris/HCl pH 8 + 50mM EDTA). Four slides were covered with each dilution of proteinase K and incubated at 37°C for thirty minutes. They were then immersed in 0.2% glycine in DEPC treated PBS for thirty seconds.

After being washed twice in DEPC treated PBS, the slides were post-fixed by immersion in 4% paraformaldehyde. They were again washed twice in DEPC treated PBS.

Prehybridisation buffer was by prepared diluting 30ml of 20X SSC in 120ml of water and adding 150ml of formamide. The slides were immersed in this buffer for two hours at 37°C

Hybridisation buffer was prepared containing

40μl of 5M Tris pH 7.5
2.5ml of Denhardt's solution
1.3μl of 30X SSC
0.5ml of 20% SDS
10ml of 100% formamide
4ml of 50% dextran sulphate
0.5ml of salmon sperm DNA (10mg/ml)
1.16ml of Water

Antisense and sense (control) riboprobes and oligonucleotide probes were used at dilutions from 1:10 to 1:100 in this hybridisation buffer which had been preheated to either 37°C or 42°C. Twenty microlitres of hybridisation buffer containing probe were placed on silicone coated cover slips and sections mounted onto these cover slips. They were then incubated in seated polythene bags with a moist paper towel overnight at either 37°C for oligonucleotide probes or 42°C for riboprobes.

The coverslips were removed by rinsing the slides in 4X SSC. The slides were then washed twice in 2X SSC for twenty minutes each time at room temperature then 0.1X SSC for twenty minutes each time at 37°C for oligonucleotide probes or 42°C for riboprobes. They were then washed in 2X SSC for five minutes.

The digoxigenin conjugated to the probe was then detected with alkaline phosphatase conjugated anti-digoxigenin antibody. Detection buffer was prepared by adding 2.36g of Tris base and 12.7g of Tris HCl to 1litre of water and, after adjusting the pH to 7.5 with HCl, adding 8.7g of NaCl. 20% human serum and anti digoxigenin antibody to a concentration of 1:500 were added to detection buffer. The slides were then incubated with the antibody solution for two hours at room temperature. They were then washed twice for ten minutes in the plain detection buffer.

A further buffer was prepared by adding 12.1g of Tris base to a litre of water and adjusting the pH to 9.5 with HCl before adding 5.8g of NaCl and 10g of $MgCl_2$. The slides were washed for five minutes in this buffer. A Nitro-Blue Tetrazolium (NBT)/ Bromochloro-Indolylphosphate (BCIP) solution was prepared as a substrate for the conjugated alkaline phosphate enzyme. This was done by adding 198μl of NBT stock solution (750mg of NBT in 70% Dimethylformamide) and 150μl BCIP stock solution (500mg of BCIP in 10ml 100% Dimethylformamide) to 45ml of this buffer and then adding 12mg of levamisole. The slides were immersed in this substrate solution overnight in a coplin jar wrapped in aluminium foil. The slides were then washed in distilled water. They were lightly counterstained with haematoxylin and washed three times in distilled water before being "blued" in Scott's tap water substitute (STWS). They were washed again in distilled water before being mounted in glycergel.

4.3 Results.

4.3.1 Preliminary Trial of Technique.

A trial of using the technique with an oligonucleotide probe (PMC) on rat pituitary, (which had previously produced staining), was attempted. The expected pattern of hybridisation was observed.

4.3.2 RNA Riboprobes.

Despite employing a range of proteinase K and riboprobe concentration the only staining that could be produced on human kidney sections was in the tubules. This was also seen when the sense control riboprobe was used and therefore appeared to be non-specific binding of probe.

Paraffin sections of normal human breast and ductal carcinoma of the breast were also used. Again only non-specific staining was seen with both the antisense and sense riboprobe.

4.3.3 DNA Oligonucleotide Probes

Oligonucleotide hybridisation was attempted using the paraffin sections of kidney. Both antisense and sense oligonucleotide probes stained the tubules. There was no difference in staining between the antisense test probe and the sense control probe, indicating that this binding was again non-specific.

4.4 Discussion

No specific staining was seen with either probe on the tissue sections used. Whilst the identity of the riboprobe template had been verified by DNA sequencing attempts at proving that it would hybridise to RNA extracts had proved unsuccessful. However given the failure of both probes, the possibility exists that the VEGF RNA in the tissues under examination had been degraded. Trials on a more extensive range of tissue samples and the creation of further probes would have been necessary to determine the cause of the problem. However it had become clear from ongoing work on an RT- PCR based assay, described in chapter 6, that a quantitative assay would be required. It was therefore felt that the development of this assay would be more fruitful and creation of further probes was not pursued.

Chapter 5 Immunohistochemistry

5.1 Introduction

Immunohistochemistry allows the identification and localisation of proteins using antibodies linked to enzymes that produce colour changes in indicator dyes. The systems are frequently multi-step. This has the advantage both of economy in allowing one enzyme-conjugated antibody to be used in the identification of several different proteins and also of building amplification into the system. Further amplification can be achieved by the use of antibodies and enzymes that combine via binding proteins such as the avidin-biotin system.

Immunohistochemistry was one of the first methods described for detection of VEGF in tissues and some workers have reported success with this technique. Brown et al in Boston [62] demonstrated cytoplasmic staining for VEGF of visceral glomerular epithelial cells but not on the adjacent endothelium. They found that in renal cell carcinomas, there was staining of both the malignant cells and tumour vessels [63].

When this research was begun, VEGF staining by immunohistochemisty in breast tissues had not been reported. The ability to use immunohistochemistry in this way was felt to be a useful adjunct to the RNA detection methods described above. It has disadvantages in being difficult to quantify and in being affected by differences in the binding of VEGF protein to the tissue substrate. However it has the potential to confirm that VEGF mRNA was being translated into protein in the tissues.

5.2 Methods

5.2.1 Tissues.

Formalin fixed, paraffin embedded sections of normal human kidney, normal human breast & ductal carcinoma of breast and frozen sections of human kidney were kindly supplied by the Department of Pathology, Western Infirmary Glasgow.

5.2.2 Antibodies

Monoclonal anti-VEGF monoclonal antibody (A 4.6.1) [119] and rabbit polyclonal anti-VEGF antibody were gifts from Genentec Inc. San Francisco.

R&D monoclonal antibody (MAB293) against the 165 amino acid variant of VEGF was purchased from R&D Systems (Abingdon).

Biotinylated goat anti-mouse antibody was used as second antibody for the monoclonal primary antibodies and biotinylated goat anti-rabbit antibody for the polyclonal antibody.

5.2.3 Preparation of Paraffin Sections

Sections were deparaffinised by two five minute soaks in Xylene then transferred 100% ethanol. They were then rehydrated down an alcohol gradient by five minutes soaks in baths containing 95%, 80% and 50% ethanol. Sections were then transferred to TBS.

5.2.4 Preparation of Frozen Sections

Sections were fixed by placing the slides in acetone for five minutes then TBS for five minutes.

5.2.5 Staining Procedure

Specimens were covered with 10% goat serum in TBS and incubated for sixty minutes at room temperature. Anti-VEGF antibody at titrated dilutions in TBS was applied to each tissue section. Control sections treated with only TBS were set up. Sections were incubated overnight at 4°C. They were then washed twice with TBS. A biotinylated species-specific second antibody against the primary antibody (diluted 1/400 in TBS) was then applied to the sections and incubated for thirty minutes at room temperature. The slides were then washed twice with TBS. Alkaline phosphatase-conjugated streptavadin was then applied and incubated for thirty minutes. The slides were again washed with TBS. New fuchsin substrate was applied and incubated for thirty minutes. Slides were then counterstained in haematoxylin for forty seconds.

5.2.6 Antigen Unmasking Techniques

When the above protocol failed to yield any staining with paraffin sections, it was repeated with sections that had been subjected to antigen unmasking. Two techniques, enzymatic digestion and microwave antigen retrieval, were used both individually and in combination.

Enzymatic Digestion

Slides were place in pre-warmed (37°C) 0.1% trypsin in PBS for times ranging from five to sixty minutes. They were then washed in distilled water.

Microwave antigen retrieval.

Citrate buffer pH 6.0 was prepared (3.8g of citric acid, 2.4g of sodium citrate per litre). Slides were immersed in this buffer in a coplin jar. They were then heated in a 800W domestic microwave on 50% power for between five and twenty minutes. The buffer was then allowed to cool and the slides rinsed in distilled water.

5.3 Results.

5.3.1 Untreated Sections

No staining was seen on any of the tissue section used with the three anti-VEGF antibodies.

5.3.2 Antigen Retrieval Techniques

Trypsin exposures in excess of thirty minutes produced significant tissue degradation. Microwave treatment followed by trypsinisation led to more tissue degradation than trypsinisation followed by microwave treatment.

No combination of trypsin of microwave treatment yielded any staining with any of the antibodies or tissues.

5.4 Discussion

With the tissues and antibodies used, no immunostaining for VEGF was observed. This may be due to tissue preparations methods. The antigen unmasking techniques used would be expected to compensate for problems resulting from fixation. No staining was seen with the frozen sections of cell lines. Discussions with other groups suggested that the available antibodies at that time were not ideally suited for immunohistochemistry. The majority of workers have used an ELISA technique to measure VEGF protein. It appeared that with the antibodies available at the time of this study, immunohistochemistry was not a viable way of assessing VEGF in breast tissues. Since completion of this work, two groups [91, 96] have however reported staining of breast carcinomas for VEGF with polyclonal and monoclonal antibodies.

Chapter 6. Development and Optimisation of Assay for the measurement of VEGF mRNA by RT-PCR

6.1 Introduction.

6.1.1 The Polymerase Chain Reaction.

The polymerase chain reaction is a technique for reproducing segments of DNA. It is able to reproduce very rapidly, large amounts of a defined DNA segment from nanogram amounts of template DNA, within a large background of irrelevant sequences. This has led to it becoming one of the most important techniques in molecular biology. Tiny traces of DNA can be amplified sufficiently to be visible on an electrophoretic gel allowing them to isolated and sequenced.

The technique relies on having unique, known sequences of DNA flanking the segment to be amplified. It is not necessary to know the intervening DNA sequence. Short DNA oligonucleotides complementary to the flanking sequences are required. These are called primers. If DNA is heated sufficiently to denature the double helix into single stands then allowed to cool, double stranded DNA will re-form. If this is carried out in the presence of a large excess of oligonucleotide primers complementary to the sequences flanking the area of interest, these primers will preferentially anneal to that area. Allowing this to happen in the presence of a DNA polymerase enzyme will result in that enzyme producing a second strand, complementary to the segment of interest, starting from the primer. This process could then be repeated by re-heating the DNA to denature it again. This re-heating would denature most DNA polymerases and further enzyme would need to be added. However the DNA polymerase of the thermophile algae Thermus aquaticus (Taq.) is stable at temperatures sufficient to cause double stranded DNA to disassociate [103]. This allows a rapidly repeating cycle of denaturation, primer annealing and sequence extension to be carried out. This is called a polymerase chain reaction (PCR). Saiki

first described an early form of the technique in 1985 [104]. He subsequently described a modified technique using Taq polymerase and an automated thermocycler in 1988 [105].

In order to generate only the required product with the highest possible yield, a PCR reaction requires optimisation of its conditions. The stage of the reaction during which the primers anneal is most susceptible to variations in conditions and most amenable to optimisation. Various conditions affect annealing. One of the most important is temperature. As the temperature is reduced, the efficiency of binding of the primers to the target DNA increases. However, the chances of a primer binding to areas of DNA that only partially match its sequence increase. This not only wastes primer but also gives rise to inappropriate products. The time allowed for annealing can also be important. It is necessary to allow enough time for all correct priming to take place but excessive times may increase the chances of incorrect priming. The concentration of magnesium ion (Mg^{2+}) also affects the efficiency and specificity of primer annealing with higher concentrations reducing specificity. Mg^{2+} is also important as a co-factor for Taq polymerase (103). The amount of primer is important. It is essential to have enough to ensure efficient annealing through to the late stages of the reaction when the number of target molecules has increased and some of the primer has been used up. However, too much can increase the chances of inappropriate priming and can lead to the formation of primer polymers. Similarly the concentration of mixed deoxynucleotide triphosphates (dNTPS), the substrates of the polymerase enzyme, is important. There must be sufficient present so that their concentration is not limiting in the late cycles but overly high concentrations can lead to nucleotide misincorporation.

The PCR reaction amplifies DNA. However, messenger RNA (mRNA) can be reverse transcribed to form a corresponding complementary DNA (cDNA) using a reverse transcriptase (RT) enzyme. These were originally derived from retroviruses and have intrinsic RNAase activity. Now RT enzymes are manufactured commercially from cloned viral genes and some such as Superscript (GibcoBRL) have the part of the gene responsible for the RNAase activity removed. This ability to generate an amplifiable cDNA version of an mRNA species means that the PCR

reaction can be used to detect very small amounts of mRNA. These combined processes are referred to as RT-PCR.

Like the PCR reaction, the reverse transcription reaction also requires optimisation to ensure maximum efficiency. Reverse transcriptase, like DNA polymerase requires priming of the target nucleic acid. This can be done either specifically using a cDNA oligonucleotide that anneals at the start of the mRNA sequence of interest or non-specifically. Non-specific methods involve the use of either random hexomers of DNA that bind at multiple sites on the RNA or Oligo-dT primers. Oligo-dT primers are deoxythymidine polymers 12- 18 nucleotides long. These bind to the polyadenine tail of mRNA. Non-specific methods are most commonly used. Oligo-dT priming has been found to yield less side products but may not work if the sequence of interest is a long way from the polyadenine tail. Whilst RT enzymes such as Superscript are functional at 37°C, higher temperatures may increase yields or facilitate the transcription of some mRNAs [106.].

6.1.2 Use of the RT-PCR Reaction for Quantitative Analysis of mRNA.

The ability of RT-PCR to detect tiny amount of RNA rapidly makes it very attractive as a method of estimating mRNA in small tissue samples. The inherent amplification makes it much more sensitive than Northern blotting. The first reported attempt to use RT-PCR for quantification of mRNA was described by Chelly in 1988 [105]. He compared expression of the scarce dystophin gene product by comparing it to the expression of aldolase mRNA.

Due to the exponential nature of amplification in the PCR reaction, small changes in reaction conditions can result in large changes in the amount of product generated. Furthermore, in the latter stages of a reaction, depletion of reaction components, accumulation of products and decreased enzyme efficiency, lead to plateauing of the amplification [108]. For PCR to be used quantitatively it is necessary that the reaction remains in the exponential phase and does not plateau across the full range of

template concentrations being assessed. Optimisation of the assay facilitates its quantitative use in two ways. The maximum possible amount of product is generated at each cycle thus minimising the number of cycles necessary for product detection and the reaction may run for more cycles before reaching plateau.

Whilst large changes in RNA expression can been measured without comparison to controls in experimental systems [109] most studies employ some control component. This may either take the form of another gene product that is expressed at a constant level in the tissues being studied, or on external template which combines with the same primers as the species being measured [110]. The use of different primers to carry out two different PCR primers in the same tube has been commonly employed [107,111] but creates several problems [112]. Having another PCR reaction competing for products and accumulating reaction products can inhibit the amplification of the main target molecule. Unless the standard is present in similar a similar concentration to the target one reaction may plateau before the other so limiting the amount of amplification of the less abundant target. This limits the sensitivity of measurement of low copy mRNA species when common housekeeper genes such as β-actin are used as controls. The reaction conditions must be a compromise between those that are optimal for the two targets. Therefore not only is the efficiency of amplification of both targets compromised but any variation in reaction conditions may shift the balance in favour of one or other target so producing an alteration in the target to control ratio. Many workers have addressed the problem of different reaction conditions by using a control target that has the same primer binding sites as the target molecule. The control should be distinguishable either by a small size difference or by inclusion of a different endonuclease restriction site. This control mRNA can be either produced synthetically [111] or mRNA from a different species used [56,114]. The inclusion of a target with the same primer-binding site results in competition for primers. The rate of amplification of one target is therefore greatly influenced by the concentration of the other [112]. This is overcome by a technique called competitive PCR. This involves running a series of PCR reactions containing a fixed amount of the unknown target molecule and varying amounts of the control. As the concentration of the control increases, more of its product and less of the unknown target product is generated. By plotting the amount of both products

generated against the concentration of the control target, the concentration of the unknown target can be extrapolated. A problem arises when using this technique to measure mRNA. If the concentration of cDNA in a reversed transcribed sample is measured against a cDNA control, this does not control for the efficiency of the reverse transcription reaction. To overcome this a titration of an mRNA standard can be used. However, this requires both a reverse transcription and PCR reaction to be carried out for each different standard concentration. Depending on the range of unknown RNA concentrations this may require up to ten RT and PCR reactions for each unknown sample. This approach was used to measure the expression of VEGF by Yonekura et al [56]. An alternative approach is to carry out a single RT reaction then use competitive PCR to measure the concentration of both the unknown mRNA and a control "housekeeper" gene product such as β-actin. This has the advantage of controlling for any errors in the estimation of the amount of mRNA present in each sample. However, it requires two sets of competitive PCR titrations to be carried out for each sample so necessitating a large number of PCR reactions [115]. The competitive PCR approach is an accurate way of measuring mRNA concentrations in experiments that require estimation of a fairly small numbers of samples. However if a large number of tissue samples are to be screened for either clinical or research purposes, the large number of PCR reactions required make it expensive and laborious.

A simpler approach would be to reverse transcribe the mRNA from each sample then amplify both the cDNA to be measured and the control gene product in separate reactions. This eliminates the problems outlined above of running two PCR reactions in the tube. Replicates are required to allow for variations in reaction efficiency between PCR reactions in different tubes but this still requires far fewer PCR reactions than the competitive method. Duplaa et al described this approach in 1993 [116]. It has been suggested that the variation between different tubes in the PCR reactions presents a problem with this technique [113]. However, since the completion of this word described in this thesis, it has been successfully employed by several groups for the measurement of VEGF mRNA in breast [87,88] and other tissues [68,117].

6.2 Methods

6.2.1 Outline of Method.

A variation on technique of separate amplification of standards, described above was used in this project. Since GAPDH may be upregulated in some breast tumours [98] β-actin was used as standard. RNA from tissues was reverse transcribed. Samples of the resulting cDNA mixture were then used in separate PCR reactions to amplify VEGF and β-actin. A sample of standard RNA extracted from the T47D breast cancer derived cell line was included with each batch of reverse transcriptions. The cDNA derived from reverse transcription of this standard RNA was serially diluted and a sample of each dilution was run with tissue-derived samples in both the VEGF and β-actin PCR reaction. The PCR products derived from both the tissues samples and serially diluted cell line cDNA was run on an agarose gel in the presence of ethidium bromide. This intercalates in the DNA helix and fluoresces when exposed to ultraviolet light. The luminescence of the band is proportional to its DNA content. Molecular weight markers were also run on each gel to ensure that the molecular weight of the product was consistent with expected product size. Each gel was imaged using the BioRad Geldoc system. The product bands from the serially diluted cell line cDNA were used by the system's "Molecular Analyst / PC" software to generate a standard curve. The relative concentrations of the VEGF or β-actin PCR products on each gel were then derived by linear regression from this curve by the software.

The standard curve ensures the linearity of amplification, luminescence and image acquisition for each gel. It also corrects for variations between different batches of PCR reactions. Therefore, if variations in master mix composition or performance of the thermocycler resulted in the VEGF reactions being more or less efficient relative to the β–actin reactions, comparison with standard mRNA processed in parallel compensates for this source of error. To control for variations in the amount of mRNA in different samples and any variations between reverse transcription reactions, the VEGF to β-actin ratio was calculated for each sample. Since this assay does not

include an external standard of a known quantity of VEGF mRNA the values it generates are relative rather than absolute. This was deemed appropriate for the purposes of this study

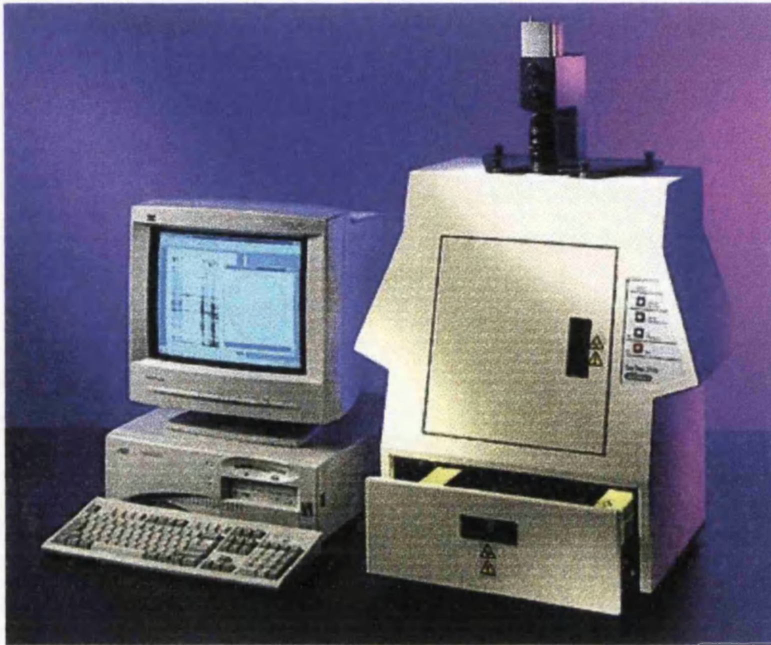


Figure 5. The GelDoc Gel Imaging System. (Photo reproduced with permission from BioRad Corp.)

The β-actin actin primers were as follows:

5' ATGCCATCCTGCGTCTGGACCTGGC (location 2310-2334)
3' AGCATTTGCGGTGCACGATGGAGGG (location 3127-3103)

They had been synthesised by Oswel DNA, Edinburgh and previously used for PCR in the Department of Surgery, Western Infirmary, Glasgow.

In order to generate a cDNA template for amplification for PCR, it was necessary to use the reverse transcription reaction. This generates a single strand of DNA using messenger RNA as a template. Superscript RNAase H reverse transcriptase (GibcoBRL) was used to carry out the reverse transcription reactions. This is produced from a cloned M-MLV RT gene from which the RNAase II sequence has been deleted, thus reducing the RNAase activity to undetectable levels [106].

Extensive effort was given to the optimisation of both the PCR and reverse transcription reactions and the verification of the linearity of amplification achieved by these.

The assay was used to estimate VEGF mRNA expression in several samples derived from archival breast tumours. This expression was then compared to the pathological and clinical data for these patients. No pathological or clinical data was obtained prior to making the VEGF measurements.

6.2.2 Tissue Culture

Two cell lines were used in this work. These were T47D (ATCC HTB-133) [118] and MCF7 (ATCC HTB-22) [119]. These grow as adherent monolayers. Both had been isolated from pleural effusions of patients with breast cancer. Both lines were purchased directly from the American Type Culture Collection (ATCC), Manssasa. Both were cultured in accordance with the recommendations in the relevant ATCC data sheets. T-47D cells were propagated in RPMI 1640 medium supplemented with 2mM L-glutamine, 1.5g/L sodium bicarbonate, 4.5g/l glucose, 10mM HEPES 1mM sodium pyruvate, 0.2units/ml insulin and 10% foetal calf serum. The medium used for the MCF7 cells was Eagle's minimal essential medium with Earle's BSS supplemented with 2mM L-glutamine 1.5g/l sodium bicarbonate, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 0.01 mg/ml insulin and 10% foetal calf serum. Cells were grown in $25cm^2$ Nunclon tissue culture flasks (Nunc). Medium was changed three times weekly and cells were subcultured with a 1:5 dilution when confluent. Cells were mobilised for subculture and harvesting by rinsing with 0.25% trypsin with 0.03% EDTA. Stocks of cells were frozen in liquid nitrogen in culture medium containing 5% DMSO.

6.2.3 Extraction of RNA

RNA extraction from both cultured cells and breast tissue was carried out using TRIzol reagent (**T**otal **RNA** **I**solation reagent) (Life Technologies Inc). This is a mono-phasic solution of phenol and guanidine isothiocyananate.

The technique for homogenisation of the sample was different for cultured cells and tissues but the remaining stages of the process were identical.

6.2.4 Homogenisation of cultured cells

A volume TRIzol reagent corresponding to 1ml per $10cm^2$ of culture surface area was added to tissue culture flasks containing confluent monolayers of cells. Cells were detached from the surface of the flask with a disposable cell scraper and the suspension pipetted to break up clusters of cells. 1ml aliquots of the suspension were placed in centrifuge tubes.

6.3.5 Homogenisation of tissues

Pieces of breast tissue that had been stored in liquid nitrogen were used. The pieces were fragmented with a steel tissue crusher (piston and cylinder) in a bath of liquid nitrogen. The fragments were then transferred to a clay pestle chilled in a bath of liquid nitrogen and ground to a fine powder.

Approximately 75mg of this powder was place into centrifuge tubes and 1ml of TRIzol reagent added to each tube. The contents were pipetted to suspend the powder.

The following steps were the same regardless of whether the material originated from cultured cells or tissue.

6.2.6 Phase Separation

The homogenised samples were incubated for five minutes at room temperature to dissociate the nucleoprotein complexes and 0.2 ml of chloroform was then added to each tube. The tubes were shaken vigorously by hand, for fifteen seconds then left to stand at room temperature for three minutes. They were then centrifuged at 12000 g for fifteen minutes in a refrigerated centrifuge at 4°C.The upper aqueous phase in each tube which contained the RNA was then carefully withdrawn and transferred to a fresh tube.

6.2.7 Precipitation and Washing of RNA

RNA was precipitated by adding 0.5ml of isopropanol to each tube and allowing it to stand at room temperature for ten minutes. The tubes were then centrifuged at 12000g for ten minutes at 4°C. The supernatant was withdrawn and the pellet in each tube washed 1ml of 75% ethanol at 4°C. The samples were mixed and centrifuged at 7500g for five minutes at 4°C. The ethanol was removed and the pellets air-dried. The pellets were then dissolved in DEPC treated water by incubating them at 55°C for ten minutes.

6.2.8 Reverse Transcription of RNA

In order to prepare a solution of T47D cDNA for use in optimisation of the PCR assay, RNA extracted from this cell line was reverse transcribed. The nucleic acid concentration of the RNA was determined by spectrophotometry. A volume equivalent to 15μg of total RNA was measured and divided equally between each of three reverse transcription reactions. Each reaction contained 5 μl of First Strand Buffer (Gibco), 5 μl of 0.1M DTT, 1.5 μl of 500 μg Oligo(dT)$_{12\text{-}18}$ (Gibco), 5 μl of 10mM mixed dNTP 2 μl of RNAguard RNAase inhibitor (Pharmacia) and 4 μl (800U) of Superscript RNAase H reverse transcriptase (Gibco) made up to a total volume of 50 μl. The products of the three reaction mixtures were pooled.

6.2.9 PCR and Image analysis.

The principle of the PCR reaction has been described above. For each stage of the optimisation of the assay all components of the reaction other than that being varied were prepared in a master mix to ensure uniformity between reactions. The total volume of each reaction mix was 50μl. Reactions were overlayed with 20ul of mineral oil to prevent evaporation. Reactions were run on automated thermocycler blocks. Gel electrophoresis was carried out in the presence of ethidium bromide as has been described in section 2.2.3 above. Gels were than placed in the Geldoc (Biorad) imaging system and a digital image of the gel acquired. This was then processed using the system's Molecular Analyst/PC v1.2 software. The software generates a value for the "adjusted volume" (counts x mm^2) of each band on the gel which corresponds to the relative amount of DNA in each band. This allows comparison between individual bands on the same gel.

6.3 Results and variations in PCR conditions.

6.3.1 Preliminary assessment of linearity and effect of cycle number.

Initially an attempt was made to determine whether a linear relationship existed between starting template cDNA concentration & the amount of PCR product and through how many cycles of PCR this was maintained.

The template used was cDNA that had been prepared by reverse transcription of RNA extracted from the T47D breast tumour cell line.

A 1μl sample of cDNA solution was serially diluted to give 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128 μl of cDNA in a total volume of 10μl per tube.

A master mix was prepared in accordance with the standard protocol in use in the laboratory. This contained PCR buffer (Gibco) at working strength, 1.5 mM $MgCl_2$

(Gibco), mixed dNTPs (0.8 µl per 50µl reaction), 100ng of 5' and 3' VEGF primers and Taq (Gibco) 1unit per 50ul reaction in a volume equivalent to 40ul per reaction. This was added to each of the diluted template samples to give a volume of 50µl.

Thermal cycler parameters were similar to those in routine use in this laboratory.

These were:
94° C for five minutes

Cycles of
94° C for thirty seconds
58° C for thirty seconds
72° C for thirty seconds

72°C for five minutes.

PCR was carried out for 25 cycles. A 10µl sample was then withdrawn from each tube. The reaction was continued for a further 5 cycles. 10 µl was withdrawn and the reaction continued for a further 5 cycles. Samples taken after 25, 30 and 35 cycles were electrophoresed on a 1.2% agarose gel along with a 100bp DNA molecular weight marker ladder.

The gel was analysed using the GelDoc acquisition and analysis system (Biorad) using volume analysis for each band with subtraction of both general and local backgrounds.

Only after 35 cycles were bands clearly visible though faint traces were seen with the higher template concentrations after 30 cycles. In the samples measured after 35 cycles, bands corresponding to 1/64µl or less of template were barely visible on either photographs or electronically captured images of the gels. Of the remaining reactions, that from the 1/4µl of template reaction gave no band and from the reaction with 1/16µl yielded a smear on the gel.

The DNA concentrations in the four visible bands from the samples taken after 35 cycles were estimated by volume analysis using the molecular analyst software (Biorad). When plotted against the relative concentration of template a straight line was obtained (r =1).

Amount of Template (ul of T47d cDNA)	Adj Volume (counts x mm²)
1	684
0.5	338
0.125	84
0.0312	11

Table 2. Preliminary assessment of linearity

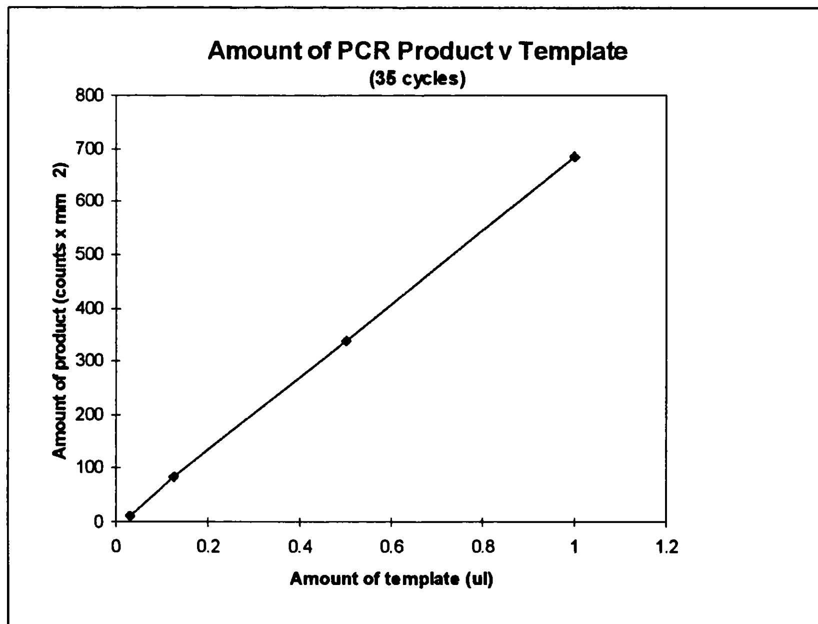


Figure 6 Preliminary assessment of linearity

Although there were only four usable data points, these were consistent with linear amplification having occurred.

6.3.2 Effect of Annealing Temperature.

Higher temperatures increase the specificity of annealing, reducing the chance of non-specific binding with consequent generation of extraneous product. However, excessive temperatures lead to poor annealing and may therefore compromise reaction efficiency. This experiment was designed to determine whether a lower annealing temperature would generate a higher reaction yield.

Sufficient master mix for four PCR reactions was prepared as above. Fifty microlitre reactions containing 1µl of T47D cDNA template were set up. Two reactions were placed on each block of the thermocycler and the programs below were run.

1)	2)
94°C for 5 minutes	94°C for 5 minutes
35 cycles of	
94°C for thirty seconds	94°C for thirty seconds
55°C for thirty seconds	58°C for thirty seconds
72°C for thirty seconds	72°C for thirty seconds
72°C for five minutes.	72° C for five minutes

The samples were run on an ethidium bromide agarose gel and analysed using the GelDoc system as before.

Annealing Temp °C	Adj Volume counts x mm²	mean	S.D
55°C	2315		
	1987	2151	232
58°C	2398		
	2371	2385	19

Table 3. Effect of annealing temperature

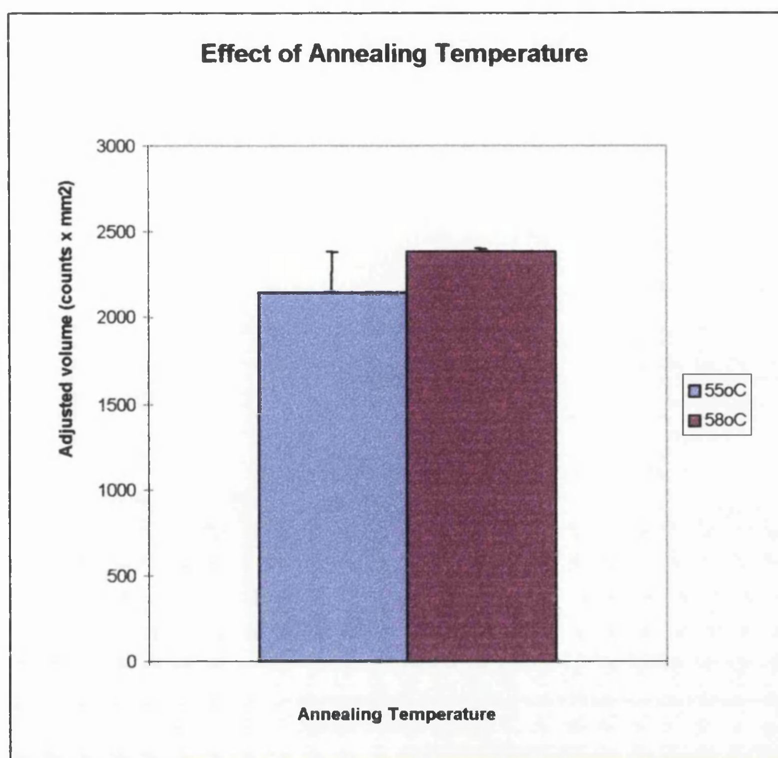


Figure 7. Effect of annealing temperature

Lowering the reaction temperature did not appear to result in an increased yield. It would not appear that the higher temperature was inhibiting annealing. No extra bands were seen at either temperature suggesting that annealing specificity was sufficiently high. It was therefore decided to continue to use 58°C as the annealing temperature.

6.3.3 Effect of Cycle Duration

Since two of the PCR reactions in the first experiment failed to yield a band when expected to do so, the possibility existed that there was a variation in the temperatures that the reactions were subjected to either due to differences in heat conduction of the tubes or the heating of the block. This is a particular problem if the cycle times are short so that the tubes do not have sufficient time to equilibrate to the set temperature and the reaction may not have time to complete once the correct temperature has been

reached. Such inconsistencies should be minimised by increasing the times spent at each temperature during the cycle. By ensuring that the reaction always goes to completion it was hoped that this might increase the overall efficiency of the reaction as well as minimising discrepancies between individual tubes.

Again, two pairs of reaction tubes with 0.5µl of T47D cDNA solution and all other components as above were set up. Using the satellite block of the thermal cycler for one pair, the PCR reaction was carried out under the following cycle settings

Pair 1	Pair 2
94°C for 5 mins	94°C for 5 mins
35 cycles of:	35 cycles of:
94°C for 30 secs	94°C for 1 min
58°C for 30 secs	58°C for 1 min
72°C for 30 secs	72°C for 2 mins
72°C for 5 minutes	72°C for 5 minutes

10µl of product from each tube was electrophoresed on a small 1.2% agarose gel as before. The gel was imaged with the GelDoc system and the bands analysed by volume analysis.

Cycle Duration	Adj Volume (counts x mm²)	mean	S.D
Short Cycle	13645		
	11744	12695	1344
Long Cycle	16879		
	18689	17784	1280

Table 4. Effect of cycle duration

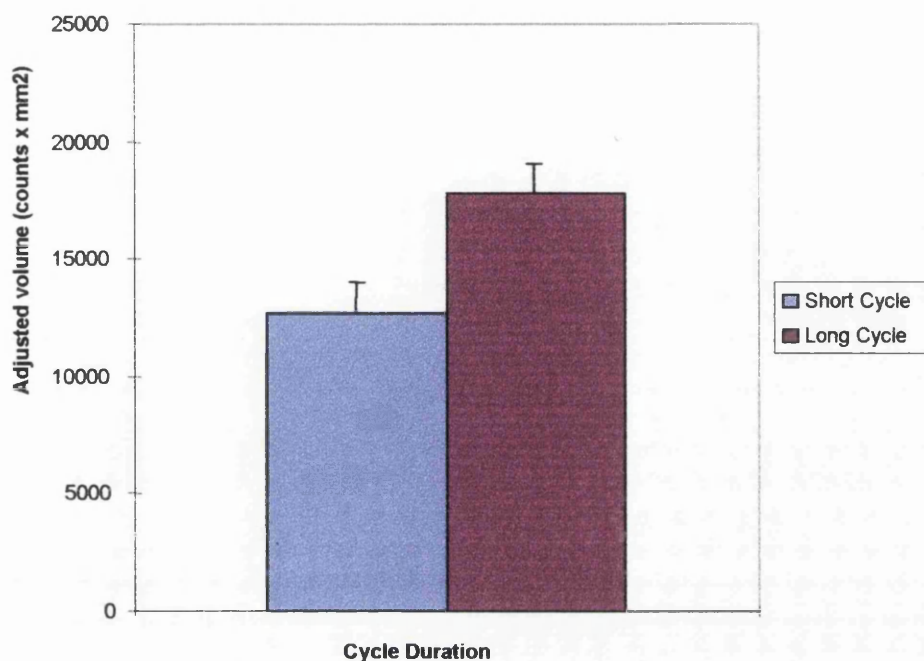


Figure 8. Effect of cycle duration

These results support the idea that using longer cycle times to ensure that each phase of the reaction consistently reaches completion does indeed increase the yield. The longer cycle durations were therefore used in subsequent work.

6.3.4 Optimisation of Reaction Mix Components.

As has been discussed in the introduction, the concentrations of the individual components of the PCR reaction are important to the efficiency of the reaction and to amplification linearity. In this set of experiments, the effect of varying Mg^{2+} concentration, Taq concentration and primer concentration was assessed. The other components of the reaction were used in the same concentrations as in the previous experiments.

For each experiment, a master mix was prepared containing all the reaction components except the one that was being titrated. PCR reactions were then carried out using the long cycle thermocycler setting described in the last experiment.

The settings were:

94°C for five minutes

35 cycles of:

94°C for one minute

58°C for one minute

72°C for two minutes

72 C for five minutes

The products of each reaction were analysed using the GelDoc system as before.

The following range of concentrations was used for each component.

Magnesium concentrations (2 units of Taq + 100ng of each primer per reaction)

1mM

1.5mM

2mM

2.5mM

3mM

3.5mM

4mM

Taq concentrations (1.5mM Mg and 100ng of each primer)

1 unit per reaction

2 units per reaction

3 units per reaction

4 units per reaction

Primer concentrations (1.5mM Mg and 2 units of Taq per reaction)

100ng of each primer

200ng of each primer

300ng of each primer.

Mg Conc mM	rel conc. (counts x mm2)	mean	S.D
1	699		
	1036	867.5	238.295
1.5	1194		
	1191	1192.5	2.12132
2	1105		
	1034	1069.5	50.20458
2.5	1124		
	1192	1158	48.08326
3	1167		
	1167	1167	0
3.5	646		
	857	751.5	149.1995
4	935		
	696	815.5	168.9985

Table 5 Effect of magnesium concentration

Effect of Mg Concentration.

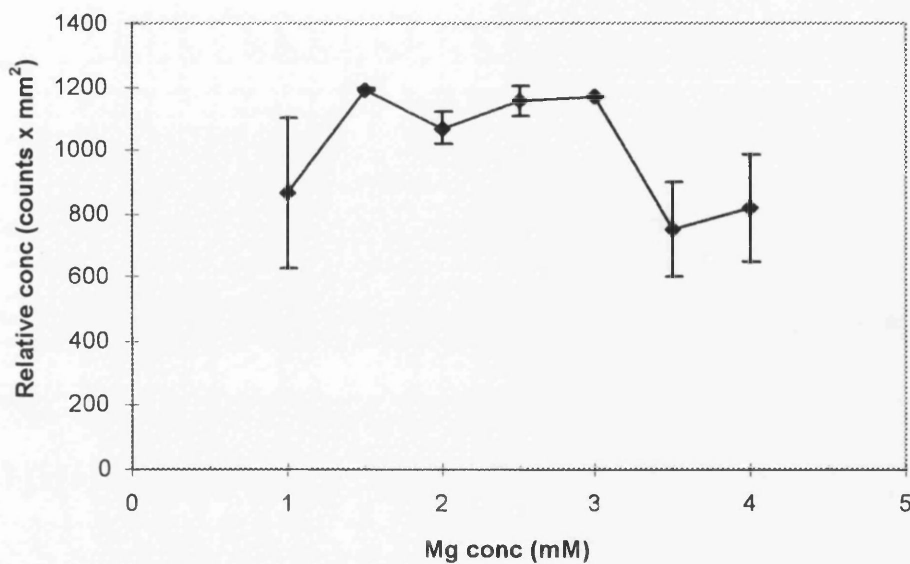


Figure 9 Effect of magnesium concentration

Amount of taq (unit / tube)	Rel. conc (counts x mm2)	mean	S.D
1	1094		
	1175	1134.5	57.28
2	1194		
	1191	1192.5	2.12
3	1622		
	2149	1885.5	372.65
4	648		
	979	813.5	234.05

Table 6 Effect of Taq concentration

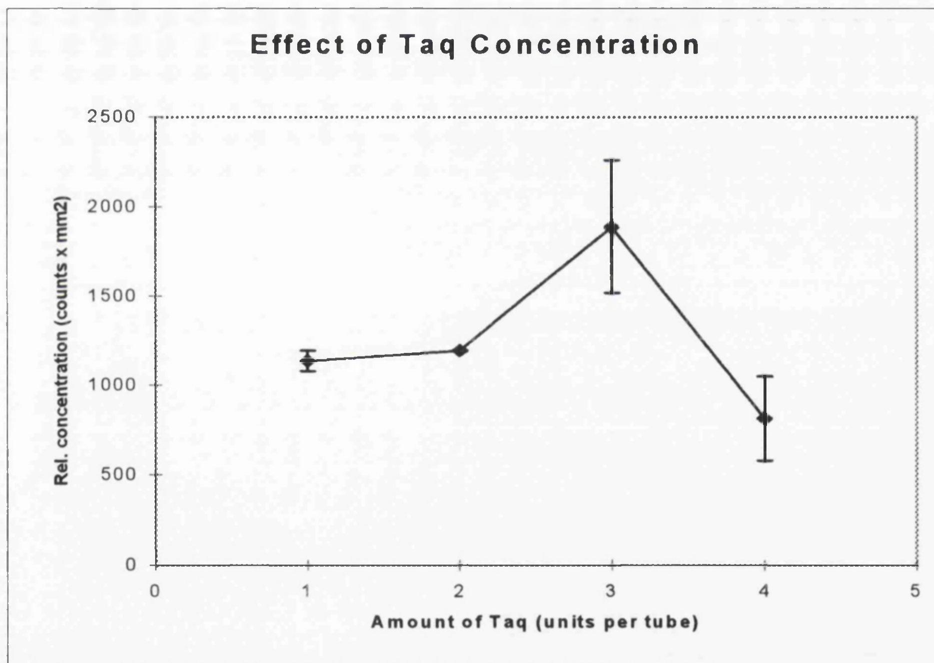


Figure 10. Effect of Taq concentration

Amount of Each Primer ng per reaction	Rel Conc.	Mean counts x mm2	S.D
100	1194		
	1191	1192.5	2.12
200	909		
	503	706	287.09
300	988		
	1009	998.5	14.85

Table 7. Effect of primer concentration

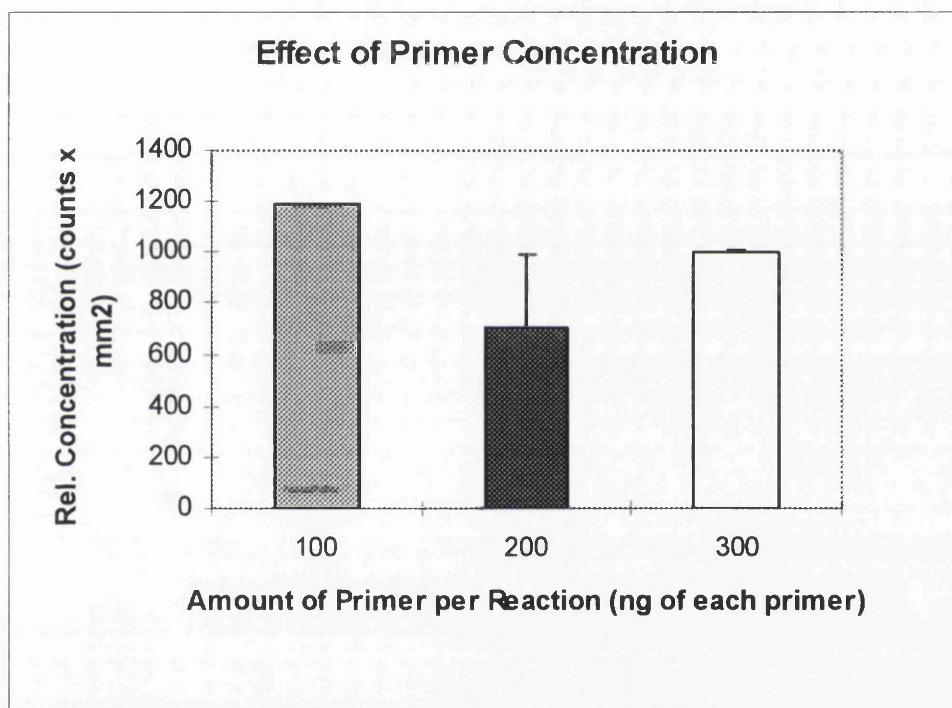


Figure 11. Effect of primer concentration

It appeared from these results that raising the concentration of $MgCl_2$ above 1.5mM gave no benefit in reaction efficiency. There appeared to be a large plateau in reaction efficiency with no substantial change in efficiency until the concentration was raised above 3mM. At higher concentrations the efficiency fell, presumably due to non-specific priming and indeed some smearing was seen on the gel. By using a concentration at the low end of this plateau, the possibility of non-specific priming was lowered. There appeared to be no benefit obtainable from increasing primer

concentrations so it was unlikely that these were limiting the amount of product generated in these reactions.

There did however appear to be significant benefit obtained from increasing the concentration of Taq with an increase in efficiency of 66% obtained by increasing the concentration from 1 to 3 units. The variations between duplicate reactions were however high and it was not clear why the efficiency apparently fell when four units of Taq per reaction were used. It was felt therefore that the possible benefit of an increased Taq concentration merited further investigation

6.3.5 Effect of Prolonging Extension time.

The need for such high concentrations of Taq for optimal efficiency raised the possibility that the reaction was still time limited. It seemed possible that if a longer extension phase was used that less Taq might be required. Therefore the effect of increasing the extension time from two to five minutes was investigated. Reactions were set up as before. Two pairs of reactions containing 1unit of Taq and two containing 3 units were prepared. One pair with each Taq concentration were subjected to the following thermocycle programs

Program 1	Program 2
94°C for five minutes	94°C for five minutes
35 cycles of:	35 cycles of:
94°C for one minute	94°C for one minute
58°C for one minute	58°C for one minute
72°C for two minutes	72°C for five minutes
72°C for five minutes	72°C for five minutes

Amount of Taq	Extension time	Rel Conc. counts x mm2	Mean	S.D
1 unit per reaction	1 min	4090 4326	4208	166
	5 mins	5347 5371	5359	17
3 units per reaction	1 min	4777 4559	4668	154
	5 mins	4279 4554	4416.5	194

Table 8 Combined effect of Taq and extension time

At low Taq concentration it appeared that prolonging the extension time did indeed increase yield. However at the higher Taq concentration, a prolonged extension time did not lead to a higher yield.

6.3.6 Effect of Incremental Extension Time

It was possible that the finding of poor yield with the combination of a high Taq concentration and long extension time was due to the generation of inappropriate non-specific product. Since this is most likely to occur during the earlier cycles when concentrations of primers and Taq are highest it is possible that a short extension time might be appropriate at this stage. A longer time would be appropriate later in the reaction when Taq concentrations are lower due to degradation and primer concentrations are reduced. An experiment was designed to investigate this using incremental extension times.

A master mix was prepared and reactions were set up as before. Two pairs of reaction tubes containing 1 unit of taq and two containing 3 units were prepared. A pair with each Taq concentration was subjected to a thermocycler program that increased the extension time by five seconds each cycle starting with a two minute extension time progressing to a time of just less than five minutes by the 35th cycle. The remaining

two pairs were subjected to a program that gave an extension time of five minutes throughout.

The thermocycler program parameters are shown below.

Program 1	Program 2
94°C for five minutes	94°C for five minutes
35 cycles of:	35 cycles of:
94°C for one minute	94°C for one minute
58° C for one minute	58°C for one minute
72°C for two mins in the first cycle increasing by five secs each cycle.	72°C for five minutes
72°C for five minutes	72°C for five minutes

Amount of Taq	Extension time	Rel Conc. counts x mm2	Mean	S.D
1 unit of Taq	5 min extension time	1543 1667	1605	88
	incremental extension time	673 803	738	92
3 units of Taq	5 min extension time	1816 1906	1861	64
	incremental extension time	1626 1179	1403	316

Table 9. Effect of incremental extension time

These results did not show any benefit in using the incremental extension time over a steady five minute extension time with either 1 or 3 units of Taq in the reactions. In this experiment there was a slightly higher product yield with 3 units of Taq per reaction.

6.3.7 Optimisation of Taq concentration

In order to elucidate further the effect of Taq concentration with the longer, five minute extension time, a series of reactions was set up with the following amounts of Taq per tube:-

1.5 units

2 units

2.5 units

3 units

3.5 units

4 units

A master mix was prepared containing 1.5mM $MgCl_2$, and 100ng of each primer per reaction. Thermocycler settings were the same as used previously with a constant five minute extension time

Units of Taq per reaction	Rel Conc. counts x mm2	Mean	S.D
1.5	3640		
	3670	3655	21.21
2	3878		
	3849	3864	20.51
2.5	3613		
	3662	3638	34.65
3	2840		
	3410	3125	403.05
3.5	2706		
	3211	2959	357.09
4	3216		
	3026	3121	134.35

Table 10 Optimisation of Taq concentration

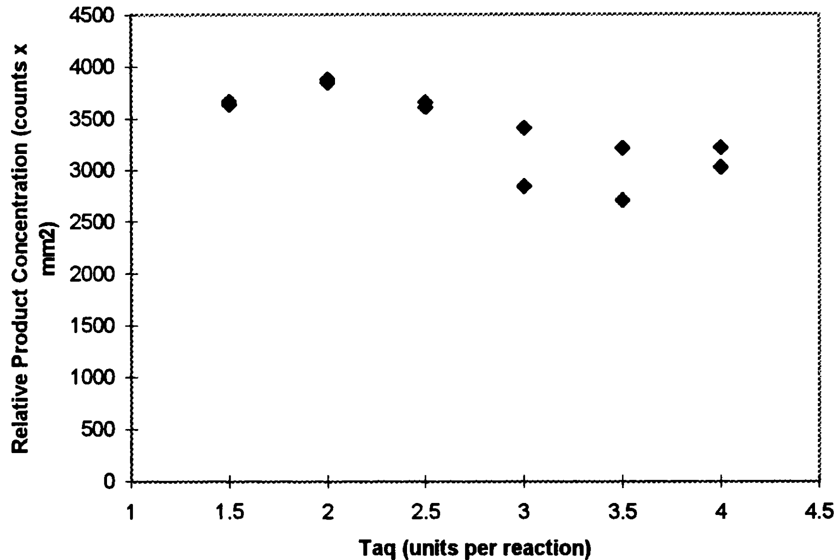


Figure 12. Optimisation of Taq concentration

In this experiment, the highest yield of product occurred in the reactions with 2 units of Taq and there was no benefit seen in increasing beyond this. It appeared that with a five minute extension time 2 units of Taq were sufficient to allow the reaction to go as near as possible to completion. This amount was therefore used in subsequent work.

6.3.8 Titration of dNTPs in the VEGF PCR reaction.

The dNTPs provide the building blocks for the synthesis of cDNA in the PCR reaction. An experiment was set up to determine whether the availability of dNTPs was limiting the generation of product.

VEGF PCR reactions were set up as in the previous experiments, i.e. 2 units of taq per reaction, 100ng of each primer and $MgCl_2$ concentration of 1.5mM in a total volume of 50 μl. Duplicate reactions containing 4, 8, 12 or 16 and 20 pmol of mixed dNTP were prepared. Reactions were run for 30 cycles of PCR using the same reaction conditions as had been previously used i.e. 94°C denaturation, 58°C annealing and 72°C chain extension.

dNTPs pmol/reaction	Adj Volume (counts x mm²)	Mean Adj Volume (counts x mm²)
4	2610 2864	2737
8	2503 2905	2704
12	2304 1721	2013
16	475 810	643
20	0 0	0

Table 11. Effect of dNTP concentration

There did not appear to be any benefit in increasing the dNTP concentration. Instead higher dNTP reaction appeared to be associated with a decreased amount of reaction product. This may be due to misincorporation of bases leading to failure of priming in subsequent cycles.

6.3.9 Optimisation of Cycle duration and Taq concentration for Actin PCR

Primers had previously been used in the laboratory, which amplify a fragment of the β-actin cDNA sequence. The reaction conditions being used with these primers were arbitrary and work was therefore directed at optimising these.

The primers were supplied by Oswel DNA, Edinburgh. The sequence of these has been described in the introduction to this chapter

As a preliminary step to gauge the potential for optimisation, the effects of using a longer cycle and a higher taq concentration as used in the VEGF PCR reactions were compared to the protocol previously used in the laboratory which used 1 unit of Taq per reaction consisted of 30 seconds of denaturation, annealing and extension in each cycle

Reactions were therefore set up using ether one or two units of Taq. Two thermocycler programs were used, a short cycle with thirty second steps as before and a long cycle as used for the VEGF assay consisting of one minute denaturation, one minute annealing and five minutes extension in each cycle. The reaction conditions were otherwise as before i.e. PCR buffer (Gibco) at working strength, 1.5 mM $MgCl_2$ (Gibco), mixed dNTPs (0.8 µl (8pmol) per 50µl reaction). 0.5µl of 10uM sense and antisense primers were added.

Amount of Taq	Cycle duration	Adj Volume counts x mm²	Mean	S.D
	Short	6007		
	cycle	8235	7121	1575
1unit taq				
per reaction	Long	8221		
	cycle	8365	8293	102
	Short			
2 units taq	cycle	9596	9045	780
per reaction		8493		
	Long	8091		
	cycle	9227	8659	803

Table 12. Effect of Taq and cycle duration on actin PCR

It appeared from these results that using two rather than one unit of taq per reaction may be of benefit in increasing the yield of the PCR reaction. No benefit was however seen in this experiment from increasing cycle times.

6.3.10 Optimisation of Magnesium Concentration Actin PCR

The concentration of magnesium in the PCR reaction is critical to the specificity and yield of the reaction. Therefore an experiment was set up to determine the optimal magnesium concentration for the actin PCR. The temperature at which annealing is carried out is linked to the magnesium concentration in that any non-specific binding that occurs at higher magnesium concentrations may be potentially be prevented by increasing the annealing temperature. Therefore, the titration was carried out at 65°C as well as 60°C. Based on the result of the previous experiment, 2 units of Taq were

used in each reaction. A range of magnesium concentrations from 1 to 4 mM was used. The master mix was otherwise as before.

PCR was carried out as follows:

94° C for five minutes

Cycles of

94° C for thirty seconds

60°C or 65°C for thirty seconds

72° C for thirty seconds

72°C for five minutes.

Samples were run on an ethidium bromide gel and analysed with the GelDoc system as before.

	60 degrees			65 degrees		
Mg Conc. (mM)	rel. vol of band counts x mm2	mean	S.D	rel. vol of band counts x mm2	mean	S.D
1.0	3523			3630		
	3763	3643	170	2682	3156	670
1.5	5092			5214		
	5497	5295	286	4109	4662	781
2.0	1924			3235		
	3671	2798	1235	3324	3280	63
2.5	1264			1107		
	1015	1140	176	933	1020	123
3.0	464			183		
	527	496	45	237	210	38
3.5	412			115		
	242	327	120	174	145	42
4.0	159			0		
	47	103	79	201	101	142

Table 13. Relationship between Magnesium Concentration and Reaction Yield

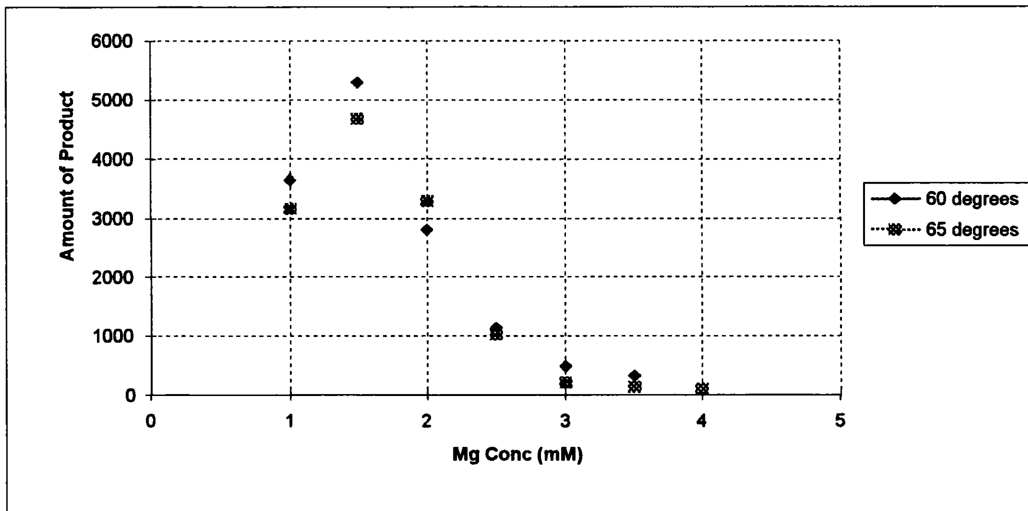
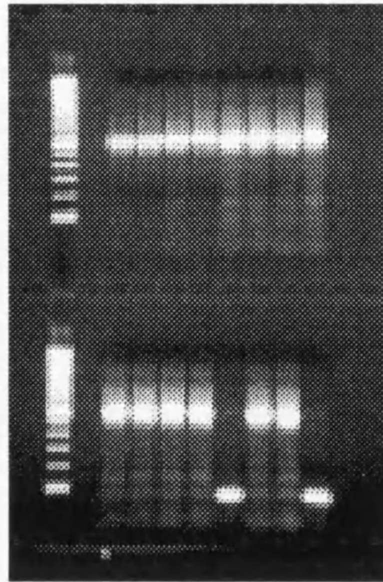


Figure 13. Relationship between Magnesium Concentration and Reaction Yield

From this experiment, the optimal reaction yield would appear to occur at a magnesium concentration of 1.5mM with a temperature of 60°C. Since these were the conditions already being employed, no improvement of reaction conditions could be made from the information obtained in this experiment.

6.3.11 Titration of Primers

The effect of varying the concentration of the PCR primers was examined to determine whether this might be a limiting factor in the reaction and whether changing this might improve reaction yield. Amounts from 5 to 40 pmol of each primer were tested. Reactions were otherwise as described previously. Thermocycler settings were as before and PCR was run over 30 cycles.



Duplicate reactions or each primer concentration

Figure 14. Primer titration- Photograph of gel

Amount of each primer (pmol)	Adj Volume (counts x mm²)	Mean Adj Volume (counts x mm²)
5	4938	
	5241	5090
10	4995	
	5447	5221
15	6056	
	6203	6130
20	5521	
	5693	5607
25	6090	
	5280	5685
30	6059	
	6010	6035
35	13	
	6740	NA
40	6711	
	58	NA

Table 14. Effect of titration of primers on actin PCR

These results suggested that some improvement in reaction yield could be achieved by increasing primer concentrations in the reactions. These improvements were however modest and did not suggest that primer shortage was a major constraint on the reactions. Moreover when 15 pmol or more of each primer was added the emergence

of extra low molecular weight bands was seen. By comparison, with a plot of migration distance of the 100 base pair markers, it is apparent that these extra bands are approximately 50, 100 and 150 base pairs compared with the main actin band of approximately 600 base pairs. Given that the primers are 24 bases in length, these extra bands may represent either primer polymers or mispriming. The appearances of this phenomenon at high primer concentrations therefore limits the increase that can be made. However given that increasing primer concentration did seem to be of some benefit, an increase from 5 to 10pmol of each primer per reaction was made in subsequent experiments.

6.3.12 Titration of dNTPs.

The effect of making two and three fold increases in the dNTP concentration was examined.

Reactions were again set up as above. The increase in the amount of each primer in each reaction from 5 to 10pmol was implemented. Duplicate reactions containing 8, 12 or 16 pmol of dNTPs were set up. Thermocycler settings were as before and 30 cycles of PCR were carried out.

dNTPs pmol/reaction	Adj Volume (counts x mm²)	Mean Adj Volume (counts x mm²)
8	23359	
	21937	22648
12	29364	
	28683	29024
16	28617	
	23010	25814

Table 15. Effect of dNTP concentration in the actin PCR

These results suggested that an increase in the amount of dNTP to 12 pmol per reaction did indeed increase the amount of product generated. However a further increase yielded no apparent additional benefit. Therefore in subsequent experiments the amount of dNTPs in each reaction was increased.

6.3.13 Effect of Length of Cycle Steps in the Actin PCR Reaction

In the preceding experiments, it can be seen that there have been considerable differences between the amounts of product from duplicate PCR reactions although these were set up by dividing a common substrate mix. This suggests that there was some difference in efficiency of the PCR reaction between tubes. It was felt that the thirty seconds allowed for each step of the reaction might not always be enough to let the reaction go to completion. Therefore the effect of doubling the step times on both the reaction yield and the variation in yield between tubes was investigated.

Actin PCR reactions were set up as before with of 10pmol of each primer and 12pmol of dNTPs per reaction. Twelve 50μl reactions were prepared and six placed in each of two thermocycler blocks.

In both blocks, the tubes were heated to 94°C for five minutes. This was then followed by 30 cycles with 94°C denaturation, 60°C annealing and 72°C extension steps. In the first block these steps were of thirty seconds duration. In the second block, each step lasted sixty seconds. Finally both blocks were held at 72°C for five minutes. Products were analysed as before using agarose gel electrophoresis and the GelDoc system. The mean and standard deviation for each group of six samples was calculated.

Thermocycler Settings	Product Adj Volume counts x mm²	Mean	S.D
30 second thermocycler steps	8767		
	11804		
	12074		
	12075		
	11599		
	12110	11405	1308
60 second thermocycler steps	11823		
	12290		
	11688		
	13015		
	11816		
	11802	12072	507

Table 16 Effect of cycle step duration in actin PCR

From these results, it appeared that increasing the time for each step of the reaction from thirty seconds to one minute not only leads to a slight improvement in reaction yield but, more importantly decreased the variation between reactions, as shown by the decrease in the standard deviation. The reaction time for each step of the actin PCR was therefore increased to one minute.

6.3.14 Assessment of linearity of VEGF PCR under modified reaction conditions

Initially, in section 6.3.1, a linear relationship was demonstrated between the amount of template present at the start of the reaction and the amount of product after 35 cycles of PCR. However, as changes had been made to the reaction times and the amount of Taq, it was necessary to test whether this linearity remained.

A serial dilution of cDNA from the T47D line was prepared to give reactions containing 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 µl of the cDNA solution. A master mix of the other reaction components was prepared and aliquoted into each tube so that each reaction contained 2 units of taq, 8mol of dNTPs, 100ng of each primer and an $MgCl_2$ concentration of 1.5mM.

The PCR reaction was carried out as before for 30 cycles i.e,

94°C for five minutes

30 cycles of

94°C for one minute

58°C for one minute

72°C for five minutes

72°C for five minutes.

A 10μl sample was then withdrawn from each tube and a further 5 cycles of the PCR reaction carried out.

Amount of cDNA per reaction (μl)	Amount of Product Rel. vol of band	
	30 cycles	35 cycles
1	751	3138
0.5	580	3183
0.25	250	2766
0.125	121	1459
0.0625	68	930
0.03125	15	628
0.015625	3	239
0.0078125	5	289

Table 17. Test of linearity at 30 and 35 cycles

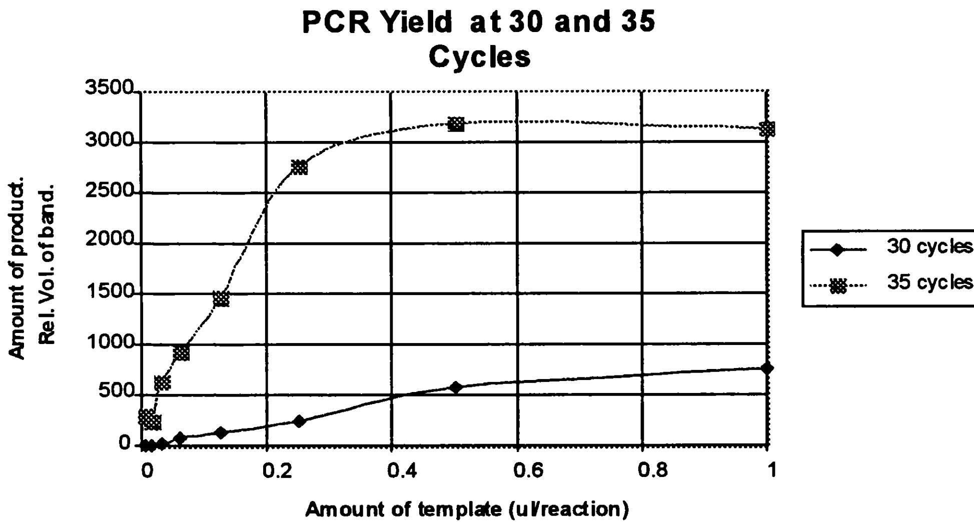


Figure 15. Test of linearity at 30 and 35 cycles

It can be seen from the graph that after 30 cycles a linear relationship appeared to exist between the amount of template cDNA and the amount of product formed with less than 0.5µl of template cDNA solution per reaction. The yield of product after 35 cycles was higher but a plateau in the product verus template plot was seen when the amount of template exceeded 0.2µl of mRNA solution. This would potentially limit the range of RNA concentrations over which the reaction was usable. Further reactions were therefore set up to assess whether a better compromise between product yield and linearity could be achieved between the 30 and 35 cycle reactions used above.

Serial dilutions of T47D cDNA were prepared and aliquoted into tubes to give 4 sets of reactions each containing the following range of cDNA amounts:- 4,2,1,1/2, 1/4,1/8, 1/16,1/32 µl.

The VEGF PCR master mix was the same as previously used. VEGF PCR was run for 32 and 33 cycles using one minute denaturing and annealing steps and five minute extension. The composition of the actin master mix reflected the modifications derived from previous experiments i.e. 2 units of taq per reaction, 12 pMol of dNTPs, 100ng of each primer and a $MgCl_2$ concentration of 1.5mM. Actin PCR was run for 26 and 28 cycles using one minute denaturing, annealing & extension steps. Relative

volumes of the bands on the ethidium bromide gel were derived from the GelDoc system and plotted against the amount of template in each reaction.

VEGF		
Amount of Template ul/reaction	Adj Volume counts x mm²	
	32 cycles	33 cycles
4	717	2563
2	256	985
1	135	561
0.5	111	198
0.25	93	51
0.125	24	39
0.0625	1	15
0.03125	0	3

Table 18. Linearity test of VEGF PCR 32 and 33 cycles

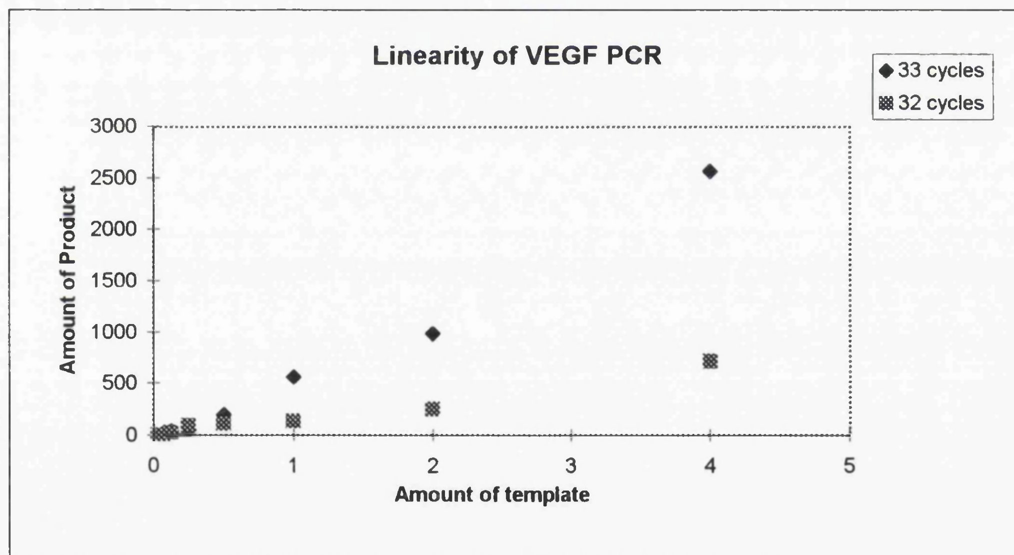


Figure 16. Linearity test of VEGF PCR 32 and 33 cycles

It appeared from the data above that VEGF PCR can be carried out up to 33 cycles with preservation of linearity over a wide range of reaction conditions.

6.3.15 Assessment of linearity of Actin PCR under modified reaction conditions

An attempt was made to determine how many cycles the actin PCR could be run for whilst preserving the linearity of the product v template curve.

The composition of the actin master mix reflected the modifications derived from previous experiments i.e. 2 units of taq per reaction, 12 pMol of dNTPs,100ng of each primer and a MgCl2 concentration of 1.5mM. Actin PCR was run for 26 and 28 cycles using 1 minute denaturing, annealing & extension steps. Relative volumes of the bands on the ethidium bromide gel were derived from the GelDoc system and plotted against the amount of template in each reaction.

Actin Amount of Template ul/reaction	Adj Volume counts x mm^2 26 cycles	 28 cycles
4	6675	7624
2	6400	8361
1	5774	8567
0.5	5535	8499
0.25	5592	7649
0.125	4540	6235
0.0625	4239	5372
0.03125	3047	3271

Table 19 Linearity test of actin PCR 26 and 28 cycles

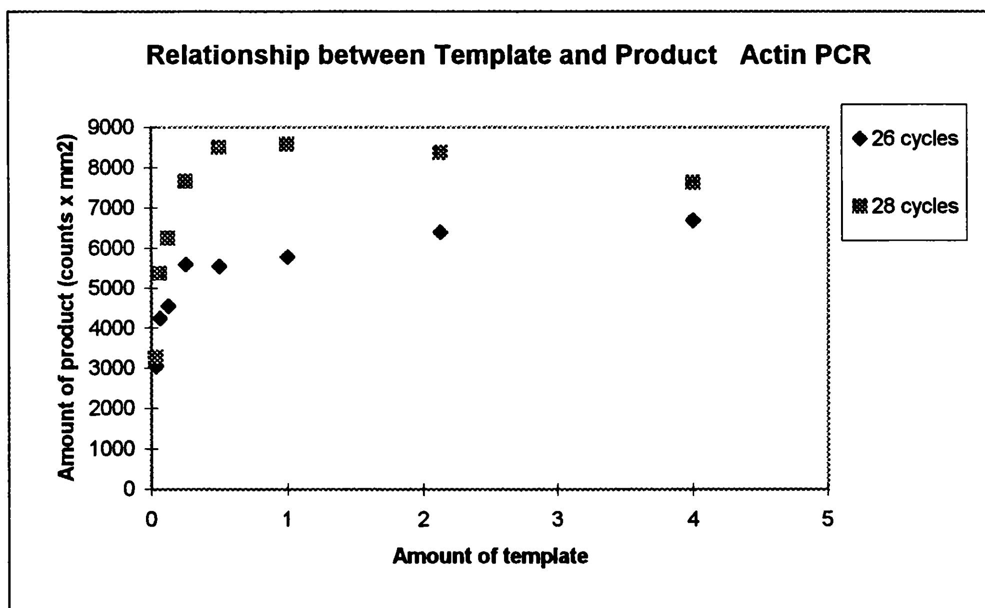


Figure 17 Linearity test of actin PCR 26 and 28 cycles.

There was a rapid rise in the amount of actin product generated with increasing template quantities but at both 36 and 28 cycles a plateau was rapidly reached. This suggested that a smaller number of PCR cycles may be appropriate to extend the usable range of template mRNA concentrations. As the ultimate aim was to achieve linearity of the entire reverse transcription and PCR process so efforts were directed at optimising the reverse transcription step and then verifying linearity of the entire process, rather than carrying out any further verification of the PCR linearity at this stage.

6.4 Optimisation of the Reverse Transcription Reaction

6.4.1 Screening of samples for DNA contamination.

Before carrying out any work on the efficiency of the reverse transcription reaction, it was important to ensure that no DNA was present in the RNA samples before reverse transcription. This could occur as a result of contamination of the samples by genomic DNA.

Samples of RNA which had been extracted from T47D cells were therefore screened. Five PCR reactions were set up for both VEGF and actin as described previously. One microgram of T47D RNA was added to duplicate actin and VEGF reactions. A positive control reaction for both actin and VEGF was created by adding T47D cDNA to the fifth tube of each set. PCR was then carried out for 35 cycles for VEFG and 25 cycles for actin. Products were run on an agarose ethidium bromide gel as previously.

Both control reactions yielded an appropriate product band on the gel. No product band was seen from any of the reactions containing only RNA.

This experiment demonstrated that the cell line derived RNA samples for use in the next part of the work did not contain contaminating DNA detectable by either the VEGF or actin reactions despite using relatively large amounts of RNA and larger than normal numbers of amplification cycles.

6.4.2 Optimisation of Temperature and Time for the Reverse Transcription Reaction

Whilst the manufacturer's protocol [106] suggests a reaction temperature of 37°C, it states that in some cases incubation at a higher temperature may increase yields or facilitate transcription of some mRNAs. The protocol recommends a reaction time of one hour. Since the time taken to achieve maximum yield may be linked to the reaction temperature, this experiment was designed to determine the effects both of raising reaction temperature and of increasing reaction time on the yield of VEGF and actin cDNAs from reverse transcription of their mRNAs.

The protocol for reverse transcription was based on that given in the Superscript data sheet except no radioactive marker was added and RNAase inhibitor was added to the reactions to protect the RNA during the course of the reaction. Pairs of reactions were run under the following incubation conditions.

1. 37 °C for one hour.
2. 37 °C for one and a half hours.
3. 45°C for one hour.
4. 45°C for one and a half hours.

Triplicate PCR reactions to amplify VEGF and β-actin were set up containing 10μl of the products of each reverse transcription reaction. There were therefore four PCR reactions for each set of reverse transcription conditions.

VEGF Reverse Transcription

Reaction Conditions	Rel Conc. (adj. vol)	Mean for each RT Reaction	Overall Mean (mean adj vol)	S.D
37°C 1 hour	3824			
	4257			
	3915	3999		
	3220			
	4019			
	3708	3649	3824	349.9
37°C 1 1/2 hours	4590			
	3560			
	3490	3880		
	3253			
	3705			
	4087	3682	3781	482.9
45°C 1 hour	2452			
	3186			
	2967	2868		
	3800			
	4485			
	3724	4003	3436	716.5
45°C 1 1/2 hours	1212			
	2027			
	2796	2012		
	3186			
	2377			
	1223	2262	2137	811.8

Table 20. Optimisation of Temperature & Time for the Reverse Transcription Reaction

This experiment was repeated and similar results wereobtained. These results do not show any benefit in either increasing the reaction time or temperature. Instead when both reaction time and temperature were increased there was a marked detrimental

effect in the amount of product formed which was confirmed when the experiment was repeated. This may be due to degradation of the cDNA in the final half hour of the RT reaction.

6.4.3 Effect of a Smaller Increase in Temperature.

It appeared from the last two experiments that raising the reaction temperature for the reverse transcription reaction to 45°C was not beneficial to reaction yields and may even be detrimental. Therefore an experiment was carried out to determine whether a smaller increase in temperature may enhance reaction efficiency.

Duplicate reverse transcription reactions were carried out as before at 37°C and 42°C. The products of these reactions were then amplified by PCR reactions for VEGF and actin as before with triplicates of each PCR reaction carried out on the products of each RT reaction. Gel electrophoresis was carried out as before.

Reaction Conditions	Rel Conc. (adj. vol)	Mean for each RT Reaction	Overall Mean mean adj vol)	S.D
VEGF				
37°C	2299			
	1721			
	1579	1866		
	2185			
	3513			
	3505	3068	2467	851.4
42°C	1890			
	2776			
	2510	2392		
	1871			
	3270			
	2070	2404	2398	558.4
Actin				
37°C	3243			
	4138			
	4712	4031		
	5326			
	5170			
	2500	4332	4182	1120.6
42°C	3647			
	3379			
	4423	3816		
	6189			
	2570			
	5843	4867	4342	1429.8

Table 21. Effect of a Smaller Increase in Temperature

In this experiment it appeared that, in the reverse transcription, reaction there was no significant difference between the yields of either actin or VEGF cDNA in those reactions run at 37°C and those at 42°C. Considering the preceding reverse transcription experiments overall, it appeared that the reactions generally reach optimum yield by one hour and that variations in temperature had little influence on the yield when the reactions are run for one hour. It was decided therefore to run future reverse transcription reactions for one hour and use 42°C as the reaction temperature.

6.4.4 Test of Linearity of Reverse Transcription Reaction by Serial Dilution

Eight reverse transcription reactions with serially halving quantities of T47D RNA from 2 µg to 0.015µg per reaction were set up and incubated as above. The transcription products were then used to set up triplicate PCR reactions for both VEGF and actin as before and products analysed using the GelDoc system.

Reverse Transcription (VEGF)

RNA /reaction ug	Adj Volume counts x mm²	Mean Vol counts x mm²	S.D
2	2271		
	2827		
	2049	2438	550
1	1412		
	1714		
	1427	1518	131
0.5	1203		
	930		
	1589	1241	232
0.25	722		
	831		
	487	680	129
0.125	162		
	261		
	169	197	42
0.0625	84		
	151		
	171	135	34
0.03125	68		
	124		
	59	84	27
0.015625	89		
	22		
	100	70	32

Table 22 Test of Linearity of Reverse Transcription Reaction by Serial Dilution (VEGF)

Reverse Transcription (Actin)

RNA /reaction ug	Adj Volume counts x mm²	Mean Vol counts x mm²	S.D
2	(2715)		
	6010		
	5887	5949	87
1	3796		
	5656		
	5031	4828	688
0.5	3909		
	1362		
	4462	3244	1255
0.25	1430		
	2411		
	2377	2073	428
0.125	262		
	386		
	1210	619	394
0.0625	306		
	138		
	1132	525	404
0.03125	217		
	168		
	235	207	26
0.015625	236		
	207		
	49	164	77

Table 23 Test of Linearity of Reverse Transcription Reaction by Serial Dilution (Actin)

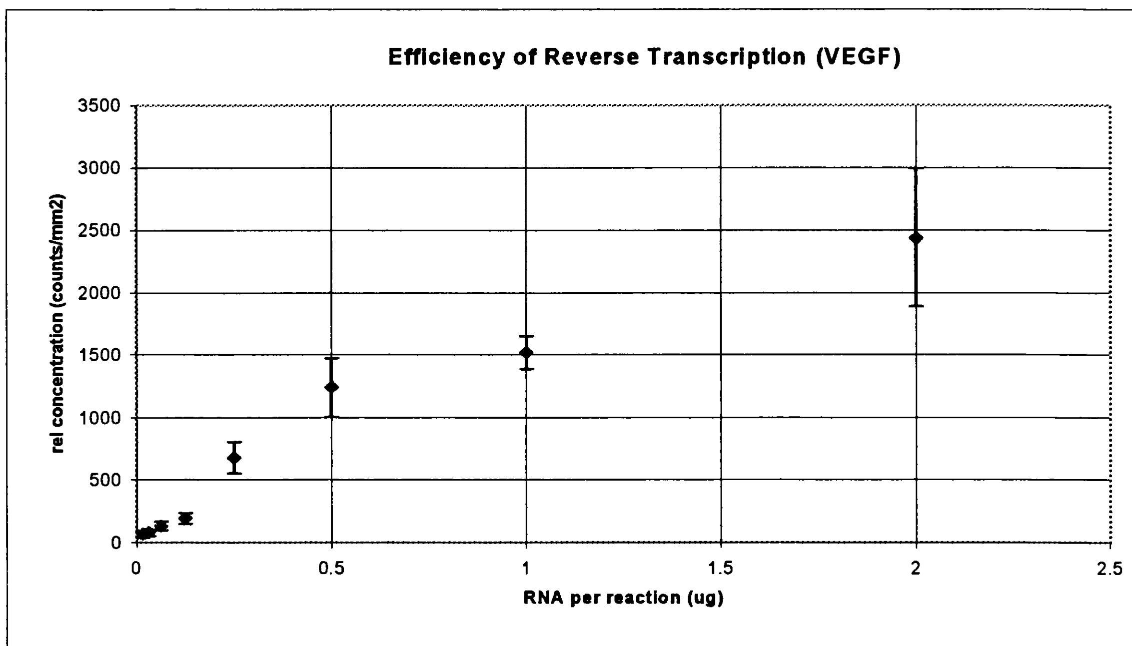


Figure 18. Test of Linearity of Reverse Transcription Reaction by Serial Dilution (VEGF)

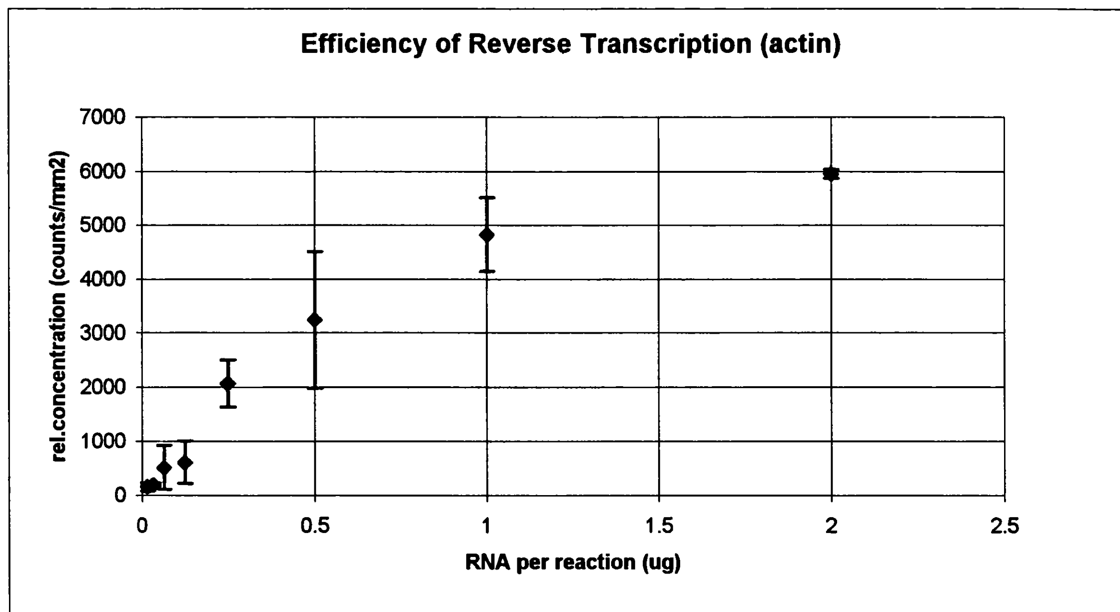


Figure 19. Test of Linearity of Reverse Transcription Reaction by Serial Dilution (Actin)

In both case a plateau effect was seen when the amount of RNA in the reactions exceeded about 0.5μg. This was not unexpected since the manufacturer's protocol for the reverse transcriptase recommends a maximum of 1μg of RNA per reaction. It would be therefore more appropriate to look closer at the reactions with lower amounts of RNA. These are plotted below.

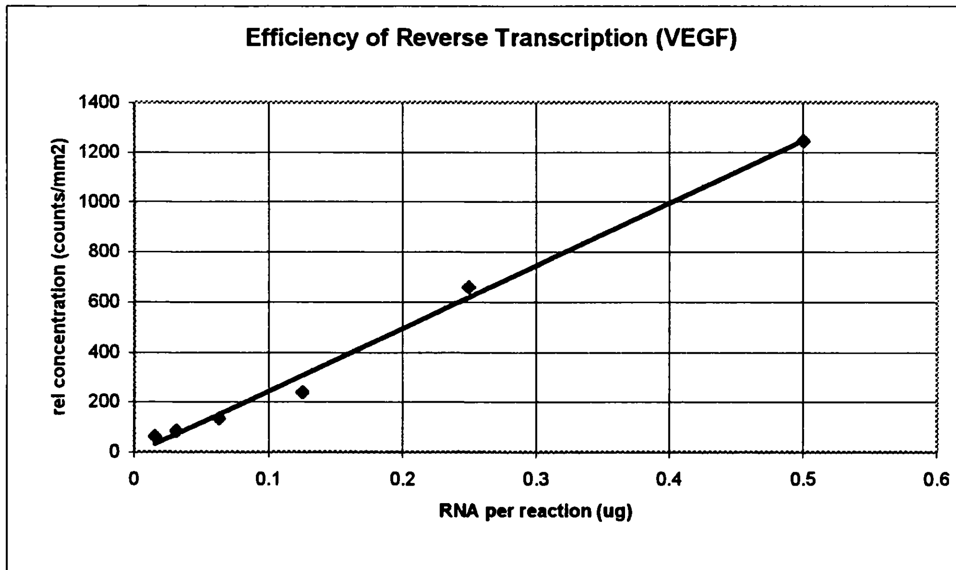


Figure 20. Linearity of Reverse Transcription Reaction (VEGF low range)

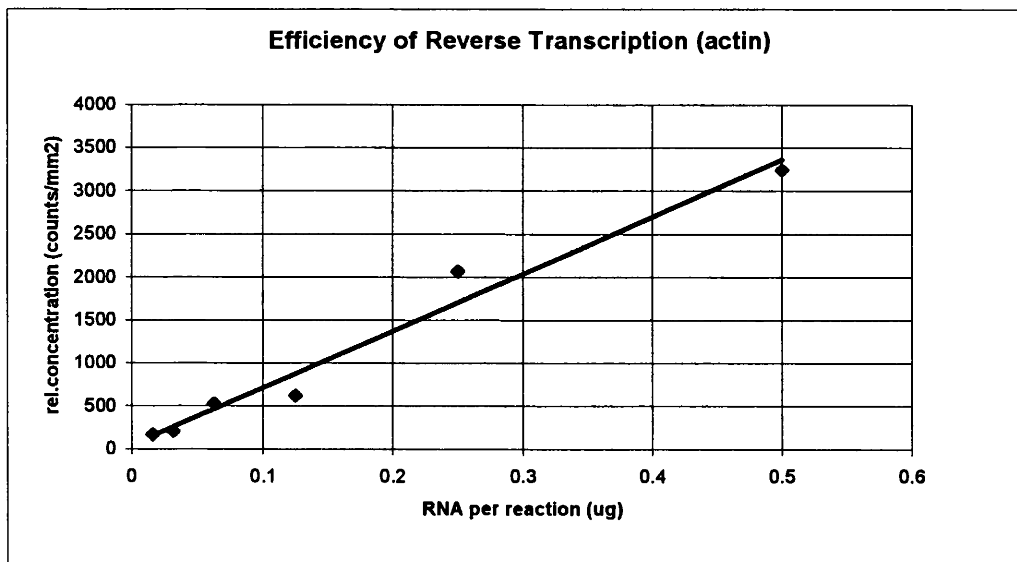


Figure 21. Linearity of Reverse Transcription Reaction (Actin low range)

With reactions containing less than 0.5μg of RNA per reaction, no plateau effect was demonstrated. It appears that in this range a linear relationship exists between the amount of RNA in the reaction and the amount of VEGF and actin cDNA generated.

6.4.5 Optimisation of the DNAase Protocol.

In the course of screening RNA samples extracted from human tissue samples for DNA contamination it was found that occasionally a faint genomic band was seen in the actin PCR reactions. Experiments were therefore conducted to assess whether DNAase treatment of the RNA samples would have any adverse effect on the subsequent RT-PCR reactions and to determine the best protocol for DNAase treatment.

One sample that had previously exhibited a genomic band in the actin PCR reaction and one that did not were used. Two micrograms of each RNA sample were diluted to 20μl. These samples were then split and half subjected to DNAase treatment. One microliter of DNAase I (Gibco) and 1μl of DNAase buffer (Gibco) were added to each sample to be DNAase treated. The tubes were then incubated at 25°C for fifteen minutes on the PCR block followed by heating to 65°C for ten minutes to denature the DNAase. The Gibco protocol recommends addition of EDTA during the denaturation stage but it was hoped that this would not be necessary in order to avoid to the possibility that carry over might affect the conditions in the PCR reaction particularly be chelating magnesium. Each sample was divided following DNAase treatment. One half was subjected to reverse transcription as before whilst the other did not undergo reverse transcription. The halves of the samples that had not been subjected to DNAase treatment were similarly split and one part subjected to reverse transcription. The final samples resulting from these divisions and treatments were then split and used in duplicate actin PCR reactions with the previously developed protocol. Products were analysed by gel electrophoresis.

DNAase + RT	Large smears on both samples.
DNAase - RT	No contaminating bands.
No DNAase + RT	Strong product bands on both samples
No DNAase - RT	Contaminating 600 & 800 base pair bands

Table 24 Effect of DNAase treatment

The sample that previously exhibited contaminating DNA bands did so again. This was successfully eliminated by DNAase. However reverse transcription of the DNAase treated samples resulted in a large smear, probably due to failure of deactivation of the DNAase.

It appeared that more stringent methods were required to denature the DNAase either by the addition of EDTA as suggested or using a higher temperature.

6.4.6 Effect of different DNAase denaturing conditions on RT-PCR reaction yields for VEGF and Actin.

An experiment was designed to compare the product yields after reverse transcription and PCR when samples were prepared by different DNAase protocols.

The experiment used the tumour RNA sample with trace contaminating DNA which was used in the previous experiment. Samples were DNAase treated for fifteen minutes at 25°C and the DNAase was then deactivated by a ten minute incubation at either 65°C with addition of 1µl of 20mM EDTA or 95°C with or without EDTA. Control samples with no DNAase treatment were also set up. Reverse transcription followed by PCR for actin and VEGF were carried out as before. VEGF and actin yields were compared between the DNAase free controls and the DNAase-treated samples using the various protocols.

	Adj Volume counts x mm²	Mean	S.D
Actin			
control (no DNAase or heat)	4676		
	3999		
	3874	4183	432
65 + EDTA	4660		
	4501		
	4330	4497	165
95	3857		
	4893		
	4650	4467	542
95 + EDTA	4930		
	4780		
	4924	4878	85
VEGF			
control (no DNAase or heat)	865		
	839		
	732	812	71
65 + EDTA	734		
	896		
	828	819	81
95	786		
	853		
	827	822	34
95 + EDTA	973		
	789		
	795	852	105

Table 25. Effect of DNAase denaturing conditions on RT-PCR Reaction Yield

There appeared to be little difference in the yield of either VEGF or actin after PCR with DNAase treatment regardless of which of the protocols was used. In view of this it was felt that it would be reasonable to use the suppliers suggested protocol that involved denaturing the DNAase at 65°C in the presence of EDTA. Since the reaction yield was so critical to quantification of the PCR, it was felt advisable however to check whether the addition of EDTA at this stage might alter the optimal magnesium requirement during the subsequent PCR.

6.4.7 Optimisation of Magnesium Concentration in the PCR reaction for DNAase treated samples.

Given the concerns expressed above regarding the effect of EDTA used in the neutralisation of the DNAase on the magnesium concentration in the subsequent PCR reaction, re-titration of the amount of magnesium added to the PCR reactions was carried out.

T47D RNA was DNAase treated as before and the DNAase denatured by incubation at 65°C for ten minutes with addition of 1μl of 20mM EDTA. The treated RNA was then reverse transcribed as before. Actin PCR reactions were set up as before except that the concentration of $MgCl_2$ was titrated from 0.5mM to 3.5mM with triplicate reaction for each concentration. PCR were carried out for 25 cycles with a 60°C annealing temperature as before. Reaction yields were plotted against magnesium concentration.

Mg Conc mM	Amount of Actin cDNA Product		
	Rel Conc	Mean	S.D
0.5	0		
	0		
	0	0	0
1	4392		
	4467		
	4662	4507	139
1.25	5901		
	6077		
	5615	5864	233
1.5	4299		
	4167		
	5974	4813	1007
1.75	4393		
	4476		
	5073	4647	371
2	2711		
	3715		
	3543	3323	537
2.25	687		
	2053		
	760	1167	768
2.5	1609		
	534		
	1495	1213	591
3	1320		
	901		
	735	985	301
3.5	1736		
	950		
	200	962	768

Table 26. Optimisation of PCR Mg^{2+} concentrations for DNAase treated samples

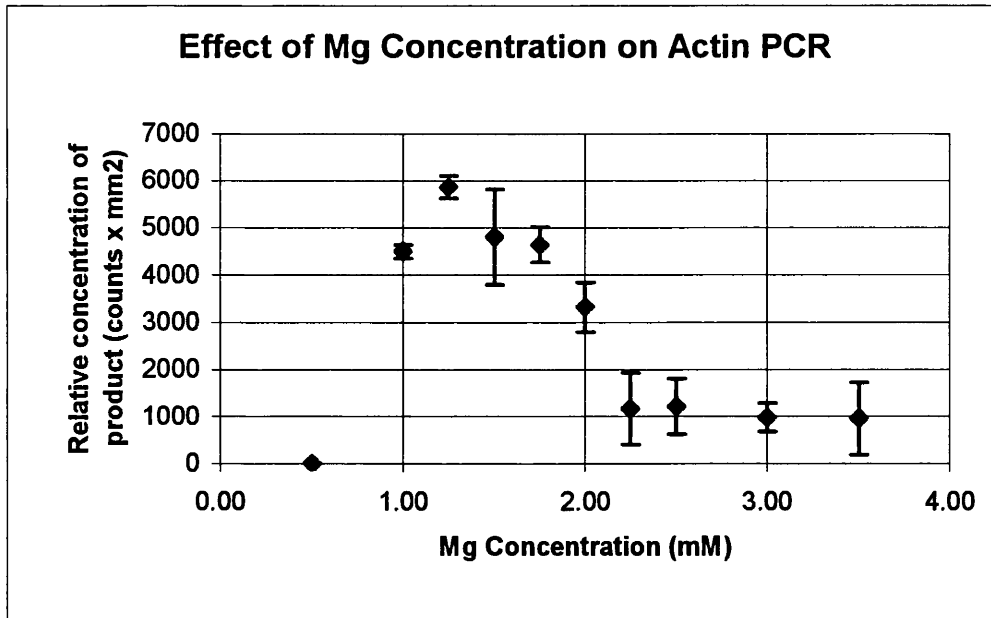


Figure 22. Optimisation of PCR Mg^{2+} concentrations for DNAase treated samples

The peak reaction yield appeared to occur at a magnesium concentration of around 1.25-1.5 mM. It appeared therefore that using DNAase and denaturing it with EDTA did not affect the magnesium requirement in the PCR reaction. No change in this protocol was therefore required.

Chapter 7. Analysis of RNA from Human Breast Samples

7.1 Introduction

The aim of the work in the preceding chapter was to develop an assay that could be used to measure the amount of VEGF RNA in breast tumours and other breast tissue samples.

This chapter describes the work carried out with samples derived from human breast tissues. The first section of the work described in this chapter was carried out at an early stage in the development of the assay. At this stage the reverse transcription and β-actin PCR reactions had not been optimised and tested. The optimal number of cycles for the VEGF PCR assay had also not been finalised. Up to that point, all of the development work had been carried out on mRNA extracted from a cell line. It was not known whether the technique being developed could detect VEGF mRNA in material from human tissue samples and how the amount of mRNA in this material would compare with the cell lines. The availability of a number of samples of cDNA, which had been prepared from RNA from normal human breast and breast tumours, gave the opportunity to determine whether the use of this assay was feasible.

The next section of the work in this chapter was carried out once the development of the assay appeared complete. Archival material that had been stored in 1989 was used. It was hoped that the use of this early material would give the longest possible patient follow-up. Whilst the samples used in this part of the work proved free of any contaminating DNA other samples subsequently tested showed traces of β-actin DNA when screened prior to analysis. The use of DNAase treatment was therefore investigated prior to analysis of further samples. This treatment was then used on the material subsequently analysed. The later material analysed was derived from tissues stored in 1993. In the interim, changes had been implemented in the method of storage of breast tumour samples in the department and it was hoped that the RNA in these samples would be better preserved.

7.2 Methods

7.2.1 Preliminary application the technique to cDNA samples from human tumours.

Prior to optimisation and testing of the reverse transcription and actin PCR, several cDNA samples prepared by reverse transcription of RNA from human breast tissues had been made available by Dr V. Speirs of the University of Hull. These were used to verify the feasibility of using the VEGF PCR assay to study human material

There were six cDNA samples derived from non-malignant breast tissue (obtained from reduction mammoplasties) and six samples from ductal carcinomas of the breast. These had been frozen in liquid nitrogen prior to extraction of RNA with TRIzol. After RNA estimation by spectrophotometry 1µg samples of total RNA had been reverse transcribed using Superscript II reverse transcriptase in accordance with the manufactures protocol.

PCR reactions were set up in triplicate for each sample. Simultaneously, reactions were prepared containing serial dilutions of T47D cDNA (1ul to 0.008µl of cDNA per reaction). PCR reactions were set up containing 2 units of Taq, 0.8pmol of dNTPs and 100ng of each primer per reaction, with a $MgCl_2$ concentration of 1.5mM. 1µl of the cDNA from each sample was placed in each reaction tube. The PCR reactions were run for 30 cycles. Two gels were required to accommodate the samples. 20µl from each reaction of serially diluted T47D cDNA was run on each gel to allow the plotting of a standard curve for that gel. The remaining triplicate samples were divided between the two gels. The amount of T47D cDNA present in the sample relative to the amount present in 1µl of cDNA derived from T47D RNA was estimated by comparison with the standard curve for that gel, using the GelDoc system.

7.2.2 Non-Quantitative PCR of cDNA from Human Breast Tissue Samples.

An experiment was set up to determine whether more of the samples used in the previous experiment would show VEGF expression given further amplification. Reaction conditions were the same as used in the above experiment. Duplicate reactions were set up for each sample and PCR carried out as before but for 35 cycles instead of 30. It had already been determined that with this number of cycles, increasing the amount of cDNA template did not necessary result in a proportional increase in the amount of product. Therefore the reaction was not quantifiable after this amount of amplification. No measurements were therefore made from the gel bands and only an approximate comparison of the samples by visual assessment was made.

7.2.3 Examination of Archival Human Breast Tumours with the Optimised PCR Based Assay

Initially an attempt was made to analyse RNA from archival breast tumours that had been stored in liquid nitrogen in 1989 and consequently had a long-term follow up data available. Samples of tumour and adjacent apparently uninvolved breast had been stored in the Department of Surgery, Western Infirmary, Glasgow at that time by wrapping 2-3cm diameter pieces in aluminium foil after surgical excision and rapidly placing them in liquid nitrogen, where they had remained. Working on a liquid nitrogen cooled tray to prevent thawing, pieces were removed from these blocks with bone nibblers. These pieces were then ground to a fine powder and subjected to TRIzol RNA extraction.

Five pairs of samples of tumour and adjacent breast were initially prepared. RNA concentrations were estimated by ultraviolet spectrophotometry. One microgram of each RNA sample was run on an RNA gel to determine which showed clear ribosomal bands. The methods for RNA gel electrophoresis have previously been described in

chapter 3. The quality of these samples appeared generally poor but the best three pairs were chosen for further analysis.

The samples were then screened for genomic DNA carryover by placing 0.3μg of RNA into VEGF and Actin PCR reactions as used previously used for analysing the reverse transcription products. Despite there being over six times as much of the original RNA solution as would normally be carried over into the PCR reactions, no product was detected in any of the VEGF or actin reactions.

Reverse transcription reactions were set up using the protocol optimised in the previous section with 0.5μg of each RNA sample in each reaction. A large batch of T47D RNA was prepared by TRIzol extraction to provide a standard. Its concentration was determined by UV spectrophotometry and it was screened for contaminating DNA by PCR. Two reverse transcription reactions containing 0.5μg of the T47D RNA were set up.

The products of the reverse transcriptions were made up to 100 μg with water. PCR reactions for VEGF and actin were set up for each sample using the protocols previously optimised. Triplicate reactions were set up from each reverse transcription reaction with 10 μg of reverse transcription product added to each. Products were electrophoresed on agarose ethidium bromide gels and these were analysed using the GelDoc system as previously described. Standard curves for VEGF and actin were produced by linear regression of the measurements from the serial dilutions T47D by the GelDoc software. Relative concentrations of the sample mRNA species calculated by comparison with this. To correct for variations in total mRNA between samples, results for each sample were then expressed as a VEGF/Actin ratio.

Case notes were extracted in December 2000 to obtain details of adjuvant therapy and the result of follow up. The total length of follow up was calculated from the number of days between surgery and the last recorded surgical review.

7.2.4 Examination of cDNA from 1993 Samples

Samples of tumour and adjacent breast from eight patients who had undergone surgical treatment of breast cancer in the Western infirmary during 1993 were extracted from the tumour bank. These samples had been cut into fragments on a liquid nitrogen cooled tray then immediately put in vials. These vials were placed in liquid nitrogen. It was hoped that this would result in more rapid and uniform freezing than had occurred in the older samples which had been frozen in large blocks wrapped in aluminium foil. A sample of tumour from an axillary lymph node was also available for one of these patients and was also processed.

Tissues were ground to a powder using equipment cooled with liquid nitrogen and the RNA extracted with TRIzol as before. Initial screening of these samples showed faint genomic actin band in some. It was therefore necessary to carry out DNAase treatment. The optimisation of protocols for this has been discussed in the preceding chapter. DNAase treatment was carried out for fifteen minutes at 25° C then 1µl of 20mM EDTA added and the DNA denatured by heating to 65°C for ten minutes. The T47D cell line control RNA was also DNAase treated. The treated samples were then checked for residual DNA contamination by VEGF and actin PCR reactions as before. No evidence of any residual contamination was seen.

As previously described, 0.5µg of each tissue RNA sample was reverse transcribed as was the T47D control RNA. The products of the reverse transcription were diluted to 100µl with water and four VEGF and four actin PCR reactions each containing 10µl of the diluted RT product set up from each. VEGF and actin PCR reactions containing serially diluted T47D reverse transcription product were also set up, as has been previously described, to create standard curves. Products were analysed by gel electrophoresis and relative VEGF and actin concentrations for each sample calculated by comparison with the T47D standard curves by linear analysis using the GelDoc software.

Case notes were examined in December 2000 to obtain follow-up information as before.

Comparisons of VEGF expressions between different groups pathological and outcome groups were made using the Mann-Whitney rank sum test. Relationship between tumour size and VEGF expression was examined by Pearson correlation. Statistic were calculated using the StatsDirect v2.0.1 statistical package (StatsDirect Ltd)

7.3 Results

6.3.1 Preliminary application the technique to cDNA samples from human tumours.

Standard Curve		
	1st gel	2nd gel
Amount of cDNA	Rel. vol of band	Rel. vol of band
per reaction (ul)	counts x mm 2	counts x mm 2
1	1626	1586
0.5	858	985
0.25	383	506
0.125	177	278
0.0625	99	187
0.0313	64	53
0.0156	27	59
0.0078	0	0

Table 27. Standard curves for gels-preliminary application of RT-PCR technique to tissues samples.

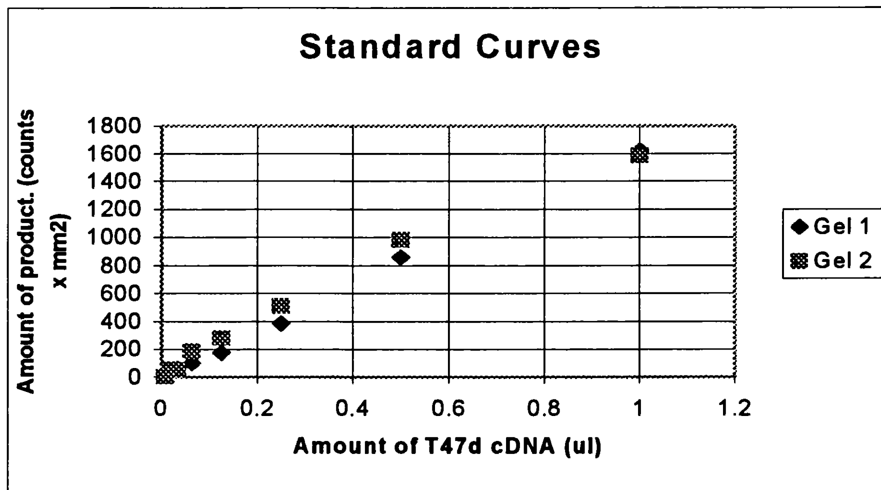


Figure 23. Standard curves for gels-preliminary application of RT-PCR technique to tissues samples.

The above plots suggest a linear relationship between the starting amount of T47D cDNA and the amount of product.

In three reduction mammoplasty specimens and three ductal carcinoma specimens, no product was detected. The amount of product obtained with the remaining samples is shown below. The amount of product obtained for each reaction was related to the amount obtained from with each was related to that obtained from T47D using the standard curves and a mean relative signal and standard deviation calculated for each sample. Sample designations are those ascribed by the Hull laboratory.

Type of Sample	Designation	Amount of Product Rel. vol of band	VEGF signal relative to T47D	Mean Relative signal	S.D
Reduction	R6	169	0.10		
Mammoplasty		219	0.13		
		242	0.15	0.13	0.02
Reduction	R195	193	0.1		
Mammoplasty		166	0.0838		
		177	0.0903	0.09	0.01
Reduction	R5	100	0.0429		
Mammoplasty		186	0.0957		
		183	0.0937	0.08	0.03
Lumpectomy	C15	128	0.0599		
		98	0.0416		
		34	0.0019	0.03	0.03
Mastectomy	M3	49	0.011		
		60	0.0178		
		8		0.01	0.00
Mastectomy	M18	304	0.1687		
		232	0.124		
		256	0.1394	0.14	0.02

Table 28. Estimation of relative VEGF expression in tissues samples-preliminary application

This experiment has demonstrated that it is possible to detect VEGF in samples of cDNA produced by reverse transcription of RNA from human tumour material. There was a large variation between samples in the amount of VEGF product obtained but no obvious association between the nature of the sample and the amount of VEGF.

The linearity of the reverse transcription reactions used to prepare these samples had been had not been verified. It was assumed that the same amount of mRNA was present in all of the samples based on standardisation of the amount of total RNA used in the reverse transcription reactions by spectrophotometry. However, no internal standards such as β-actin or GAPDH were included.

6.2.2 Non-Quantitative PCR of cDNA from Human Breast Tissue Samples.

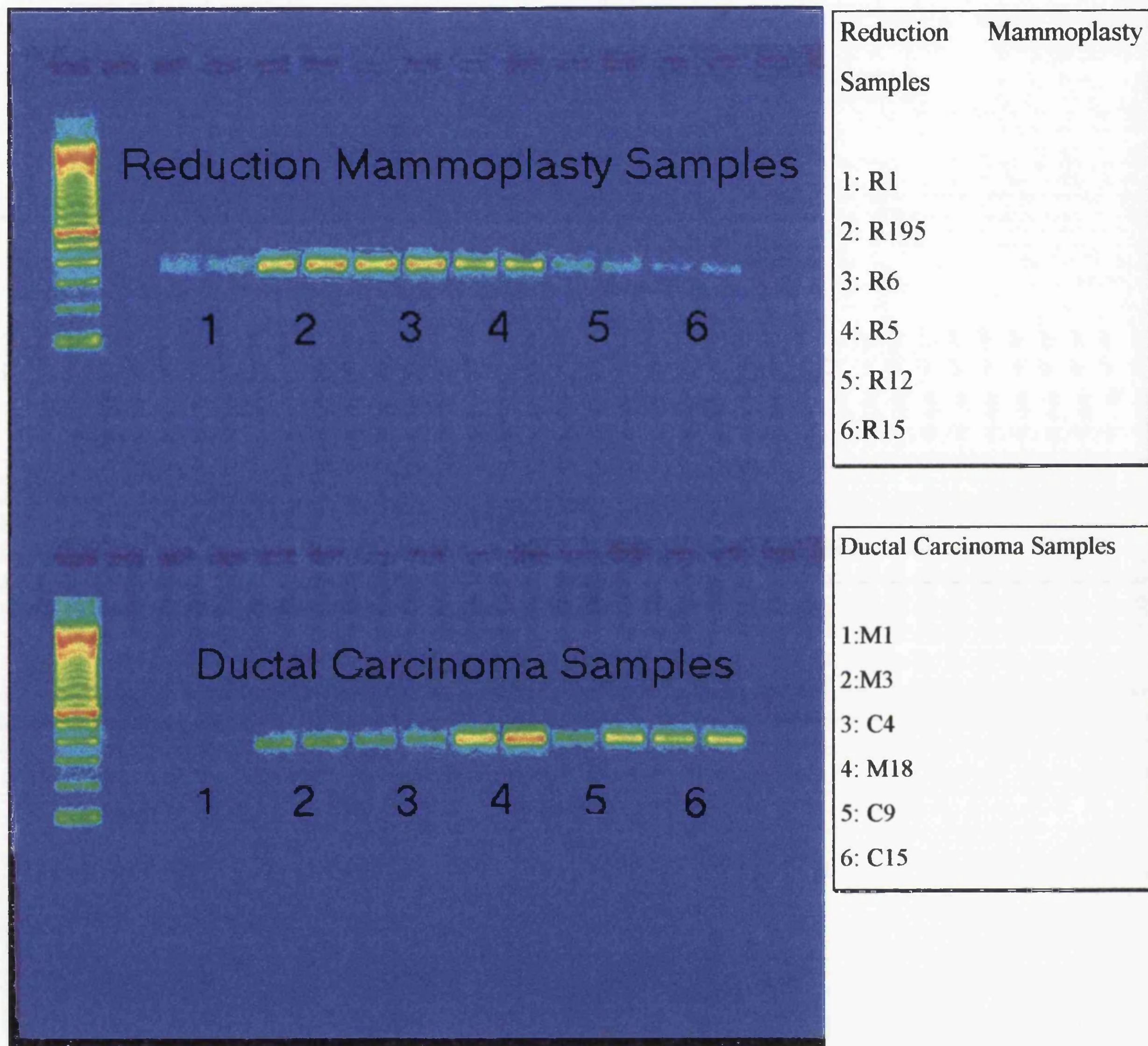


Figure 24. Gel Electrophoresis of RT-PCR Amplified VEGF from Reduction Mammoplasty and Ductal Carcinoma Specimens.

It can be seen that after 35 cycles, a band consistent with the VEGF PCR product was amplified from the cDNA derived from all of the reduction mammoplasty tissue and all but one of the tumour samples. Furthermore the bands obtained from some of the reduction mammoplasty tissue were of a similar size to those obtained from the tumour samples. The demonstration of the expression of VEGF RNA by benign breast tissue suggested that to investigate differences in expression of VEGF between benign and malignant breast tissue, an assay that allows quantitative comparison was needed.

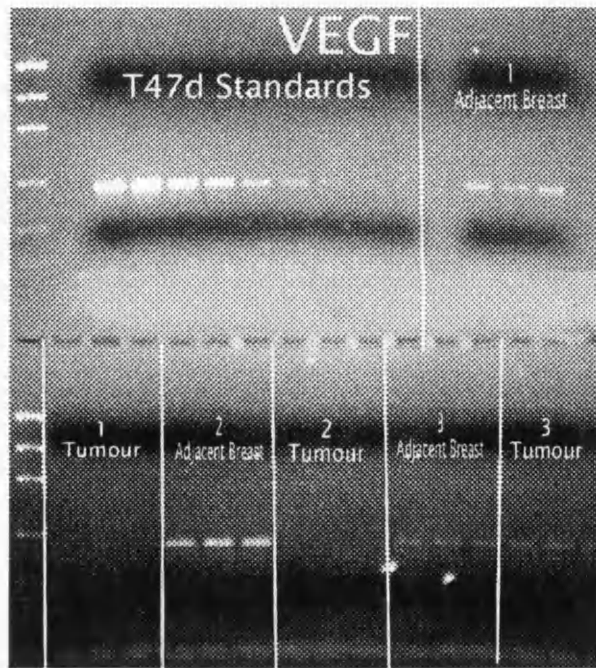
Prior to carrying out any further work on human material, improvements were required to the assay system. The conditions for the reverse transcription assay were optimised and the linearity of this stage of the reaction tested. A PCR assay for β−actin was optimised to provide an internal standard. Further work was carried out on the VEGF PCR reaction to improve its sensitivity by increasing the amount of amplification that could be achieved whilst still maintaining a linear relationship between template and product quantities. This work has been described in chapter 6 above.

6.3.3 Examination of Archival Human Breast Tumours with the Optimised PCR Based Assay (1989 patients)

Pathological and clinical data for these patients is shown in the table below.
Photographs of the gels produced by electrophoresis of the PCR products and the results of Geldoc analysis of these gels is also shown.

Patient	Age	menopausal status	Type of Surgery	Tumour type	Tumour Grade	Size (mm)	number of involved lymph nodes	oestrogen receptor	Adjuvant Therapy	Recurrence	Site of rec	Follow-up (days)	time to first recurrence (days)	time to death (days)
1	64	post	Mastectomy	Ductal	2		0		None	n		781		
2	62	post	lastectomy	Lobular		40	11	positive	RT	y	bone	2185	1667	2185
3	31	pre	Mastectomy	Ductal	3		11		CMF	y	bone, liver	1075	824	1075

Table 29. Pathological and clinical data -1989 patients.



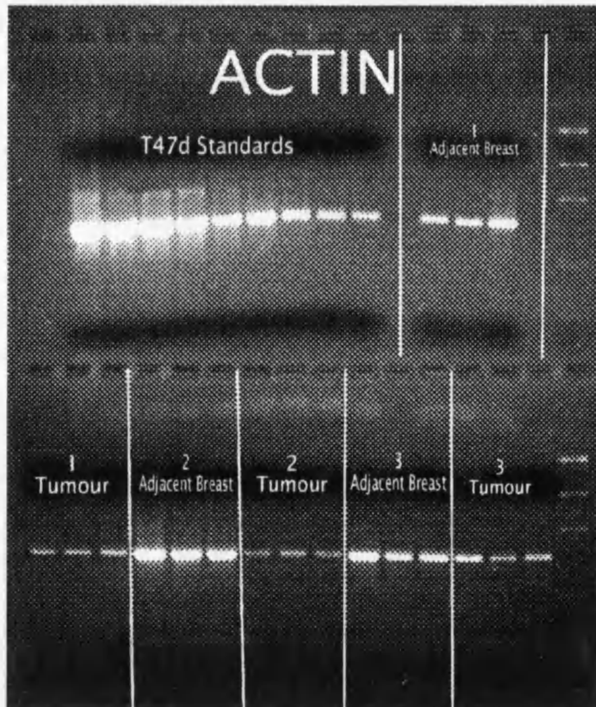


Figure 25. Gel Electrophoresis of RT PCR products-1989 patients.

Sample	VEGF Rel. Conc.	VEGF Mean	VEGF S.D	Actin Rel. Conc.	Actin Mean	Actin S.D	VEGF/ Actin Ratio
1 Adjacent Breast	0.225			0.271			
	0.237			0.300			
	0.241	0.234	0.008	0.600	0.390	0.182	0.600
1 Tumour	0.011			0.081			
	0.049			0.102			
	0.030	0.030	0.019	0.163	0.115	0.043	0.261
2 Adjacent Breast	0.259			0.753			
	0.261			0.562			
	0.259	0.259	0.001	0.580	0.632	0.105	0.411
2 Tumour	###			0.064			
	###			0.097			
	###	###	###	0.063	0.075	0.020	###
3 Adjacent Breast	0.101			0.651			
	0.123			0.400			
	0.110	0.111	0.011	0.453	0.501	0.132	0.222
3 Tumour	0.073			0.282			
	0.081			0.121			
	0.046	0.066	0.018	0.166	0.189	0.083	0.351

Bands not visible on gel

Table 30. Semi-quantitative analysis of VEGF expression-1989 patients

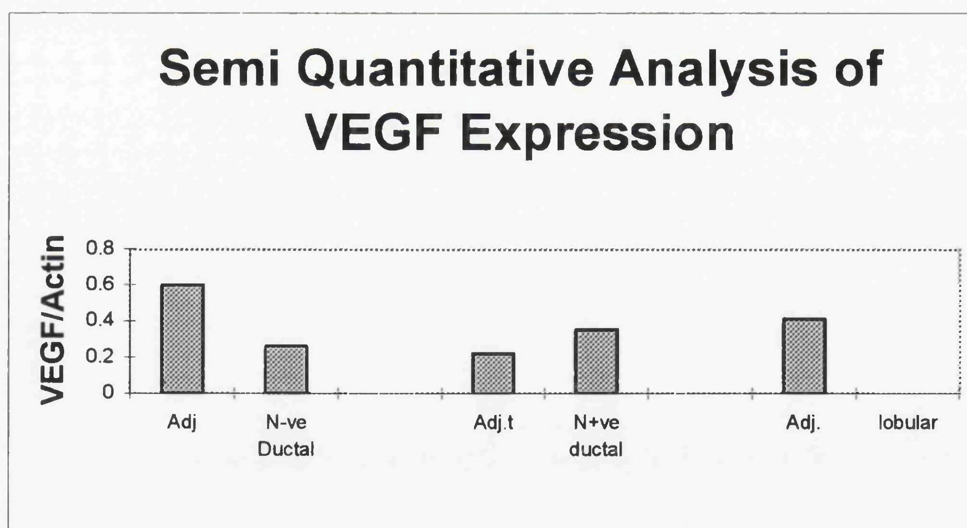


Figure 26. Semi-quantitative analysis of VEGF expression-1989 patients

6.3.4 Examination of cDNA from 1993 Samples

RNA was extracted from tumour and adjacent breast tissue obtained from patients who had undergone surgery in 1993 as described above. These samples had been frozen and stored under better conditions than the previous samples and it was hoped that the RNA would be better preserved.

Clinical and pathological data for these patients whose tumours were analysed in this study is shown below as are the results of the semi- quantitative RT PCR on the 1993 patient samples are shown below. As before, relative concentrations of VEGF and actin were used to calculate a VEGF/actin ratio to compensate for variations in readable mRNA between samples. A further ratio was obtained by dividing the VEGF/actin ratio for each tumour by the VEGF/actin ratio for the adjacent breast.

Patient	Age	menopausal status	Type of Surgery	Tumour type	Tumour Grade	Size (mm)	number of involved lymph nodes	oestrogen receptor	Adjuvant Therapy	Recurrence	Site of rec	Follow-up (days)	time to first recurrence (days)	time to death (days)
1	53	post	Mastectomy	Ductal	2	15	0	negative	Not known	Not known	Not known	Notes lost	Not known	Not known
2	84	post	Mastectomy	Ductal	3	40	11	negative	None	Not known	Not known	None	Not known	Not known
3	75	post	Mastectomy	Ductal + DCIS	3	55	1	negative	RT + Tam	y	lung	23	20	23
4	53	post	Mastectomy	Ductal	3	25	1	positive	Tam	y	bone, liver	38	19	38
5	47	pre	Mastectomy	Ductal + DCIS	3	20	11	negative	CMF	y	local, bone	48	7	49
6	46	pre	Mastectomy	Ductal + DCIS	3	55	1	negative	RT + CMF	n	no recurrence	77	no recurrence	alive
7	49	pre	WLE + Axilla	ductal within phyllodes	1	4.5	0	unknown	RT + Tam	n	no recurrence	69	no recurrence	alive
8	45	pre	WLE	Ductal + DCIS	2	12	0	positive	RT	n	no recurrence	63	no recurrence	alive

Table 31. Pathological and clinical data -1993 patients

Sample	VEGF Rel Conc	VEGF Mean	VEGF S.D	Actin Rel Conc	Actin Mean	Actin S.D	VEGF/Actin ratio
	0.098			0.003			
1	0.161			0.011			
Adjacent Breast	0.251			0.003			
	0.164	0.169	0.063	0.003	0.005	0.004	35.309
	1.089			0.009			
1	1.870			0.008			
Tumour	1.519			0.009			
	1.963	1.610	0.397	0.004	0.007	0.002	215.405
	0.000			0.000			
2	0.000			0.000			
Adjacent Breast	0.000			0.000			
	0.000	0.000		0.000	0.000		na
	0.382			0.088			
2	0.271			0.056			
Tumour	0.604			0.079			
	0.264	0.380	0.159	0.074	0.074	0.013	5.106
	0.905			0.223			
2	0.973			0.001			
Lymph Node	0.928			1.344			
	0.849	0.914	0.051	1.097	0.666	0.654	1.371
	0.000			0.000			
3	0.000			0.000			
Adjacent Breast	0.000			0.000			
	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	1.949			0.074			
3	1.684			0.128			
Tumour	0.132			0.103			
	1.603	1.342	0.820	0.073	0.094	0.026	14.206
	0.660			0.043			
4	0.811			0.044			
Adjacent Breast	1.198			0.008			
	0.443	0.778	0.318	0.015	0.027	0.019	28.575
	0.957			0.045			
4	0.803			0.045			
Tumour	0.810			0.033			
	0.743	0.828	0.091	0.037	0.040	0.006	20.640
	1.387			0.171			
5	2.821			0.193			
Adjacent Breast	1.680			0.233			
	2.447	2.084	0.664	0.233	0.207	0.031	10.058
	0.000			0.000			
5	0.864			0.000			
Tumour	0.896			0.000			
	0.802	0.640	0.429	0.000	0.000	0.000	na
	2.440			0.104			
6	2.888			0.102			
Adjacent Breast	0.515			0.100			
	1.494	1.834	1.054	0.060	0.092	0.021	20.023
	3.200			0.119			
6	2.214			0.142			
Tumour	3.180			0.163			
	3.158	2.938	0.483	0.177	0.150	0.025	19.556
	0.113			0.068			
7	0.124			0.070			
Adjacent Breast	0.078			0.055			
	0.125	0.110	0.022	0.032	0.056	0.017	1.956
	2.110			0.300			
7	0.800			0.261			
Tumour	0.013			0.150			
	1.915	1.209	0.984	0.233	0.236	0.064	5.125
	0.132			0.000			
8	0.067			0.000			
Adjacent Breast	0.105			0.000			
	0.034	0.084	0.043	0.000	0.000	0.000	na
	0.173			0.113			
8	0.106			0.117			
Tumour	0.110			0.115			
	0.002	0.098	0.071	0.073	0.104	0.021	0.937

Table 37-Semi-quantitative analysis of VEGF expression-1993 patients

Tumour	Tumour VEGF/Actin	Tumour to Adj. Breast ratio
1	215.40	6.10
2	5.11	-----
3	14.21	-----
4	20.64	0.72
5	-----	-----
6	19.56	0.98
7	5.12	2.62
8	0.94	-----

Table 32 Semi-quantitative analysis of VEGF expression in 1993 Patients- comparative ratios

6.4 Discussion.

With the samples from the 1989 patients it was possible to demonstrate VEGF expression in two of the ductal carcinomas and adjacent breast. No VEGF bands were visible in the sample from a lobular tumour. It is interesting that the sample from the node negative tumour showed VEGF expression at a lower level than the surrounding breast whilst the node positive tumour showed a higher level than the surrounding breast. However it would be inappropriate to draw conclusions from this on the basis of two samples. This is particularly true since the quality of RNA extracted from these samples was poor. It was decided that samples that had been frozen more recently would be more useful for further work. These more recent samples had been cut into much smaller pieces before being placed in liquid nitrogen and had therefore frozen faster and more evenly.

In the 1993 samples, VEGF was detectable in RNA from all of the tumour samples and from six samples of adjacent breast. The two samples of adjacent breast that failed to yield detectable VEGF did not show any product with the actin PCR. A further sample of adjacent breast that yielded VEGF PCR product failed to yield any actin. This appeared therefore to be a reflection of the poor quality of the mRNA in these samples despite standardization of the total RNA in the reaction by spectrophotometry. Surprisingly, one of the tumour samples and one of the samples of adjacent breast that exhibited product in the VEGF PCR reaction failed to yield any actin product on any of

the replicate PCR reactions, despite the actin and VEGF PCR reactions being carried out on products from the same reverse transcription reaction.

There was a considerable difference in the VEGF/actin ratios between the tissue samples derived from patients who underwent surgery in 1989 or 1993. The mean of the VEGF products measured in the 1993 group was almost twice that of the 1989 group whilst the mean of the actin products in the 1993 samples was a third of that of the 1989 samples. The only difference in the analysis of the two groups of samples was that the 1993 RNA samples were treated with DNAase prior to reverse transcription. The 1989 samples were screened for contaminating DNA prior to use and none was demonstrated. It has been shown that the DNAase treatment does not affect the yield of products from either the actin or VEGF RT-PCR. Certainly even within each group there were large differences in the yield of actin between different tissue samples suggesting that there were differences in the quantity of intact mRNA capable of being reverse transcribed. This occurred despite standardization of the amount of total RNA in the samples by spectrophotometry. The difference in actin yield between the 1989 and 1993 group was statistically significant ($p<0.0001$). This suggested that either the RNA in the two groups of samples has been preserved to a different extent or that there is an overall difference in the efficiency of the PCR reactions between the two groups. For this reason, the data from the two groups of samples could not be assessed together.

Of the eight patients from the 1993 group, pathological data was available for all. All samples were ductal carcinoma though one of these was arising with in a phyllodes tumour. Five of the tumours had positive lymph nodes. Follow up data was available for six of the patients. Mean length of follow up was 53 months (range 23-77 months). During this time three out of six patients developed distant recurrence of their tumours and subsequently died. One elderly patient had not been followed up and a further had no case notes on file.

Usable VEGF quantification (VEGF/actin ratio) was obtainable for seven of the tumour samples and five of the samples of adjacent normal breast.

A statistically significant difference in between pre and postmenopausal patients in VEGF expression (VEGF/actin ratio) could not be demonstrated in either the tumours or adjacent breast (p=0.4 and p=0.2). No significant difference could be demonstrated between VEGF expression in node negative or positive tumours (p=0.85) or between tumours of high histological grade (3) or those of low grade (1&2) (p=0.86). There was no significant correlation between tumour size and VEGF expression (r=0.23 p=0.59).

There was no significant difference between VEGF expression in normal breast and tumours (median 20 v 14.2 p=0.64, 95.2% CI = -18.6 to 23.4.) Whilst patients who developed distant recurrence had a mean VEGF/actin ratio higher than those and those who did not the small number of samples meant that the possibility that this was due to chance could not be excluded. (median 5.13 v 17.42, p=0.4).

It has been demonstrated that VEGF mRNA is stabilized by hypoxia and therefore accumulates in hypoxic cells. It is likely that surgically excised tumours are subjected to a period of hypoxia. This starts during mobilisation of the tumour or entire breast as the blood supply is progressively withdrawn. There is then a period between the tumour being removed from the patient and placed in liquid nitrogen during which it is hypoxic. It is possible that the time taken to surgically remove the tumour and the transport and processing times may influence the amount of VEGF mRNA in the tumour. The protocols regarding tumour processing in place at the Western Infirmary were such that tumour material could not be removed for research purposes before the specimen had been processed in the pathology department. There had therefore been some delay in freezing of all of the material in the tumour bank. It is notable that if all of the tissues, benign and malignant from mastectomy specimens are compared with those from wide local excision (lumpectomy) specimens, a statistically significant difference exists (median 20.02 v 1.19 p=0.026). When only tumours from mastectomy and lumpectomy specimens are compared the difference does not reach statistical significance (median19.66 v 3.03 p=0.19). The lumpectomy tumours were smaller and of lower grade. It is not clear whether this represents a true difference in the biology of the tumours or a difference in

the conditions prevailing in the tumour, particularly cellular hypoxia during and immediately after surgery. Further work is needed to determine the changes that occur in tissue VEGF mRNA levels in tissue samples after devascularisation.

Chapter 8. Final Discussion

When this study was embarked upon in 1994, there had been extensive debate in the literature over the preceding three years about the significance of microvessel density, a histological measure of angiogenesis in breast cancer. As discussed in the introduction, most workers found that a high microvessel density predicted for poor outcome in breast cancer. Whilst potentially useful, the assessment of microvessel density was fraught with problems of observer variability and was a rather time consuming technique for routine clinical pathology. Furthermore it relies on having a sufficiently large block of tissue for preparation of suitable tissue sections and could therefore only be assessed following surgical removal of the tumour.

Given the suggestion from measures of microvessel density that angiogenesis could predict for tumours with a worse prognosis, it may be useful to have a technique that would identify these tumours with a high potential for angiogenesis. Ideally, if this phenotype could be identified prior to surgery, it would then be possible to assess whether alterations in the management of these patients such as neo-adjuvant chemo-radiotherapy might alter their outcome. Therefore a technique that could be applied to small amounts of tissue was potentially advantageous.

In 1989, Napoleone Ferrara had described Vascular Endothelial Growth Factor (VEGF). This seemed to be potentially the most important angiogenic factor yet described. It had been reported that in tumours of the central nervous system and kidney, VEGF was present at higher levels that in the tissue of origin. There had not been any report of VEGF expression in breast tumours. However, if any single factor was able to identify those breast tumours with a high angiogenic potential, VEGF seemed to be the most promising. This study was therefore designed to determine whether VEGF could be identified and quantified in breast tumours.

The few studies that had been published at that time reporting VEGF expression in tumours had used either immunohistochemistry or *in situ* hybridisation. Both of these techniques on their own have inherent problems. Immunohistochemistry identifies that a substance is present within a tissue. As previously stated, VEGF has several forms which bind to the tissue substrate to a varying extent. Immunohistochemistry will give an indication of the amount of VEGF bound to tissue components. This does not necessarily reflect which cells in the tissue are actually producing it or even how much is being produced since the soluble forms may be lost from the tissue. *In situ* hybridisation identifies the expression of mRNA by cells. This has the advantage of identifying the cells that are probably producing the substance and since the RNA does not escape from the cells, it is not influenced by differences in tissue binding. However, the expression of mRNA by a cell does not necessarily prove that it is actually producing the protein. The two techniques are therefore complementary. Since these techniques had been reported as able to identify VEGF in tissues, attempts were made in this work to employ them. It should be noted however that both of these techniques have the disadvantages previously noted with microvessel counting. They both employ sections of tissue though could potentially be applied to cytological preparations. Both techniques require a visual analysis of the stained slides and an assessment of the degree of staining.

A technique that could rapidly give an estimate of VEGF expression from a very small sample of tissue and which was not subject to observer variations would be ideal. RNA in tissue samples can be measured by Northern blotting but this requires relatively large amounts of tissue. The polymerase chain reaction (PCR) offered the potential to identify VEGF in tiny samples of tissue. The initial work in this study to develop a VEGF riboprobe had demonstrated that VEGF mRNA could readily be amplified by RT-PCR. PCR was however regarded as largely a qualitative technique. Methods for using it quantitatively have however have been described and these have been discussed already in the introduction to chapter 6. Whist competitive techniques have been described their use requires multiple reverse transcription and PCR reactions. This greatly increases the amount of template RNA required as well as the assay cost. These techniques are therefore less suitable for application to routine clinical use. It was felt that with close

attention to optimisation of assay conditions and careful verification of the relationship between template and product that a simpler PCR based system could be employed.

The development of this assay is described in this work. A PCR based assay has been developed which yields a linear relationship between the amount of mRNA in a starting sample and the relative amount of VEGF product measured by computer analysis of the PCR products. The assay system includes an internal standard in the form of β-actin to control for variations in the quantity and quality of mRNA contained in the samples and any differences in the efficiency of reverse transcription between samples. The assay is sufficiently sensitive to allow estimation of VEGF using only 0.5μg of total RNA from each tissue sample and could be used with even smaller amounts of RNA. It gives an opportunity to study VEGF expression in tissues in either a research or clinical context where the amount of tissue available for study is restricted. It could be applied to small amounts of archival material or to small biopsy samples such as fine needle aspirates from patients.

After the completion of this work, Anan et al [87] in Japan described a very similar semi-quantitative PCR based system. They too compared VEGF and β-actin amplified by PCR from tumour RNA with the same products amplified from a standard sample of RNA from a breast cancer cell line. The main difference in methodology was that they initially employed a radioactive label and southern blotting to quantify the product bands rather than direct computer analysis of the gel used in this study. The method described in this study eliminates the safety and a logistical problem associated with the use radioactivity and eliminates a time-consuming blotting stage. In a subsequent publication, the same group used photographs of the gels scanned on a flatbed scanner and analysed by computer [88]. They showed that the results obtained with this technique correlated closely to their radioactive method.

Among the aims of the in the present study, it had been intended to compare VEGF measurements from tumour samples with the tumour microvessel counts and to assess the feasibility of using the assay on tumour samples abstained by fine needle aspiration. Time

did not permit the achievement of these objectives in this study. Annan et al however did use the semi-quantitative PCR assay for this purpose [87, 88]. They demonstrated a correlation between high microvessel density and increased expression of VEGF mRNA and a correlation between VEGF mRNA estimations from FNA samples and from larger samples of the tumour.

As previously stated, the transcription and stability of VEGF RNA in cells is affected by tissue hypoxia. With any surgically excised material there will be a period between the tissue being deprived of its blood supply and the complete cessation of metabolism by fixation or freezing. With the material used in this study, this study had been compounded by the transfer of the tissue to the pathology department prior to it being available for freezing. The effect of this delay on the VEGF mRNA concentration in the cells is unknown. Further work to determine the change in VEGF mRNA conditions in devascularised tissues would be valuable.

Comparisons were made between tumours and the histologically normal breast adjacent to the tumour. It has however been found previously [17] that apparently normal tissue from tumour bearing breast is more angiogenic *in vivo* than tissue from breasts without tumours. Therefore, the lack of a demonstrable difference between the tumours and adjacent breast tissue may be at least partly due to the breast tissue adjacent to the tumours producing more VEGF than would be produced by normal breast tissue.

Due to time constraints, the assay has only been used to analyse a small number of archival tissue samples. No statistically significant associations were seen between the expression of VEGF in these samples and any of the pathological or outcome variables examined. The statistical power of the study however is not sufficient to exclude the existence of such associations. Subsequently several researchers have found that high VEGF expression in breast carcinomas predicts for poor outcome. The results of these studies have been outlined in the introduction.

Attempts to demonstrate VEGF by immunohistochemistry proved unsuccessful despite the use of antigen unmasking techniques such as microwaving and trypsin digestion. Although demonstration of VEGF by immunohistochemistry has been reported [77] subsequent discussions with other workers has revealed that they too have had difficulty demonstrating VEGF by this method. It may be that the tissue fixation influences the success of this technique but staining was also not seen with the frozen sections and cytological material used. It was felt that, with the antibodies available at the time of this study, immunohistochemistry was not a viable method for assessing VEGF expression.

In this study an RNA riboprobe was produced by cloning of part of the first exon of the VEGF cDNA into an expression vector and transcription of a digoxigenin labelled antisense mRNA molecule. Despite verification of the sequence of the cloned vector, attempts to use this probe to detect VEGF mRNA in either northern blots or *in situ* hybridisation proved unsuccessful. Subsequently, a sample of plasmid DNA cloned with VEGF RNA was obtained from Genentec. However it was felt that the PCR based assay held greater potential and attempts to detect VEGF RNA using a riboprobe has not as yet been pursued further by either *in situ* hybridisation of northern blotting.

Commercial kits have now become available to detect VEGF protein by enzyme linked immunoseroassay (ELISA). These kits allow rapid measurement of VEGF protein and have contributed to the rapid expansion of the literature regarding VEGF in cancer in the last few years. There must be some caution regarding the measurement of VEGF protein in tissues given the variable binding of different forms of the protein to the extracellular matrix. The technique has however facilitated the rapid processing of large numbers of patient samples and yielded data on links between VEGF expression and clinical outcome in cancer. It is also possible that measurements of VEGF in the blood of patients with breast cancer may yield prognostic information. Lantz et al [122] have demonstrated a correlation between pre-operative serum VEGF and microvessel density in 46 breast cancer patients. The prognostic value of measurements of circulating VEGF are as yet unknown.

RT-PCR based techniques such as that described here cannot compete with ELISA in for economy or ease of use. However, when only small amounts of material such as fine needle aspirates or small biopsies are available to study, the amplification inherent in this technique means that it may prove valuable. The ability to study the production of individual splice variants of VEGF by using different PCR primers also contributes to its potential usefulness.

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Appendix.

Comparison of the VEGF sequence derived with each sequencing primer in the sequenase reaction with the published VEGF sequence (Section 2.2.15)

Expected Sequence		Sequence from primers		
Sense	Antisense	T3	T7	m40
A	T	A		
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