The influence of tumour angiogenesis on the metastatic potential of colorectal carcinomas

A thesis submitted in partial fulfilment of the requirements of Kingston University for the degree of Doctor of Philosophy.

In collaboration with:
St George's Hospital Medical School, University of London

May 2007

By
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Abstract

Colon cancer is the fourth most common cause of cancer death in the world. It kills approximately 529,000 people annually and around two thirds of these deaths are in the developed world. These figures can be attributed to the fact that colorectal cancers are well advanced before they are detected. Effective prevention of the disease is early detection and removal of pre-cancerous polyps. In cases where the cancer has already developed, early detection still significantly improves the chances of a cure by surgically removing the cancer before the disease is able to metastasise.

However, in metastatic colorectal cancers, in order to ascertain an effective treatment regimen and determine the prognosis of a patient after surgery a prognostic tool that accurately portrays the extent of the disease needs to be developed. Currently, the most commonly used prognostic tool to assess colorectal cancer spread is the clinicopathological staging system. However, there is a need for additional markers of metastasis as nearly one-third of patients with a clinical diagnosis of Dukes' B, a commonly used pathological staging system for colorectal cancer prognosis, will die of the disease despite complete resection of the primary tumour.

In several human cancers such as breast, prostate and the lung, tumour vascularity has been shown to be of prognostic value. However, studies correlating the significance of the number of tumour vessels to prognosis in colorectal cancers are relatively lacking or show conflicting results. Moreover, it is not yet fully understood if factors involved in the complicated cascade of tumour blood vessel formation (tumour angiogenesis), such as proteases cathepsin B and dipeptidyl peptidase IV aid colorectal cancer progression. Two of the most potent angiogenic growth factors: vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) play a significant role in the development of tumour vessels; however, their influence on the production of these proteases is not clear.

Therefore, this study set out to i) establish a reliable method to quantify colorectal tumour vessels ii) to assess the correlation between vessel counts and prognostic parameters associated with metastasis iii) to ascertain the pattern of spatial distribution of tumour vessels in order to define the relationship between the positions of the vessels in relation to areas of tumour growth iv) investigate the levels of the enzyme expression of tumour associated proteases cathepsin B and dipeptidyl peptidase IV, at both protein and mRNA level, in colorectal tumours of varying clinicopathological grades and to v) assess the influence of VEGF and bFGF on CB and DPPIV proteolytic enzyme activity in human colon, rectal cancer cells and normal human umbilical vein endothelial cells.

The study established that an effective method to examine tumour vascularity, was by immunolocalising tumour endothelial cells by CD31 and quantifying all of stained vessels present within each cross-sectional area. As a result of tumour endothelial cell marker assessment it was noted that CD34 was highly specific to certain vessels and distinctly immunonegative to others and that the same vessels were positive to CD31. These vessels were morphologically assessed by using the standard histological criteria identified as lymph vessels.

Tumour blood and lymph vessels were quantified and a positive correlation to prognostic parameters relating to cancer spread was identified. The pattern of distribution of colorectal tumour vessels was also examined and a distinct pattern was observed in metastatic tumours with greater numbers of vessels in the peripheral regions and the invasive fronts.

Qualitative analysis of protease expression and mRNA intensity, in colorectal tumours, revealed increased levels of CB and DPPIV in tumours with clinicopathological parameters associated with metastasis. mRNA localization near tumour vessels highlighted the potential for, not only cancer cells but also tumour endothelial cells, to produce proteolytic enzymes to aid the process of tumour angiogenesis and hence tumour growth and metastasis.
The study also demonstrated that the angiogenic factors VEGF and bFGF up-regulated CB and DPPIV activity in human colon, rectal cancer cells and normal human umbilical vein endothelial cells.

The presence of tumour lymph vessels and their positive correlation to metastatic parameters signifies an important role for such vessels in the process of colorectal cancer spread. The positive relationship of tumour blood vessels to parameters associated with metastasis re-instates the important role of tumour angiogenesis in colorectal cancer. Other factors that were investigated such as tumour associated proteases CB and DPPIV and the influence of the potent angiogenic proteins upon their activity suggests that these factors have a direct role in colorectal cancer metastasis.

Therefore, it can be concluded from the results of this thesis that tumour blood vessel counts can be used as a reliable prognostic marker in colorectal cancer. Tumour angiogenic factors as well as the colorectal tumour lymph vessels have the potential to be used as additional prognostic markers in clinical prognostic evaluations. This has important implications in allowing clinicians to accurately assess post-operative survival rates and to devise an effective treatment regimen in order to prolong the lives and possibly cure colorectal cancer patients.

Presentations

Humma Khan, Asim N. Sahraouei, Craig N. Kennet, Andy Jewell, Babar Abbas. Determination of cathepsin B enzyme activity under the influence of angiogenic proteins VEGF and bFGF in human colon carcinoma (HT29) and human umbilical vein endothelial cells (HUVEC). **Poster presentation at the American Association for Cancer Research (AACR) meeting, Vail Colorado, U.S.A. Publication in 'Cytokines and Cancer: Regulation, Angiogenesis and Clinical Applications,' September, 2000.**

Humma Khan, Peter Dalton, Andy Jewell, Babar Abbas. The assessment of tumour vessel density in relation to colorectal cancer progression. **Poster presentation at the European School of Oncology (ECO), Milan, Italy. Publication in 'Biological basis for anti-angiogenic therapy,' November 1999.**

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Acknowledgements.

I would like to thank my supervisors Dr B. Abbas (Director of studies) and second supervisor Dr A. Jewell for their intellectual guidance and support as well as perseverance during my Ph.D.
Thank you both, as the knowledge I have gained has enabled me to be a more confident person and succeed in my career within the pharmaceutical industry.

I am also grateful to Professor A. Russell for all her wisely advice and also Dr R. Manly for his support in funding my research as well as attendances to medical conferences which enabled my work to be presented in front of renowned scientists in the field of oncology.

I would like to express my appreciation to Dr. A. Seddon for his help on statistical analysis and also to Gurm, Michael and Jayne for their technical support.

Many thanks to: Dr M. Grahn (Royal London Hospital) – for colo26 mouse tumours.
Dr A. Sahraouei (Royal London Hospital) – help and guidance for the work on CB and DPPIV.
Dr C. Finlayson (St. Georges Hospital) - for her advice and direction in studies on tumour vascularity its and influence on clinicopathological parameters.
Mr P. Dalton (St. Georges Hospital) – for his generosity and allowing me to obtain human colorectal samples from the hospital's histopathology archives.
This work would not have been possible without their help.

A special thank you goes to my beloved parents and my brother and sister who have supported me both financially as well as emotionally throughout my Ph.D. I am greatly indebted to you for your loyalty and faith in me and also all your patience.

Humma Khan, 2007.

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Chapter 1

1.1 General Introduction

Cancer is still one of the leading causes of death around the world therefore, research into the mechanisms as well as its treatment has led the precedence in finding a cure for the disease. Once a patient is diagnosed with cancer, the clinician can give a prognosis and treatment regimen that will treat or keep the condition under control. However, management and the survival rate evaluations are not precise.

With the recent discovery of tumour angiogenesis and its significance in cancer spread, its possible role as an independent prognostic marker has been investigated in the breast, prostate, and the lung with positive results. However, due to the lack of data, some of which is contradictory, the assessment of tumour angiogenesis in colorectal cancer is important. Therefore, this study has set out to investigate the role of colorectal tumour vessels and associated angiogenic factors and their relation to pathological parameters linked to cancer spread.

The study aims to firstly, explain the biology of colorectal cancer including the known causes as well as the process of initiation and progression of the disease. Tumour angiogenesis is now known to be a fundamental process in certain cancers therefore, chapter one also looks at the cascade of events that are involved in the process.

To effectively assess the extent of colorectal tumour angiogenesis chapter two of the study looks at producing a standard method to highlight and then quantify tumour vessels in both a mouse tumour model and human colorectal carcinomas. The chapter assesses the specificity of various tumour endothelial markers and also describes a novel method to identify human tumour lymph vessels. This sets the foundation for chapter three which investigates the relationship between the number of human tumour blood and lymph vessels to clinicopathological variables associated with metastasis. The chapter also analyses the spatial distribution of tumour vessels and if this parameter has any bearing on the spread of the cancer.

The second half of the study examines the presence of tumour associated proteases cathepsin B and dipeptidyl peptidase IV in human colorectal carcinomas. Chapter four explores the association between the levels of enzyme protein release and the presence of their mRNA to the extent of colorectal cancer spread. Further to this, chapter five investigates the influence of two potent angiogenic growth factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) on the release of the enzymes.
From this study, the thesis aims to define the role of tumour vessels and associated angiogenic proteins in colorectal cancer progression in order to establish colorectal tumour vessel counts as an independent prognostic marker.

1.2 Colorectal Cancer

Cancer is a disease characterised by un-regulated cell growth and consequently the invasion of the cancer cells to surrounding tissue as well as spreading to other parts of the body (King 2000). The process of cancer development is a multi-step mechanism resulting from the accumulation of errors in vital regulatory pathways. It is initiated in a single cell which then multiplies and acquires changes that gives it a survival advantage over its neighboring normal cells. The altered cells must be amplified to generate billions of cells that make a cancer (De Flora and Ferguson 2005).

Carcinoma of the colon and rectum, also termed as colorectal cancer, occurs with great variation in frequency in different parts of the world. The cancer is common in the West ie. highly developed countries, with the highest incidence of the disease occurring in the United States (Weitz *et al,* 2005). The mean age that this disease occurs in patients is approximately 63 years (Golfinopoulos *et al,* 2006).

Worldwide, colorectal cancer is the fourth most common cause of cancer death closely, following behind lung, breast and prostate cancers. The World Health Organisation (WHO) estimates that 945,000 new cases occur every year and that 529, 000 of those patients die from the disease. However, there is evidence that screening of asymptomatic average-risk

individuals can reduce the number of deaths. Hence, detection and subsequent assessment of the degree of spread of the cancer is ultimately the most effective method of reducing mortality (Weitz *et al*, 2005).

Due to effective screening and the availability of modern drug therapies as well as education on lifestyle the incidence in mortality has seen a decrease in recent decades. Further research in the understanding of cancer spread is therefore, essential to control and even provide a possibility for a cure of the disease (Fernández *et al*, 2005).

The following pages aim to provide an insight into the mechanisms of colorectal cancer progression and how certain pathological characteristics such as histology, cancer cell proliferation, metastasis and vascularity of the tumour can influence clinical prognosis.

1.2.1 Epidemiology

Cancer is a major cause of morbidity in the U.K. with nearly 341,000 new cases registered in 2002 (fig.1.0). Death from the disease accounts for 26% of all mortality in people under the age of 75 years with 153,397 reported deaths in 2004 (Fig 1.1). There are more than 200 different types of cancers, four of which account for over half of the new cases, which are cancers of the lung, breast, prostate, and the large bowel (colorectal) (Cancer Research. UK, 2006).

The incidence of colorectal cancer in the U.K., for 2004, was approximately 34,900 registered cases and 16,148 deaths reported. This accounts for 11% of the total number of known cases of cancers recorded in this year and the disease ranks as one of the top three most common cancers in Britain (Cancer Research UK, 2006) (Fig.1.0).

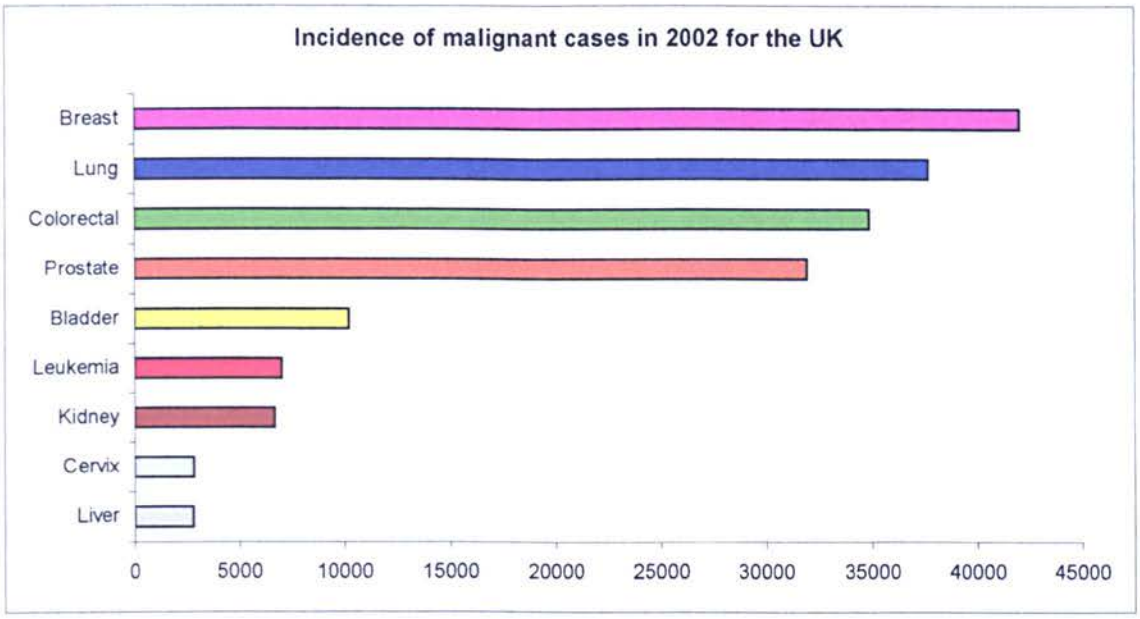


Fig. 1.0 A graph showing the incidence of various cancers in 2002 for the U.K. (data obtained from Cancer Research U.K, 2006)

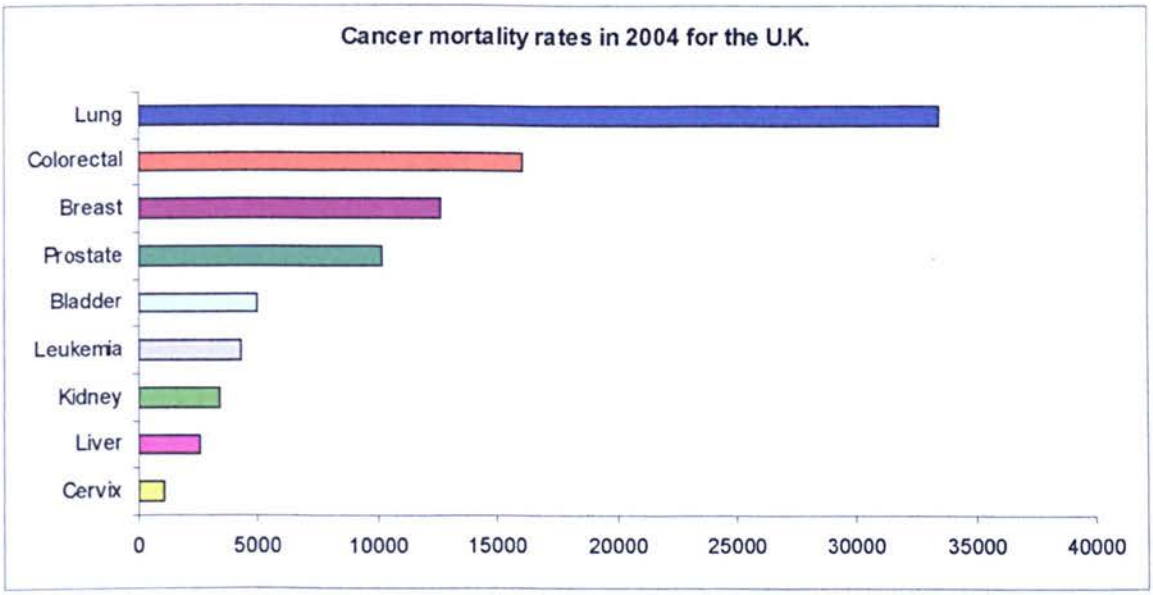


Fig. 1.1 A graph showing the mortality rate of the various types of cancers registered in 2004 for the U.K (data obtained from Cancer Research U.K, 2006).

1.2.2 Causes of colorectal cancer

There are two major forms of this disease, one where there is a known hereditary component and the other which tends to occur sporadically and can be caused by external factors. Sporadic colorectal cancers are prevalent in patients over the age of 70 years and develop from adenomas. With the influence of genetic abnormalities in the tumour suppressor genes and oncogenes these tumours can develop into metastatic carcinomas (Walker and Quirke, 2001).

Inherited colorectal cancers

The inherited forms of colon cancer include familial adenomatous polyposis coli (FAP) and hereditary non-polyposis colon cancer (HNPCC). FAP is characterised by thousands of benign, adenomatous polyps at an early age, which if untreated progress to invasive cancer (Tomlinson *et al*, 1997). HNPCC is also an inherited disease that has a high degree of penetration. Both familial types occur before the age of 50 which is earlier than the sporadic cancers (King 2000).

Sporadic colorectal cancers

Diet is a major determinant of human carcinogenesis for sporadic colorectal cancer. Carcinogens are involved in the formation of cancer and act by damaging the DNA (Fig 1.2). They are also known as genotoxin chemicals which are agents that have electrophilic groups which exert their effect by forming covalent adducts with the bases in DNA. The period during DNA synthesis, in a normal cell, is when the cell is at its most vulnerable point to carcinogenic attack. Adduct formation distorts the DNA structure and hence DNA replication is disrupted and mutations arise in the new strand (King 2000).

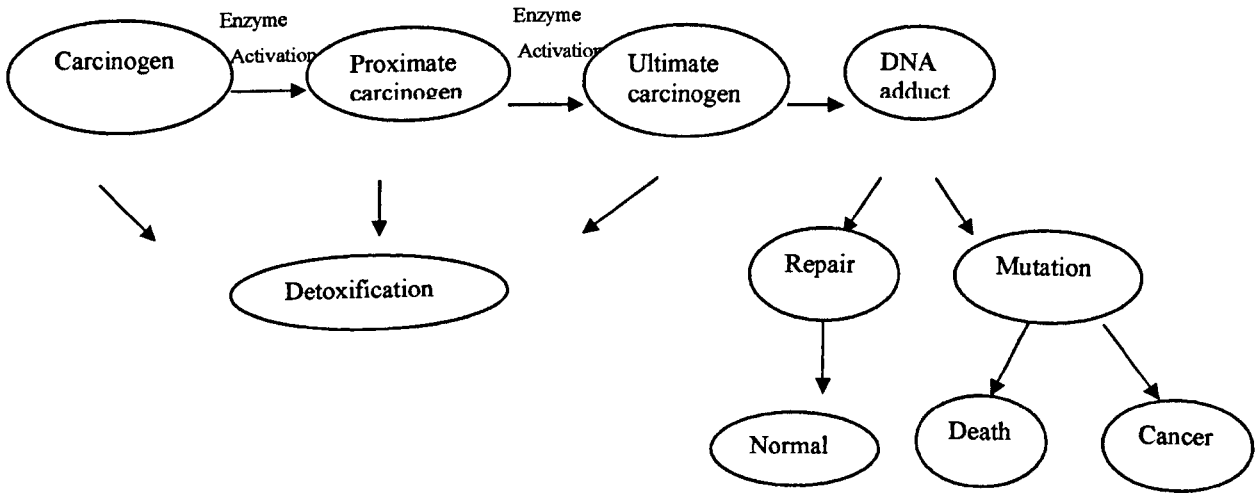


Fig 1.2 This diagram shows the sequence of events induced by a carcinogen, during mutation of DNA (Adapted from King 2000).

The carcinogens in the diet are thought to come from meat. Polycyclic aromatic hydrocarbons (PAH) are carcinogens that have a potential impact on humans. PAH's have been widely used in experimental carcinogenesis models and are known to be generated in biological materials such as grilled meat (Kato *et al*, 1999).

PAH's are made up of an inactive parent compound called phenanthrene which is composed of three fused aromatic rings. The addition of another benzene ring converts the compound into a carcinogen. These interact with purine bases especially guanine but only after enzymatic activation via proximate and ultimate carcinogens as shown in the above diagram (Fig 1.2) (King 2000).

High meat consumption is linked to the elevated risk of colon cancers. Epidemiological studies comparing the dietary habits of the world have suggested that dietary fats, especially of animal origin, are the predominant causal agent. As meat is a major source of animal fat in the Western diet, therefore, the Western countries, especially Eastern Europe and the U.S.A have the highest incidence of the disease (Marques-Vidal *et al*, 2006).

It is hypothesized that the more fat you eat the more the liver has to produce bile acids. The liver secretes bile acids to breakdown the fats consumed and diets with a high animal fat intake retain the bile acids in the large intestine. This causes bacteria in the gut to convert bile acids to a cancer causing agent: 3-methyl-cholanthrene. The sooner the bile acids and bacteria are removed there is a lesser chance of carcinogens forming. Therefore, high fibred diets such as those consumed in African and Asian countries decreases the contact rate of the carcinogens in the tract thus preventing it attacking the mucosal walls of the intestine (Marques-Vidal *et al,* 2006).

Dietary fibre has been demonstrated as having a protective characteristic against colorectal cancer by not only diluting or adsorbing fecal carcinogens, reducing colonic transit time, altering bile acid metabolism, but also reducing colonic pH, or increasing the production of short-chain fatty-acids (Fuchs *et al,* 1999).

There are two types of dietary fibre: insoluble fibre which has mechanical effects that involve shortening colonic transit of ingested contents and soluble fibre which delays gastric emptying and affects absorption. By delaying gastric emptying and unstirred water in the small bowel, soluble fibre may produce satiety and delay peak glucose concentration. Short chain fatty acids are a product of fermentation of soluble fibre in the colon. They influence the movement of water and electrolytes in the colon, serve as a source of energy, and provide nutrients to the colonic mucosa. (Bennett and Cerda, 1996).

Various naturally occurring constituents in vegetables and fruits have been studied for their anti-cancerous potential. Folate has recently been the subject of interest as epidemiological studies show that individuals with a high folate intake have a lower risk of developing cancer (Emmons *et al,* 2005).

Numerous studies have also proposed that high levels of physical activity are related to lower levels of colon cancer. Again epidemiological studies illustrate that activity is not merely a marker for a healthier lifestyle, but it also exerts an independent protective effect (Benzoumig 2004).

There have been conflicting reports that cigarette smoking is linked to colorectal cancer. This hypothesis is plausible because many carcinogens are formed during the burning of tobacco, and they could reach the bowel mucosa either by direct ingestion or via the circulatory system (Emmons *et al*, 2005)

1.2.3 Colon Cancer Pathology

90% of colon cancers are epithelial in origin and start as protuberant mucosal outgrowths into the lumen of the gut which are called polyps (Goligher 1984). These polyps are termed as adenomas and are direct precursors of adenocarcinomas which follow a predictable course of metastasis unless interrupted by treatment. They are presented as either sessile or pedunculated in form and histologically, are characterized as tubular or villous (Bloom and Fawcett 1997) (Fig 1.3).

According to the World Health Organization (WHO) tubular adenomas are encountered most frequently with 80-86% exhibited in colorectal cancer. They have a lobulated surface and are almost always sessile and consist of long papillary structures covered by the epithelium (Ramji 2006).

Villous adenomas occur less frequently and are often associated with larger ademonas with severe degree of dysplasia. They tend to arise, more often, in the rectum, although they may occur anywhere in the colon. Villous adenomas are generally sessile in structure and appear as velvety or 'cauliflower-like' projections (Ramji 2006).

Adenocarcinomas arising in the ascending colon and the caecum tend to be exophytic (cauliflower-like) and the descending and sigmoid regions present with lesions that are often circumferential and strictured (Cotton 1992).

The number of polyps and the degree of dysplasia contribute to the extent of malignancy. Well differentiated carcinomas have recognizable glandular structures and have a better prognosis than the poorly differentiated ones. However, morphological features of malignancy are not absolute as some tumours can demonstrate a deceptively benign appearance, yet are able to metastasise to distant organs (Cotton 1992).

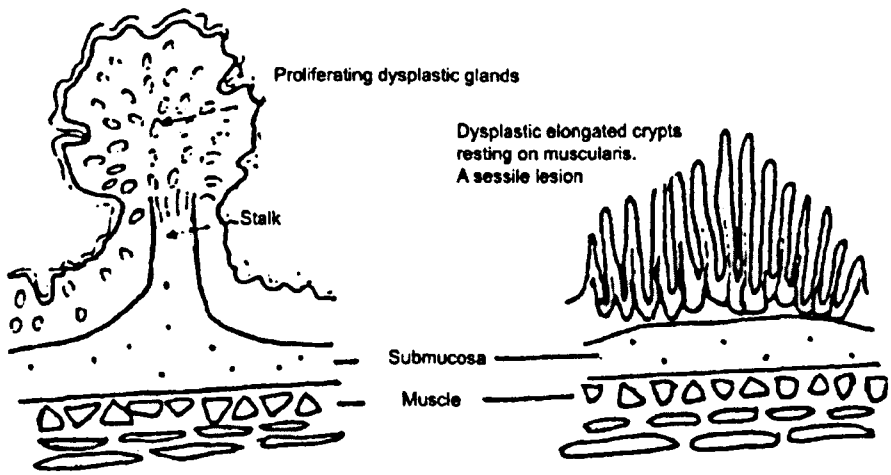


Fig. 1.3 The two main subtypes of adenomas presented in colorectal cancer: tubular (left) and villous (right) (Bloom and Fawcett 1997).

1.2.4 Presentation, diagnosis and prognosis

Communication between the surgeon, pathologist and the oncologist is essential for effective diagnosis and prognosis of colorectal cancer (Weitz *et al,* 2005). The disease can present with symptoms such as: blood or mucous in the stool, a change in normal bowel habit such as persistent diarrhea or constipation, weight loss, pain in the abdomen or rectum and blockage or obstruction in the bowel causing sickness, constipation, griping pain and bloating (Winawer *et al,* 2003).

These symptoms can be caused by conditions other than cancer of the large bowel therefore it is imperative that patients are screened so tumours can be discovered when they are small and treatment is more likely to be successful (Cancer Research. UK, 2006).

Diagnosis involves physical examination as well as assessment of faecal samples for blood. Further tests include endoscopy or sigmoidoscopy which involves examination of the large bowel and rectum by inserting a tube with a light into the patient. Another method of detection is colonoscopy which is carried out only if the whole length of the bowel needs to be examined. It is a tube made up of flexible fibres that can easily pass around curves of the entire length of the large intestine. The flexible tube is passed in to the rectum and then the colon of the patient which enables the doctor to examine for any abnormal areas. During this procedure photographs and samples of the bowel wall can be taken (Kahi and Rex, 2004).

A barium enema can also be used for detection of cancer lesions. This process involves the use of a barium mixture which is also passed into the rectum and then colon, the barium shows up in x-rays and hence the contours of the large bowel can be visualized (Cancer Research. UK, 2006).

Once a diagnosis is made the type of treatment needed for a patient depends on the stage of the cancer. The initial course of action for treatment of colorectal cancer is surgery. Surgical excisions can help to identify the stage (ie. position, size and whether it has spread) of the cancer (Winawer *et al*, 2003).

1.2.5 Staging of bowel cancer

Staging classifications for cancers of the large bowel depends on the depth of the primary tumour invasion both within and beyond the bowel wall as well as the presence or absence of nodal or distant metastasis. The combined outcome of these categories is one of the strongest prognostic factors available (Nagtegaal and van Krieken 2002).

Historically, the Dukes' classification system has taken prominence in staging colorectal cancers and many pathologists and clinicians still prefer it as their system of choice. This has been the case with the human colorectal samples obtained from the histopathology department, St George's hospital in Tooting. Therefore, all prognostic evaluations with in this study have been conducted using Dukes' stage. However, the Tumour Node Metastasis (TNM) system is now predominantly used. This is the standardized classification system in use today which has been agreed on and published by the International Union Against Cancer (UICC) (Hermanek 1999).

Dukes' Staging:

- Dukes' A - the cancer is contained within the bowel wall.
- Dukes' B - the cancer has spread through or as far as the muscle of the bowel wall, but the lymph nodes are not affected.
- Dukes' C - the cancer has spread to one or more of the local lymph nodes. Lymph nodes are usually the first place the cancer spreads to.
- Dukes' D - the cancer spreads to another site in the body forming secondary deposits.

(Cooper 1992)

The significance of staging is relevant to prognosis. The five year survival rate is greater (approximately 90% survival) in patients exhibiting Dukes' stage A and B. At these stages the cancer may be cured with treatments such as radio- or chemo- therapy along with surgery. However, prognosis is relatively poor if there is lymph node involvement as in tumours that are Dukes' C and D. The five year survival rate falls to 40-50% and patients with detectable

metastasis are usually no longer curable, although, appropriate treatment can significantly prolong life (Cooper 1992).

Tumour Node Metastasis staging:

- Tis - carcinoma in situ

- T_1 - tumour invades submucosa
- T_2 - tumour invades muscularis propria
- T_3 - tumour invades through the muscularis propria into the subserosa or non-peritonealized pericolic or perirectal tissues
- T_{4a} - tumour perforates the visceral peritoneum
- T_{4b} - tumour directly invades other organs or structures

- N_0 - no regional lymh node metastasis
- N_1 - metastasis in 1 to 3 pericolic or perirectal local lymph nodes
- N_2 - metastasis in ≥ 4 pericolic or perirectal local lymph nodes
- N_3 - distant nodal metastasis

- M_0 - no distant metastasis
- M_1 - distant metastasis present (Gunderson *et al*, 2002)

Staging systems are models that take into consideration clinical and pathological findings with proven independent influence on prognosis. Such systems calculate prognostic indices for the individual patient which provides information on the risk of recurrence of the cancer to the survival rate of the patient. Staging also provides customized information on the type of treatment regime needed by the cancer sufferer (Nagtegaal and van Krieken, 2002).

Dukes'	TNM	T	N	M
A	I	T_1	N_0	M_0
		T_2	N_0	M_0
B	II	T_3 T_{4a}	N_0	M_0
		T_{4b}	N_0	M_0
C	III	T_1 T_2	N_1 N_2	M_0
		T_3 T_{4a}	N_1 N_2	M_0
		T_{4b}	N_1 N_2	M_0
D	IV	Any T	Any N	M_1

Table 1.0 Comparison of the two staging systems used in assessing colorectal cancer. (Adapted from Gunderson *et al*, 2002).

1.3 Mechanism of Cancer Development

1.3.1 Initiation

Cancer results from changes in the DNA sequence called mutations, the pathway leading to cancer formation is depicted in fig. 1.4. The mutations are reflected in proteins which have an altered amino acids sequence, and these altered proteins ultimately change the cell function. Such mutagens can be endogenous (ie. changes occurring from normal intracellular metabolic events) or exogenous agents diet and lifestyle (Tomlinson *et al*, 1997).

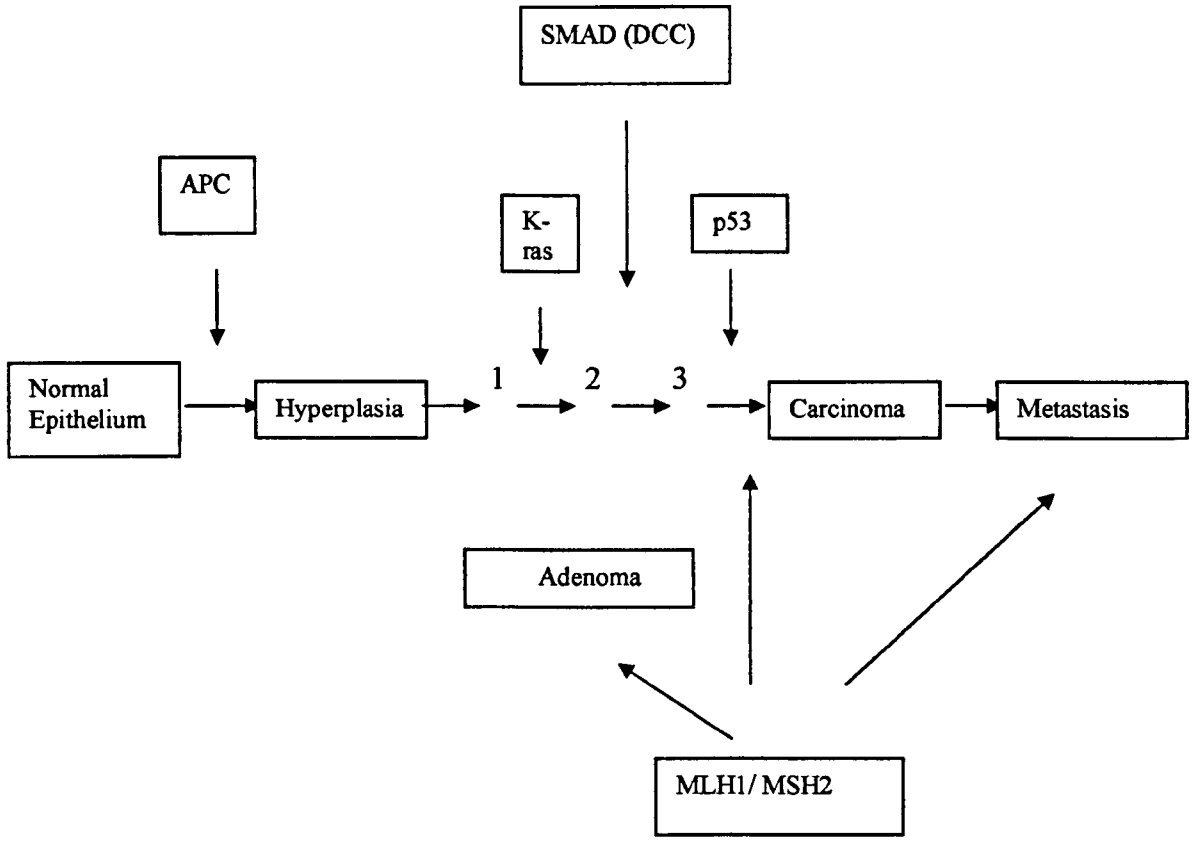


Fig 1.4 Displays the sequence of events that contribute to different stages of development in cancer formation. It demonstrates the gene changes during colon carcinogenesis (Adapted from Michor *et al,* 2005).

Many cancers are of the sporadic type and arise from somatic cells with no evidence of inherited links. However, a small percentage of common cancers arise from inherited defects. Such defects are passed on from parent to off-spring via male and female gametes (King 2000).

The inherited mutation in the adenomatous polyposis coli (APC) gene is involved in both familial and sporadic forms of colon cancer. Familial adenomatous polyposis coli (FAP) patients inherit a defective APC repressor gene that results in multiple benign polyps within which cancers develop. (Michor *et al,* 2005).

Hereditary non-polyposis colon cancer (HNPCC), in which polyp formation is not observed, has subgroups that have been identified. The majority of HNPCC cases have germline mutation in the MSH2 gene with a smaller number having a mutation in the MLH1 gene. The MSH2 and MLH1 genes are involved in the formation of nuclear proteins that are required to repair mismatched bases in the DNA double helix (Allen and Johnston, 2005).

There are three consequences to DNA damage that occur in a cell. These are i) repair and return to normality; ii) extensive damage leading to cell death iii) misreading of modified bases at the next cycle of DNA synthesis. The commonest result of chemical damage is a point mutation in which one base is replaced by another. Assessment of the p53 repressor gene, in colon cancers, demonstrates that there is a high purine base change ie. guanine to adenine transition (Weitz *et al,* 2005).

In sporadic cancers the decreased efficiency of repair of altered DNA sequences by mutagens in normal cells leads to the changes required for cancer formation. Cells with a faster mutation rate gain survival advantage over neighboring normal cells hence, the inability to normalize damaged DNA would accelerate the accumulation of errors in DNA bases. Mutation rates also increase with age in normal cells therefore, the phenomenon that carcinogenesis is a normal consequence of ageing in human life holds true (Castagnola and Giaretti 2005).

Mutations are more likely to occur in stretches of DNA containing repeat sequences because of misalignment of multiple repeats on the template and daughter strands than in a gene with few repeats. It is noted that the high frequency of cancers arising from epithelial cells of the colon is due to the elevated mutation rates caused by the repeated contact with mutagens from digested material (Walker and Quirke, 2001).

1.3.2 Promotion

Although a decreased ability to repair DNA is an important factor in carcinogenesis many cancers do not have a defect in the repair process. They may incur damage in the ancillary pathways that are involved with regulation, repair and the transfer of DNA to daughter cells. This is also termed cell proliferation and therefore, a defect in proliferation is a determinant of accumulation. Accumulation of mutated cells, over time, generates cancer (King 2000).

In normal tissue, the cell number remains constant because of a balance between proliferation, death and differentiation. However, in abnormal situations, increased cell number can result from the blocking of cell death and/or differentiation or an increase in proliferation (Castagnola and Giaretti, 2005).

Cancer cells are thought to proliferate faster than their counterparts and more importantly, they lack the stop signals that maintain normal tissue stasis. As tumours progress proliferation rates increase. Cancer cells also undergo cell death: necrosis and apoptosis (Castagnola and Giaretti, 2005).

If eventual cell death is the only factor involved then cancers would be more contained and treatable. However, in some cases cell division is asymmetrical in that one daughter cell accumulates errors in its DNA that results in senescence after 40 doublings whilst the other cell retains the potential of unlimited proliferation (King 2000).

In normal cell proliferation and regulation, DNA must be replicated and distributed equally to daughter cells (mitosis). These series of events occur in the cell cycle and are separated by 'gaps' (King 2000) as illustrated in Fig 1.5. 'Gaps' are time periods during which RNA and protein are made and the cell re-organizes itself for the next cycle of division.

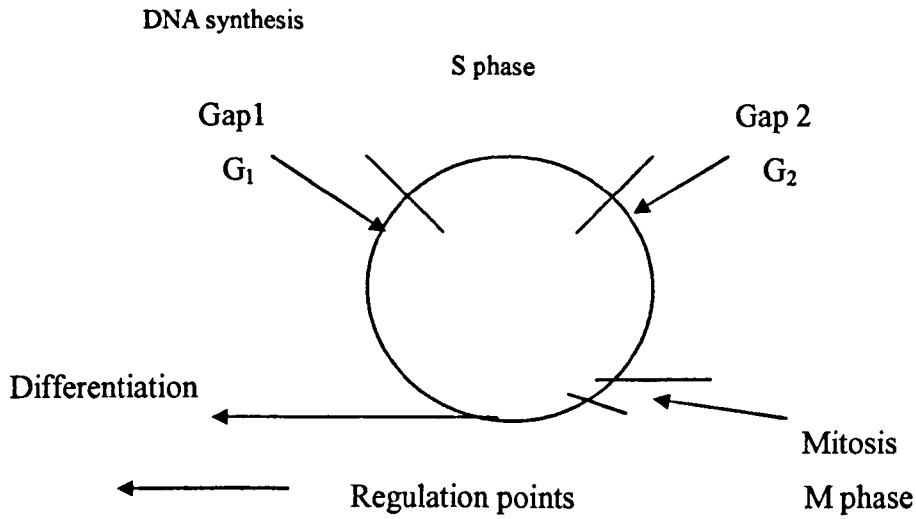


Fig 1.5. A diagram showing the series of events called phases in a cell cycle which are tightly regulated in the synthesis of DNA for new daughter cells (King 2000).

G_1 phase (Fig 1.5) is the period in the cycle that determines future division, differentiation or death is the focal point of regulatory signals. If these signals are altered then the cell cycle is affected. It is here that many gene products have been identified and the activation of oncogenes can be detected at this point (King 2000).

Primary regulation of G_1 phase involves three proteins: 1) cyclins 2) cyclin dependant serine/threonine protein kinases (CDKs) and 3) cyclin dependant kinase inhibitors (CKIs). They play a regulatory role with cyclins activating proliferation and the CKIs inhibiting cell division. An important substrate of the kinase family is the protein product of the retinoblastoma gene Rb. This protein blocks cell proliferation at the G_1 phase this repression is released by protein phosphorylation and regained by dephosphorylation (Fig 1.6).

This mechanism provides a rapid reversible switch mechanism for altering proliferation rates. G_1 phase is also involved in DNA repair. DNA damage is detected by the repressor gene p53 which blocks cycle progression until repair is completed (Fig. 1.6) (Enders and Maude, 2006).

The S phase (Fig 1.5) is the DNA synthesis phase. And during this phase DNA strands are separated, bases are exposed and therefore sensitive to external agents such as mutagens. Therefore synthesis is completed as fast as possible once it has started. As DNA repair is also part of the S phase it plays an important part in preventing the generation of cancers (O'Farrell 2001).

In the G_2 phase (Fig 1.5), the double complement of DNA and chromatin proteins formed during S phase condense and then are packaged into sister chromatids. The G_2 phase monitors any unreplicated or damaged DNA that has escaped repair by the p53 gene.

In the M phase (mitosis) (Fig 1.5) sister chromatids must be aligned correctly so that they are retracted to opposite poles of the dividing cell. If any part of this event goes wrong for example, cells pass through S but not M then an increase in ploidy (amplification of DNA) occurs. Advanced cancers often have an increased DNA content per nucleus (O'Farrell 2001).

Cancer cells are characterised by their unregulated proliferation. Such defects are brought about in the cell cycle system at the G_1 phase. The alteration of cyclin and CKI genes are important in the formation of cancers however, it is the p53 and the Rb genes that are frequently altered (Fig 1.6) (O'Farrell 2001).

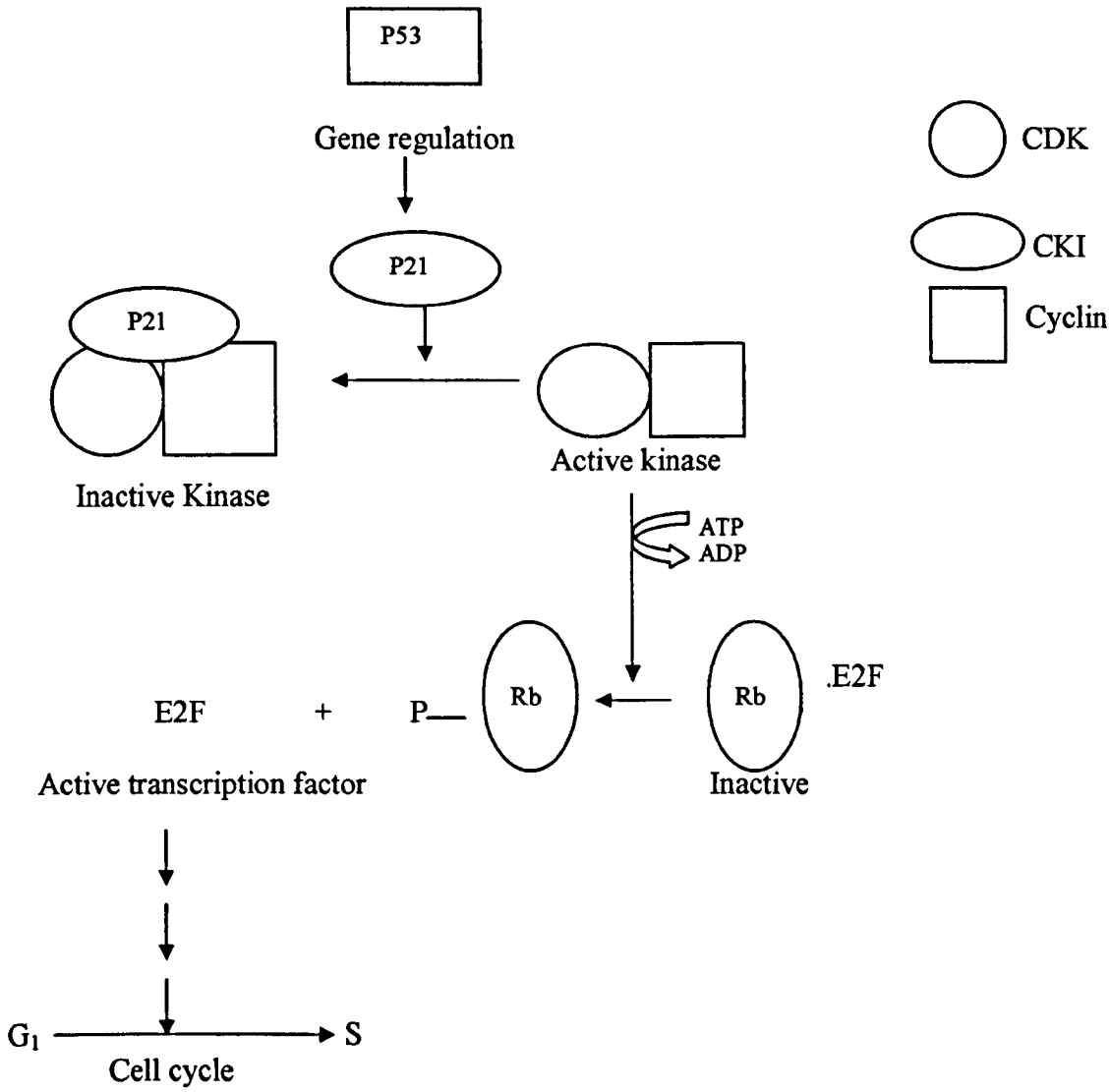


Figure 1.6 Shows the inhibition of the cell cycle by p53 and Rb. Each of the boxed proteins shown can be altered in cancer (King 2000).

1.3.3 Cell death

There are two types of cell death, apoptosis and necrosis. Apoptosis is programmed cell death that occurs in both normal and cancer cells. In cancer cells the gene changes that lead to decreased apoptosis contribute to an increase in cell number. Depending on the need for cells in a tissue structure, apoptosis is either activated by the BAX protein or inhibited by the Bcl2 protein (Michor *et al*, 2005). The p53 gene and certain growth factors regulate the apoptotic proteins. Loss of p53 influence in the cell cycle reduces the apoptotic removal of abnormal cells which results in cancer (Watson 2006).

Necrosis on the other hand, is a result of an increase in cell volume and loss of membrane integrity in tumours. It is triggered by the poor supply of nutrients and commonly occurs in the central regions of a tumour (Jain 2005).

1.4 Tumour Formation and Progression

If the equilibrium between proliferation and death is altered then abnormal growth occurs. In carcinogenesis, unregulated proliferation or decreased cell death will generate a tumour (Watson 2006). Metastatic tumours consist of a population of abnormal cells showing unrestricted growth over their normal counterparts. These abnormal cells invade surrounding tissues and may metastasize to distant areas as demonstrated in Fig 1.7 and Fig 1.8 (Shamsiddin 1990).

The initial onset of a lesion usually presents itself as a focal proliferation that is orderly in form and temporarily restricted in its growth and is characterized as a benign tumour.

If lesions with abnormal form and cytology and biological properties of a primary cancer appear then the sequential events lead to tumour progression and metastasis (Cotton 1992). Neoplastic lesions can be divided into classes on the bases of growth characteristics: temporally restricted, or temporally unrestricted, and whether lesional growth is confined to a single tissue compartment or two or more tissue compartments (Clark 1991).

Class I: is the precursor lesion, its growth is restricted and confined to the tissue compartment of origin. The lesion grows to surrounding tissue but growth does cease eventually.

Class IA: this initial lesion is composed of an organised collection of neoplastic parenchymal cells. It is biologically benign and has a tendency to disappear, however, if a cancer is to develop the initial lesion fails to disappear. Initial lesions are numerous and progression to the next lesional class is rare. Example of a class 1A lesion is adenomatous colonic polyps (tubular adenomas).

Class IB: Any lesion that fails to follow the programmed pathway of development, evolution, and disappearance via differentiation is classed as aberrant differentiation in the initial lesion. The characteristics of such a lesion is disorganised growth, cytologic abnormalities, they demonstrate a neoplastic parenchyma and mesenchyme. The lesions are indolent and their progression to the next class is rare. Example of one is small tubular adenomas of the colon.

Class IC: These lesions show the aberrant differentiation of class 1B with atypical cells. These cells have prominent hyperchromatic nuclei. Progression to class II is rare but does occur and a typical example of a class IC lesion is colonic adenoma.

Class II: This is the intermediate lesion and it is not temporally restricted however, it does not metastasise. Its growth is slow and rarely ceases, however, there is high probability of progression to class III.

Class III: The lesions are temporally unrestricted, grow in two or more tissue compartments and have the ability to metastasise.

Class IIIA: This is a primary cancer without metastasis. Carcinoma of the colon that does not extend to the muscularis propria is not associated with metastasis.

Class IIIB: This is a neoplastic primary cancer lesion that manifests the ability to metastasis. It has the ability to grow in three or more tissue compartments: the site of origin of the primary tumour, the mesenchyme of the primary site and one or more distant sites.

Class IV: These lesions are malignant neoplasms that demonstrate metastasis via blood or lymphatic vascular channels or across body cavities eg. through the peritoneum (Clark 1991).

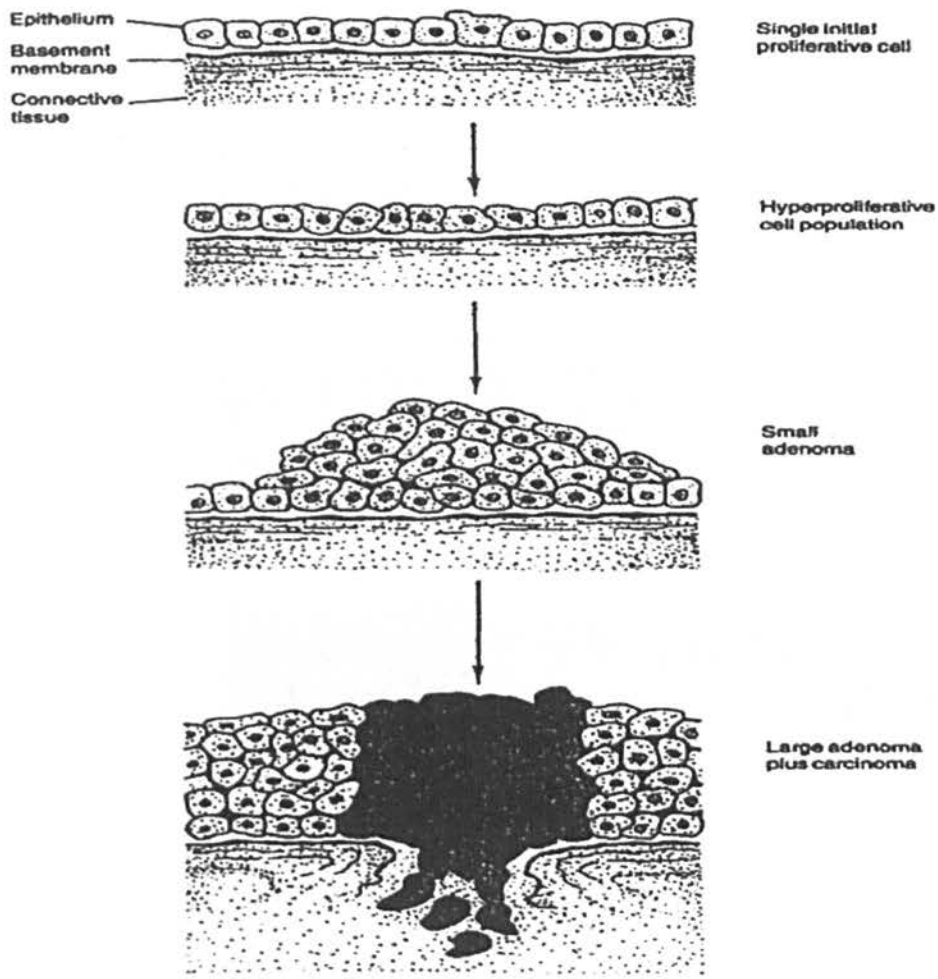


Fig.1.7 Diagramatically portrays the sequence of events ranging from tumour initiation to formation (Cooper 1992).

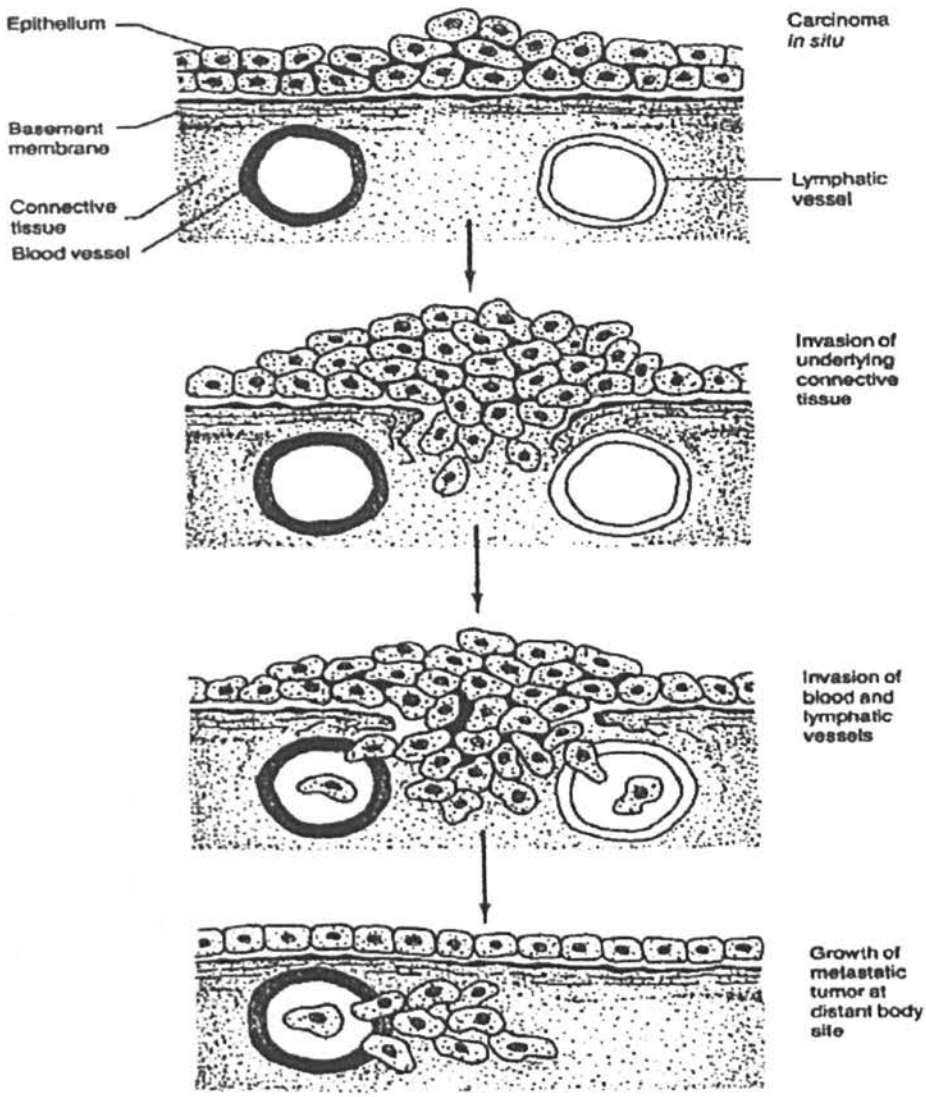


Fig 1.8 Demonstrates the sequence of events following tumour growth and how cancer cells infiltrate vascular channels to undergo metastasis (Cooper 1992).

1.4.1 Metastasis of colorectal cancer cells

In common epithelial cancers, the cells detach and traverse the basement membrane until they are able to migrate through the stroma in order to reach blood or lymphatic vessels (Fig. 1.9). The vessels then carry the cancer cells to distant sites in order to establish new foci of growth (Fig 1.10); this is the fundamental difference between a benign and malignant tumour (Palm *et al,* 2005).

The initiation of metastasis can be attributed to the mutations in the ras gene in colon carcinogenesis. In familial adenomatous polyposis coli the mutation in the APC gene (protein that codes for cell-cell adhesion) is a prerequisite for escape of cancer cells from the primary growth site. Hence both oncogenes and repressor gene products are involved in metastatic spread. A progression to the invasive state also has clinical implications in that metastatic cancers are life threatening (Castagnola and Giaretti, 2005).

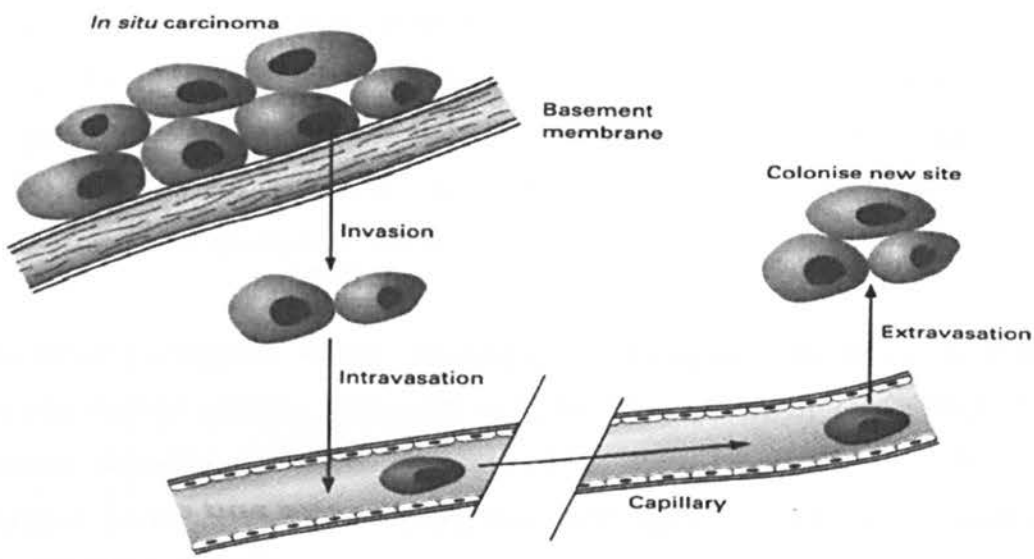


Fig 1.9 Demonstrates the escape of cancer cells to a metastatic site (King 2000).

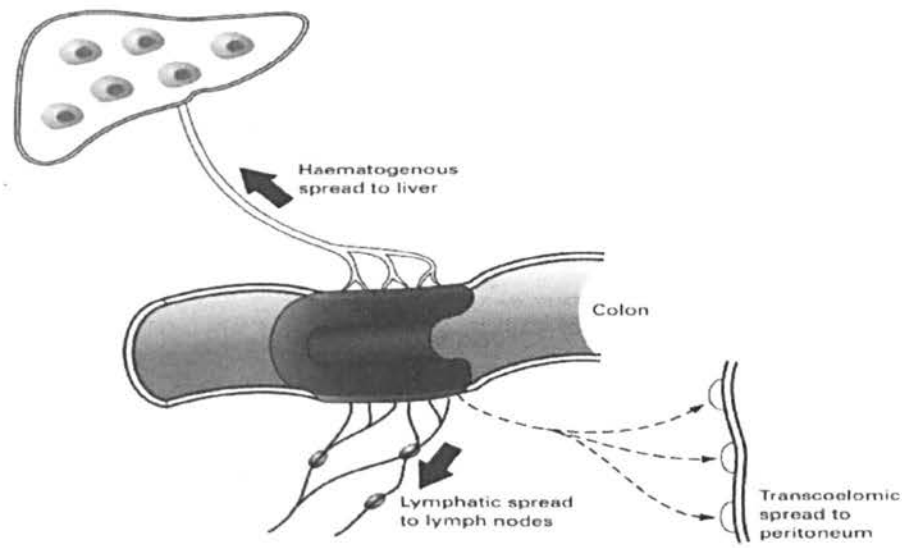


Fig 1.10 Shows the preferential site for metastasis in colon cancers is lymphatic spread to local lymph nodes and haematogenous spread to the liver (King 2000).

The process of epithelial cancers breaking links with adjacent epithelial cells and migrating through the extracellular matrix (ECM) to invade vessels requires the recognition of protein receptors on other cells and the ECM. These proteins are called cell adhesion molecules (CAMs). It is noted that cancer cells have a different profile of receptors to their normal counterparts (Yamaguchi *et al,* 2005).

The ability to recognise similar (homotypic) or dissimilar (heterotypic) cell types or ECM proteins such as collagen, fibronectin or laminin is mediated by four classes of membrane receptor: integrins, cadherins, immunoglobulin family and selectins. These membrane receptors have extracellular domains that bind ligands to specific cytoplasmic proteins (Yamaguchi *et al,* 2005).

Integrins provide a major mechanism whereby cells recognize proteins in the ECM and basement membrane. These groups of receptors are formed from a range of α and β subunits.

The ligands include: collagen IV, laminin, and fibronectin (basement membrane), and collagen I, laminin, and fibronectin (stroma) (Kurschat and Mauch 2000).

Cancers have different types and different distributions of these receptors compared with their normal counterparts. In cancer, intergrins involved in tissue organization are decreased where as those needed for migration are not (Damsky and Ilić, 2002).

Integrin	Ligand	Function
$\alpha_2\beta_1$	Laminin, collagen	Lost in anchorage-independent cells, lost in breast cancer.
$\alpha_3\beta_1$	Laminin, collagen, fibronectin	High in some metastases.
$\alpha_4\beta_1$	V-CAM, fibronectin	Arrest of cells in capillaries.
$\alpha_5\beta_1$	Laminin	Angiogenesis, extravasation of cells from capillaries.
$\alpha_6\beta_1$ or $\alpha_6\beta_4$	Most ECM proteins	High in cancers of the bladder, lung, colon.
$\alpha_v\beta_3$	Matrix metalloproteinase-2	High in metastatic melanoma, angiogenesis.
LFA1 ($\alpha_1\beta_2$)	ICAM	Immune response.

Table 1.1 Lists integrins relevant to carcinogenesis (adapted from King 2000).

$\alpha_v\beta_3$ has a broad ligand specificity, is little expressed in normal cells but is increased in normal endothelium participating in cancer directed angiogenesis. The ability to recognize virtually any of the glycoproteins of the ECM means that cells expressing $\alpha_v\beta_3$ can migrate over whatever matrices they encounter on their route to distant parts. The ability of the integrin to bind metalloproteinases also helps digest a path through the ECM. Up-regulation of $\alpha_v\beta_3$ coincides with the appearance of more aggressive growth patterns (Damsky and Ilić, 2002).

1.5 Tumour Angiogenesis

The process of tumour metastasis is a complex cascade of events which includes 1) invasion by tumour cells 2) intravasation and extravasation of the cells from the circulatory system 3) colonisation and 4) angiogenesis (Fig 1.11) (Lichtenbeld *et al,* 1996). Since tumour growth and metastasis is dependant upon angiogenesis and is demonstrated to be directly or indirectly induced by a variety of cytokines, it is therefore imperative that the underlying mechanisms, implications and the potential therapeutic applications of angiogenesis are assessed (Folkman 2003).

The hypothesis that a tumour is dependent on angiogenesis was first formulated in 1971 by Judah Folkman who famously stated that "once a tumour take has occurred, every further increase in tumour cell population must be preceded by an increase in new capillaries which converge upon the tumour". However, the idea that solid tumour growth is dependent on the induction of neovascularisation originated in the 1960's from experiments done with tumours grown in isolated perfused organs (Lichtenbeld *et al,* 1996).

Angiogenesis is the process by which new blood vessels are formed in i) a developing embryo ii) developing or growing tissue iii) wound healing and normal repair iv) inflammation, and v) pathological processes such as cancer termed as tumour angiogenesis (Presta *et al,* 2005).

In the developing embryo, the first endothelial cell precursors appear along with primitive blood cells or hemangioblasts in zones known as blood islands. Most embryonic blood vessels form as a result of fusion or anastomosis of adjacent blood islands that form the initial vasculature (Mangi 2000). The formation of early blood vessels is referred to as 'vasculogenesis' and is considered to be distinct from angiogenesis. This is because angiogenesis is where new vessels arise as outgrowths from pre-existing vessels (Bicknell *et al,* 1997).

The further growth of blood vessels in the developing organs of the embryo takes place largely as a result of the invasion of capillaries from the primitive vascular network into the new organs. Considerable changes to the pattern of vasculature such as vessel extension, branching, loop formation and vascular regression occur after vessels have formed in a given organ (Norby 1997)

Much of the vasculature is in place at birth however, further vascular expansion occurs along with tissue growth. Here, vascular growth occurs largely as outgrowths of new capillaries from pre-existing vessels (Zetter 1988).

In healthy adults, vascular turnover is extremely low and angiogenesis rarely occurs. One of the few places where angiogenesis is necessary is in the female reproductive cycle where vascular invasion is a component of the normal development of the corpus luteum.
Angiogenesis also occurs in wound healing where, it is thought signals for wound associated angiogenesis are sent by activated platelets and macrophages which are cells that are the first to respond at a wound site (Reynolds *et al,* 2005).

Angiogenesis also plays a key role in the maintenance of diseases such as diabetic retinopathy, psoriasis, rheumatoid arthritis and tumour growth (Folkman 1995). It has been proposed that it is the angiogenic switch that converts *in situ* tumours into lethal cancers (Kwon and Libutti, 2006).

The production of angiogenesis in tumours is elicited from pre-existing host vessels and the process is excessive and dysregulated in comparison to normal angiogenic processes such as wound healing and reproductive functions. The equilibrium between pro- and anti angiogenic factors affecting endothelial cells in normal physiological angiogenesis is tightly regulated where as this balance is lost in tumour blood vessel formation. Therefore, the physiology of tumour angiogenesis is distinctly different and the resultant outcome is an abnormal vascular structure, altered endothelial cell-pericyte interactions, increased vessel permeability as well as abnormal blood flow (Garcea *et al,* 2004).

Without angiogenesis, tumours are limited in size to the distance that oxygen can diffuse, for example 1-2 mm in diameter. These avascular tumours stay dormant by reaching a steady state between tumour cell proliferation and apoptosis. It is believed that only a very small subset of dormant tumours convert to the vascular phase which aids the exponential growth of these tumours. This also leads to the greater probability of haematogenous embolisation leading to metastasis (Folkman 1990, 1995).

The conversion of the tumour to the vascular phase is a result of the angiogenic switch which has many effects on the target capillary. Pericytes, positioned in the vascular basement membrane of vessels, retract and an increase in the endoplasmic reticulum and Golgi apparatus is observed. The endothelium then releases pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) by tumour or inflammatory cells (Kwon and Libutti, 2006).

This release is induced by secreted growth factors, hypoxia, release of inflammatory proteins and activation of oncogenes as well as the inactivation of tumour suppressor genes (Martínez 2006). The process of down-regulating anti-angiogenic factors such as thrombospondin-1 is also recognized as part of the angiogenic process in some tumours (Watnick *et al*, 2003). The release of pro-angiogenic factors, such as VEGF, leads to their binding to endothelial cell membranes. This causes endothelial cell proliferation and the subsequent invasion of the basement membrane. Proliferating endothelial cells start to form tube-like structures that eventually develop into tumour vessels and then a disorganized vascular network (Garcea *et al*, 2004).

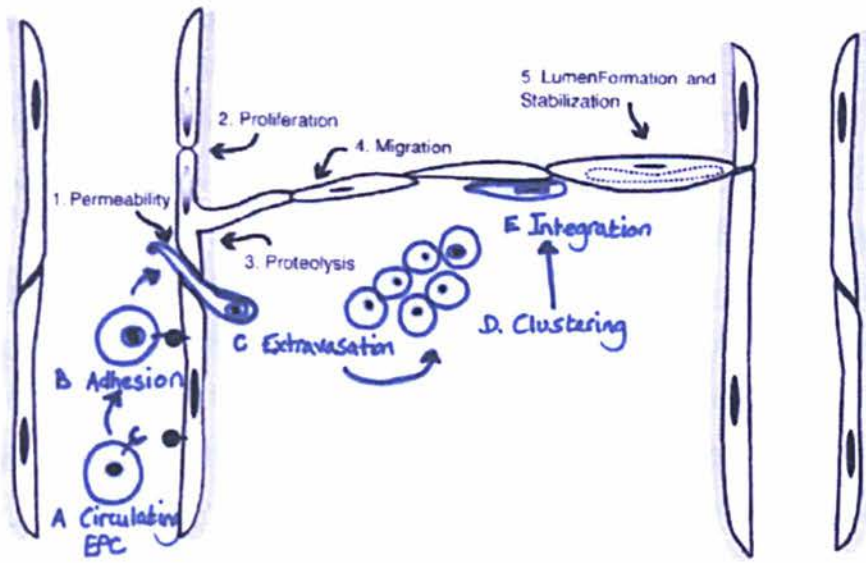


Fig. 1.11 Diagram represents the sequence of events in the process of tumour sprouting. The sequence of events shown are: 1) permeability of endothelial cells (e.c.) increase followed by 2) e.c. proliferation. 3) Proteolysis of the basement membrane and ECM by proteases to 4) enable sprouting of e.c.s into interstitial spaces. 5) Sprouts eventually form a lumen. The diagram also shows another mechanism for tumour sprouting termed as the recruitment of endothelial cell precursors. Circulating endothelial cell progenitor cells (EPCs) (A) adhere to the activated e.c. (B). The EPCs then extravasate through the e.c. layer (C) and form clusters in the interstitium (D). EPCs will then integrate into an existing sprout and comprise a part of the newly formed capillary (E) (also see Fig 1.12C) (adapted from Milkiewicz *et al*, 2006).

1.5.1 Angiogenic switch

Angiogenic switch is associated with a marked decrease in overall tumour cell apoptosis with the proliferation rate of the tumours remaining the same. This is caused by the paracrine release of anti-apoptotic factors from tumour vessel endothelial cells (Streit *et al*, 1999).

The angiogenic switch in human tumours is driven by four factors: 1) angiogenic oncogenes which up-regulate pro-angiogenic factors and down-regulate endogenous angiogenic inhibitors; 2) tumour-associated hypoxic conditions which activate hypoxia-inducible factor-1 (HIF-1) which inturn up-regulates angiogenic proteins; 3) fibroblasts induced by tumour cells

to produce pro-angiogenic proteins; and 4) bone marrow derived progenitor endothelial cells which traffic to tumours (Folkman 2003).

Studies show that the angiogenesis switch can be induced by tumour oncogenes. Udagawa *et al,* (2002) transfected non-angiogenic osteosarcoma with the ras oncogene and found that neovascularisation appeared within 2 weeks. Evidence of the influence of pro-angiogenic factors on endothelial cell formation has recently been shown by Abdollahi *et al,* (2004). They demonstrated that the application of endostatin, a known inhibitor of angiogenesis, resulted in the down-regulation the oncogenes NF-κB (nuclear factor-kappa B) and Ets-1 (erythroblastosis virus E26 transformation specific sequence gene 1) as well as HIF-1 (hypoxia inducing factor-1) protein.

Oncogenes reponsible for the stimulation of tumour angiogenesis

The development of tumour vascularity coincides with the activation of proto-oncogenes which form oncogenes as a result of mutation. It is the oncogenes that provide the signals to enhance the secretion of pro-angiogenic factors and stimulate the degradation of the extracellular matrix (Bicknell *et al,* 1997).

The *ras* oncogene, a guanosine triphosphate (GTP) protein expressed by endothelial cells, plays an important role in endothelial cell activation and its function appears to be necessary for endothelial cell motility. The K-*ras* gene is an activated mutation of the *ras* gene and the highest incidence of the mutation occurs in the colon and small intestine (Michor *et al,* 2005).

Tumour cell oncogenes also stimulate the production of angiogenic factors or may directly encode for such angiogenic promoters. The most commonly expressed tumour angiogenic factors that act both as mitogens and attractants for endothelial cells are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Rak and Yu, 2004).

The expression of the *ras* oncogene and v-*src* oncogene stimulates the expression of VEGF from tumours. It is a protein that plays a critical role in the development of tumour vascularity. The *src* oncogene also stimulates the production of VEGF and can also down-regulate thrombospondin-1 (Fox *et al*, 2001). The oncogenes responsible for the production of bFGF are int-2, which stimulates the release of FGF-3 (an isomer of bFGF) and hst which is responsible for the release of FGF-5 (also an isomer of bFGF) (Bicknell *et al*, 1997).

The release of proteolytic enzymes which play a fundamental role in the spread of tumours is also regulated by oncogenes. Activated endothelial cells mediate the proteolytic degradation of the extracellular matrix (ECM) which is an essential step in the budding of new vessels produced from host vasculature into tumours. The oncogenic transcription factors, which function to induce specific genes that code for certain proteases are c-ets-1 and c-*rel*/NF-κB. The c-ets-1 regulates the expression of a number of genes that code for proteases such as collagenases, stromelysin, and urokinase-type plasminogen activator. The *ras* and *src* oncogenes are also responsible for the stimulation of the protease group called metalloproteinases (Bicknell *et al*, 1997).

Tumour suppressor genes responsible for the stimulation of tumour angiogenesis

Tumour suppressor genes stimulate the cells, in which they are expressed, to secrete high levels of angiogenic inhibitors. Wild-type p53 (a tumour suppressor gene) normally inhibits tumour angiogenesis by up-regulating thrombospondin-1, inducing HIF-1-alpha, suppressing transcription of VEGF and down-regulating bFGF-binding protein. Therefore, the loss of p53 function would result in the increase of a tumours angiogenic output (Ren *et al*, 2006).

The mt (mutant) form of the protein has a longer half-life than the wt (wild type) form thus is frequently expressed at relatively high levels in tumour specimens with p53 mutations. However, the mutant form up-regulates VEGF production whereas, the wild-type has the opposite effect by decreasing VEGF and increasing thrombospondin-1 (Fox *et al*, 2001).

Mutated forms of p53 are common in colorectal cancer but their prognostic role is disputed. In many tumour systems, mutation of this gene has been associated with poor prognosis. As neo-vascularity of a tumour is a strong indication of tumour progression, it only made sense to conduct studies linking the expression of p53 to angiogenesis (Georgiou *et al,* 2006). Sarioglu *et al,* (2001), assessed the correlation between a prominent angiogenic factor VEGF, vascularity and p53 expression in human colon cancer tissue by immuno-histochemical staining. As their predecessors found there was a strong association between the three parameters. Sarioglu *et al,* (2001) also found that increased expression of mutant p53 correlated to angiogenesis in colorectal tumours.

Influence of hypoxia on tumour angiogenesis

Angiogenesis is also driven by the response of the tumour to hypoxia, and the protein hypoxia-inducible factor-1 (HIF-1) plays a critical part in the process. The protein consists of two subunits: HIF-1α and HIF-1β. In normal oxygen concentrations HIF-1α is rapidly degraded however during hypoxia its destruction is suppressed. Due to this HIF-1α accumulates which causes the activation of genes whose protein products act to increase tissue oxygen such as the pro-angiogenic protein VEGF. This suggests that genetic changes in a pathway directly regulating angiogenesis can cause cancer (Milkiewicz *et al,* 2006).This protein has also been demonstrated in colorectal cancer and has been associated with the metastatic potential of colorectal tumour vascularisation (Matínez 2006).

1.5.2 Angiogenic stimulators

The presence of a humoral mediator of tumour angiogenesis had long been suspected and it was Folkman (1971) identified a soluble angiogenic factor which was named tumour angiogenesis-factor (TAF). With progressive studies basic fibroblast growth factor was identified and subsequently other angiogenic promoters were discovered (Fox *et al,* 2001).

Tumour progression is dependent on the balance of angiogenic activators or inhibitors in the tumour environment. Important activators include: vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF), granulocyte colony stimulating factor (G-CSF), interleukin -1, -6 and -8, and tumour necrosis factor α (TNF α) (Moehler *et al,* 2003).

VEGF and bFGF are known to be the most potent inducers of angiogenesis (Veikkola and Alitalo, 1999; Javerzat *et al,* 2002). Therefore, this chapter will briefly concentrate on the angiogenic properties of these two pro-angiogenic factors (also see chapter 5.1.2 and 5.1.3).

Vascular endothelial growth factor (VEGF)

VEGF has various properties: it increases vascular permeability, it possesses vasodilatory abilities, stimulates the synthesis of proteolytic enzymes, has anti-apoptotic properties, as well as facilitating endothelial cell proliferation and tube formation. It is a family of isomers (VEGF A, B, C, D, and E) and VEGF-A is known to be exclusively expressed on endothelial cells (Nagy and Senger 2006).

The VEGF-A binds to high-affinity endothelial cell tyrosine-kinase receptors such as VEGFR-1 (flt-1), VEGFR-2 (KDR) and VEGFR-3 (flt-4). VEGFR-1 functions in the migration of endothelial cells where as VEGFR-2 induces the permeability and proliferation of endothelial cells (Fox *et al,* 2001).

VEGF-A has been shown to be highly expressed in tumours of the lung, brain, gastrointestinal tract and the urogenital tract. Takahashi *et al,* (1995) demonstrated the up-regulation of VEGF-A in colorectal carcinomas and in gastric cancers VEGF-A expression was correlated to microvessel density and pathological variables such as lymphatic and blood vessel invasion (Bikfalvi and Bicknell 2002).

VEGF-C has been implicated in the formation of lymph vessels and has been found to be an independent factor in predicting lymph node metastasis in patients with cervical cancer (Yonemura *et al,* 2001). VEGF-C binds to and induces tyrosine phosphorylation of VEGFR-3 and a correlation between the expression of VEGF-C, tumour lymphangiogenesis and metastasis in regional lymph nodes has been described in lung, breast and colorectal cancers (Veikkola and Alitalo 1999).

Fibroblast growth factor (FGF)

Amongst the vast array of fibroblast growth factor molecules, acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF), are the most extensively studied due to their ability to strongly stimulate angiogenesis. They mediate their effects via the tyrosine kinase receptors FGFR-1, -2, -3 and -4 with FGFR-1 being the main receptor expressed on endothelial cells (Javerzat *et al,* 2002).

Activation of these receptors induces endothelial cell proliferation, migration and tubulogenesis. FGFs have been found to be over expressed in the urine of patients suffering from a wide range of cancers. Increased expression has also been demonstrated in blood samples of colorectal cancer patients in particular levels of bFGF have been associated with the metastatic spread of colorectal tumours (Akabulut *et al,* 2002). Therefore, assessment of bFGF in metastatic colorectal cancer may have a predictive value to the outcome of the disease (Garcea *et al,* 2004).

Other growth factors

The platelet derived growth factor (PDGF) isomer PDGF-BB is a potent oncogene. It stimulates the production of VEGF-A, the migration of smooth muscles and regulates vasoconstriction. The PDGF-CC isomer has been implicated in accelerated tumour growth (Garcea *et al*, 2004).

The platelet-derived endothelial cell growth factor (PD-ECGF), thymidine phosphorylase, acts as a chemo-attractant for endothelial cells, induces vascularisation in rat sponge models and accelerates the growth of breast cancer xenografts in mice. The levels of expression of growth factor is correlated to increased microvessel density in colorectal tumours and may be an important pro-angiogenic factor in individuals with low levels of VEGF-A (Garcea *et al*, 2004).

Hepatocyte growth factor (HGF) is secreted by mesenchyme derived cells as an inactive precursor that is activated by proteolytic cleavage either by urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA). It binds to a tyrosine kinase receptor on endothelial cells and is classed as an important angiogenic mediator. This is because HGF induces VEGF expression in the endothelium as well as down-regulating the anti-angiogenic protein thrombospondin-1 in tumour cells (Milkiewicz *et al*, 2006).

Over expression of epidermal growth factor (EGF) in cancer cells has been shown to correlate with the increase of VEGF levels as well as angiogenesis. EGF signaling induces VEGF production by increasing the synthesis of the HIF-1α protein (Milkiewicz *et al*, 2006).

Interleukin-8 (IL-8) is a cytokine that directly stimulates tumour growth and may also influence the stimulation of angiogenesis. It is regulated by hypoxia in the tumour microenvironment. Concentrations of IL-8 in both tumour and serum tissue of colorectal cancer patients have been reported to be associated with increased microvessel density and levels have shown to be significantly higher in patients with liver metastasis (Bicknell *et al*, 1997).

Another cytokine tumour necrosis factor α (TNFα), produced from macrophages, activates the transcription factors that express IL-8 as well as bFGF and VEGF. On the other hand, interferon γ (INF γ), a cytokine produced from either T cells, NK cells and or macrophages, mediates the production of IL-12 which induces an anti-angiogenic effect (Lee *et al,* 2006).

Transforming growth factor β 1 (TGF-β1) is a cytokine that acts as a regulator of angiogenesis as well as an anti-oncogenic factor. Activated TGF-β1 can stimulate monocytes and macrophages to secrete pro-angiogenic cytokines. It is secreted in a latent form but it is converted to an active form by thrombospondin 1 to enable it to regulate tumour growth. The effects of TGF-β1 can be either inhibitory or stimulatory depending on the activity level of the cytokine or the stage of tumour progression. In colorectal cancer tumours activated TGF-β1, by thrombospondin 1, was shown to have an anti-angiogenic effect and reduce tumour growth (Ren *et al,* 2006). However, Sumpio *et al,* (2002) demonstrated that the over expression of both proteins increased the malignancy of gliomas.

1.5.3 Proliferation and migration of endothelial cells

In 1971 Folkman postulated that tumour angiogenesis is the growth of new vessels into the tumour from pre-existing ones. However, recent advances in the study of how vessels are recruited have changed opinions on the mechanisms involved in such a process (Auguste *et al,* 2005).

The mechanisms identified are: sprouting angiogenesis, intussusception, cooption and mosaic vessels (Fig 1.12). These processes may exist concomitantly in the same tumour or may be involved only in a specific tumour type or host environment (Hendrix *et al,* 2003). It is important to bear in mind that little is known on how lymph vessels are formed as research in tumour lymphangiogenesis is still in its infancy (Streit and Detmar, 2003).

The first mechanism that accounts for tumour vascularisation is sprouting. This involves proliferation and migration of endothelial cells from pre-existing vessels to eventually form tubular vascular structures. The VEGF family is the major player in this process and begins with the inducement of dilation of the pre-existing capillaries. This increases permeability of

the vessels which enables fibrinogen to extravasate and form a provisional matrix for later use in endothelial cell migration and tubulogenesis (Auguste *et al,* 2005).

Angiopoietin-2 (Ang-2) is produced and released by endothelial cells and provokes Angiopoietin-1(Ang-1) activity on the tyrosine kinase Tie2 receptor at the endothelial cell surface. Ang-1 is known to mediate endothelial cell stability, whilst Ang-2 is involved in vascular remodeling. Ang-2 is also thought to be an initiating factor in angiogenesis by priming endothelial cells for mitogenic signals (Milkeiwicz *et al,* 2006).

This inhibits pericyte-endothelial cell interaction and produces an immature vascular network. VEGF-A not only induces endothelial cell proliferation but also influences the release of proteases to degrade the extracellular matrix (ECM) to aid endothelial cell migration (Berger *et al,* 1999).

Proteolysis

During angiogenesis the basement membrane and the ECM liberate growth factors VEGF and bFGF which induce the release of enzymes called proteases. This allows the creation of space for endothelial cells to migrate and align into new vessels. Here, the fibrin provides the temporary scaffold that is later replaced by mature basement membrane and ECM (Moehler *et al,* 2003).

Many proteases have been identified in both tumour and endothelial cells. However, due to the vast number of studies conducted on the proteases matrix metalloprotease (MMP) and plasmin more is known on their functions in angiogenesis (Auguste *et al,* 2005).

MMPs are strongly implicated in capillary sprouting (Haas 2005). Production of the two isomers of MMPs, MMP-1 and MMP-2, is stimulated by mechanical forces as well as the growth factors bFGF and VEGF (Milkiewicz *et al,* 2006). The importance of MMP enzymes have been shown by Guedez *et al,* (2003) in animal studies where they demonstrated that inhibition of MMP suppressed angiogenesis and capillary growth in tumours.

Plasmin is an active protease derived from the cleaving of plasminogen by tissue or urokinase plasminogen activators (tPA or uPA). This activation is facilitated by the interaction of plasminogen and PA with cell surface receptors uPAR. Both uPAR and uPA are up-regulated by VEGF, bFGF and hypoxic conditions (Pepper 2001). The PA system has been discovered to play an essential role in angiogenesis (Bajou *et al*, 2004).

Migration of endothelial cells

It is postulated that the signal that guides host vessels into the tumour comes from VEGF (Bouïs *et al*, 2006). Gerhardt *et al*, (2003) demonstrated that endothelial cells present at the tip of invading blood vessels constituted clusters of the VEGF receptor VEGFR2 which was up-regulated in the presence of VEGF. This has not yet been established in tumour angiogenesis (Auguste *et al*, 2005).

Cellular attachments to the matrix molecules determine the migratory properties of endothelial cells; integrins play an important part in this process. $\alpha v\beta 3$ and $\alpha v\beta 5$ is shown to increase in proliferating endothelial cells and also by bFGF and VEGF stimulation (Nisato *et al*, 2003).

Migration is also stimulated by mechanical processes initiated by the proteases. Proteolysis of the basement membrane results in local thinning which distorts the cell membrane due to altered tractional forces (Ingber 2002). Theses forces are translated into biochemical signals within the cell which then initiates the process required for cell motility (Shin *et al*, 2001).

Cascone *et al*, (2003) suggested that the enzymes from the GTPase family are found to be active in the early stages of endothelial cell network formation and cell alignment.

Lumen formation and stabilization

Capillary sprouts (Fig 1.12A) form a lumen by intracellular canalization which occurs through fusion of cytoplasmic vesicles or by intercellular canalization in which a lumen is created by two different cells. In vitro studies have shown that the presence of integrin $\alpha 2\beta 1$ is critical for lumen formation (Davis *et al*, 2002).

The next stage in the completion of the angiogenic process is stabilization. This step is achieved through the production of factors that promote differentiated and stable capillary structures. Angiopoietins 1 and 2 participate at this stage of angiogenesis. Angiopoietin 1 binds and activates the endothelial cell specific receptor Tie2 whereas angiopoietin 2 competes with angiopoietin 1 for the binding to the Tie2 receptor. Angiopoietin 2 is expressed at low levels in quiescent endothelial cells, however, its production is noted to be increased at sites of blood vessel remodeling (Milkiewicz *et al*, 2006).

The stabilization stage in angiogenesis involves the recruitment of pericytes and coverage. Both placental growth factor (PlGF) (Milkiewicz *et al*, 2006) and platelet derived growth factor B (PDGF-B) (Auguste *et al*, 2005) assist in this process. Furthermore, transforming growth factor β (TGFβ) and sphingosin-1-P also participate in capillary stabilization by interacting with endoglin and the S-1-P receptor. In tumours this mechanism is not regulated therefore, it leads to the loosening of endothelial cell/pericyte interactions which may contribute to the fact that tumour capillaries are leaky and have large diameters (Auguste *et al*, 2005).

Intussusception

This is another process of vascular generation whereby an interstitial tissue column is inserted into the lumen of pre-existing vessels creating a partition of the lumen (Fig 1.12 B). This process has been found in nearly all of the organs studied as well as in tumour angiogenesis. During intussusception, two opposite endothelial cell membranes make contacts which form interendothelial junctions. In the middle of the contact junction the membranes are thinned and pressure induced by the cytoplasm opens them into two separate vessels. In a mouse human colon cancer tumour model 50% of the vessels were formed by intussusception (Patan *et al*, 2001; Djonov *et al*, 2003).

Cooption

Highly vascularised tumours such as tumours of the brain and lungs use cooption to form vessels. Coopted vessels are surrounded by tumour cells and no vessel sprouts are observed (Fig 1.12 D). Here, the endothelial cells synthesize angiopoietin 2 and its receptor Tie2. Angiopoietin binds to the endothelial cell specific receptor and induces the dissociation of the mural cells from the endothelial cells. Its activity results in a large decrease of tumour vessels but an increase in vessel diameter of the ones present.
This leads to hypoxia which causes the up-regulation of VEGF hence, angiogenesis is reinstated predominantly in the peripheral areas of the tumour (Leenders *et al*, 2002).

Mosaic vessel

It is hypothesized that tumour cells may localize in the wall of tumour blood vessels. bFGF and VEGF activate the MMP enzymes to degrade the basement membrane to allow the tumour cells to position themselves in the capillary wall. These cells may be used by activated natural killer (NK) cells to penetrate inside the tumour (Auguste *et al*, 2005). Chang *et al*, (2000) demonstrated that 4% of the total tumour vascular surface displayed mosaic vessels in colon carcinoma xenografts (Fig 1.12 E).

Vascular mimicry

This mechanism portrays a type of tumor vessel that has been observed in aggressive uveal melanoma. The angiogenic vessels are only present on the border of the tumour, however, inside the tumour tissue spaces are present and surprisingly, no necrotic regions are observed. The tissue spaces or 'vascular channels' are lined with a thin basal lamina and endothelial cells are not present. Inside the channel red blood cells may be observed and whether theses channels are functional is debatable (Fig 1.12 F) (Auguste *et al*, 2005).

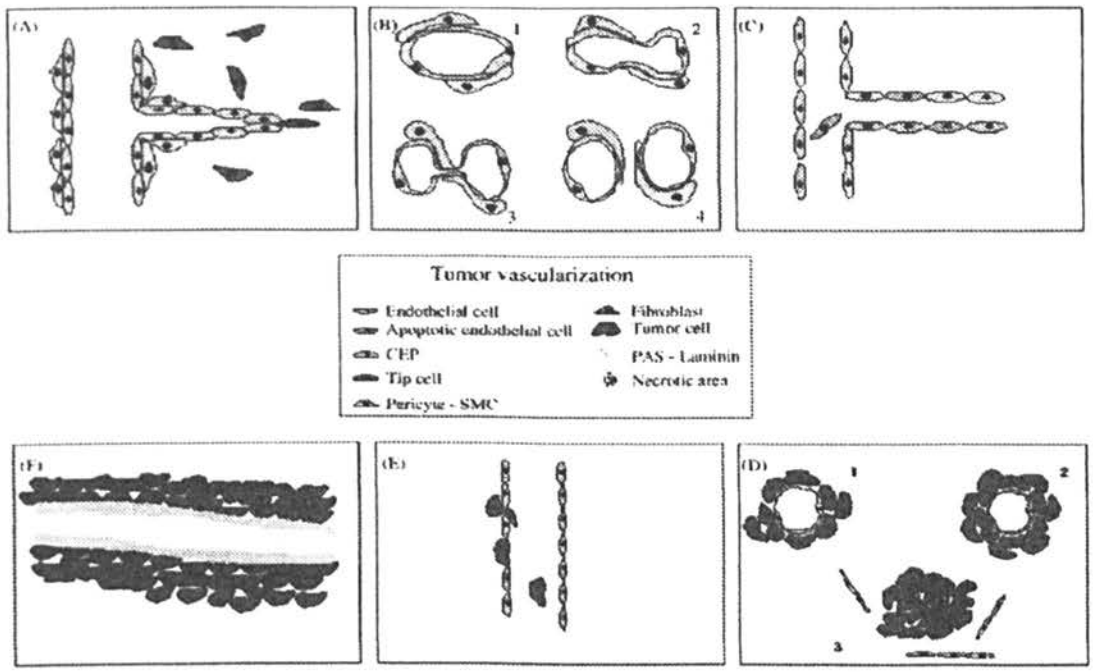


Fig 1.12 demonstrates the different mechanisms involved in the vascularisation of tumours. These include (A) sprouting; (B) intussusception; (C) recruitment of circulating endothelial precursors; (D) cooption; (E) mosaic vessels; (F) vascular mimicry. Diagram taken from Auguste *et al*, 2005.

1.5.4 Tumour vessel structure

Tumour blood vessels, like normal vessels, consist of endothelial cells, mural cells (pericytes or smooth muscle cells) and a basement membrane, however, these are abnormal in tumour vessels. The vasculature is variable in size, shape and branching patterns and vessels are not organized into categories such as arterioles, venules and capillaries (Munn 2003).

Tumour vessels lack the tight endothelial monolayer that is important for normal barrier function. This abnormality results in leakiness and with tumours presenting few or no functional lymphatic vessels, tumours possess high interstitial fluid pressure. Due to this tumours display a compromised blood flow (Nagy and Senger, 2006).

Pericytes are structures that are closely associated with endothelial cells and positioned with in the basement membrane of the vasculature. These structures are key to the development, stabilization and remodeling of normal vessels. Due to the fact that in tumour vessels pericytes are loosely attached to the endothelial cells, this may contribute to the abnormality of tumour vasculature (Baluk *et al*, 2005).

The basement membrane in tumour vessels is also known to present defects which may be because of the dynamic nature of the endothelial cells. Its structure in tumour vessels is irregular in thickness and may present with focal holes as well as extensions into the tumour stroma. The basement membrane has been highlighted as a source of angiogenic and anti-angiogenic factors (Baluk *et al*, 2005).

This abnormality is also reflected in the vessel networks of the tumour with haphazard branching patterns, larger but irregular diameters of vessels and irregular blood flow which all may contribute to less effective drug delivery (Munn 2003).

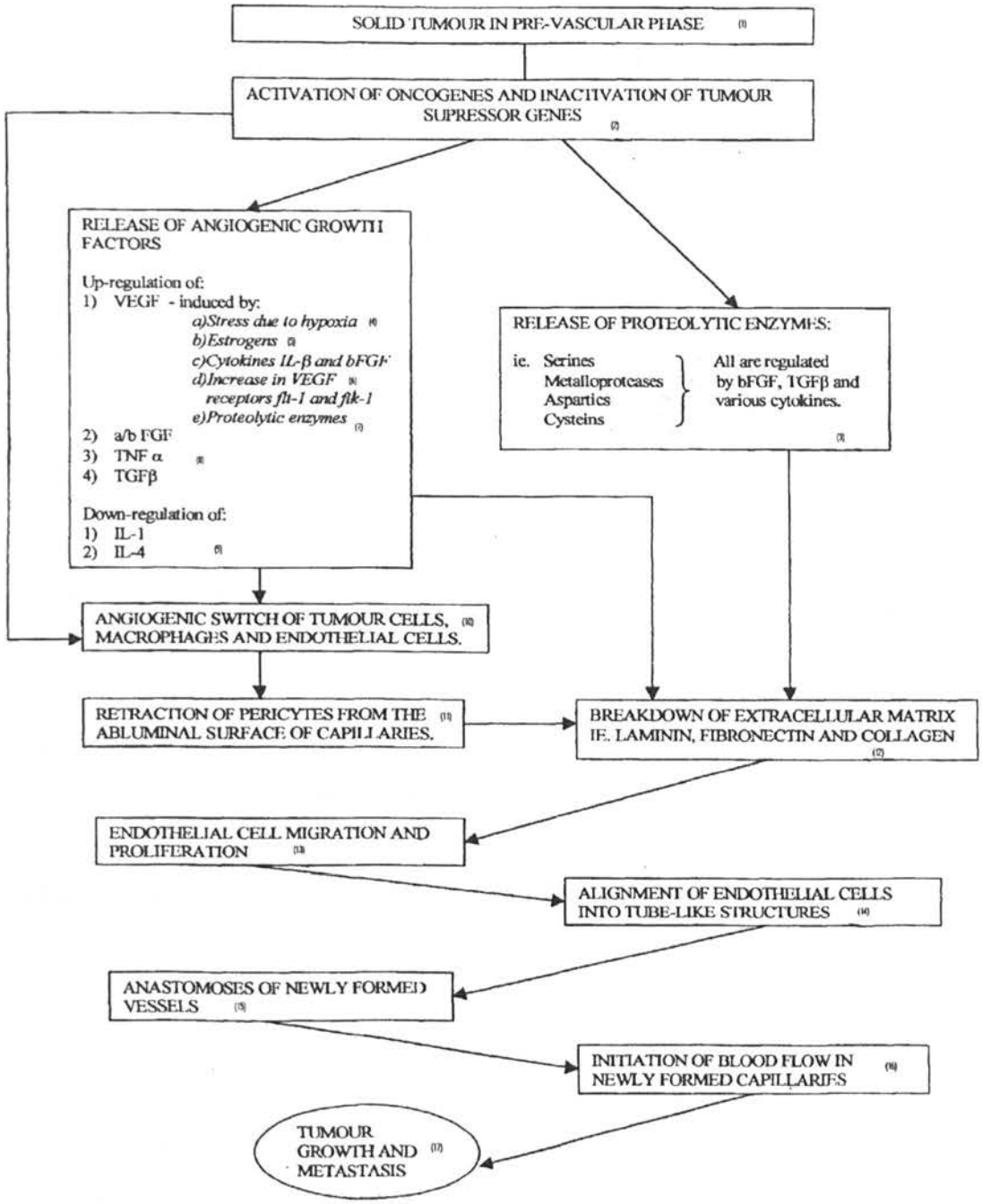


Fig 1.13 Flow chart summarizing the known steps involved in the process of angiogenesis. It is based on the information derived from the following references.

Fig. 1.13 References.

(1) Munn (2003).

(2) Ren *et al*, (2005); Milkiewicz *et al*, (2006).

(3) Auguste *et al*, (2005).

(4) Pin *et al*, (2004).

(5) Fox *et al*, (2001).

(6) Fox *et al*, (2001).

(7) Garcea *et al*, (2004).

(8) Sumpio *et al*, (2002); Lee *et al*, (2006).

(9) Lee *et al*, (2006).

(10) Udagawa *et al*, (2002).

(11) Bergers *et al*, (2000).

(12) Moehler *et al*, (2003).

(13) Auguste *et al*, (2005).

(14) Milkiewicz *et al*, (2006).

(15) Leenders *et al*, (2002); Auguste *et al*, (2005).

(16) Jain (2005).

(17) Munn (2003).

1.6 Aims of the thesis

The primary objective of this study was to assess the angiogenic potential of colorectal tumours and to ascertain the relationship between tumour angiogenesis and metastasis. Therefore, the aims of this thesis were to:

i) Establish a tumour endothelial marker that would provide optimum results. A panel of endothelial cell specific antibodies: CD31, CD34, vWF, ICAM-1, and UEA-1 were used to highlight vessels in human colorectal tumours and their endothelial cell specificity to both tumour blood and tumour lymph vessels were examined.

ii) To assess the number of tumour blood and lymph vessels and whether the number of vessels correlated to clinicopathological variables associated with metastasis in human colorectal carcinomas.

iii) To analyse the spatial distribution of vessels in both human colorectal tumours as well as in a mouse tumour model (colo26). The position of vessels in both tumour tissue and the adjacent normal tissue were investigated to determine areas of increased angiogenic activity and whether regional differences in the number of vessels may have had a bearing on the growth of the tumour. Tumour vascularity over specific time points was also studied in colo26 tumours to ascertain the levels of tumour vascularity from early stage to later stages of colo26 tumour growth.

iv) To determine the presence of proteolytic enzymes associated with tumour growth such as cathepsin B (from the cysteine family) and dipeptidyl peptidase IV (from the serine family) in human colorectal cancer and to analyse if increased levels were associated with metastatic tumours. The study also set out to investigate the possible influence of potent angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) on the levels of the proteolytic enzymes in human colon cancer, human rectal cancer and normal endothelial cells.

The assessment of the angiogenic factors such as tumour vascular counts, their spatial distribution, proteolytic enzyme activity and the influence of the well known potent angiogenic growth proteins: VEGF and bFGF on proteases and their relation to cancer spread may possess prognostic relevance. Therefore, they may have the potential to be markers for colorectal cancer progression. Hence, this study has aimed to define the significance of these parameters in colorectal carcinomas and has set out to achieve a better understanding of the angiogenic potential of colorectal tumours.

Chapter 2

Methods of identification and differentiation of tumour vascular endothelium using immuno- and enzyme- histochemical techniques.

2.1 Introduction

The vascular endothelium has a varying antigenic profile depending on the type of vessel and the tissue in which it lies. This is due to the fact that the endothelial cells (ECs) in different tissues possess distinct functions. They also display variations in appearance in relation to their function. For example, the brain and retina are lined by continuous ECs connected by tight junctions that help to maintain the blood-brain barrier. The liver, spleen and bone marrow sinusoids are lined by discontinuous ECs that allow cellular trafficking (Invernici *et al*, 2005).

A classical example of EC heterogeneity is depicted in the expression of homing receptors or EC surface proteins, such as antigens, during cell trafficking. The diversity of EC surface proteins, commonly known as antigens, has led investigators to characterize them by raising antibodies to the varying antigens expressed by the endothelium and then testing their immunoreactivity (Bicknell *et al*, 1997). The endothelium also displays diversity in enzyme activity which also enables cytochemical differentiation between vessel types such as lymph and blood vessels (Weber *et al*, 1994).

Such a distinct endothelial profile has lead to the establishment of markers to organ-specific endothelium. Due to its significance in pathology methods to assess vascular endothelium and its relation to disease processes have been carried out (Dua *et al*, 2005).

2.1.1 The vascular endothelium

The vascular endothelium plays an important role in both physiological and pathological processes (Parums *et al*, 1990). It plays a fundamental part in a) coagulation, b) inflammation and the immune response, c) synthesis of stromal components, d) vascular tone regulation and e) angiogenesis (Invernici *et al*, 2005).

a) In the process of coagulation ECs have pro-coagulant (inhibition of anti-coagulants or fibrinolytic substances) and anti-coagulant roles. The cells also participate in platelet aggregation by secreting von Willebrand Factor (a factor VIII associated antigen). This antigen helps the adhesion of the platelets to the basal lamina of damaged vessels (Fajardo 1989).

b) An important step in inflammation is leukocyte adhesion to the endothelium. This is regulated by specific receptors such as ICAM-1 which is present on the membranes of the involved cells ie. inflammatory and endothelial cells (Kawano *et al*, 2005).

c) Endothelial cells also participate in the production of stromal elements such as components for the formation of the basal lamina: collagen IV, V and laminin. As well as formation of the basement membrane ECs also participate in its degradation by secreting proteases to enable EC migration and proliferation in angiogenesis (Bicknell *et al*, 1997).

d) In the process of vascular tone regulation, endothelial cells contribute to the regulation of contraction and relaxation of the vascular smooth muscle by secreting vasoactive substances such as angiotensin, bradykinin, prostacyclin or endothelium derived relaxing factor (EDRF) (Fajardo 1989).

e) Angiogenesis is the most dynamic function of the endothelium whereby endothelial cells proliferate to form new blood vessels in response to an angiogenic stimulus. This process has also been hypothesized in tumour angiogenesis however, most of the molecular mechanisms identified today have been conducted in endothelial cell lines derived from normal blood vessels (Schmeisser *et al*, 2001).

Accumulating evidence, from *in vivo* experiments, now suggest that ECs from malignant tumours possess distinct features which may be responsible for its diverse vessel architecture (see section 1.5.4). Therefore, *in vitro* models of ECs isolated from normal blood vessels may not fully represent the properties of ECs that have been nurtured in the microenvironment of a tumour. It would therefore, be more beneficial to be able to study the role of tumour endothelial cells from malignant tumours (Bian *et al*, 2006).

Certain distinct features of angiogenic ECs include the distinct antigenic profile they possess and their assessment may contribute to the better understanding of tumour pathogenesis. Intravasation of tumour cells, from the primary tumour to secondary sites, takes place via angiogenic and lymphangiogenic vessels rather than through quiescent microvascular or lymphatic systems. Therefore, it is important to investigate the antigenic profile of angiogenic endothelial cells to assess the hypothesis that changes in expression of cell adhesion molecules are required to mediate intravasating tumour cells (Dua *et al*, 2005).

The identification of endothelial cell specific adhesion receptors have also aided the assessment of tumour vessels *in vivo* and how tumour vasculature may play a role in cancer spread and hence influence prognosis (Hamid *et al*, 2003).

A pivotal role is played by the vascular endothelium in both health and disease. Hence, its identification in routine diagnostic practice would be very valuable. A number of markers for blood vessel endothelium do exist and are used extensively for the assessment of the vasculature in pathological tissue (El-Gazzar *et al*, 2005).

2.1.2 Vascular endothelial markers

The vascular endothelium has a very different antigenic profile, depending on the type of vessel and the tissue in which it lies (Ribatti *et al*, 2003). Due to the expression of such antigens antibodies against them have been developed for use in clinical as well as experimental purposes; this technique is called immunohistochemistry and the antigens are termed as markers. However, there is no reliable method to highlight vessels for quantitative

assessment of vascularity. Reported protocols vary which could account for the discrepancies in the results (Jordan *et al*, 2002; Vermeulen *et al*, 2002).

Various angiogenic markers have been identified that could provide diagnostic and prognostic information about cancers. The current markers that have shown promising results in highlighting tumour angiogenesis are vascular endothelial growth factor (VEGF), CD105 (endoglin) and the integrin αvβ3 (Vermeulen *et al*, 2002).

It is known that VEGF is a potent angiogenic growth factor which is a key growth protein that aids initiation of tumour vascularisation and proliferation. Hence, many studies have shown immunoreactivity to VEGF in tumour microvessels, invasive fronts and in tumour tissue surrounding vascular hot spots (Lee *et al*, 2000; Chin *et al*, 2005).

Two isomers of VEGF: VEGF-A and VEGF-C, are known to be highly expressed in vascular endothelium. The expression of VEGF-A has been demonstrated to be elevated in tumours of the lung, brain and gastrointestinal tract. Increased levels of VEGF-A has been associated with tumour microvessel density and poor prognosis hence, supporting the importance of VEGF-A in malignant disease. VEGF-C has been reported to play an important role in lymphangiogenesis. Its receptor VEGFR-3 has been shown to be up-regulated in tumour lymph vessels and has been used to highlight lymphatics in tumours (Fox *et al*, 2001).

The antibody to CD105, a receptor for transforming growth factor β (TGFβ) and LM609 the antibody to the integrin αvβ3, is able to distinguish proliferating from quiescent ECs. Both CD105 and αvβ3 are known to be up-regulated in most tumour endothelium and have demonstrated their ability to give better prognostic evaluations (Brekken *et al*, 1998; Kumar *et al*, 1999).

The traditional markers (table 2.0) are currently still used to aid quantification of tumour vessels. Despite conflicting opinions on which marker displays greater specificity to the tumour endothelium these EC specific antibodies are still recommended for tumour microvessel density assessment in study protocols (Freitas *et a*l, 2005). According to literature, the five commonly used markers are von Willebrand factor (vWF), CD31, CD34, Intercellular adhesion molecule-1 (ICAM-1) and Ulex Europaeus Agglutinin-1 (UEA-1) (Fox *et al*, 2001; Hamid *et al*, 2003; Freitas *et al*, 2005).

EC Marker	Molecular Weight (kDa)	Properties of EC marker
von Willebrand Factor (vWF)	500-10,000 (Schmugge *et al*, 2003)	Also known as factor VIII RAg. It is one of the first markers used to stain ECs. Participates in platelet adhesion and aggregation. It is stored in Weible-Palade bodies of ECs. It is not expressed in all blood vessel ECs however, it known to be present in lymph vessel endothelia (Romani de Wit et al, 2003; El-Gazzar *et al*, 2005).
CD31	130 (Jackson 2003)	Also known as platelet-endothelial-cell adhesion molecule 1 (PECAM-1) that is commonly used EC marker. It belongs to the immunoglobulin superfamily of cell adhesion molecules and expressed on vascular ECs. The adhesion molecule plays a role in mediating EC interaction and promoting the adhesion of leucocytes in an immune response. It is highly expressed in ECs of existing and newly formed blood and lymph vessels (Ilan and Madri, 2003).
CD34	115 (Müller *et al*, 2002	Antigen recorded to be present on mature microvascular endothelium. It has a functional role in 1) angiogenesis and EC proliferation 2) leucocyte binding and migration to extravascular regions and 3) cell-cell or cell-matrix interactions (Hristov *et al*, 2003).

Intercellular adhesion molecule-1 (ICAM-1)	90-115 (Tachimori *et al*, 2005)	A member of the immunoglobulin superfamily is expressed on ECs and its expression is elevated by cytokines produced by tumours. High serum levels of ICAM-1 have been described in patients with colorectal cancer. It serves as a ligand for leucocyte adhesion to blood vessels in inflammation (Kamezaki *et al*, 2005).
Ulex Europaus Agglutinin-1 (UEA-1)	46 (Leader *et al*, 1986)	A sensitive marker in endothelial derived neoplasms. It has been described as being present on membranes of human erythrocytes (blood group O), epithelial cells of the colon, bronchus and tonsil as well as ECs of normal and neoplastic tissue (Leader *et al*, 1986).

Table 2.0 shows a summary of the properties of recommended endothelial markers used to assess tumour vascularity.

2.1.3 Tumour endothelial markers and their role in prognosis

The role of the endothelium in pathological conditions is of clinical significance as it has a fundamental role in the process of angiogenesis (Lee *et al*, 2000). The range of pathologies in which angiogenesis occurs includes cancer as one of the major killers of the Western population (Gasparini *et al*, 1994).

Folkman first proposed in 1971 the dependence of tumours on angiogenesis for growth beyond 2mm and that for every increase in tumour diameter there must be a corresponding increase in vascularisation of the growing tumour. Since then an exponential number of

publications have sought to answer the question of whether tumours are angiogenic dependant (Folkman 1990).

The most clinically significant observation incurred has been the report that the vascular density of primary tumours has now been correlated with survival in many tumour types. The most widely used method to assess angiogenesis in human neoplasia is the quantification of intra-tumoral microvessel density by using specific markers for endothelial cells which include vWF, UEA-1, ICAM-1, CD31 and CD34 (table 2.0) (Weidner *et al*,1991; Viera *et al*, 2005).

To date, CD31 is the most sensitive marker which recognizes a greater number of microvessels than anti-vWF or anti-CD34, therefore, this marker is widely used and has provided the most significant prognostic information to date (Müller *et al*, 2002).

2.1.4 Lymphatic endothelium

Compared to the blood vasculature, little is known about the biology of lymph vessels in tumours. It is postulated that peri-tumoral lymphatic vessels contribute to tumour metastasis by providing a passage for tumour cells to spread to distant sites. However, opposing views exist as to whether intratumoral lymphatics play any role in tumour metastasis (He *et al*, 2004).

The increased expression of vascular endothelial growth factor-C (VEGF-C) (its receptor VEGFR-3 is known to be present on lymphatic endothelial cells) correlates with lymph node positive tumours in breast cancer. Rodent models have provided evidence that tumour lymphangiogenesis facilitates lymphatic metastasis. Experiments conducted in transgenic mouse models showed the elevated expression of VEGF-C in the β cells of the pancreatic islets increased lymphangiogenesis around the primary tumour which enhanced tumour-cell spread to the draining lymph nodes. These studies support the theory that tumour lymph vessels may have an important role to play in the pathogenesis of cancer (Saharinen *et al*, 2004).

It was first hypothesised that lymph vessels are present in tumours by Witte and Witte in 1986. Light microscopic identification of lymphatic vessels is essential in studying the fine distribution of lymph vessels in tissue sections. However, basic staining such as hematoxylin and eosin do not discriminate between blood and lymph vessel types or even connective tissue spaces (Sawa *et al*, 1997).

Identification under light microscopy is made difficult due to lymph vessels displaying thin walls and occasionally they may exhibit collapsed lumens. Another complicating factor is that lymphatic capillaries look like blood capillaries under a light microscope. The distinctive features of blood and lymph vessels can be visualized, with certainty, by electron microscopy only (Sawa *et al*, 1997).

The structural make-up of lymphatic capillaries are 10-60 μm in diameter and are comprised of an endothelial cell layer, typically made of one or two non-fenestrated, highly attenuated cells in a cross-section. They have a discontinuous or an absent basement membrane (Swartz 2001). Blood capillaries are 8-20 μm in diameter and also consist of a single layer of

endothelial cells, however, they possess a complete basement membrane enveloped by pericytes. They also lack a smooth muscle layer unlike the larger blood vessels such as the veins, arterioles and arteries (Pugsley and Tabrizchi, 2000).

On the other hand, tumour blood vessels, like normal vessels, consist of endothelial cells, mural cells (pericytes or smooth muscle cells) and a basement membrane, however, these are abnormal in tumour vessels. The vasculature is variable in size, shape and branching patterns and vessels are not organized into categories such as arterioles, venules and capillaries (Munn 2003). Tumour lymphatic vessel morphology has not been assessed as there has been the assumption that solid tumours do not possess lymph vessels. However, this hypothesis has recently been corrected with the advent of specific lymphatic markers (table 2.1) (Swartz 2001). These structural characteristics make the identification of vessels in both normal and tumour tissue very difficult.

It is also important to note that these features cannot be assessed under light microscopy therefore, methods such as enzyme histochemical differentiation between blood and lymph vessel endothelium was devised to enable easier determination of lymphatics (Kato and Miyauchi, 1989). Kato and Miyauchi (1989) proposed that these vessels can be histologically distinguished by highlighting their differences in enzyme activities with blood vessels expressing high levels of 5'-nucleotidase activity and lymph vessels expressing alkaline phosphatase activity.

Endothelial markers such as CD31 (Jackson 2003) and von Willebrand factor (Di Nucci 1996; El Gazzar *et al*, 2005) have also been found to be present in lymph vessel endothelium of normal tissue, however, such markers have not been used to assess lymph vessels in tumours (Dua *et al*, 2005).

The main limitations to research on lymphatic vessels have been the lack of histological, ultrastructural and immunohistochemical markers specific to lymph vessel endothelial cells. However, specific markers have been identified (table 2.1) and may prove to be promising in the assessment of tumour lymph vessels (Al-Rawi *et al*, 2005).

Lymphatic Marker	Molecular Type	Site of expression	Biological activity
Podoplanin	Glomerular podocyte mucoprotein. (molecular weight: 43 kDa surface glycoprotein)	Co-expressed with VEGFR-3 in lymphatic capillaries, vascular tumours and renal podocytes.	Involved in maintaining lamellar permeability and the shape of podocyte foot processes in the kidneys.
Prospero related homeobox gene-1 (Prox-1)	Transcription factor (spans more than 40kb)	Heart, liver, pancreas and nervous system.	Homebox gene involved in the development and differentiation of lymphatic vessels. Prox-1 difficient neonates have failure of lymphatic sprouting and differentiation.
Lymphatic endothelial receptor-1 (LYVE-1)	HA receptor-1 (molecular weight 60 kDa)	Kidney, pancreas, adrenal glands and thyroid glands.	Transport of hyaluronan from the extracellular matrix to lymph nodes.
Vascular endothelial growth factor receptor-3 (VEGFR-3)	Receptor tyrosine kinase	Mainly on lymphatic vessels but also in activated neovasculature in tumours	Receptor for isomers of VEGF: VEGF-C and VEGF-D
Desmoplakin	Endothelial adhering junction	Small lymph vessels although absent from large lymphatics such as the thoracic duct.	Provide gaps through which macromolecules and circulating cells pass.

Table 2.1 shows the specific markers for the lymphatic endothelium, their expression sites and their main biological functions (adapted from Al-Rawi *et al*, 2005).

2.2 Aims

The aim of this study was to determine the optimal immunohistochemical staining method in paraffin embedded human colorectal carcinomas in order to assess tumour vessels. The method was applied by using a panel of five endothelial cell markers that are commonly used to highlight tumour angiogenesis such as CD31, CD34, vWF, ICAM-1, and UEA-1. Due to the lack of data on tumour lymph vessels the study also set out to identify a relevant lymph vessel endothelial marker to highlight the possible presence of tumour lymph vessels in order to enable the subsequent analysis of the impact of tumour lymph vessels in colorectal cancer progression.

2.3 Methods and materials

Immunohistochemistry and enzyme-histochemistry were used to enable the identification of vessels within human colorectal tumours. These methods were tested in order to determine a procedure which would effectively identify most of the blood and lymph vessels in the tissue samples.

2.3.1 Assessment of human colorectal tumour vasculature using a panel of endothelial specific antibodies

Twelve excised human colorectal tumours were obtained from the histopathology department at St.George's Hospital, London following the ethical approval guidelines of the hospital. Malignant tumour tissue and adjacent normal colon tissue were surgically excised from patients prior to further treatments such as chemo- or radiotherapy. Tissue from Dukes' graded tumours: Dukes' A (n=3), Dukes' B (n=5) and Dukes' C (n=4) were used in the following experiments.

Sections from the paraffin embedded tumour tissue blocks were cut and mounted on poly-L-lysine-coated slides in preparation for immunohistochemistry. Sections were then deparaffinised with two changes of xylene (BDH, Poole, U.K.) and then rehydrated, firstly in absolute and then in 95% ethanol. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide solution for 30 minutes followed by antigen retrieval (table 2.2). Slides were immersed in 0.1% bovine serum albumin/Tris-HCL for 20 minutes to block non-specific binding of antibodies.

Slides were then incubated with one of the following antibodies: mouse anti-human CD31 (clone JC/70), mouse anti-human CD34 (clone QBEND/10), rabbit anti-human vWF, mouse anti-human CD54 (ICAM-1) and biotinylated Ulex Europaeus Agglutinin-1 (UEA-1) (dilutions and source shown in table 2.3). All slides were incubated at room temperature in a humidity chamber for one hour. Biotinylated secondary IgG antibody, specific to the primary antibody under investigation, was applied to the tissue sections and incubated for 30 minutes at room temperature (for dilutions see table 2.3). This was followed by the addition of the avidin-biotin-peroxidase complex for 30 minutes at room temperature. All antibodies in this

and the following experiments were titrated for optimum dilutions rather than the standard recommended dilutions taken into account.

Staining was observed using 3, 3'-diaminobenzidine (Sigma Fast ™ Dorset, U.K.) until the reaction products were visible. Tissue sections were counter stained with Gill's hematoxylin (BDH, Poole, U.K.) then dehydrated in 95% ethanol followed by absolute ethanol. Sections were finally immersed in xylene before being mounted. The above method was carried out in normal vascular endothelium of the human colon, which was used as the positive control. Negative controls were carried out by using the same protocol, however, this time omitting the primary antibody. Sections were then examined and photographed using a light microscope.

Primary antibody / lectin	Antigen retrieval method	Incubation period
Rabbit anti-human Vwf	Predigestion with trypsin	1 hour
Mouse anti-human CD34 (QBEND/10)	Microwave oven irradiation for 15 minutes	1 hour
Mouse anti-human CD31 (JC/70)	Microwave oven irradiation for 25 minutes	1 hour
Mouse anti-human CD54 (ICAM-1)	Microwave oven irradiation for 25 minutes	1 hour
Biotinylated UEA-1	Predigestion with trypsin	30 minutes

Table 2.2 A summary of antigen retrieval methods and incubation times specific to the primary antibodies used.

Primary antibody/ lectin	Dilution	Source	Secondary antibody	Dilution	Source
Rabbit anti-human vWF	1:200	Dako	Goat anti-rabbit IgG	1:300	Dako
Mouse anti-human CD34 (QBEND/10)	1:40	Serotec	Horse anti-mouse IgG	1:50	Vector laboratories
Mouse anti-human CD31 (JC/70)	1:10	Dako	Horse anti-mouse IgG	1:50	Vector laboratories
Mouse anti-human CD54 (ICAM-1)	1:50	Dako	Horse anti-mouse IgG	1:50	Vector laboratories
Biotinylated UEA-1	1:10	Vector laboratories	-	-	-

Table 2.3 Shows the panel of primary antibodies and the secondary antibodies used to highlight vessels, in human colorectal tumours, by immunohistochemistry.

Differentiation of blood and lymph vessels using endothelial markers CD31 and CD34

Eight serial sections of 5μm thickness were cut from each block and mounted on poly-L-lysine-coated slides. Sections were then deparaffinised with two changes of xylene (BDH, Poole, U.K.) and then re-hydrated, firstly in absolute and then in 95% ethanol. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide solution for 30 minutes followed by antigen retrieval by microwaving slides (Sanyo 800W) in citrate buffer pH 6.0 for 12 minutes. Slides were immersed in 0.1% bovine serum albumin/Tris-HCL for 20 minutes to block non-specific binding of antibodies.

Slides were numbered from 1-8. Odd numbered slides were incubated with the antibody CD31 (clone JC/70) (Dako, Buckinghamshire, U.K.) at a dilution of 1: 10. The slides labeled with even numbers were incubated with the CD34 antibody (clone Qbend/10) (Serotec, oxford,

U.K.) at a dilution of 1: 40. All slides were incubated at room temperature in a humidity chamber for one hour. Biotinylated anti-mouse IgG secondary antibody (Vector Labs., Peterborough, U.K.) diluted to 1:50 was applied to the tissue sections and incubated for 30 minutes at room temperature. This was followed by the addition of the avidin-biotin-peroxidase complex for 30 minutes at room temperature.
Staining was observed by using the same method as above and sections were then examined and photographed under a light microscope.

The above method was also carried out in normal vascular endothelium of the human colon, which was used as the positive control. Negative controls were carried out by using the same protocol, however, this time omitting the primary antibody.

The criteria used to morphologically distinguish blood from lymph vessels was based on the classic features possessed by the lymph vessels. The features included: larger lumens which appeared in some sectional profiles as partially collapsed, irregular in shape than blood vessels, lymph vessels were generally devoid of cellular components as opposed to blood vessels which usually contained red blood cells.

Identification of lymphatic endothelium using anti-VEGFR-3 and anti-laminin

The above method was used to identify tumour lymphatic endothelia therefore, to enable the confirmation of the results vessels were highlighted by VEGFR-3 and anti-laminin.
A series of 37 sections were used to obtain optimum results for VEGFR-3 staining and a further 29 sections were used to assess anti-laminin specificity.

Sections were incubated with anti-humanVEGFR-3 (anti-human Flt-4) (Autogen, U.S.A.) at a dilution of 1:50 overnight at 4°C. The rest were incubated with rabbit anti-laminin (Serotec, U.K.) at a dilution of 1:200 for 1 hour at 37°C. Prior to incubation sections were treated with the microwave antigen retrieval method (heat treatment for an optimum time of 5 minutes in citrate buffer).

Following incubation with primary antibodies the slides were then incubated with secondary antibodies: biotinylated goat anti-rabbit IgG at a dilution of 1:100 (Autogen, U.S.A.) (applied

to slides treated with anti-humanVEGFR-3) and biotiylated sheep anti-rabbit IgG laminin (Serotec, U.K.), dilution 1:400 (applied to slides treated with rabbit anti-laminin).

Incubation time for both secondary antibodies was 1 hour and the remaining immunohistochemical steps, stated above, were carried out. Human breast carcinoma was used as the positive control tissue for anti-VEGFR-3 and the human kidney for anti-laminin.

Identification of blood and lymph vessels using enzyme-histochemistry

Eight serial sections of human colorectal carcinomas were de-paraffinised with two changes of xylene (BDH, Poole, U.K.) and then rehydrated, firstly in absolute and then in 95% ethanol. Sections were then immersed in distilled water before incubating slides in standard media to obtain a specific reaction for 5' nucleotidase (5'Nase) activity in lymphatic vessels or a standard media to obtain a specific reaction for alkaline phosphatase (ALPase) in blood vessels. Slides were numbered from 1-8. Odd numbered slides were incubated with 5' Nase medium and the slides labeled with even numbers were incubated with the ALPase medium.

The medium for 5'Nase consisted of 0.1M of Tris-Maleat Buffer (pH 8.5), 2.9mM adenosine 5' monophosphate (a substrate), 3.6mM $MgSO_4$ (enzyme activator), 3.6mM Pb $(NO_3)_2$ (capture agent), 2mM L-tetramisole (inhibitor of non-specific ALPase) and 7% sucrose. All reagents were purchased from Sigma U.K. Slides were incubated under optimum conditions which were 150 minutes at 37°C.

Samples were rinsed with distilled water and then immersed in 1% ammonium sulphide solution for 1 minute at room temperature. The sections were then immediately mounted and examined and photographed under a light microscope.

The medium for ALPase contained 0.04g naphthol AS-MX phosphate. This was dissolved in a mixture of 2ml of N'N- dimethylformamide and 0.04g of fast blue BB salt made up in 40ml of 0.1M Tris-HCl buffer (pH9.2). All reagents were purchased from Sigma U.K. Slides were incubated under optimum conditions which were 90 minutes at 4°C. The sections were also immediately mounted and examined and photographed under a Ziess light microscope.

Results

A panel of endothelial cell markers were used to assess endothelial cell specificity in human colorectal carcinomas. A standard protocol was devised after optimizing methods to highlight blood and lymph vessels in order to assess colorectal tumour vascularity.

Antibody assessment to blood vessel endothelium in human colorectal tumours.

A panel of endothelial markers: anti-vWF, anti-CD31, anti-CD34, anti-ICAM-1 and anti-UEA-1, were investigated for immunospecificity in paraffin wax tissue sections of 12 malignant colorectal tumours of varying grades. The criteria used to identify prominent tumour blood capillaries were: intact endothelial lining and contents of the lumen, whereby, erythrocytes were the main bodies present. The results are summarised in table 2.4.

Strong positivity to the antigen vWF by the vWF specific primary antibody was detected in a majority of endothelial cells. However, these results were inconsistent and were not reproducible in all sections. Nevertheless, in the immunopositive cases tumour vascular endothelium was intensely stained. The greater problem of non-specific background staining repeatedly hindered the visualisation of many vessels (fig. 2.2).

Similar staining intensity and specificity was observed in the adjacent normal colon and the pattern of staining was observed to be uniform in both normal and tumour tissue.
Negative controls showed no staining making it evident that the antigen vWF was the only protein being highlighted by this staining method (fig 2.1).

Histology	Anti-vWF	Anti-CD31	Anti-CD34	Anti-ICAM-1	Anti-UEA-1
Normal human colon (n=12)	++	++++	+++	+++	+/-
Dukes' A Well differentiated (n=3)	++	++++	+++	+	+/-
Dukes' B Well differentiated (n=1) Moderately differentiated (n=4)	++	++++	+++	+/-	+/-
Dukes' C Poorly differentiated (n=4)	++	++++	+++	+	-

Table 2.4 A summary of results obtained by staining varying grades of colorectal tumours and normal human colon tissue with the endothelial markers. Staining is graded according to specificity and intensity.

Key: ++++ staining is intense and highly specific with all vessels highlighted; +++ is intense and specific however, not all vessels are stained; ++ is specific however, marred by background staining; + staining is weak with not all vessels highlighted; +/- staining is very weak and not reproducible; - no staining observed.

Anti-UEA-1 showed endothelial staining in very few cases. Endothelial cell staining was weak and many vessels failed to react with anti-UEA-1 in certain sections examined (fig. 2.5). Results were inconsistent and observations of vessels were made difficult due to the low immunoreactivity of the antibody within colorectal tumour tissue. No reactivity was observed in negative controls (fig 2.4) and staining was evident in normal colon endothelium (fig 2.3), however, staining was relatively very weak in comparison to other markers used in this study. The intensity and specificity of the endothelial markers were scored as demonstrated by table 2.4. Scoring ranged from ++++ for highly intense and specific staining to – where no staining was observed.

Reactivity with anti-ICAM-1, in vessel endothelium, was intense especially in vessels adjacent to and within the invasive tumour edge (fig 2.8). In contrast, central tumour regions showed little or no reaction to the antibody. Nevertheless, the vessels that were highlighted were

prominent but weakly stained and minimal background staining was observed. Immunopositivity of anti-ICAM-1 within normal colon was strong and focal (fig 2.6) and negative controls displayed no reactivity (fig 2.7).

Immunoreactivity of tumour blood vessel endothelium to both anti-CD31 (figs 2.11) and anti-CD34 (figs 2.14) was specific and intense with minimal background staining. These antibodies displayed the greatest reactivity to vessel endothelium than any other markers used in the study. Such results were consistent in all sections examined (figs 2.11 and 2.14). The intensity and specificity of the staining made the identification of vessels very easy to locate under light microscopy.

The reactivity of blood vessel endothelial cells to the marker CD34 was intense and specific with minimal background staining in both normal colon and colorectal tumour tissue. This greatly enhanced the visualisation of the arterioles, venules and the blood capillaries present in normal tissue. The strong expression of CD34 on endothelial cells was consistent in all sections. However, a number of vessels located in close proximity to the arterioles were weakly positive or completely negative to anti-CD34. This was especially notable in the submucosal regions of the normal colon (fig 2.12). These faintly stained vessels were also noted amongst clusters of intensely stained blood capillaries in tumour tissue. In some areas almost a complete absence of CD34 staining in the endothelium was observed.

By contrast, anti-CD31 displayed highly specific staining in all of the vessels present in normal and tumour tissue. Consistent results were achieved in all of the sections stained (fig 2.11). The antibody staining reactions, with both anti-CD31 and anti-CD34 were weaker in normal colon tissue than in tumour tissue. However, specificity to the endothelium remained consistent.

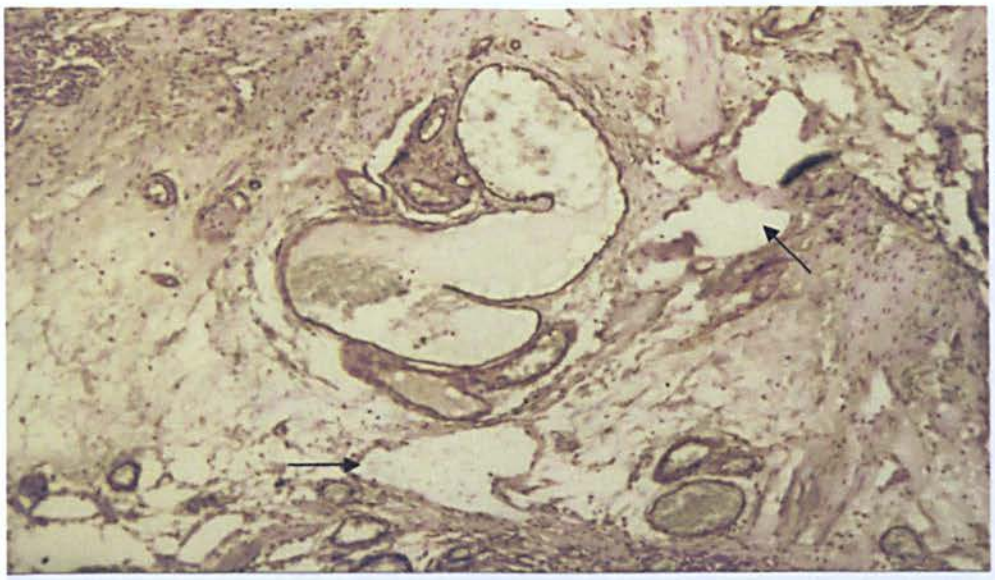


Fig 2.0 Magification of x 700 of a section from normal human colon tissue stained with the antibody anti-vWF. Section was used as a positive control for vWF antigen, note all vessels have been highlighted by the antibody (arrows).

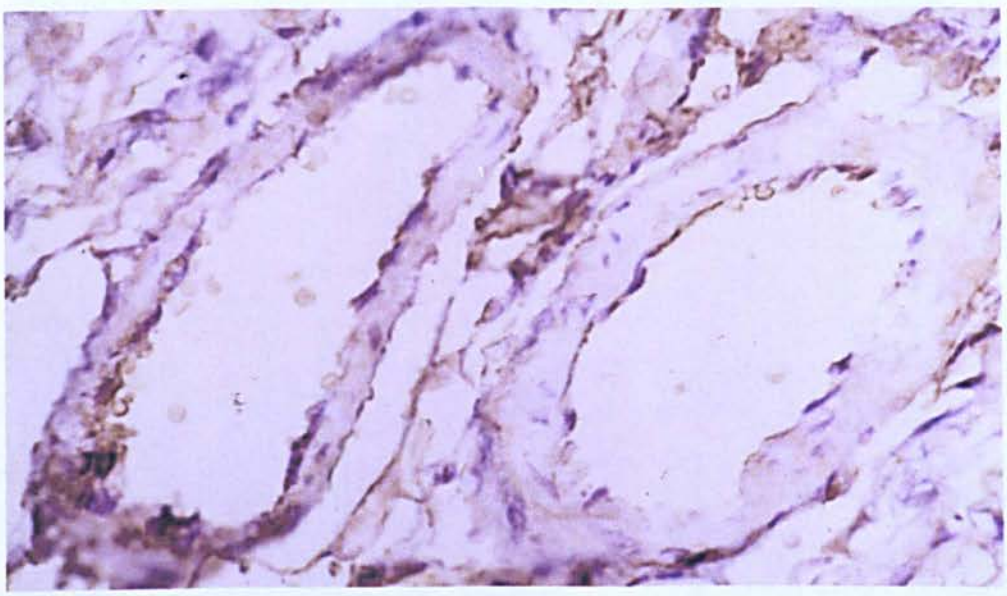


Fig 2.1 Magnification x1160**.** Negative control to vWF, no staining is observed in vessels of normal human colon tissue. Primary antibody is omitted in the immunostaining process to ascertain if staining is a direct result of primary antibody localisation.

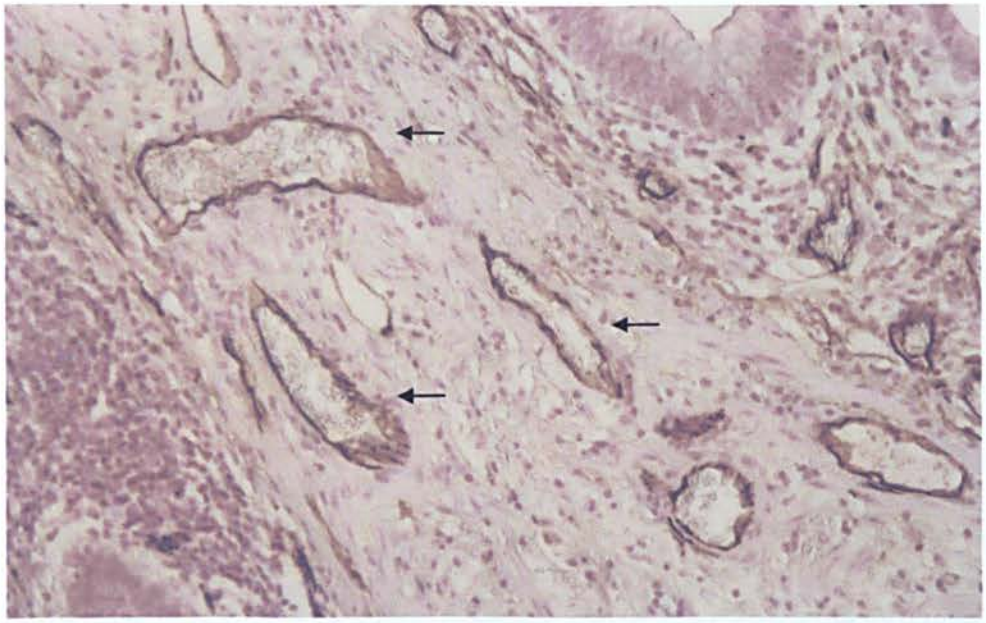


Fig. 2.2 Magnification of x 840 of a human colorectal cancer tissue, Dukes' grade C, stained with vWF. Reactivity of vessels (arrows) to the antibody shows to be strong however, ample background staining is observed.

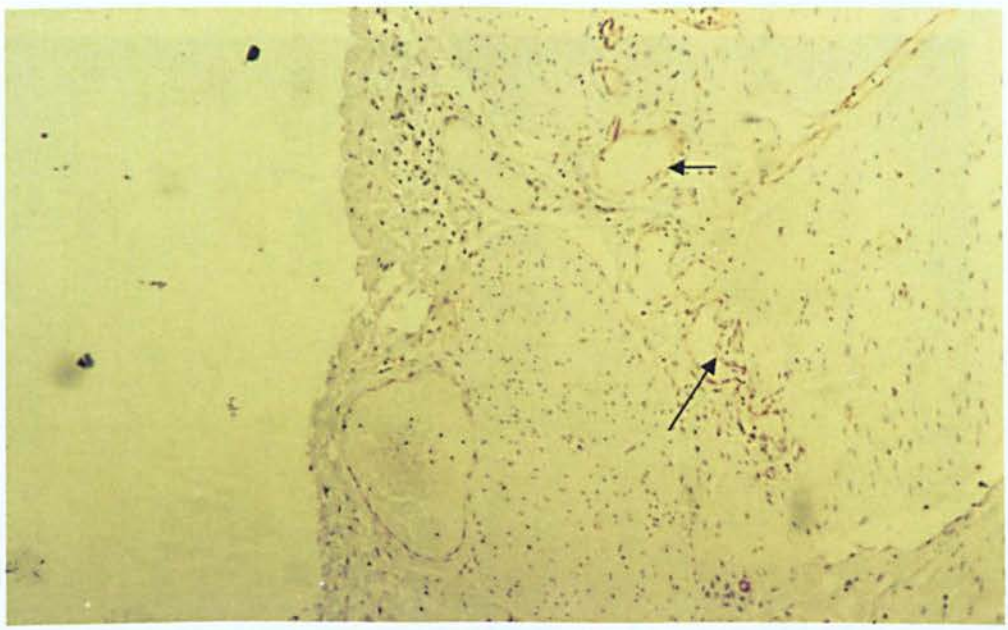


Fig 2.3 Magnification x280. Staining of vessels by UEA-1 in normal human colon tissue. The positive control exhibits localised staining of vessel endothelium (arrows) however, not all vessels are highlighted.

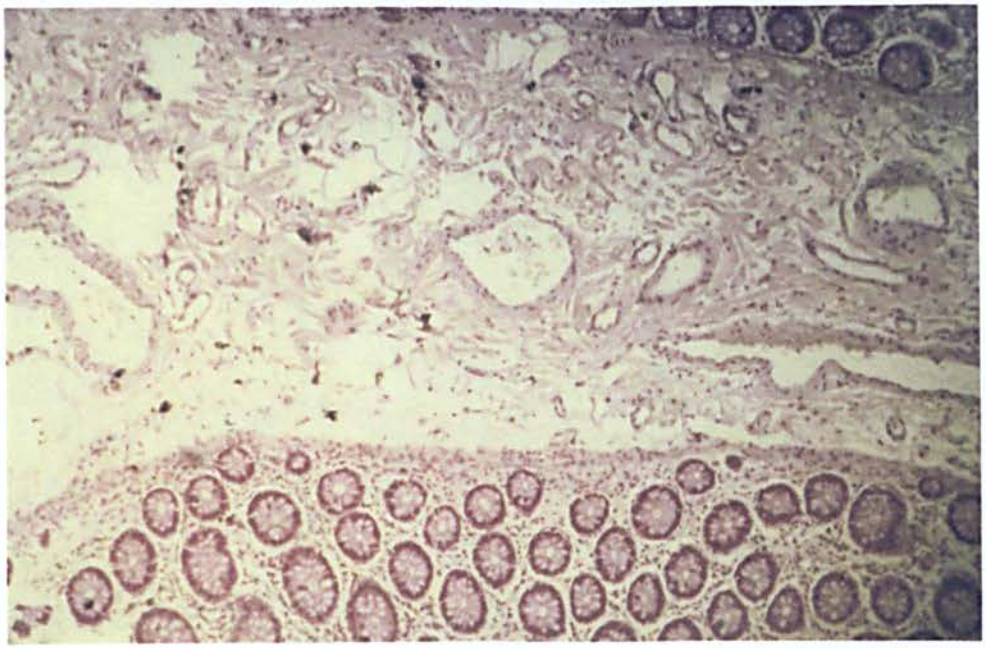


Fig. 2.4 Magnification x 280. Negative control for UEA-1 in normal human colon tissue.

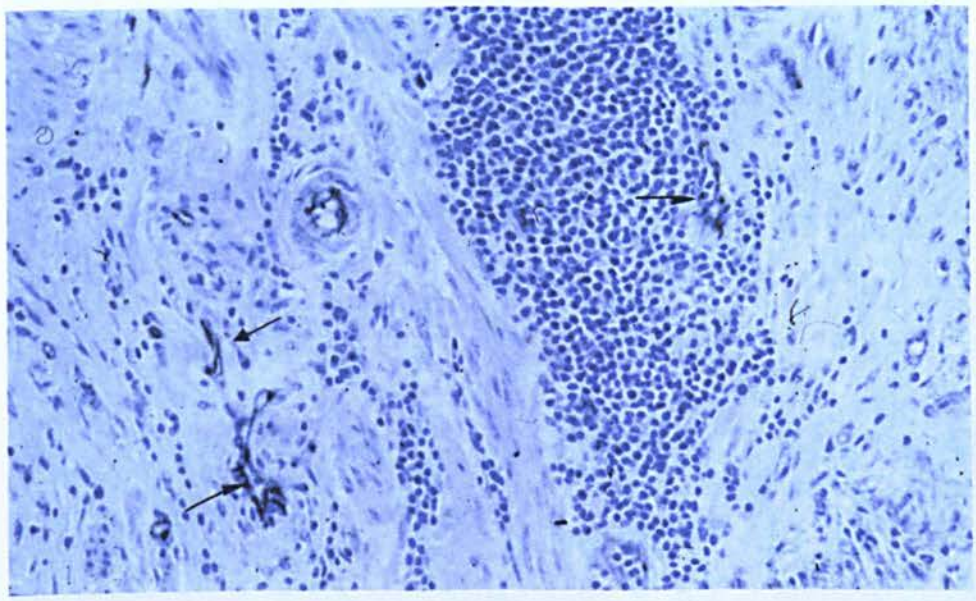


Fig 2.5 Magnification x700. Vessels present in colorectal cancer tissue, graded Dukes' C, have been highlighted by UEA-1 (arrows). Staining is very weak however, no background staining is observed. Contrast between the hematoxylin (BDH, Poole, U.K.) and the 3, 3'-diaminobenzidine (Sigma Fast ™ Dorset, U.K.) stain, used to highlight the avidin-biotin complex (see methods) is minimal therefore making the identification of vessels very difficult.

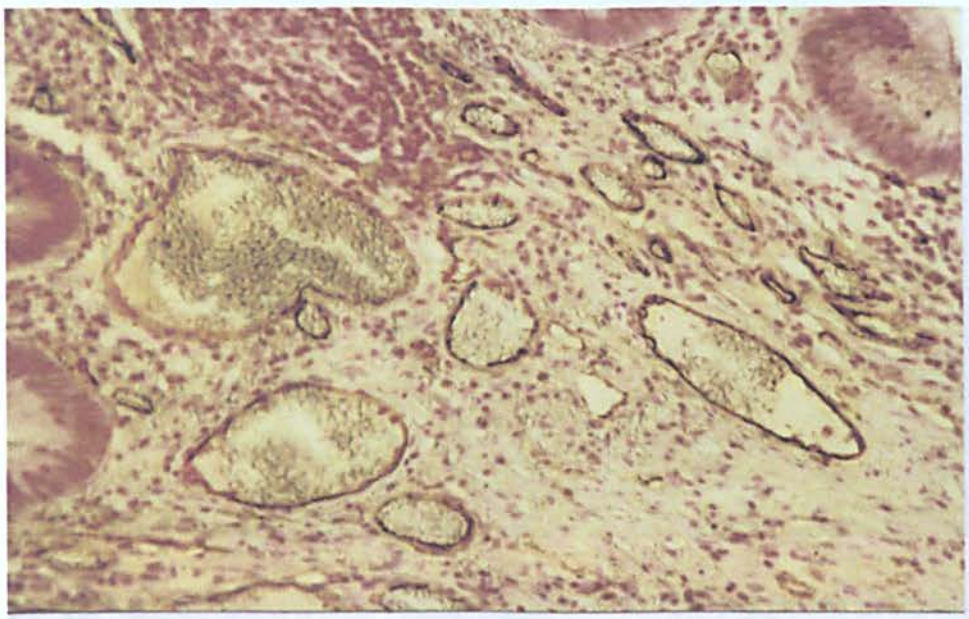


Fig 2.6 Magnification x1120. Positive control for ICAM-1 using normal human colon.

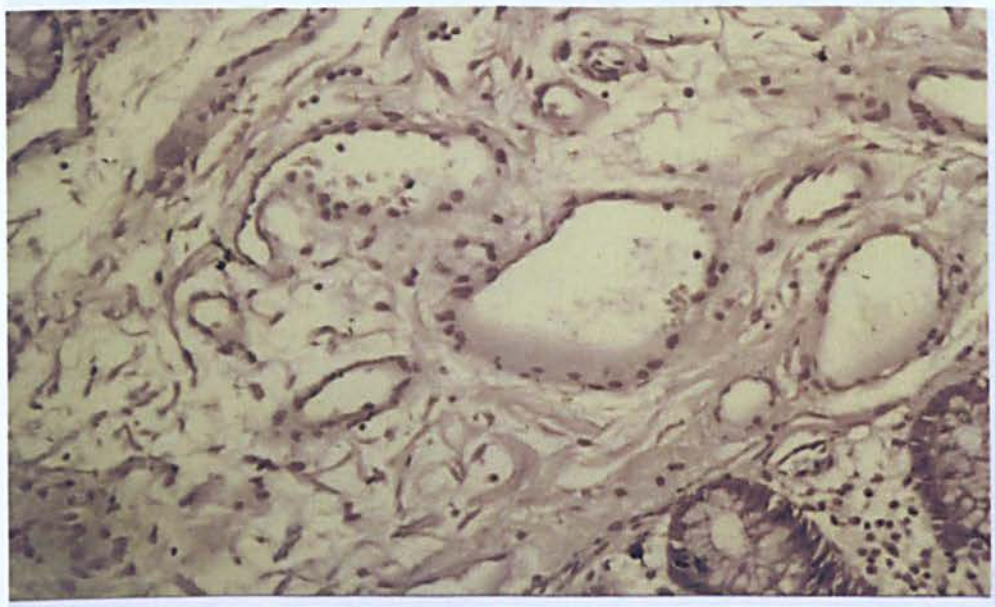


Fig 2.7 Magnification x1120. Negative control for ICAM-1 using normal human colon tissue.

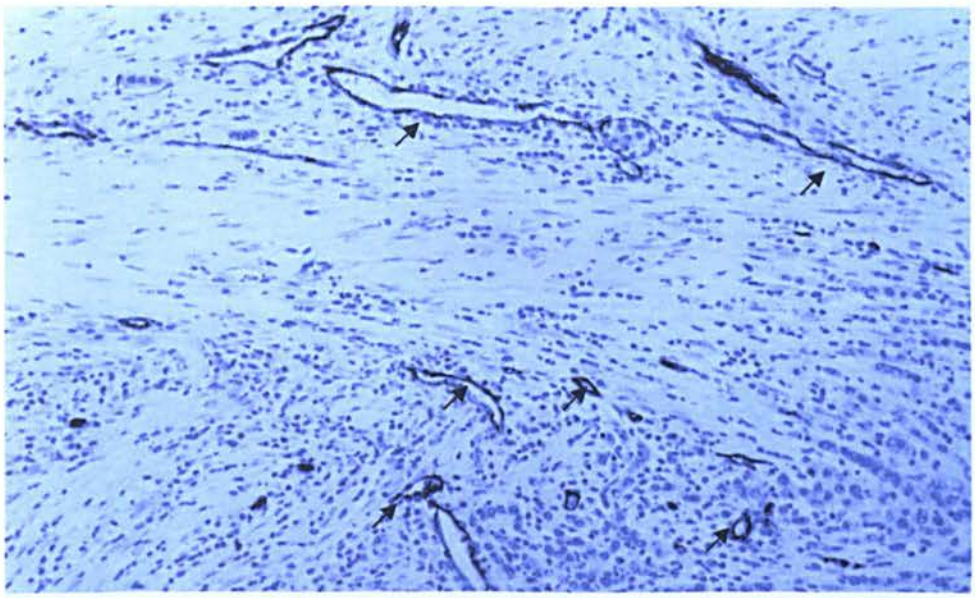


Fig 2.8 Magnification x 336.Vessels stained by ICAM-1 (arrows) in Dukes' C graded tumour. Staining is specific and relatively intense. However, results with the antibody have not been reproducible.

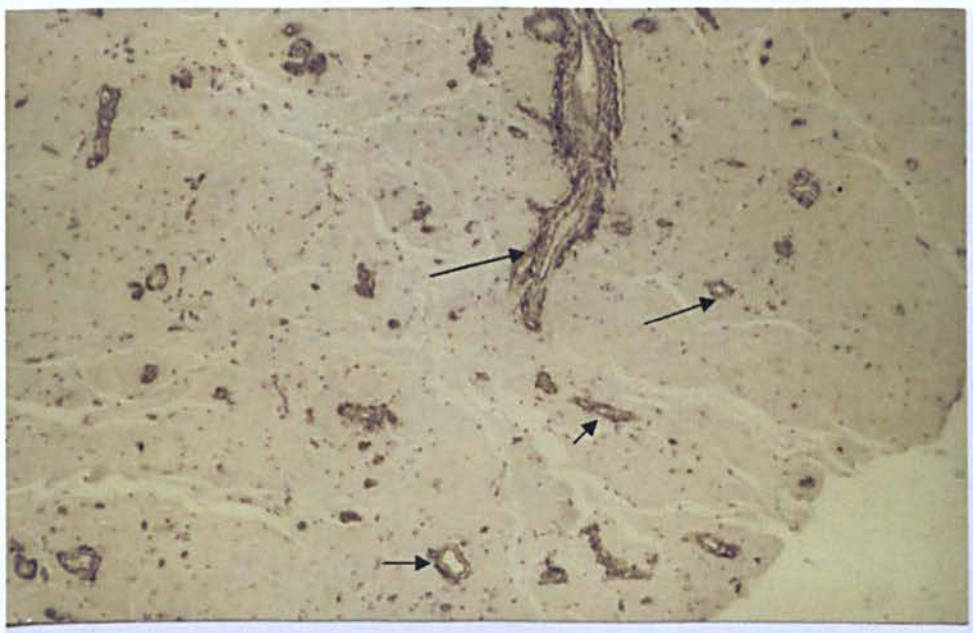


Fig 2.9 Magnification x336. Positive control for CD31 in normal human colon tissue. Note staining is specific and majority of the vessels have been highlighted (arrows).

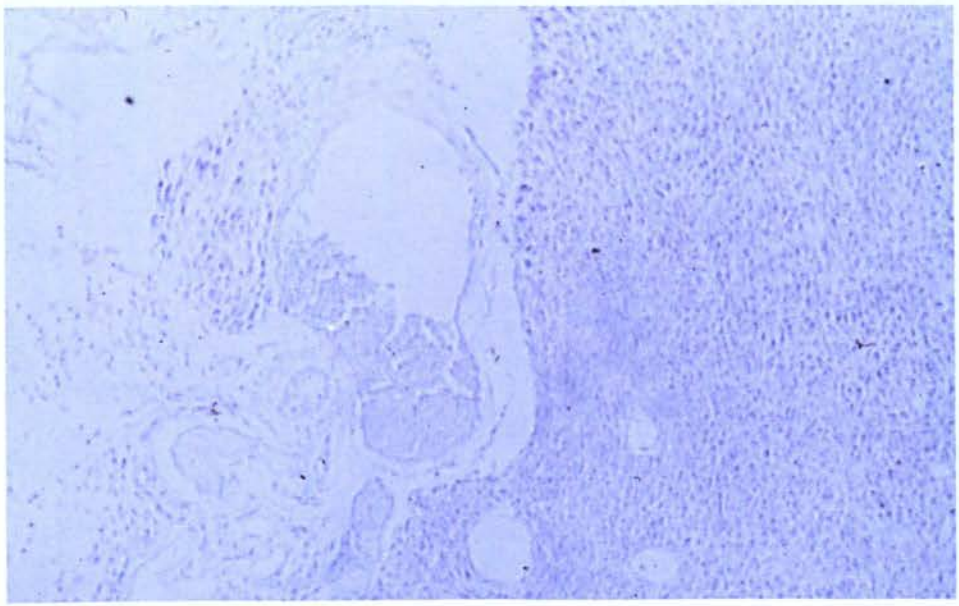


Fig 2.10 Magnification x280. Negative control for CD31 using normal human colon tissue. Note: no staining is observed.

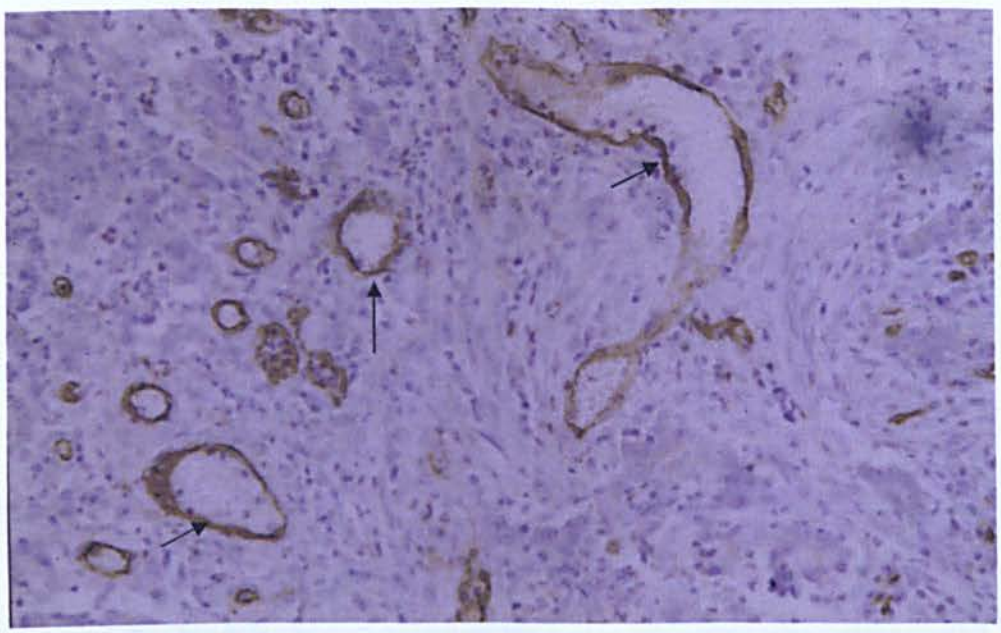


Fig 2.11 Magnification x 1120. Vascular endothelium in human colorectal tissue, graded Dukes' C, was strongly immuno-reactive to CD31 (arrows). The staining was specific and a maximum number of vessels were observed to be highlighted. CD31 proved to be an excellent marker to highlight tumour blood vessels.

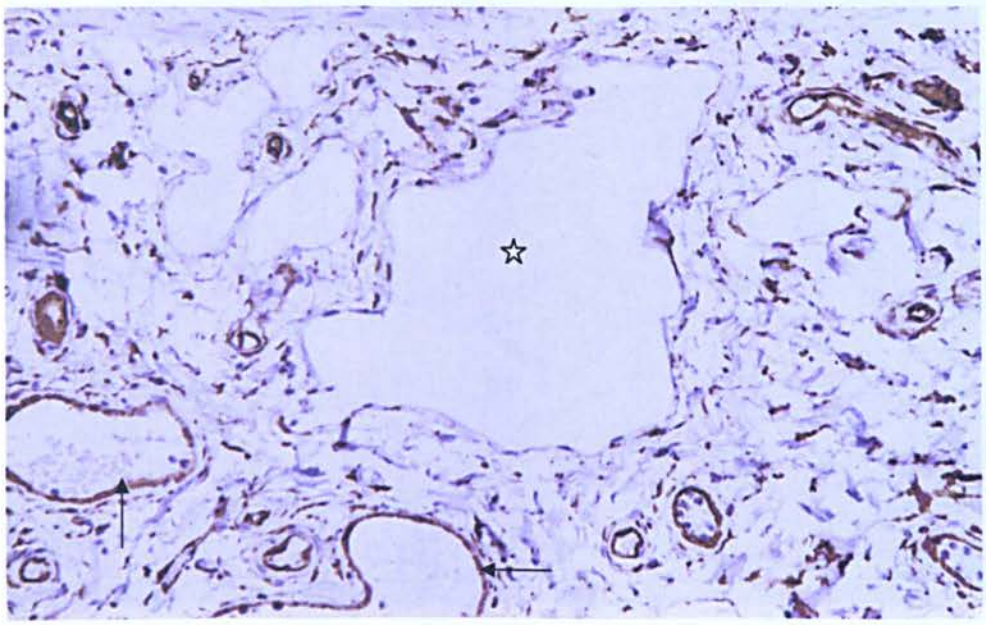


Fig 2.12 Magnification x1120. Positive control for CD34 using normal human colon tissue. Note: blood vessels have been highlighted for CD34 however, lymph vessels have been left unstained (star).

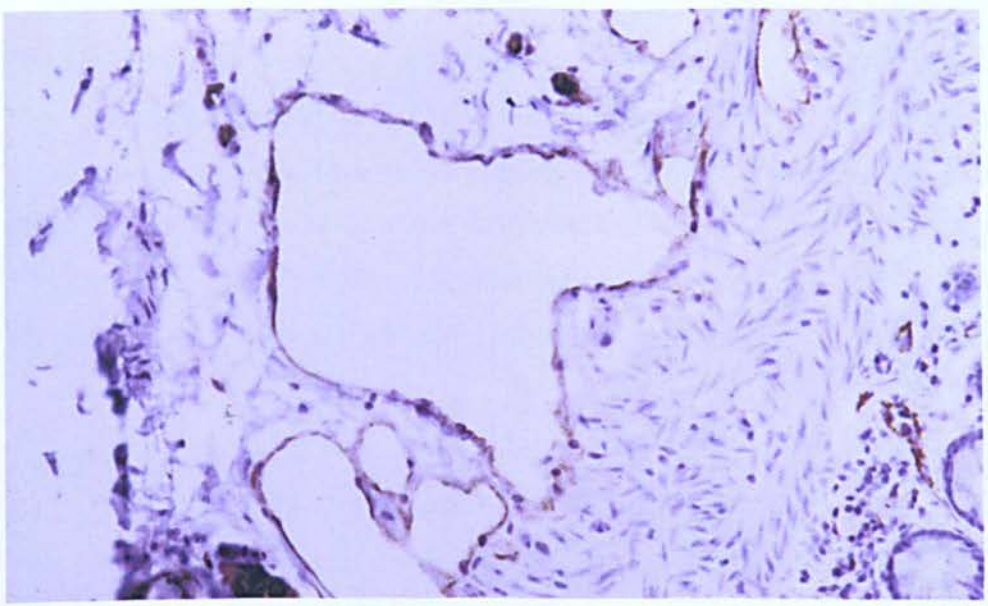


Fig 2.13 Magnification x1120. Negative control for CD34 using normal human colon tissue. No staining is observed.

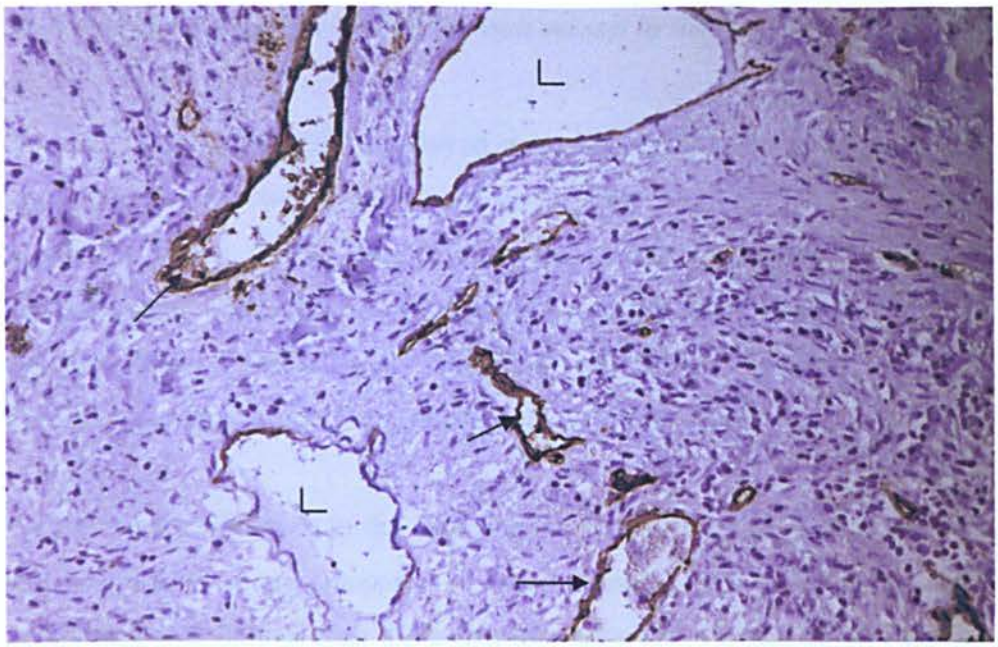


Fig 2.14 Magnification x 560. CD34 staining in Dukes' C graded tumour demonstrates to be highly specific to the vascular endothelium in some vessels (arrows). It was clearly noted to be immuno-negative in other tumour vessels (L).

Differential staining of blood and lymph vessels in the normal colon and tumour tissue

Anti-CD31 and anti-CD34 displayed a marked difference in the types of vessels that they highlighted in normal human colon tissue (figs 2.15 and 2.16). CD31 stained all vessels present, however, CD34 clearly left some vessels unstained. The unstained vessels were examined and morphologically assessed to certify their identity. With light microscopical analysis these vessels were identified as lymph vessels using the criteria which morphologically distinguishes lymphatic from blood vessels (see page 56).

Lymph vessels observed in the submucosal areas of the normal colon were larger in diameter and irregular in shape compared to blood vessels. Under closer examination the lymphatic vessel wall was thinner than the arterioles, veins and venules as they lacked a smooth muscle layer and in many cases were noted to be in a collapsed or partially collapsed state. These vessels were generally positioned adjacent to blood vessel and displayed minimal lumen content consisting mainly of plasma proteins (fig. 2.14). Based on these morphological features these vessels were considered to be lymphatic in origin.
Distinct immunolocalisation of CD31 and CD34 was also observed in blood and lymph capillaries.

The differential staining was also noted in tumour tissue (figs 2.17, 2.18, 2.19 and 2.20). However, morphological examination could not be used to identify the vessel types as tumour vessels are heterogeneous with no definite characteristics (Munn 2003). On the basis of the staining reactivity in normal colon with anti-CD31 and anti-CD34 it was suggested that the differential staining obtained in tumour tissue also distinguished blood from lymph vessels. No reaction to vessel endothelium was observed in the negative control experiments.

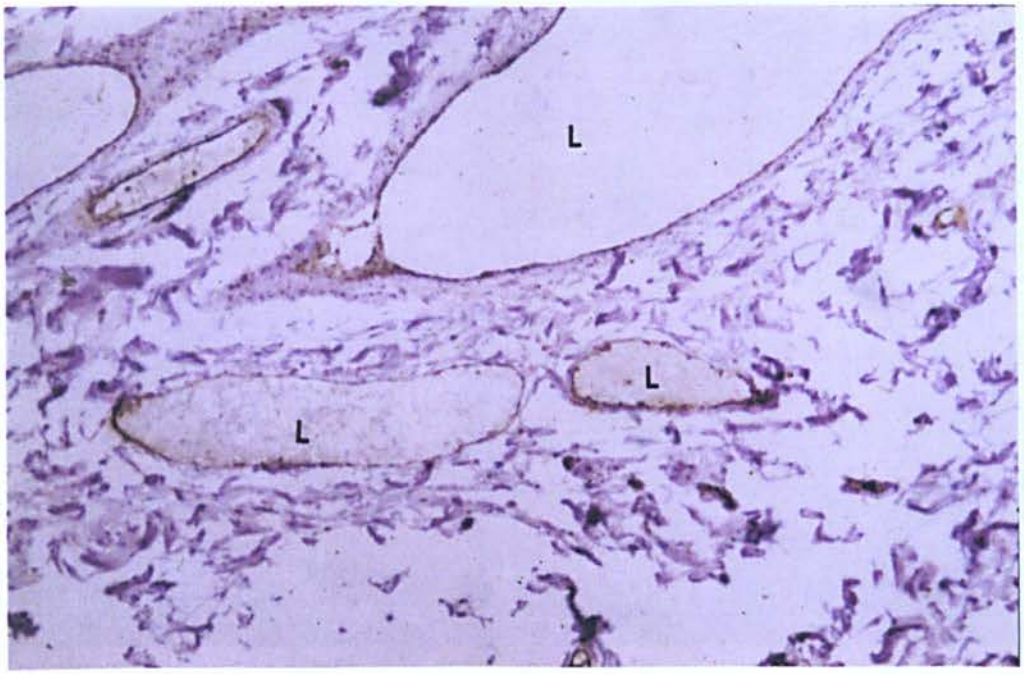


Fig 2.15 (Magnification x 1120). Staining with CD31 shows lymph vessels (L) highlighted by the antibody in the submucosal area of normal human colon tissue.

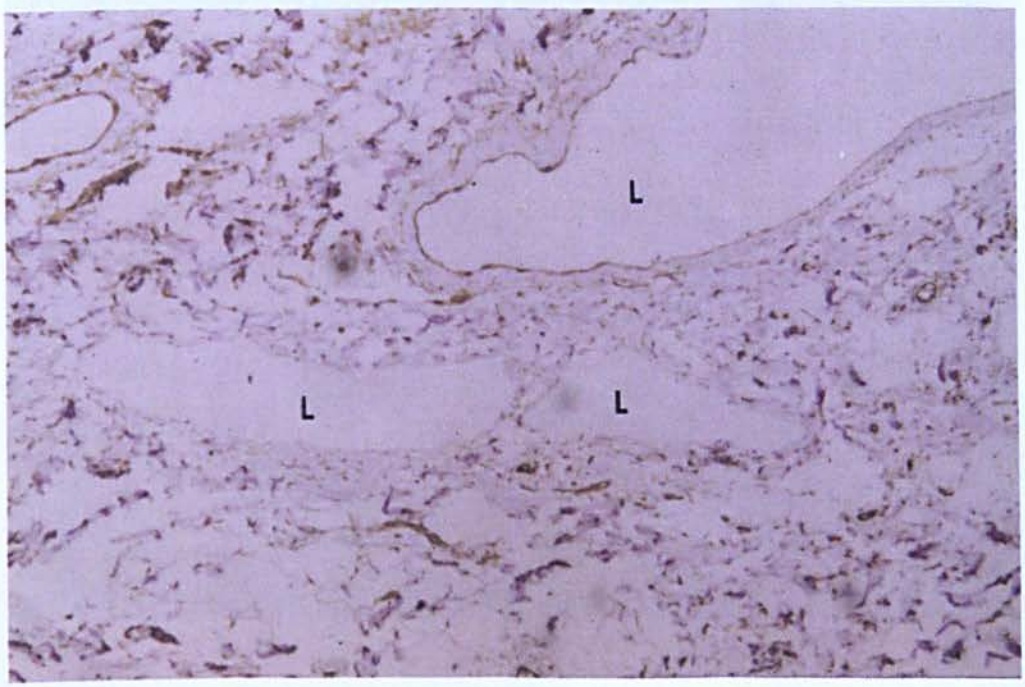


Fig 2.16 (Magnification x 1120). Staining with CD34 is shown to be very weak or non-existent in the lymph vessels (L) in the submucosal area of normal human colon tissue (this is the adjacent tissue section to the above section).

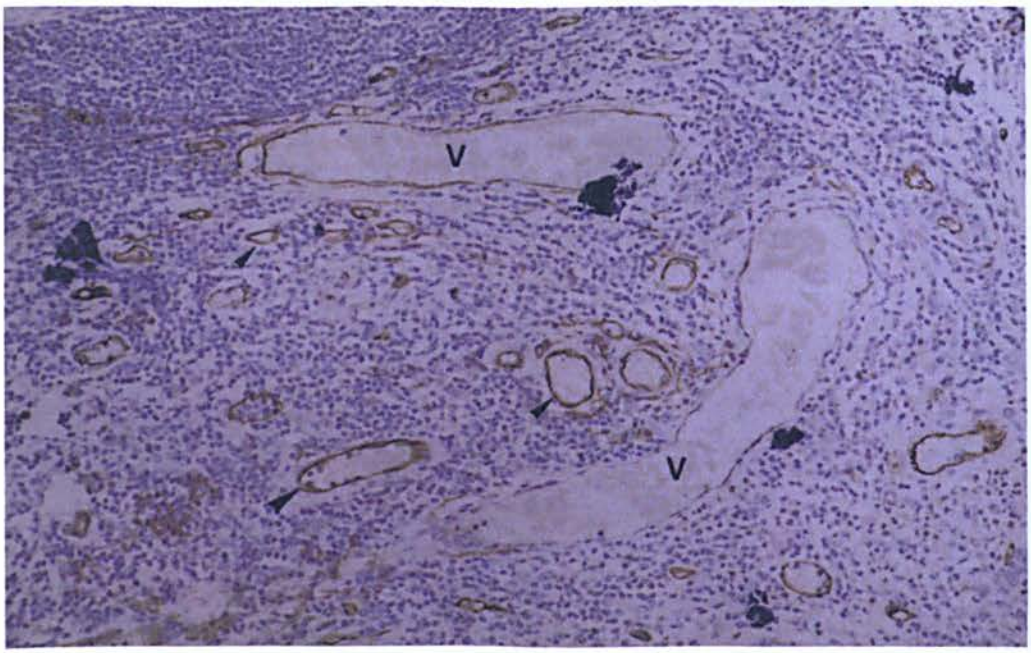


Fig 2.17 (Magnification x 700). Shows blood vessels in human colorectal tumours (arrow heads) highlighted by CD34 however, the lymph vessels (V) have been clearly left unstained.

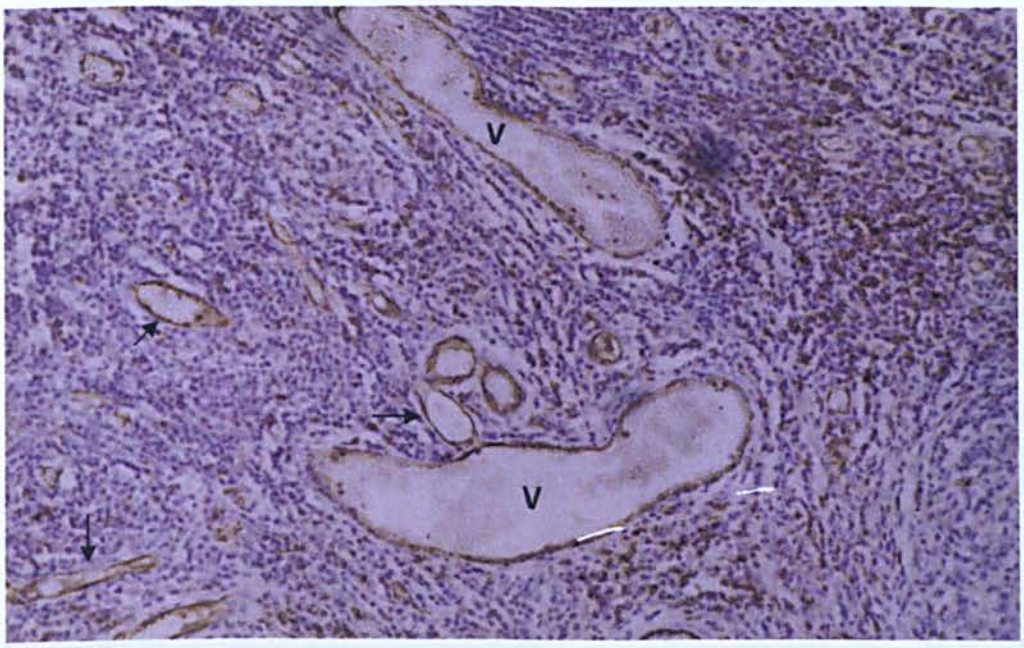


Fig 2.18 (Magnification x 700). Both blood vessels (arrows) and lymph vessels (V) have been high lighted by CD31 in colorectal tumour section.

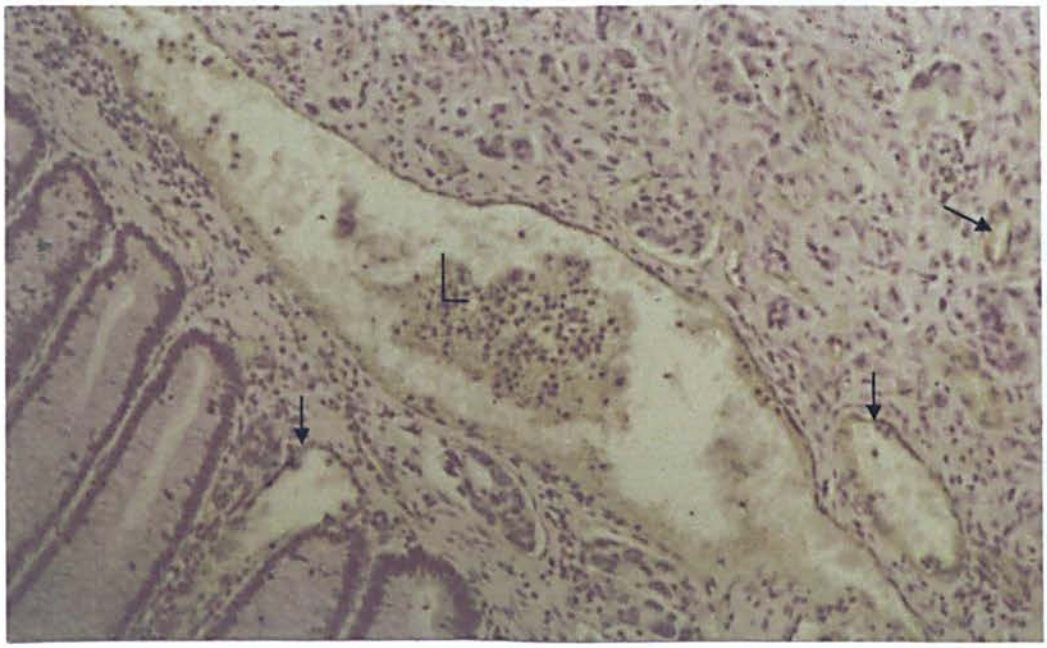


Fig 2.19 (Magnification x 1120). Section displays a lymph vessel (L) which is large and irregular in shape consisting of plasma protein in its lumen. It is clearly highlighted by CD31 with surrounding blood capillaries (arrows) also positive to the antibody.

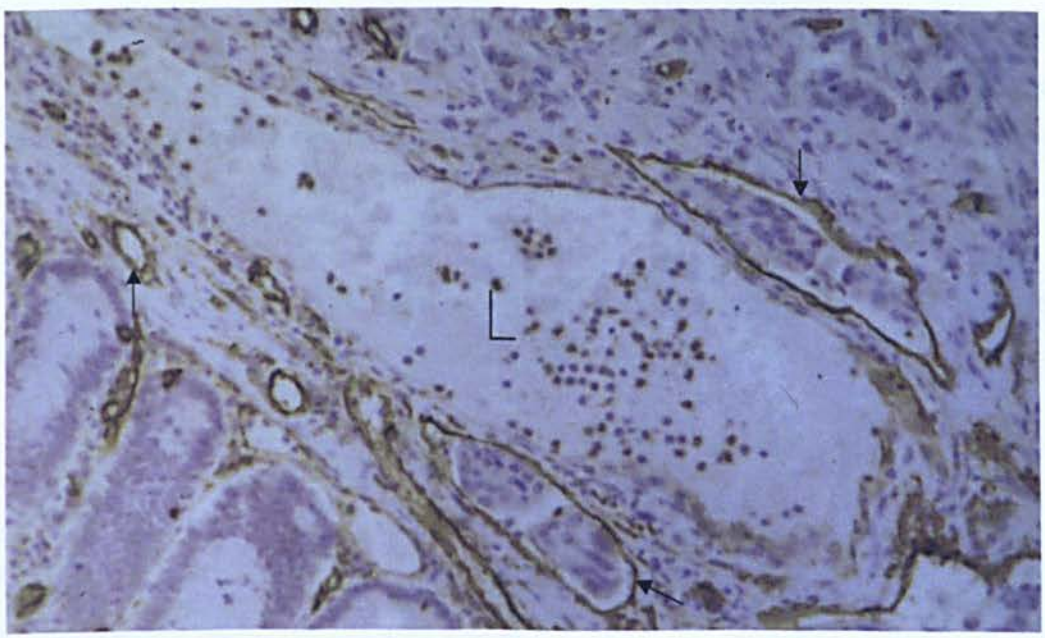


Fig 2.20 (Magnification x 1140). Lymphatic endothelium (L) is clearly immunonegative to CD34 in tumour tissue, however, it is strongly specific to the surrounding blood capillaries (arrows).

Staining with anti-VEGFR-3 and anti-laminin

To further investigate the staining specificities of anti-CD31 and anti-CD34 within tumour tissue, a lymphatic endothelial specific marker was used to prove whether the weakly stained vessels by anti-CD34 were actually lymph vessels.

In all of the sections examined, variable staining patterns were observed. The reaction of VEGFR-3 to lymph vessel endothelium was intense however, blood vessel were also stained. Staining with VEGFR-3 was inconsistent and background staining hindered the visualisation of vessels (fig. 2.23). The positive control tissue used was the human kidney, both blood and lymph vessels were shown to be immunopositive (fig.2.21). No staining in the negative controls for anti-VEGFR-3 was observed (fig. 2.22).

In sections stained by anti-laminin, very few showed endothelial staining and these results were not always reproducible. Endothelial cell staining was weak and many vessels failed to react with anti-laminin in most of the sections (fig. 2.26). Anti-laminin in positive control tissue (breast carcinoma) did not highlight lymph vessels however, stained positively for laminin around breast carcinoma cells (fig. 2.24). The negative control showed no staining (fig 2.25).

The lack of specificity of VEGFR-3 and anti-laminin may have been caused by the inability of the antibodies to detect lymph vessel endothelium in paraffin wax sections.

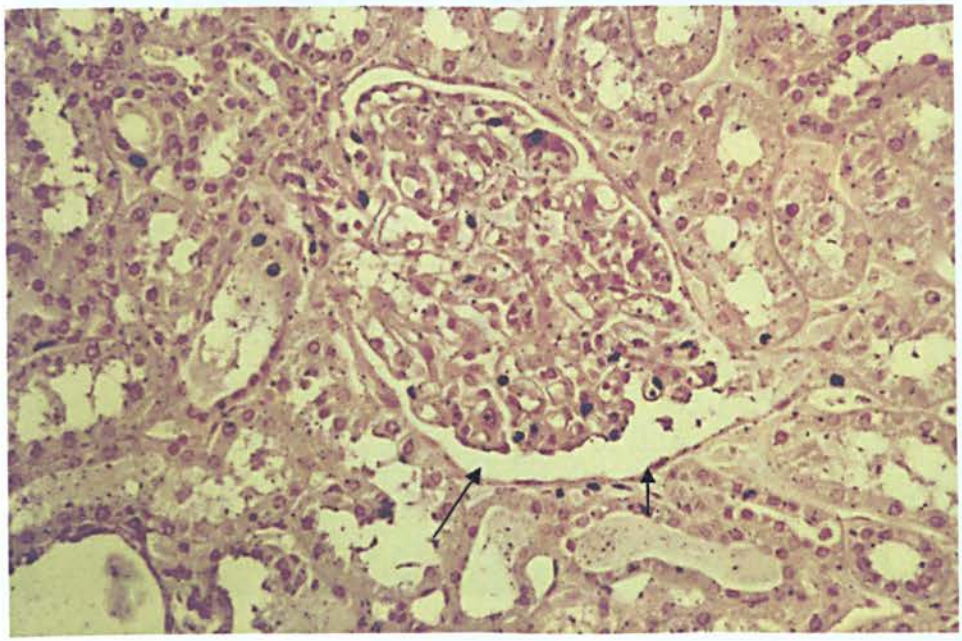


Fig 2.21 Magnification x1120. Positive control for VEGFR-3 using normal human kidney tissue. Note staining of basement membrane of Bowen's capsule (arrow).

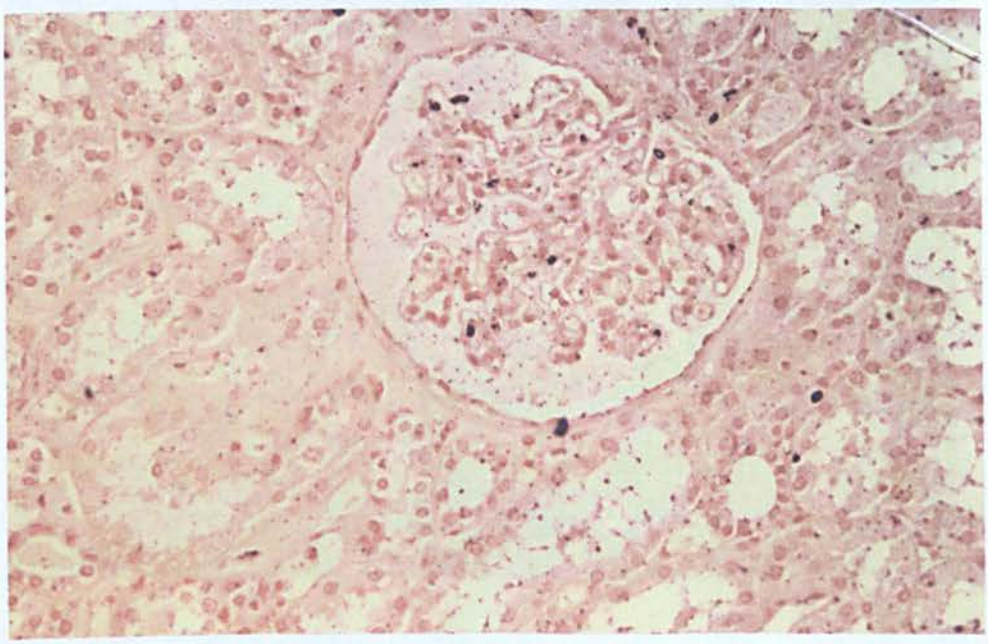


Fig 2.22 Magnification x1120. Negative control for VEGFR-3 using normal human kidney tissue. No staining observed.

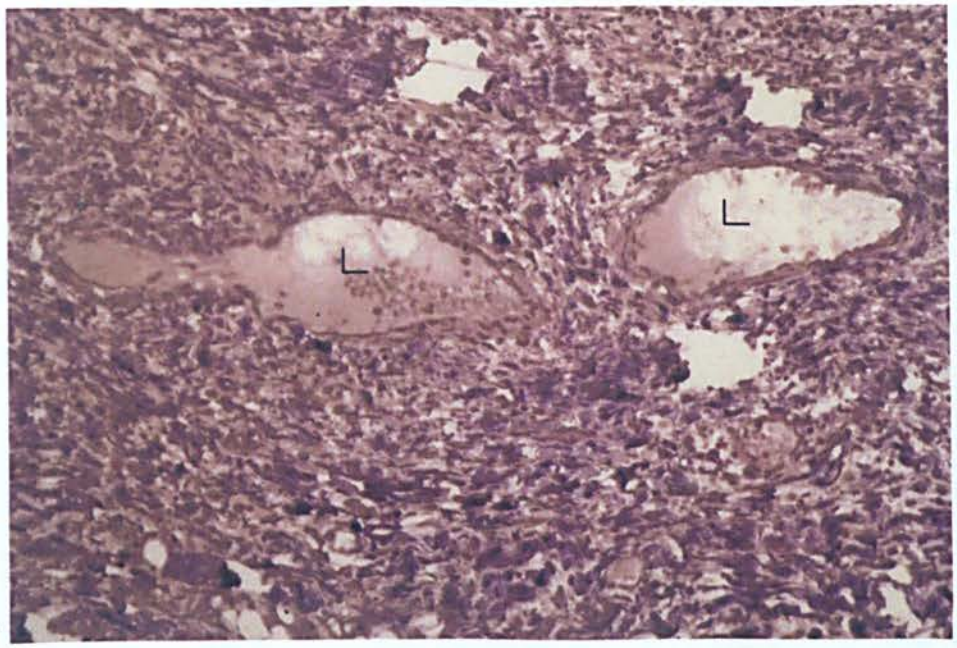


Fig 2.23 Magnification x 1120. Shows lymph vessels (L) highlighted by VEGFR-3 however, staining was non-specific and blood vessels as well as stromal tissue were also detected by the antibody.

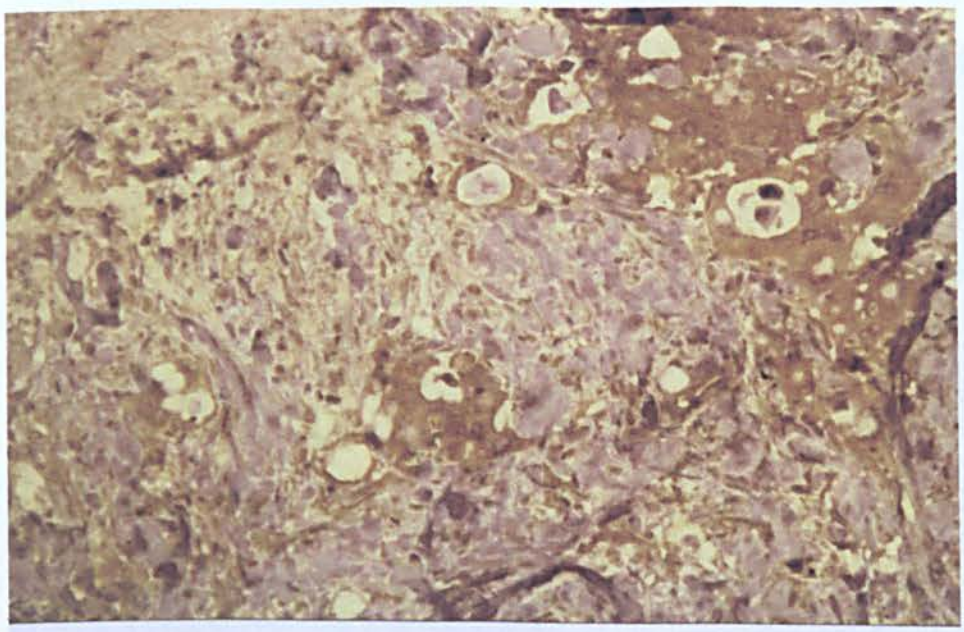


Fig 2.24 Magnification x1160. Positive control for laminin using human breast carcinoma tissue.

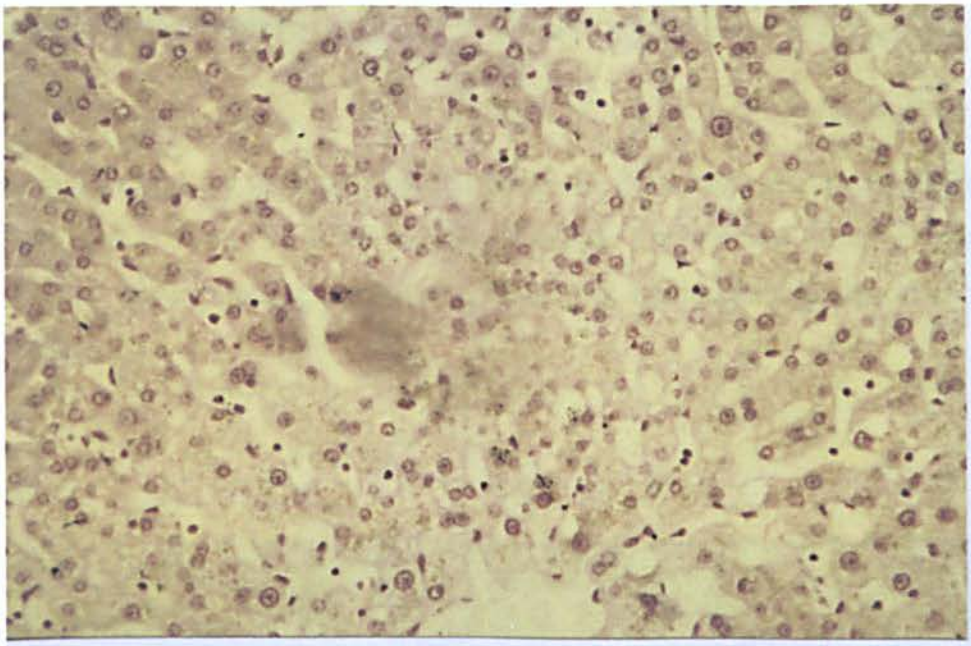


Fig 2.25 magnification x1160. Negative control for laminin using human breast carcinoma. No staining observed.

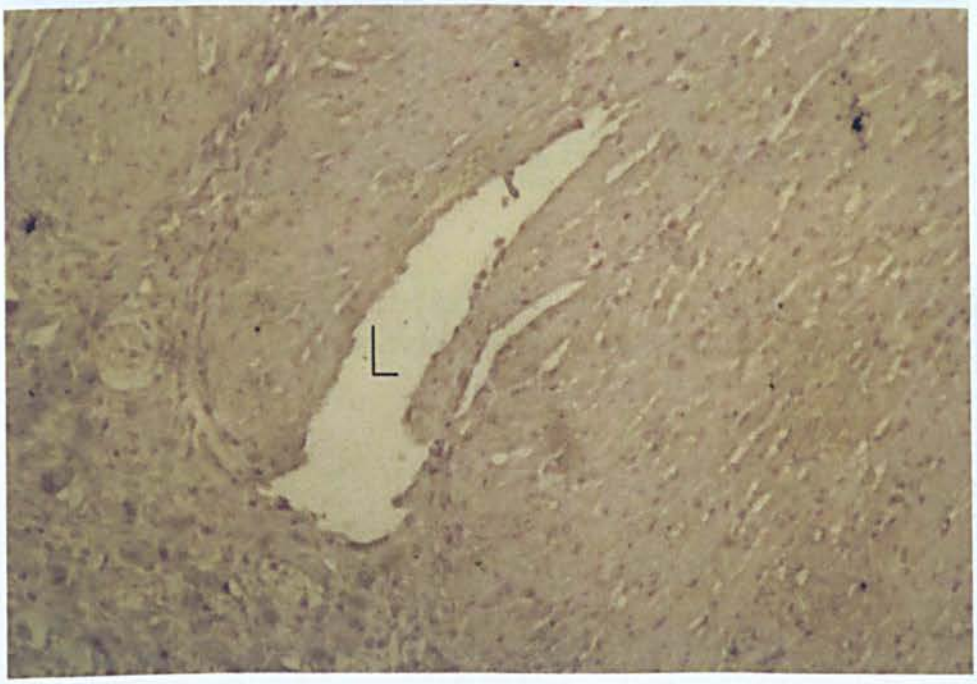


Fig 2.26 (Magnification x 1120). Depicts very weak staining in the lymphatic vessel (L) with anti-laminin. Background staining hindered the visualisation of all vessels and staining was non-specific.

Enzyme reactivity in human colorectal tumour and normal vessel endothelium to ALPase (fig. 2.27) and 5'Nase (fig 2.28) was not detected. The method produced immense background staining of surrounding connective tissue which marred the identification of blood and lymph vessels.

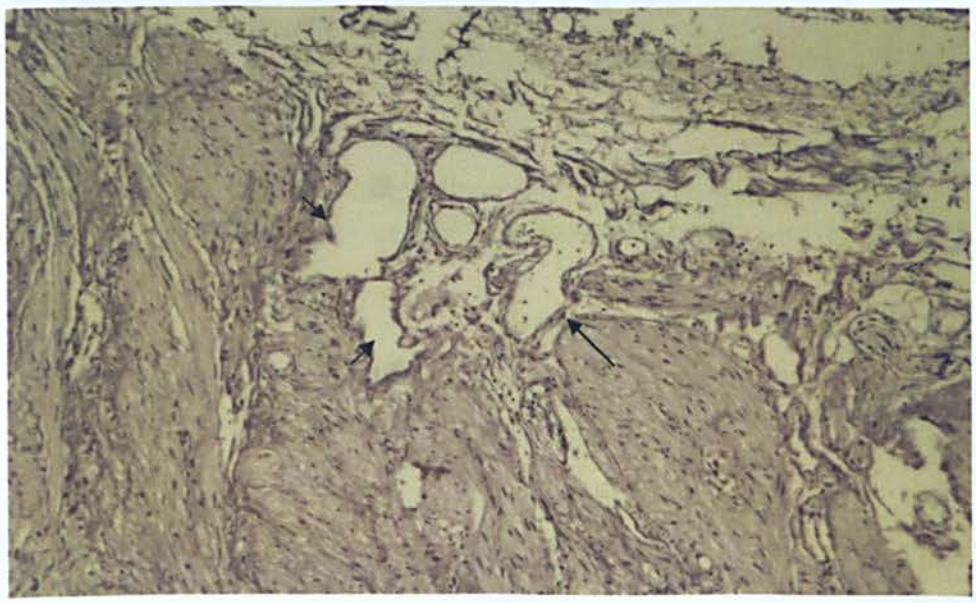


Fig 2.27 (Magnification x 280). Detection of alkaline phosphatase using enzyme-histochemistry in blood vessel endothelium (arrows) of colorectal tumours was not possible as no reaction was seen.

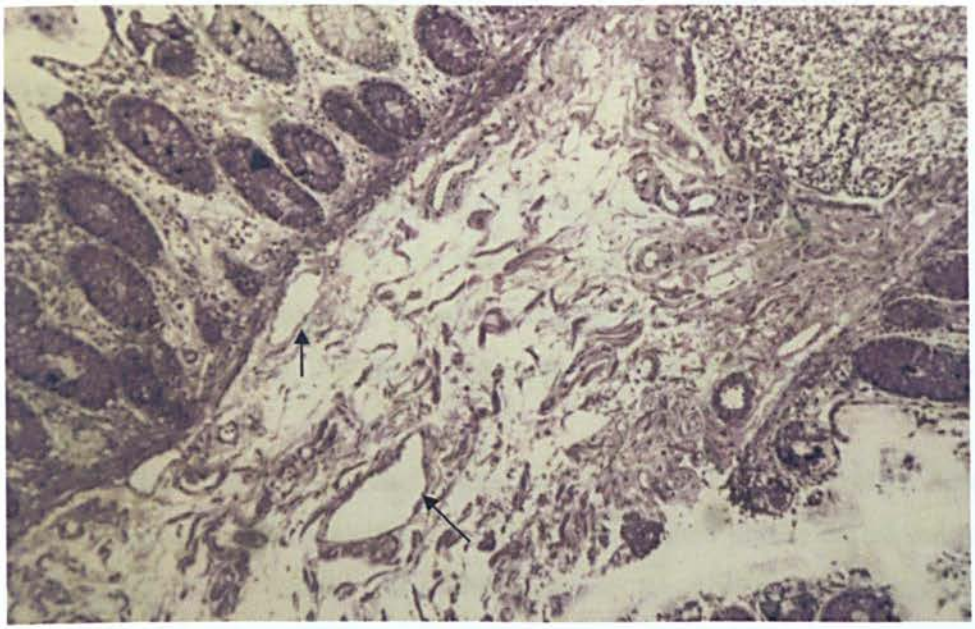


Fig 2.28 (Magnification x 280). Detection of 5' nucleotidase using enzyme histochemistry in lymph vessel endothelium (arrows) of colorectal tumours was not possible as no reaction was seen.

Discussion

Tumour growth and the formation of metastasis is a multistep process involving complex interactions between tumour cells and the vascular endothelium (Alexiou *et al*, 2001). These interactions are mediated by several cell-surface adhesion receptors including the selectins, integrins, and immunoglobulin-like adhesion molecules (Pinatelli and Vessey 1994).

The metastatic cascade includes angiogenesis as one of its many steps. Angiogenesis has now been indicated, in many studies, to aid tumour growth and is correlated to patient prognosis (Nakayama *et al*, 2001). Since Weidner *et al*, (1991) demonstrated that intratumoral microvessel density (iMVD), assessed by immunohistochemical staining for endothelial cells, was an independent prognostic factor in breast carcinoma, iMVD has now been reported to be a possible prognostic indicator in many cancers including colorectal carcinomas (Tanigawa *et al*, 1997).

Although angiogenesis can not be measured directly, it can be inferred by quantification of tumour vasculature. Studies have investigated the expression of various acknowledged markers of angiogenesis by comparing normal tissue with tumour tissue (El-Gazzar *et al*, 2005).

The most widely used method for the detection of vascular endothelium is immunohistochemistry. Here the vascular endothelial cell antigens are targeted by monoclonal antibodies which allow the structures under investigation to be highlighted with a certain degree of specificity (Viera *et al*, 2005). Conflicting opinions exist as to which immunohistochemical marker offers the best results in highlighting tumour vessels (Freitas *et al*, 2005). Therefore, this study investigated a panel of antibodies: vWF, CD31, CD34, ICAM-1 and UAE-1, which are commonly used in studies assessing tumour microvessel density, in order to find the optimal endothelial marker that would detect a larger number of vessels in colorectal neoplasms.

The results of this study demonstrated that CD31 highlighted a greater number of vessels with consistency and endothelial cell specificity. CD34 also demonstrated superior results, however, the antibody was not able to detect all of the vessels within the colorectal tumours. This pattern of staining was also observed in the positive control tissue (normal human colon). These results coincide with Tanaka *et al,* (2003) and Jacquemier *et al,* (1998) who stated that from their results their marker of choice was CD31.

The study by Freitas *et al,* (2005) also demonstrated effective staining with CD31. Oral hemangiomas and pyogenic granulomas expressed focal imunohistolocalisation with CD31, but unlike the results from this study, endothelial labelling with vWF was more intense and uniform. This may be due to a number of reasons, firstly, different tissue types present with differential antigen profiles therefore, human colorectal tumour endothelial cells may display greater levels of CD31 than vWF. Freitas *et al,* (2005) used a monoclonal vWF antibody and incubated the tissue with the antibody overnight whereas this study used a polyclonal, only due to availability, and incubated the tissue for one hour. This may also contribute to the disparity in the results.

Studies have shown that in *in vitro* as well as *in vivo* the expression of PECAM-1/CD31 is strong and homegenous in normal human endothelial cells (HUVEC) (Müller *et al,* 2002). It is generally accepted that CD31 is uniformly expressed on all endothelial cells. However, this is questioned due to fact that endothelial cells in different vascular regions, even in the same organ, as well as tumour tissue express variable amounts of CD31 (Hristov *et al,* 2003). Nevertheless, endothelial cells isolated from fetal and adult aorta which are typically used to confirm endothelial origin display a high percentage CD31 antigens which suggests that it is an efficient endothelial cell marker (Invernici *et al,* 2005).

CD31 receptor is involved in the initial formation and stabilisation of cell-cell contacts at the lateral junctions of endothelial cells. It also plays a pivotal role in the modulation of cell migration, transendothelial migration of monocytes and neutrophils as well as formation of new blood vessels in angiogenesis (Jackson 2003). Therefore, the presence of CD31 in blood vessel endothelium of both normal colon and colorectal tumours suggests that not only does the receptor serves its function in normal tissue but also maintains such processes in cancer.

Investigators have proposed that CD31 should be the standard marker used for microvessel assessment in paraffin embedded tissue, however, optimal staining results are not always achieved (Vermeulen *et al,* 1996; El-Gazzar *et al,* 2005).

CD34 is known to primarily be expressed by mature microvessels but can also be present on newly formed ones. Its expression is elevated in neovasculature during wound healing and tumour growth (Müller *et al,* 2002). This suggests that its participation in blood vessel formation in tumours may be due to circulating endothelial progenitor cells which comprise of a subset of $CD34^+$ cells that give rise to endothelial cells. These may develop into tumour blood vessels whose endothelial cells exhibit CD34 phenotype (Nakul-Aquaronne *et al,* 2003).

CD34 has also received good reviews on its ability to highlight blood vessels in both normal and tumour tissue. However, it has also been categorised as a less specific marker to evaluate angiogenesis (Hristov *et al,* 2003). Viera *et al,* (2005) demonstrated that CD34 also marks the muscle layer of the aorta, it highlights fibroblasts, stroma of the uterine cervix, adipose tissue as well as the endothelium of lymphatic vessels. By contrast, Russell Jones *et al,* (1995) reported lymph vessels to be negative for CD34.

This study demonstrated that CD34 was highly specific to blood vessel endothelium of normal and neoplastic tissue of the colon however, certain vessels were clearly left unstained by the antibody. After examination of the unstained vessels in normal colon tissue, they were established to be lymphatic in origin as they fit the criteria set (page 56). Differential staining was also noted in tumour tissue therefore, suggesting that colorectal tumours possess lymph vessels and that these tumours not only undergo tumour angiogenesis but also lymphangiogenesis.

These findings led to further investigations of the CD34 immunonegative vessels. Known markers, specific to lymph vessels, such as VEGFR-3 and anti-laminin were used to stain lymphatics in normal and neoplastic colorectal tissue. Specific results could not be obtained by these antibodies which may have been due to the fact that receptors for these markers may have been denatured during the process of fixing. Negative results were also obtained for enzyme-histochemical differential staining also suggesting that the processes performed to

obtain paraffin wax sections for histology may cause damage to certain endothelial cell specific proteins; thereby hindering sensitive detection methods such as immunohistochemistry and enzyme-histochemistry. The technical difficulty can possibly be resolved by repeating the study using frozen cryostat sections of colorectal tissue instead. This method is known to preserve the antigen profile of tissue and therefore, is known to exhibit greater immunolocalised staining than paraffin embedded tissue.

The present study demonstrated strong detection of endothelial cells by vWF, however, results were inconsistent and not reproducible with major background staining. vWF is a polyclonal antibody, this may attribute to the non-specific binding to connective tissue of normal and tumour tissue of the colon. Polyclonal antibodies have multiple binding sites therefore, are prone to exhibit non-specific staining whereas monoclonal antibodies have greater specificity (Naish *et al*, 1989). Nevertheless, Toi *et al*, (1995); de Waal *et al*, (1997) and Soin-Vardy *et al*, (2001) recommended vWF as their marker of choice over CD31 and demonstrated uniform and easily interpretable results.

Staining with ICAM-1 produced inconsistent results with staining relatively weaker than vWF, CD31 and CD34. Vessels in distinct areas of colorectal tumours, namely the invasive edges, were highlighted by the marker. Vessels in central regions of the tumour were noted to be negative. Vessels surrounding invasive edge of the tumour were also noted to be weakly positive.

ICAM-1 expression in the tumour microvasculature allows cytotoxic lymphocyte trafficking towards the tumour and facilitates transmigration of monocytes and T lymphocytes across the endothelium enhancing tumour angiogenesis (Alexiou *et al*, 2001). Circulating ICAM-1, on the other hand, interferes with natural killer and lymphocyte-activated killer cell reactivity to tumour cells. So the shedding of adhesion molecules by endothelial or tumour cells can block counter ligands or immuno-competent recognition of lymphocytes, there by promoting metastasis. Hence, studies report increased concentration of ICAM-1 in advanced stage cancers in the colon, melanoma and breast (Maurer *et al*, 1998; Kawano *et al*, 2005; Tachimori *et al*, 2005).

As the endothelial cell adhesion molecule is stipulated as an antigen that favours metastasis it is not surprising to find more intense vessel staining in the invasive edge and surrounding regions highlighted by the marker.

Hamid *et al,* (2003) demonstrated that UEA-1 labelling is useful in identifying endothelial cells in culture. However, this study produced very weak to negative staining in sections of colorectal cancer and normal colon tissue. UEA-1 has been shown to be well suited for staining of endothelial cells in formalin fixed, paraffin embedded tissue sections, although sections from this study did not exhibit reliable staining therefore, UEA-1 was not classified as a reliable marker.

Vessels can be identified in formalin fixed tissue by the use of various antibodies to antigens expressed by vascular endothelial cells, following appropriate antigen retrieval. However, slight variations in protocols such as the influence of tissue processing, the antibodies used and for cancer prognostic studies the method of quantification, may invalidate comparisons between studies (El-Gazzar *et al,* 2005). Schor *et al,* (1998) found that statistically significant differences in vessel counts were most likely to arise from failure to optimise the staining protocol and methods to assess tumour vessels. Therefore, it was imperative that the endothelial markers were assessed for specificity to colorectal tumour endothelium before tumour vessels were quantified to establish their role in colorectal cancer progression.

This study demonstrated that the endothelial cell markers anti-vWF, anti- CD31 and anti-CD34 were reliable markers for highlighting tumour blood vessels and therefore, as a result these markers were used in the following studies (chapter 3).

Chapter 3

Qualitative and quantitative analysis of colorectal tumour vasculature: association of tumour vessel counts with prognosis

3.1 Introduction

The prognosis of colon cancer is influenced by a variety of clinical and morphological features present at the time of initial diagnosis. Such factors include: age, gender, duration of symptoms, and tumour location. In addition, vascular and lymphatic invasion, ploidy, degree of differentiation and preoperative levels of plasma carcinoembryonic antigen have been shown to be of prognostic significance. However, the simplest and the most widely applied prognostic feature is the presence of lymph node metastasis in a surgically resected tumour. This forms the basis of all staging systems and has shown to have more prognostic value than any other feature (Cotton 1992; Beets-Tan *et al*, 2005).

Traditionally, the clinical outcome of colorectal cancer patients may be predicted by pathological staging. Staging the disease helps to define the clinicopathological features and provides information on the prognostic out come of a patient (Lee *et al*, 2000). The traditional method of staging colorectal cancer, that is still in current use today, especially in the diagnosis of colon cancer is called Dukes' stage. However, by world wide agreement the Tumour, Lymph nodes, Metastasis staging system (TNM) has now been set as the standard prognostic system which is proving to be the system of choice (Nagtegaal and van Krieken 2002). It is important to emphasize that clinical assessment of patients with the disease, especially ones with significant risk of recurrence, are predominantly the ones who would benefit from adjuvant therapy (Offersen *et al*, 2003).

It has been noted that patients with lower grade tumours may redevelop the cancer. Dukes' stage A (approximately 10% of all colorectal cancer patients) and Dukes' stage B patients (30-40%) will develop local recurrence or distant metastasis years after receiving surgical treatment (Lee *et al*, 2000). Therefore, it is important to find other parameters which are m

suitable and possibly more accurate in predicting patient outcome to identify high-risk patients in order to customize relevant therapy (Bamias and Dimopoulos, 2003).

Assessment of tumour vasculature has shown to be a promising reliable prognostic indicator in a variety of malignancies including malignant melanoma, breast carcinoma, ovarian and prostatic carcinoma (Takei *et al*, 2000; Nakayama *et al*, 2001; Fox *et al*, 2001). The distribution of blood vessels and increased vessel counts in solid tumours has been shown to be related to tumour growth and metastasis in these cancers thereby indicating that tumour vasculature may be a relevant target to inhibit tumour progression (Rüegg *et al*, 2006).
However, the number of studies correlating angiogenesis to prognosis in colorectal cancer is relatively lacking, and to a certain extent controversial, in comparison to the extensive research conducted for the cancers of the breast, lung, prostate and ovaries (Wild *et al*, 2000). Therefore, a greater knowledge of the role of angiogenesis in colorectal cancer should improve the current understanding of colorectal cancer biology and hence help create avenues for new therapeutic strategies.

3.1.1 Tumour heterogeneity.

A prognostic marker is defined as a variable that provides information regarding patient outcome which is complementary to the data obtained by histopathology and on which therapeutic decisions can be guided (Laking *et al*, 2006). To-date, the most important clinicopathological parameter for the assessment of colon cancer is the stage of the disease at diagnosis, with depth of tumour invasion and infiltration of lymph nodes or distant organs being used to define patient prognosis (Acikalin *et al*, 2005).

Angiogenesis is emerging as one of the more potentially useful prognostic indicators to assess the extent of cancer progression and a large number of experimental and clinical data demonstrate the relationship between tumour microvessel density and clinicopathological variables (Baluk *et al*, 2005). It is becoming increasingly more apparent that tumour growth is dependent on angiogenesis (tumour vessels facilitate the exchange of nutrients, oxygen and waste products to aid tumour progression) (Weidner 1995). Folkman (1971) first recognised that the assessment of tumour vasculature would yield important information and bear clinical

significance for the management of cancer patients and hence become a fundamental marker for cancer progression.

However, majority of solid tumours are heterogenous with diverse biological aggressiveness. The cell heterogeneity of the solid tumours is the result of genomic instability due to genetic alterations that may include: mutations, deletions, chromosomal rearrangements, activation of oncogenes, down regulation of tumour suppressor genes, and gene amplification. Tumour cell heterogeneity may confer different properties of growth, immunogenicity, ability to metastasise, and differential sensitivity to various treatment regimens (Ergun *et al*, 2006).

Tumour cell genomic instability and heterogeneity are the biological basis of the clinical observation that both the outcome of the patients and their responsiveness to anti-cancer therapy may be different among tumours classified as having the same pathological or clinical stage. Thus, for several solid tumours it is difficult, by using conventional clinicopathological criteria, to assess the prognosis or the likelihood of response to a specific form of anti-cancer treatment. This important clinical problem stimulated the search for several novel markers to obtain more precise and selective prognostic information (Bicknell *et al*, 1997).

Movement of molecules through tumour vasculature is governed by the blood flow rate and vascular morphology which includes: the number of vessels, their length, diameter, and geometric arrangement. However, tumours have spatial and temporal heterogeneities in blood supply coupled with variations in the vascular morphology therefore, the uptake and distribution of therapeutic agents in tumours is also heterogenous. Hence, it is important to analyse tumour pathophysiology as it affects the above mentioned steps which are involved in the delivery of various anti-cancer agents (Jain 2005).

Therapeutic agents that enter the tumour vasculature reach cancer cells via distribution through the vascular compartment, transport across the microvascular wall, and transport through the interstitial compartment. For a molecule of a given size, charge and configuration, each of these transport processes may involve diffusion and convection as well as the non-specific or specific binding of therapeutic molecules to proteins or other tissue components (Laking *et al*, 2005).

3.1.2 Spatial distribution and architecture of tumour vessels.

Methods are being developed to determine the *in vivo* distribution of probes targeted to the vasculature. The use of labeled probes, which can be visualised by fluorescence, transmission electron or light microscopy, is helping to define tumour vasculature (Baluk *et al*, 2005).

Such methods reveal that tumour vessels are heterogeneous and are tissues that display variable architecture; they may also develop changes during tumour progression (Jain 2005). Qualitative analysis on the vascular architecture of human tumours was initially carried out in the first half of the last century by Goldmann in 1907. However, this early work received little interest, until reports by Weidner *et al*, (1991) stated that vascular density may provide prognostic information in human tumours.

It is well documented that the tumour vasculature originates from the host vasculature and the mechanisms of angiogenesis are similar in both cases. However, the organisation of tumour vasculature may be completely different to other tumours depending upon its growth rate and location (Bicknell *et al*, 1997). Tumours are architecturally complex differing regionally in vasculature. Such an aspect of tumour vasculature makes the uniform delivery of conventional chemotherapeutics difficult. Improving the situation would require directing the tumour vessels into a more normal branching pattern and also encouraging a functional lymphatic network to form inside the tumour (Laking *et al*, 2005).

However, this goal requires a fundamental understanding of network formation, maturation in normal physiology, and how these processes go wrong in tumours. Because we are still far from achieving this goal, therapies relying on drug delivery to the cancer cells will suffer from limitations in the knowledge of the tumour vasculature for years to come (Munn 2003).

What is abnormal in tumours is the dysregulation of angiogenesis: it does not follow the organized sequence that produces normal vessels in development, wound healing or the female reproductive cycle. Studies show that tumour vessels are leaky because of unusually large gaps between adjacent endothelial cells, an abnormal basement membrane and extensive fenestration (Hashizume *et al*, 2000). High levels of vascular endothelial growth factor

(VEGF) produced by tumour cells contribute to the high permeability of tumour vessels. Structural analysis shows that effective pore size for endothelial gaps is around 200-2000 nm. Tumours are therefore, highly permeable with large pore size, they also possess high interstitial fluid pressure which is roughly equivalent to the intravascular pressure (Hashizume *et al*, 2000).

Tumour vessels have a disorganized branching structure and lack a functional lymphatic system which results in pockets of cancer cells that are not accessible by blood-borne drugs. It is thought that cancer cells in these regions of compromised nutrient delivery survive because they are genetically protected against apoptosis. Furthermore, oxygen and nutrients, which are generally much smaller than the injected molecular therapeutics and particles, might be able to diffuse into these isolated regions more easily. Blood flow in individual tumour segments is highly variable, often stopping or reversing temporarily (Tyrrell *et al*, 2005).

Vessel recruitment during tumour angiogenesis involves capillary sprouting, in which endothelial cells in a pre-existing vessel proliferate and migrate to form additional vascular segments. However, tumours can also recruit new blood vessels by intussusceptive growth (see chapter 1), a mechanism by which existing vessels are split into smaller looping segments, resulting in a mesh-like network that fills the tissue. Perivascular cells are thought to play a crucial role in intussusception by directing the vasculature through the extra cellular matrix. They may also be responsible for burrowing ahead of endothelial sprouts in sprouting angiogenesis (Patan *et al*, 2001; Djonov *et al*, 2003).

Three types of blood vessels have been reported to exist in tumours: a) new sprouts which, structurally only possess endothelial cells, poorly differentiated or non-existent basement membrane b) capillaries lined by endothelial cells which are anchored to a partially developed basement membrane and partially assembled pericytes and c) capillaries with larger lumen diameter and a basement membrane with anchored endothelial cells and pericytes. The last category of vessels is known as 'stabilized vessels' (Baluk *et al*, 2005).

Four defined areas can be recognized in a solid tumour by the number of vessels present: an avascular, necrotic region, a semi-necrotic region, a stabilised microcirculation region and an advancing front (fig 3.0). This information has been brought about by studies assessing the temporal and spatial heterogeneity of tumour blood flow. The difference in blood flow is a result of elevated geometric resistance in tumour vessels coupled with high vascular permeability hence, based on perfusion rates, distinct tumour regions can be identified (Jain 1997).

In the peripheral regions of the tumour a dense network of vessels are present with clusters of vessels observed in the invasive fronts. No necrotic areas are seen. However, in semi necrotic regions partially developed vessels are noted. These vessels survive because they are separated by other tumour vessels that are a maximum distance of 5-9 layers of cells in distance from each other. Vessels further away undergo necrosis. Tumour necrosis is generally exhibited in central zones of malignant tumours (Ergun *et al,* 2006).

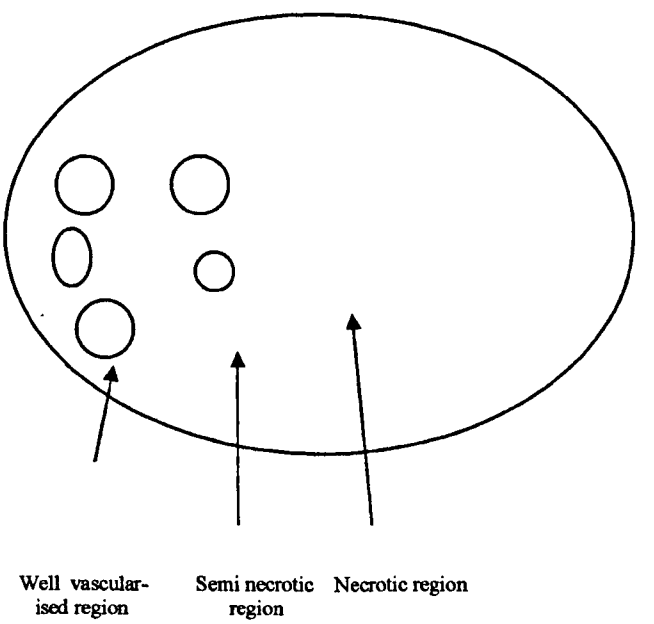


Fig.3.0 Schematic diagram showing heterogeneously perfused tumour showing a well vascularised periphery; a semi necrotic, intermediate zone; and an avascular necrotic central region (Diagram adapted from Bicknell *et al,* 1997).

3.1.3 Assessment of angiogenesis in rodent models.

A better understanding of angiogensis and its implication in disease should help to reveal specific targets for therapeutic agents to combat progression of disease states. A variety of *in vivo* bioassays of angiogensis have enabled investigators to make rapid progress in the understanding of the mechanism of angiogenesis and the discovery of promoters and inhibitors of the process (Dellian *et al*,1996).

Classical *in vivo* models to study angiogenesis include the hamster cheek pouch. This method observed the growth of new vessels within regenerated tissue. This allowed for direct observation of angiogenesis in semi-transparent tissue (Klintworth, 1973). *In vivo* studies of angiogenesis, in normal and neoplastic tissue, was first assessed by injecting neoplastic cells into the dorsal skin of mice. This method proved to be limiting in terms of the inaccuracy of evaluating dermal angiogenic responses such as the extreme variability in vessel counts and the inability to distinguish new vessels from existing ones.

The dorsal air sac model was the predecessor to the above method; here cells were introduced into an air pocket created on the dorsum of a rat. It also proved to be limiting due to the above reasons as well as the inability of the examiner to monitor the evolution of an angiogenic response. However, this model provided the groundwork for the eventual development of the corneal bioassay (Bicknell *et al,* 1997).

The observation of angiogensis in the iris and the cornea of the rodent eye is proved to be the most versatile bioassay and an invaluable system to investigate tumour neovascularisation (Auerbach *et al.* 1991). Folkman first established that tumours were angiogenesis dependent by using this method in the early 1970's. He reported that tumour spheroids, that were initially free floating in the anterior chamber, remained biologically dormant. However, once they came into contact with the surface of the iris they became vascularised and grew in size quite rapidly. This bioassay is mainly regarded as a qualitative assay, however, quantitative analysis was also conducted by recording positive responses when growths of capillary loops or sprouts are detected. Negative scores were given when an occasional sprout or hairpin loop was detected without evidence of sustained growth.

Other tumour models have been developed to test anti-angiogenic and anti-cancer agents. For these tumours, investigators inject tumour cells subcutaneously into the flank of BALB/c mice and the agents are administered intravenously. The tumour is measured at regular intervals to assess whether the anti-angiogenic or anti-cancer agents have an inhibitory effect on tumour growth in a dose dependent manner (Bicknell *et al*, 1997).

This model has been used in my study to assess whether tumour growth is dependent on angiogenesis and also to investigate tumour vascularity in relation to time. Tumour vessels, in both cases, were quantified by histological methods. Detection methods for vascular endothelium include basic histological and immuno-histological staining.

3.1.4 Prognostic value of intra-tumoral micovessel density (iMVD) in human solid tumours.

The association between intratumoral microvessel density and tumour aggressiveness can be explained by the fact that a greater number of tumour vessels increase the opportunity for tumour cells to enter the circulation (Weidner 1995). First, a highly angiogenic primary tumour with a high intratumoral microvessel density is more likely to seed distant sites with highly angiogenic clones (Weidner 1993). Second, solid tumours are composed of two discrete, yet interdependent components (ie. the malignant cells and the stroma that they induce), and measuring intratumoral microvessel density could be a valid measure of the success that a particular tumour has in forming this very important stromal component. Thirdly, the density of intra tumoral microvessels is likely to be a direct measure of the spread of tumour cells to other sites for instance, it can be hypothesized that the larger the number of vessels the greater the number of tumour cells there are circulating with the potential of metastasis. Hence, it is not surprising that many studies have found a correlation between iMVD and patient survival (Vermeulen *et al*, 2002).

Breast carcinoma is a tumour on which more independent studies have been performed to verify the prognostic value of iMVD than any other tumour (Ahlgren *et al*, 2002). The link between iMVD and patient prognosis of breast cancer was first confirmed by Bosari *et al*,

(1992). iMVD was assessed in node-negative and node-positive tumours and the findings of the group were that node-positive tumours showed significantly higher vessel counts than in node-negative tumours (Weidner1991). Because nodal status is presently the most powerful prognostic factor in breast cancer, the positive correlation of microvessel counts with nodal status indicates that tumour vessel density is a reliable marker in predicting the prognosis of breast cancer (Takei *et al*, 2000).

Studies conducted on breast carcinomas show a significant association between vascularisation and clinical outcome of patients and therefore, studies confirm that there is a strict association between the degree of angiogenic activity of primary tumours and their potential to develop distant metastasis (Nakayama *et al*, 2001).

Jacquemier *et al*, (1998) reported that iMVD has independent prognostic value in predicting disease free and overall survival. They noted that not only iMVD but mitotic index, tumour size, hormone receptor status, p53 over expression, c-erb B2 expression, Ki-67 labelling index, and histological grade all correlate with disease free survival. For overall survival iMVD ranked third as an independent prognosticator coming behind Ki-67 labelling index and progesterone receptor expression.

Preliminary studies in such tumour tissues also suggest that angiogensis may be used as a marker to predict responsiveness in some forms of conventional anti-cancer therapy (Gasparini 2000). Studies carried out on squamous cell cancer of the oropharynx also demonstrates how powerful iMVD is as an independent predictive factor for treatment failure in irradiated patients for the disease (Aebersold *et al*, 2000).

A strong correlation between iMVD and metastasis has also been reported in patients with prostate, head and neck, non-small cell lung, gastric and colorectal carcinomas (Bicknell *et al*, 1997; Lee *et al*, 2000; Ushijima *et al*, 2001; van Moorselaar and Voest, 2002).

Colorectal cancer is one of the most prevalent tumours. Traditionally, and still primarily used today, the clinical outcome of colorectal cancer patients may be predicted by pathological staging such as Dukes' and TNM staging (Lee *et al*, 2000). These systems assess histological grade, lymph node involvement, and depth of tumour involvement. This information allows specific treatment regimens to be selected according to the extent of the disease. Therefore,

staging is currently the most important prognostic factor in determining the clinical outcome and survival in colorectal cancer (Boyle 2003).

However, some patients who have a favorable clinical outcome, still develop local recurrence or distant metastasis. Therefore, it is important to find some other indicators that can predict for recurrence or the development of metastasis so that high-risk early stage patients can be treated effectively (Weyn *et al*, 2004).

Microvessel quantification may prove to be an additional factor that when used together with these already established clinical parameters could help cancer patient management (Jesionek-Kupnicka *et al*, 2002). However, quantitative data on the vascularity of human colorectal tumours is largely lacking and the use of iMVD in clinical practice is limited by the methods employed for microvessel identification and quantification (Vermeulen *et al*, 1996, Tanigawa *et al*, 1997, Akagi *et al*, 2002) (see chapter 2).

The results in the studies exploring the prognostic value of microvessel counts in colorectal carcinomas conflict (Banner *et al*, 1998). Saclarides *et al*, (1994) assessed microvascularity in 48 rectal carcinomas and found significant association between high vessel numbers and tumour invasion through the bowel wall and death within 5 years. There was also a trend towards high microvessel counts and increased tumour size and incidence of metastasis that was observed. However, this study did not compare microvascular density with tumour stage therefore, Frank *et al*, (1995) investigated the association of angiogenesis to prognosis. The group found a significant link between high angiogenic scores to death within 5 years and tumour recurrence.

These and other studies (table3.0) found a correlation between higher counts and more aggressive cancer progression. However, Lindmark *et al*, (1996) declared that they found higher microvessel counts in patients with longer survival rates. The reason for the discrepancy between results of this study and the previous studies (table 3.0) is not clear and such a study casts a doubt upon whether microvessel density can be considered as an independent prognostic marker in colorectal cancer.

Nevertheless, more studies need to be conducted, as have been in breast cancer, before reaching a conclusion on the significance of angiogenic scoring on prognosis of colorectal cancer sufferers.

Source	*Number of patient samples*	*Site*	*Stage*	*Correlation*
Saclarides *et al*, 1994	48	Rectum	Astler-Coller A-D	High MVC correlated with higher stage, poor survival, larger tumour size, and metastasis
Frank *et al*, 1995	105	Colon	Astler-Coller B2	High MVC correlated with survival <5yrs and tumour recurrence.
Takebayashi *et al*, 1996	166	Colorectal	Dukes' A,B,C	High MVC correlated with larger tumour size, relapse, lymph node metastasis, vessel invasion and poor survival.
Lindmark *et al*,1996	227	Colorectal	Dukes' A,B,C	High MVC correlated with longer survival; no correlation between MVC and tumour differentiation or Dukes' stage.
Engel *et al*, 1996	35	Colorectal	Dukes' A,B,C	High MVC correlated with recurrence.
Tanigawa *et al*, 1997	133	Colorectal	Dukes' A,B,C	High MVC correlated with hematogenous metastasis, grade, depth of invasion, lymphatic invasion, lymph node metastasis, and peritoneal metastasis.
Banner *et al*, 1998	22	Colorectal	T3 N0 M0	Trend toward higher MVC with longer survival.

Table 3.0 Summary of studies conducted for the assessment of microvascular counts (MVC) as a prognostic marker in colorectal carcinoma (adapted from Banner *et al*, 1998).

3.1.5 Assessment of tumour lymphangiogenesis.

Tumour metastasis to regional lymph nodes represents the first step of tumour dissemination in many common human cancers and serves as a major prognostic indicator for the progression of the disease. However, it has been difficult to determine whether cancer metastasis to lymph nodes is achieved through the formation and invasion of new lymphatics within and around the tumour or expansion and invasion of pre-existing lymphatics (Cunnick *et al*, 2001).

Once lymphangiogenesis proves to be a reliable indication of lymph node metastasis, then its detection could be a valuable prognostic tool for patients at high risk of recurrent disease, and a useful criterion for planning subsequent treatment such as radio- or chemotherapy (Farnsworth *et al*, 2006).

The concept of tumour lymphangiogensis aiding metastasis has raised a great deal of skepticism. Folkman stated that tumours do not possess lymphatics however, van Netten *et al*, (1996) published contrary findings that breast cancers display 'lymphatic-like labyrinths.' It has also been argued that tumours do not generate lymphatics and also do not require a lymphatic supply because they drain their fluid in the lymph vessels of surrounding tissue. It is also suggested that lymph vessels that are identified within tumours cannot be functional because tumours exhibit greater hydrostatic pressures than normal tissue.

However, in tumours where microvascular density does not correlate with metastatic spread for example, decreased number of vessels associated with metastasis, it is thought that tumour lymph vessels may have a key role to play in metastasis. It is also now accepted that haematogenous tumour spread is associated with the presence of tumour lymph vessels ie. tumours from patients with lymph node positive metastasis is thought to be preceded by tumour lymphangiogenesis (Vermeulen *et al*, 2002).

Knowledge of intratumour lymphatics and their relationship to tumour spread is scarce which may be due to the fact that markers for the detection of lymphatics have only recently been described (Al-Rawi *et al*, 2005).

3.1.6 Methods used to quantify tumour vessels.

From the vast number of studies published on the prognostic value of angiogenesis in malignant tumours it is clear that the methods involved are equally as important as the final results (Boyle 2003). The College of American Pathologists investigated the prognostic factors in breast cancer and depending on the number of published data ranked each category. Despite discrepancies in methodological variations such as: antibody selection and methods of counting tumour vessels; quantification of tumour angiogenesis came third in their ranking (Vermeulen *et al*, 2002).

The identification of tumour vessels using a variety of antibodies has aided the accurate detection of tumour vasculature. Immunostaining has now enabled investigators to distinguish between mature and developing microvessels. Amongst the established endothelial markers vWF is expressed by mature endothelium where as CD31 and CD34 detect more actively growing microvessels (Boyle 2003). However, CD105 (a transforming growth factor β (TGF β) receptor) is selectively expressed by angiogenic vessels and hence appears to be a better marker of active angiogenesis than CD31 and CD34 (Akagi *et al*, 2002).

Counting microvessels in a microscopic field of a tumour section gives an estimate of the angiogenic potential of a tumour. Graticule-based vascular morphometry such as Chalkey counting quantifies vessels by measuring the number of grid points that hit stained vessels (Fox *et al*, 1994). Therefore, this method gives a relative area count rather than a true vessel count (Vermeulen *et al*, 2002).

The method introduced by Weidner *et al*, (1991) was based on identifying the most densely vascularised area of the tumour ('hot spots') under the microscope and the tumour area with the greatest number of vessels (also termed as microvessel density MVD) in this hot spot was counted and used to characterise the tumour. The two methods are not equivalent (Boyle 2003) however, Offersen *et al*, (2003) presented paired data comparing prognostic outcomes from MVD and Chalkey counts in prostate, breast, bladder and lung cancer. The study highlights that the two methods yield counts that are correlated to prognosis suggesting that the two methods are effective in quantifying tumour vessels.

Automated systems are able to improve the objectivity of microvessel evaluation, however, the costs involved in acquiring such equipment is a drawback, hence impede the widespread application of such methods (Vermeulen *et al*, 2002).

3.2 Aim

The purpose of this investigation was to assess the distribution of tumour vasculature in subcutaneously grown human tumours in BALB/c mice. The influence of time over vessel development was also investigated by resecting tumours at time points between 8 to 22 days and quantifying the number of vessels present.

Further clinical studies were carried out to obtain qualitative and quantitative data of tumour vessels from human colorectal cancers. Quantitative studies involved assessing the number of blood and lymph vessels present in different graded tumours. The results were correlated to the clinicopathological variables to ascertain a relationship between tumour angiogenesis and lymphangiogenesis to prognosis.

3.3 Methods and materials

The following histological and quantification techniques allowed the investigation of vessel densities in both murine and human colorectal tumours.

3.3.1 Mouse tumour (colo26) study

Mouse tumour (colo26) production method

The colo26 tumour model was used to assess the relationship between vascularity and tumour growth and its post-inoculation times. Fifty two tumours were obtained from the Academic Department of Surgery, Royal London Hospital, Whitechapel, London following the Royal London Hospital ethical regulations. The tumours were subcutaneously grown in BALB/c female mice, weighing between 18 and 23g, which were bought from Charles River U.K. and housed in accordance with Home Office guidelines.

The tumour cell line (colo26), used for the establishment of the tumours, was grown as monolayers and incubated in a humidified atmosphere at 37°C with 5% CO_2. RPMI supplemented with 10% heat activated foetal calf serum, 5mM of L-glutanin, 100U/ml penicillin and 100 Fg/ml streptomycin (Gibco Life technologies, Paisely, Scotland) were used to sustain the gowth of the cell-line. Cells were then passaged until a concentration of $40x10^6$ viable cells were reached.

0.05ml of cell suspension (approx. 2 million cells) were injected subcutaneously in the top of the hind leg, 1cm lateral to the spine, of the mouse. The cells were allowed to develop into solid tumours and excised at various post-inoculation times ie. 08, 16, 18, 21 and 22 days for use in this study.

Tissue preparation

The excised tumours were immediately fixed in 10% buffered formalin for a minimum of two days. The tumours were cut into four parts and labelled regions 1-4 before placing in embedding cassettes. These were then processed according to the standard procedure using an automatic tissue processor (Shandon Elliot U.K.) for 22 hours. The processed tissues were embedded in paraffin wax (58-60°C) and left to solidify on a cold plate (Shandon Histocenter).

Serial sections were cut from the prepared blocks at 3-4 um and placed on a water bath (30°C) to remove any creases from the tissue sections. They were then gently lifted from the water with glass slides and left to dry over night on a hot plate at 38°C.

Tumour sections were dewaxed in histoclear and rehydrated in a series of graded alcohols in preparation for staining. Sections were numbered in sequence and the even numbered slides were stained with hemotoxylin and eosin and the odd numbers were stained with Masson's Trichrome. Following staining, sections were dehydrated in a series of alcohols and finally cleared in histoclear and mounted with a cover slip using histomount. Blood vessels within the tumour sections were located and viewed under the x10 and x40 objective lenses of a light microscope.

Morphometrical analysis

A total of 52 tumours were used in the study, 31 tumours were used for quantification analysis. Prior to processing, tumours were immediately cut in half from which three orthogonal diameters D1, D2, D3 were measured. The volume was calculated by using these measurements in the formula $V=\pi/6$ (D1XD2XD3). Each of the four regions of the tumour (cut prior to processing as previously stated) was systematically scanned for blood vessels under low power of a light microscope.

Vessels from each section were counted and the mean number of vessels per mm^3, from all of the sections of each tumour was considered. Note, the criteria for the identification of vessels was: i) vessel demonstrating an intact endothelium and ii) a lumen preferably displaying the presence of red blood cells or plasma.

Statistical analysis

The correlation between vessel number and post-inoculation time was analysed by the Spearman's rank correlation coefficient. The data collated was not normally distributed, therefore, a non-parametric test such as Spearman's rank test was used. The test is applied when considering the relationship between two variables by ranking both sets of data and comparing the orderings. The two variables in this study were: vessel counts and post-inoculation times. A p value ≤ 0.05 was taken as significant.

3.3.2 Human colorectal carcinoma tissue

Thirty one primary tumours from colorectal cancer patients were obtained with consent from the St. George's Histopathology archives under the St. George's Hospital ethical regulations. Clinical profiles of patients from which the tumour specimens were excised were also obtained. Details such as age, sex, tumour histology (venous and lymphatic invasion) and tumour grade (Dukes' stage) were noted (see table 3.1 for the number of tumours in each category). These were surgical resections from patients of varying histological grades of colorectal carcinomas. All specimens used in the study were from patients who had no prior chemo- or radio- therapy.

Twenty one sections of tumour tissue were cut at 5um thickness and mounted on positively charged slides. These were incubated at 37°C before being stained with vWF, CD31 and CD34 (see chapter 2 methods). Three sections from each tumour were used to assess the number vessels highlighted by the endothelial markers vWF, CD31 and CD34 and two sections were used to for the positive and negative controls. Further eight sections were used to investigate the mean number of vessels in each region of the tumour by highlighting the vessels using CD31 and CD34. The tumour regions were categorised into four areas: tumour center (TC), tumour periphery (TP), adjacent host tissue (AH) and distant host tissue (DH).

The remaining eight were used to assess the correlation between mean vessel counts per tumour to the clinicopathological variables listed in table 3.2. Vessels in these tumour sections were also highlighted by CD31 and CD34.

Positive and negative controls were assessed in normal colon tissue, however, in negative controls incubation with the primary antibody was omitted.

Quantitative analysis

Sections were scanned for blood vessels and scored by two independent observers under x10 objective and x10 occular lens of a light microscope. Invasive margins of the tumour, when present in the section, were included in the scanned area. Specific criteria, based on those of Weidner et al, 1993 were applied when counting vessels: 1) vessels had to be in the stroma within the tumour; 2) vessel containing stroma had to occupy more than 50% of the x10 field to be counted; 3) vessel diameter had to be 50um or less. The mean counts in each section stained by each antibody were used in the statistical analysis.

Statistical analysis

The Kruskal Wallis test was used to examine the difference in vessel counts between the four regions of the human colorectal tumours used in the study. The data from the study was not normally distributed therefore, a non-parametric test was applied to analyse the data.

The Kruskal Wallis test was used as more than three groups of variables were compared. The test looked at the number of blood vessels in the different regions of the tumour ie. tumour centre, tumour periphery, adjacent host tissue and distant host tissue and assessed whether regional distribution of vessels was correlated to: a) tumours excised from male or female patients, b) tumour pathology (well, moderate, poorly differentiated), c) lymphatic invasion, d) venous invasion, e) Dukes' stage (A,B,C).

The Kruskal Wallis test was also performed to assess the statistical significance and the correlation between tumour blood vessel counts in the overall tumour to the clinicopathological variables: tumours excised from male or female patients, b) tumour pathology (well, moderate, poorly differentiated), c) lymphatic invasion, d) venous invasion, e) Dukes' stage (A,B,C). The same test was also used to assess the correlation between tumour lymph vessel counts and clinicopathological variables. A p value ≤ 0.05 was taken as significant.

3.4 Results

3.4.1 Murine colorectal tumour model (colo26)

Tumours demonstrated key characteristic features in terms of spatial distribution of vessels. Due to the fact that colo26 tumours grow subcutaneously, they interface murine skin on one side and murine muscle on the other. Blood vessels within normal murine tissue were, mainly, noted in the cutaneous regions.

Serial sections were methodically scanned for neovascularisation. Vessel counts were performed at a magnification of x40 with an eye piece graticule. The number of tumour blood vessels increased as the post-inoculation time progressed. Tumours of earlier time points displayed a progressive increase in the number of blood vessels: tumours aged 8 days had a mean value of 6 vessels ±3 and tumours aged 16 days 10 vessels ±6. A sharp increase in the number of vessels was noted for day 18 with vessel number 85 ± 64 and day 21 with 67 ±50 vessels. However, the latest post inoculation time, age 22 days, demonstrated a decline in the number of blood vessels: 27 ±8. The study showed a correlation between blood vessel counts and post-inoculation times ($P<0.001$) (Fig 3.1).

A typical characteristic of the colo26 tumours was the presence of capillaries in large clusters within the peripheral regions of the tumours (Fig 3.2). These clusters of vessels were, notably, in close proximity to the subcutaneous regions in the host tissue. Tumours rarely contained arterioles or other large vessel types such as venules. However, in exceptional cases where such structures were present in the tumours, similar patterns of distribution were observed for example, arterioles and venules surrounded by numerous capillaries (Fig 3.3).

Within the overall area of tumour sections a greater number of blood vessels were displayed in the peripheral zones although vessels within central regions were scarce. Necrosis and extravasated red blood cells were commonly evident in many tumour specimens (Fig. 3.2 and 3.4).

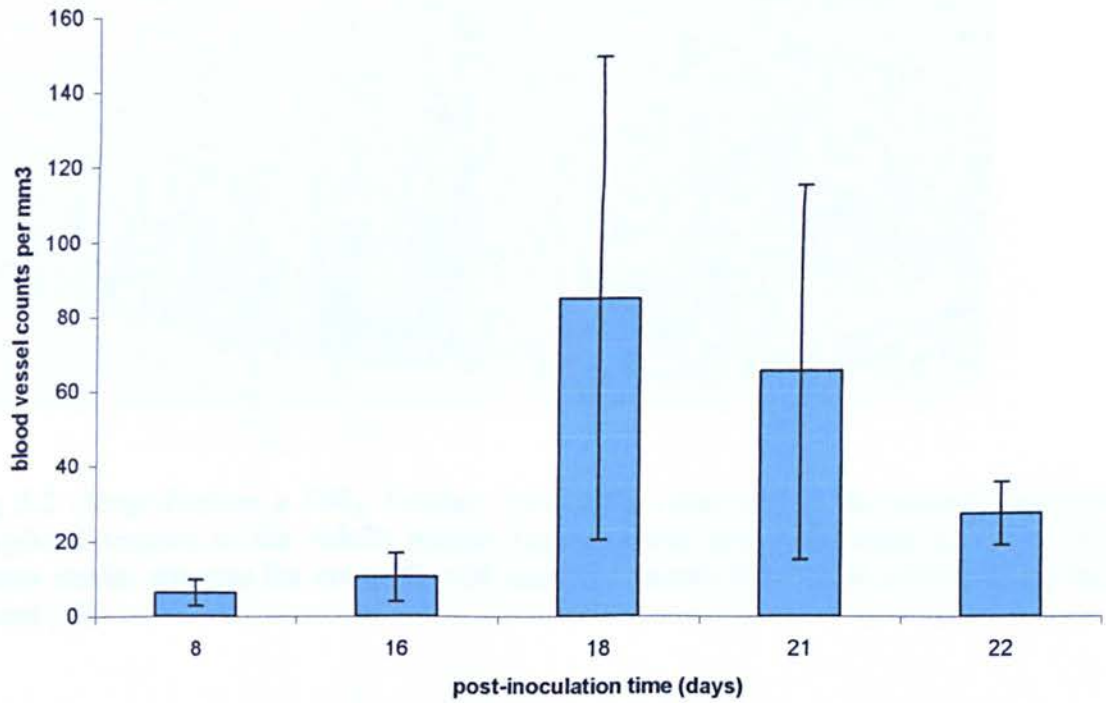


Figure 3.1 showing the association of the mean blood vessel number per colo26 tumour with post-inoculation times. Thirty one colo26 tumour sections stained with Masson's Trichrome were quantified for the mean number of vessels per mm^3 for time points 8, 16, 18, 21 and 22 days. A correlation between blood vessel counts and post-inoculation time is demonstrated with a statistical significance of $p<0.001$.

Colo26 microvessel density was assessed using basic stains ie. hematoxylin and eosin and Masson's trichrome with Masson's trichrome showing prominent staining of vessels. Negative results from antibody staining were achieved and basic staining failed to effectively highlight or even distinguish many tumour vessels from the rest of the tissue in colo 26 tumour. Due to the lack of a specific marker for colo 26 tumour endothelium it was decided that vascularity should be investigated in tumour tissue from the human colon.

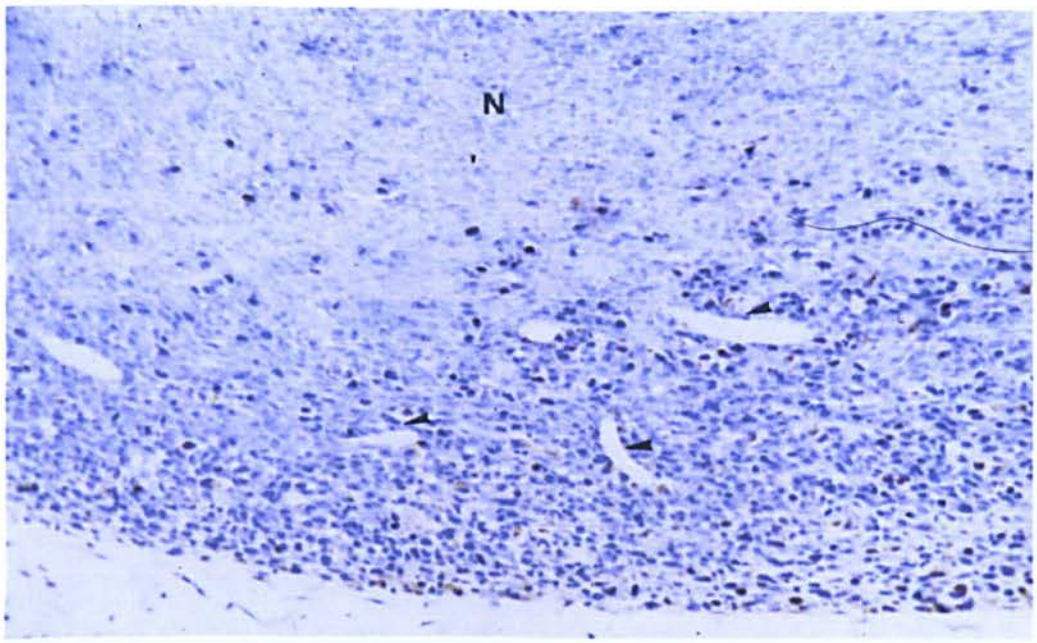


Fig 3.2 (Magnification x 560). Section through the outer part of the central areas and the peripheral regions of the colo26 mouse tumour. Outer peripheral zone is highly vascular (arrow heads) whereas the zone adjacent (central regions) exhibits no vessels and a necrotic region (N).

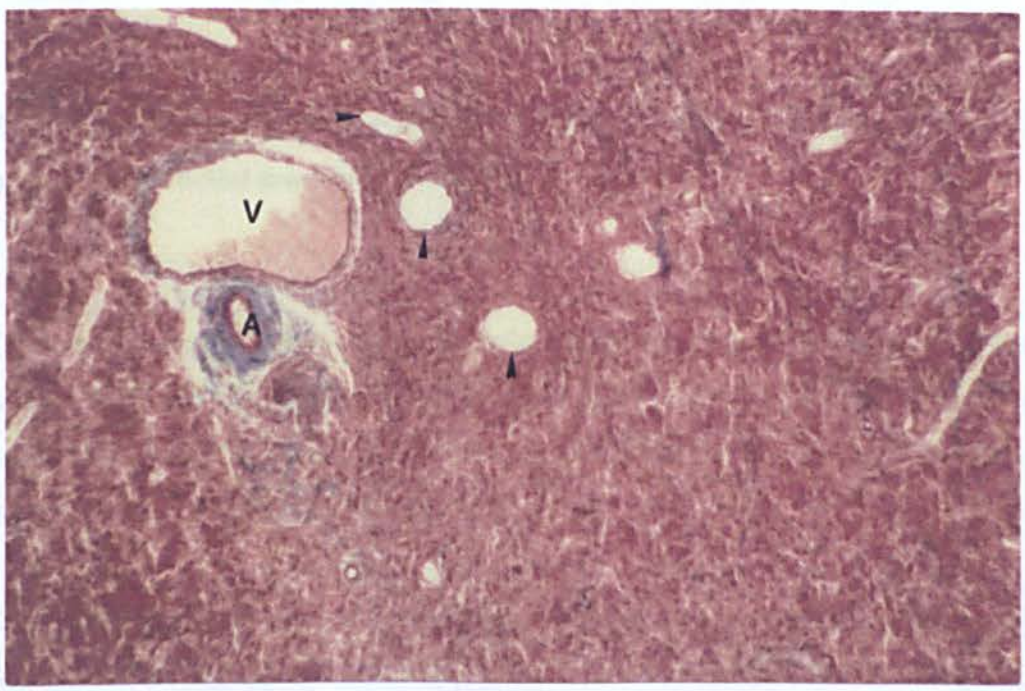


Fig 3.3 (Magnification x 336). Section through tumour displaying an arteriole (A) adjacent to a venule (V) present in the peripheral regions of the colo 26 tumour. These vessels were rarely present in this tumour tissue. Clusters of capillaries (arrows) surround the prominent vessels.

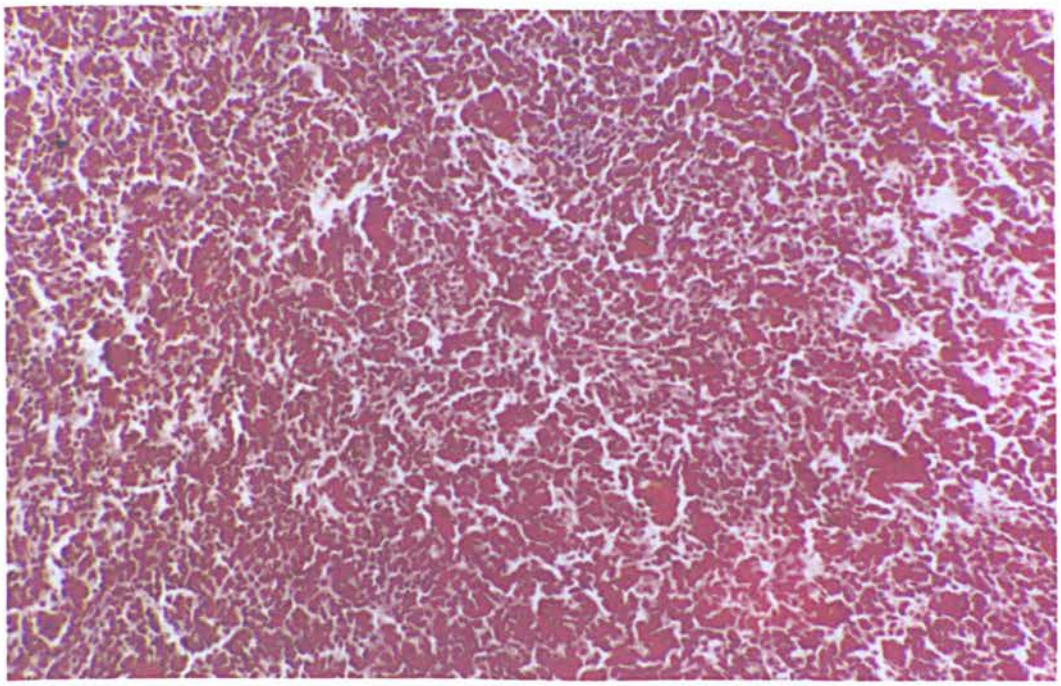


Fig 3.4 (Magnification x 700) A photomicrograph of the central zone of the colo 26 tumour. The region is densely packed, necrotic and mostly avascular.

3.4.2 Assessment of vascular density in human colorectal tumours using the three most specific endothelial markers: vWF, CD34 and CD31.

Optimal staining of tumour blood vessel endothelium was achieved with the three antibodies: vWF, CD34 and CD34 (see chapter 2). However, the results obtained using the antibody vWF were poor (fig. 3.6). Blood vessels were stained by the antibody however, strong background staining with vWF often led to difficulties in the identification of individual blood vessels.

CD31 demonstrated uniform specificity and highlighted a maximum number of vessels in all sections observed (fig. 3.7). Staining of the endothelial cells was intense, specific and easy to visualise. Such results were consistent in all sections stained with the antibody. In comparison, a lower number of vessels were detected by anti-CD34 (fig. 3.9).

Quantification of vessels in colorectal carcinomas was undertaken to determine which antibody provided the highest blood vessel counts. A greater number of vessels (328±138) were identified in sections stained with anti-CD31, in comparison to anti- CD34 stained sections in which the vessel count was lower (260±95) (fig. 3.5). An even lower number of vessels (75±22) were detected with anti-vWF (fig. 3.5). Anti-CD31 was technically superior for staining blood vessels in human colorectal tumours therefore, this antibody was used for the remainder of the studies.

A significant correlation between the number of blood vessels stained with vWF and CD34 was obtained (p=0.0002). This was also observed between vessels stained with vWF and CD31 (p=0.0003). The association between CD34 and CD31 stained blood vessels was highly significant (p=0.0001) (fig. 3.5).

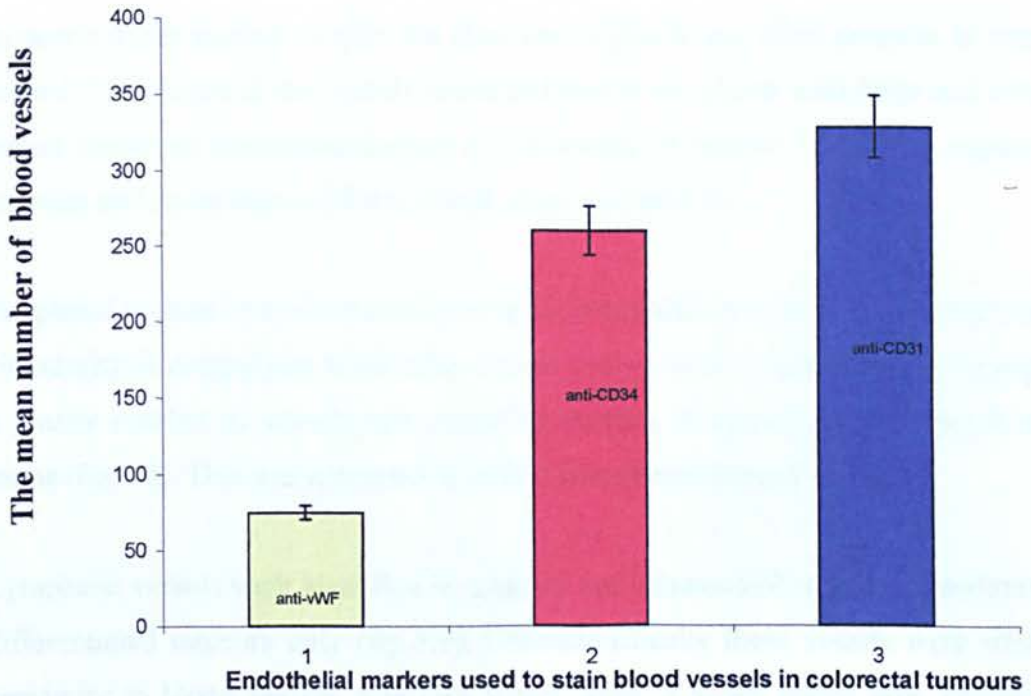


Figure 3.5 showing the mean number of human colorectal tumour vessels highlighted by endothelial cell specific markers vWF, CD31 and CD34. Human colorectal tumours graded Dukes' A-C were immunohistologically stained with anti-vWF, CD31 or CD34 and highlighted tumour vessels were quantified under light microscopy. The results are expressed as the mean ± S.D. number of vessels.

3.4.3 A qualitative and quantitative analysis of blood and lymph vessels in colorectal carcinomas

Spatial distribution of blood vessels in human colorectal tumours

Qualitative analysis of tumour blood vessels in and around tumour sections displayed characteristic spatial distribution in all sections observed. The histology of the mucosal regions of well to moderately differentiated tumours demonstrated spherical and structured neoplastic glands. Surrounding these glands were a number of vessels however, the submucosal region was highly vascular in comparison (fig 3.6). In contrast, the central regions of tumour sections exhibited densely packed neoplastic cells with groups of vessels clustered in the outer areas of the central region (fig.3.7). However, a fewer number of vessels were present in this region and the inner most central regions were generally necrotic.

In poorly differentiated samples the structure of glands was often irregular in shape as well as dilated. The shapes of the vessels mimicked that of the glands with large and irregular shaped lumen. However, this region showed a high density of vessels. The central regions were rarely vascular and sometimes exhibited a high degree of necrosis.

Peripheral regions in moderate and poorly differentiated tumours demonstrated regions of high vascularity in comparison to all other tumour areas. Areas of tumour invasive edges exhibited a greater number of vessels surrounded by clusters of vessels in the adjacent normal colon tissue (fig.3.8). This was not noted in well differentiated tumour tissue.

Lymphatic vessels were identified in mucosal and submucosal regions of moderate and poorly differentiated tumours only (fig 3.9). Characteristically these vessels were situated in close proximity to blood vessels with thin vessel walls, a larger lumen size, presence of plasma material within the lumen was also occasionally noted.

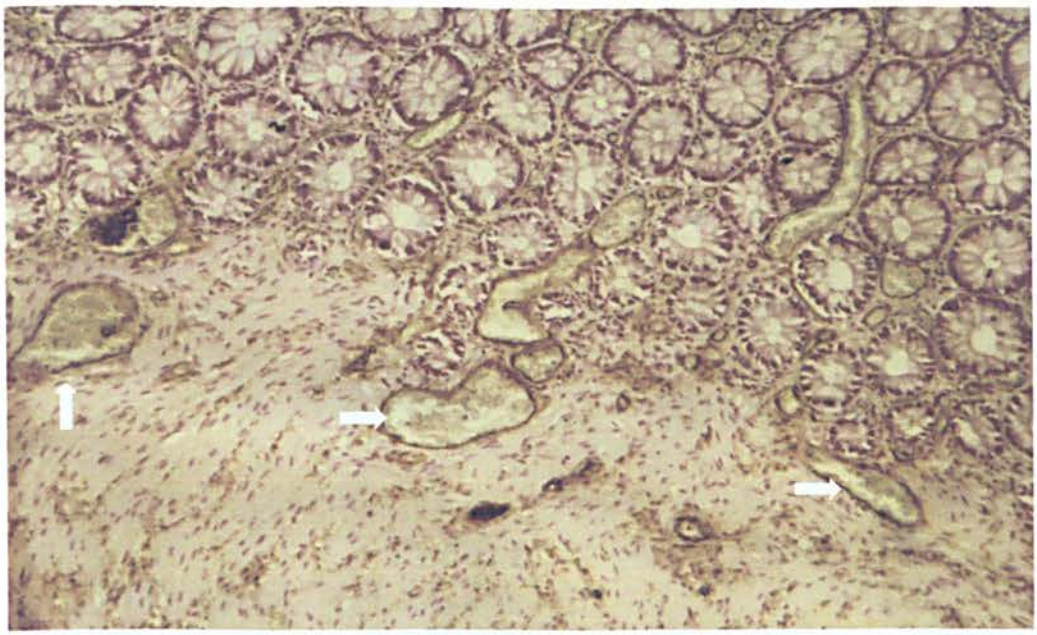


Fig 3.6 (Magnification x 560). A Section through a well differentiated human colorectal tumour (Dukes' A). The tumour in the mucosal region shows structured vessels (arrows) highlighted by vWF.

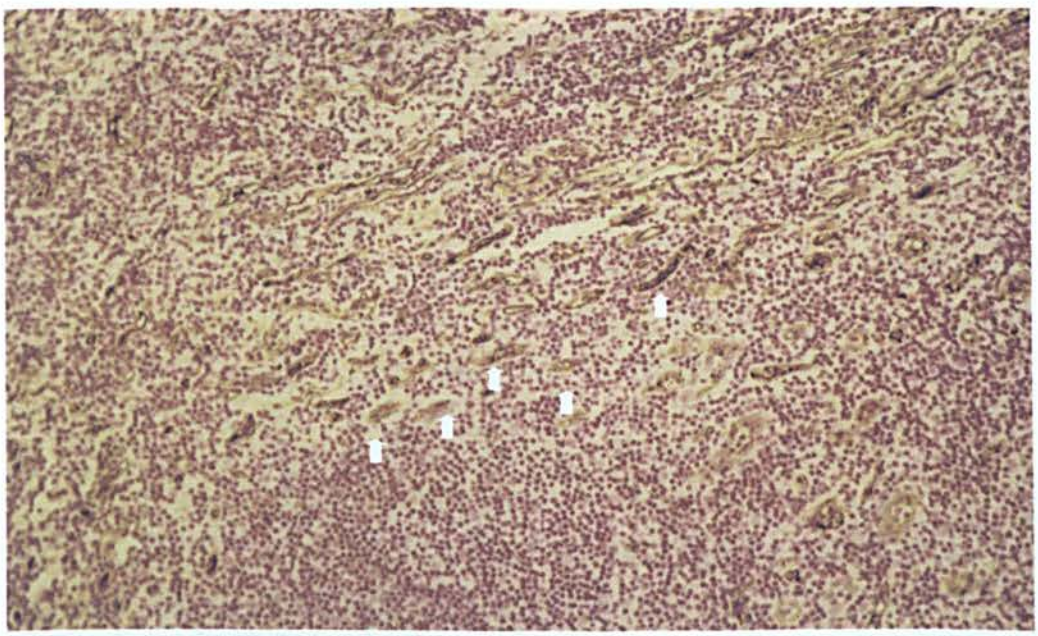


Fig 3.7 (Magnification x 336). Outer regions of the central zone of a moderately differentiated tumour (Dukes' B) exhibits clusters of vessels (arrows) located in close proximity to each other. Vessels are highlighted by CD31. Further towards the centre of the tumour the area is relatively avascular.

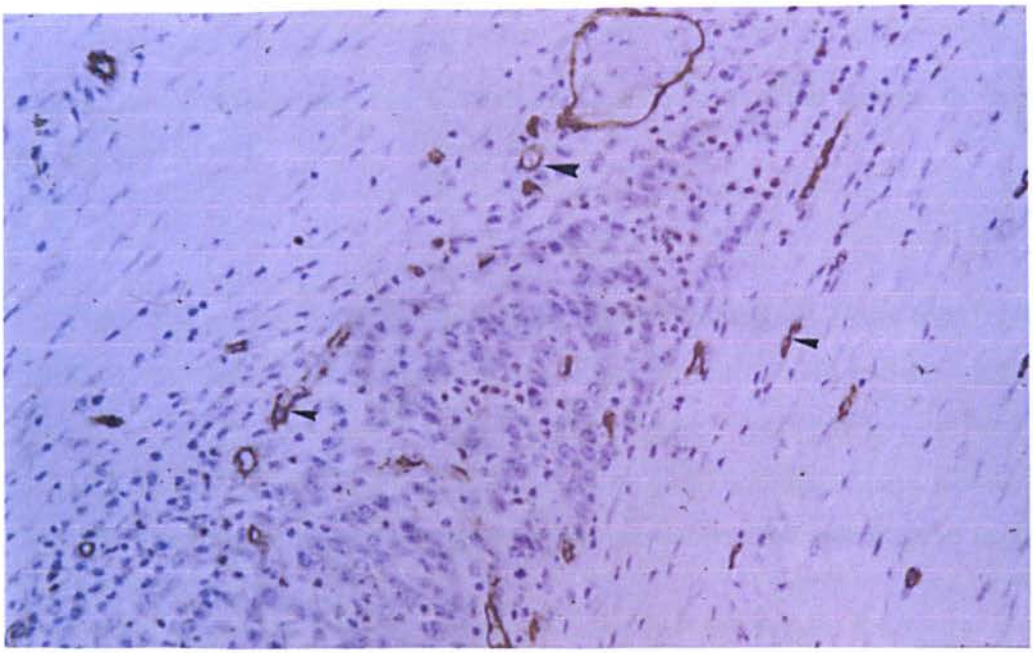


Fig 3.8 (Magnification x 1120). Demonstrates the extreme invasive edge of a Dukes' stage C tumour. There is an increased number of vessels (arrow heads) surrounding the metastatic front.

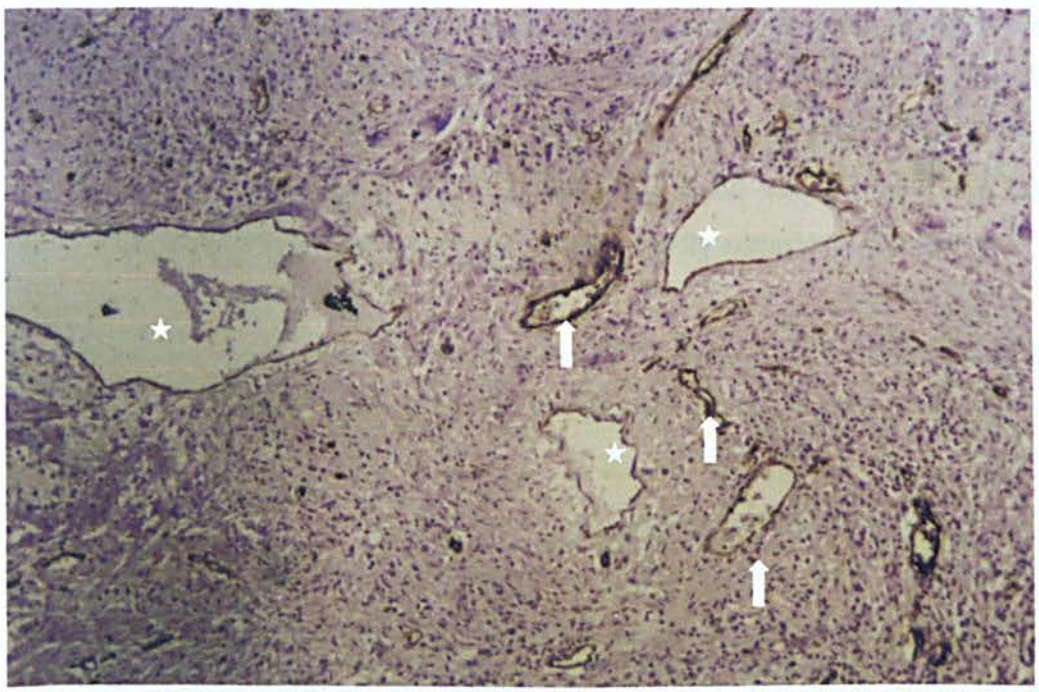


Fig 3.9 (Magnification x 280). Section through a Dukes' C tumour showing the presence of blood vessels and lymph vessels. Blood vessels have been stained with CD34 (arrows) however, lymph vessels are CD34 negative (stars).

A quantitative analysis of the spatial distribution of tumour vessels in human colorectal carcinomas

The following series of graphs depict the number of vessels quantified from the different regions in the cross-sectional areas of each tumour which also included normal host tissue adjacent to tumour tissue. The tumour regions under investigation were: the tumour centre (TC), tumour periphery (TP), adjacent host tissue (AH) and distant host tissue (DH). Regional vessel numbers were taken from tumours that came from the pathological categories listed in table 3.1.

Colorectal tumours taken from female patients (fig. 3.10) displayed variable counts of vessels in the designated tumour areas. The tumour periphery had the greatest number of vessels (100 ± 16) with the central regions showing the second highest count (64 ± 6). The number of vessels sited in the adjacent host tissue was five times lower than the tumour peripheral regions (22 ± 8) with host vessels distant from the tumour bearing the least number (7 ± 5). This pattern of distribution was also noted in tumours from male patients (fig. 3.11) however, the overall number of vessels per region was lower: (TC 51 ± 10), (TP 89 ± 10), (AH 12 ± 4), (DH 6 ± 4). The difference between the regional blood vessel counts and tumours excised from either male or female patients was not statistically significant ($p \leq 0.41$).

The above mentioned regional pattern of vessel numbers: such as high vascular density in the periphery, lower vessel numbers in the tumour centre with a marked decrease in vascularity noted in the adjacent host tissue and in distant host tissue were observed in several clinicopathological categories. This was noted in the regions of poor: (TC 85 ± 15), (TP 145 ± 28), (AH 22 ± 5), (DH 8 ± 4) (fig 3.12), moderate (TC 47 ± 10), (TP 77 ± 7), (AH 17 ± 8), (DH 6 ± 3) (fig 3.13) and well differentiated (TC 14 ± 7), (TP 17 ± 8), (AH 2 ± 2), (DH 6 ± 4) (fig 3.14) tumours. However, well differentiated tumours displayed greater numbers of vessels in distant host tissue compared to host tissue adjacent to tumour tissue. The results indicated that there was statistically a significant difference between histology groups for blood vessel counts in the different tumour regions ($p \leq 0.001$).

In tumours excised from patients with either no venous invasion (fig 3.15), with venous invasion (fig 3.16) ($p \leq 0.001$), without lymphatic invasion (fig 3.17) or with lymphatic invasion (fig 3.18) ($p \leq 0.001$) the regional tumour densities also followed the pattern of vessel distribution observed in the male, female and histological categories. Similar observations were also made in tumours from Dukes' B (fig 3.20) and Dukes' C (fig 3.21) ($p \leq 0.001$). However, in Dukes' A tumours (fig 3.19), although peripheral regions were more vascular, central regions also displayed high numbers of vessels and the distant host tissue was also more vascular than the adjacent host tissue. Dukes' A tumours portrayed less number of vessels per tumour region than Dukes' B tumours and the highest counts were noted in Dukes' stage C.

The results in most clinicopathological groups indicate that there is evidence of highly significant differences between the four regions: tumour centre, tumour periphery, adjacent host and distant host of human colorectal tumour tissue and the number of vessels that were present. In all of the clinicopathological categories examined the highest vessel counts were obtained in the peripheral regions of the tumour with significantly lower vessel counts observed in the adjacent host tissue and the least number of vessels exhibited in distant host tissue in comparison.

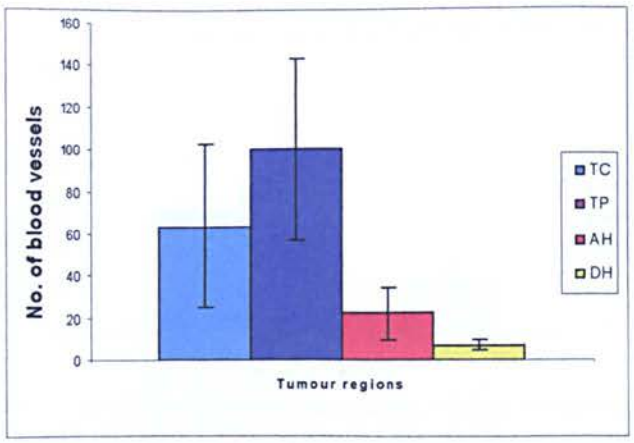


Fig 3.10 shows the number of vessels per tumour region in in colorectal carcinomas from female patients (n=13).

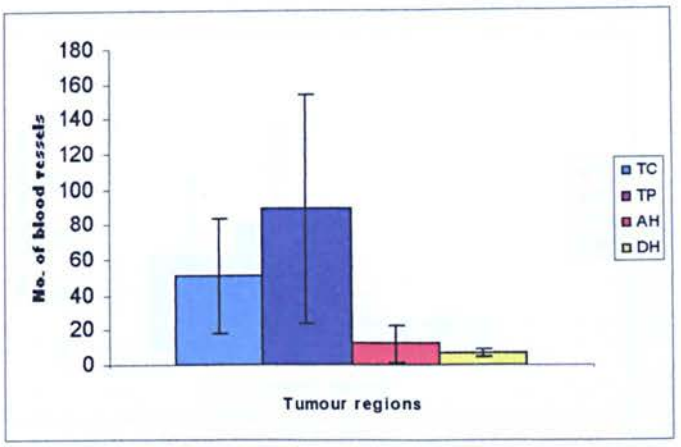


Fig 3.11 shows the number of vessels per tumour region colorectal carcinomas from male patients (n=18).

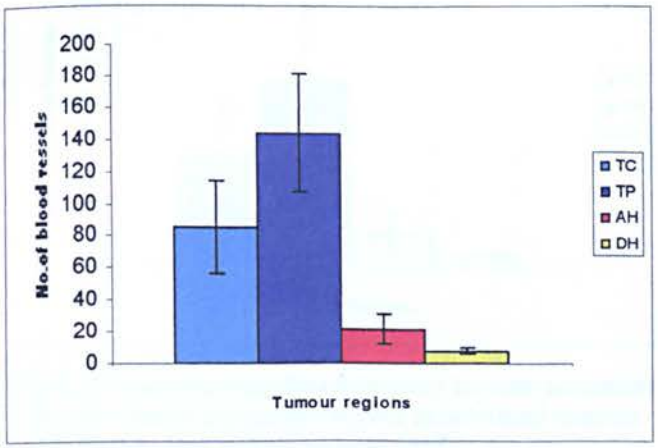


Fig 3.12 shows the relationship between intratumoral microvascular density of colorectal tumours and tumour histology moderately (poorly differentiated tumours) (n=12).

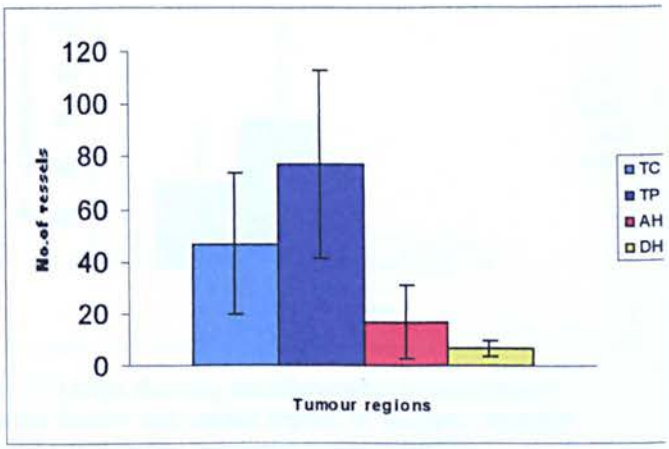


Fig 3.13 Graph showing the number of vessels in varying tumour regions of colorectal carcinomas within differentiated tumours (n=14).

Key: TC- Tumour centre

TP- Tumour periphery

AH- Adjacent host tissue

DH- Distant host tissue

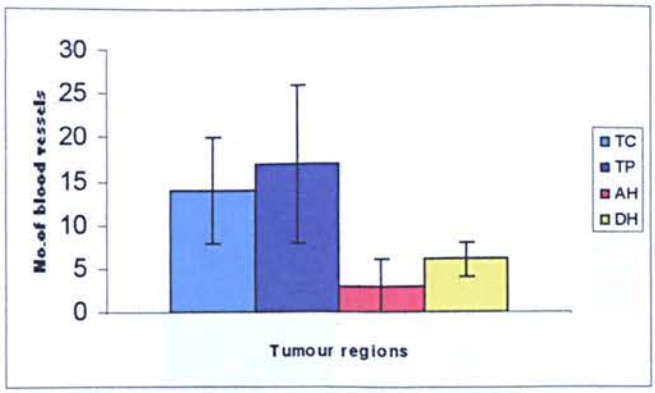


Fig 3.14 depicts the number of vessels in different tumour regions of well differentiated colorectal carcinomas (n=5)

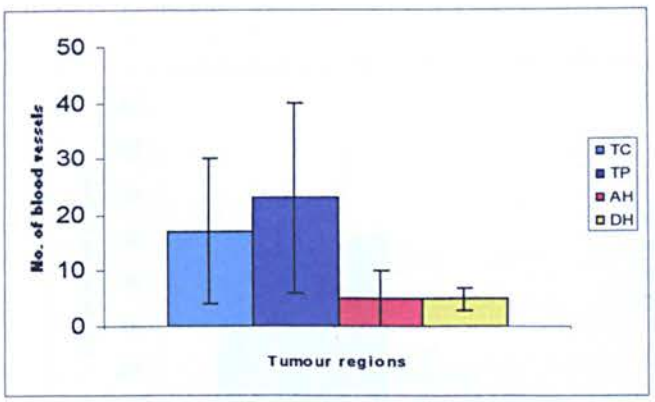


Fig 3.15 Graph showing the relationship between micro vascular density and tumour regions in colorectal tumours with no extratumoral venous invasion (n=24)

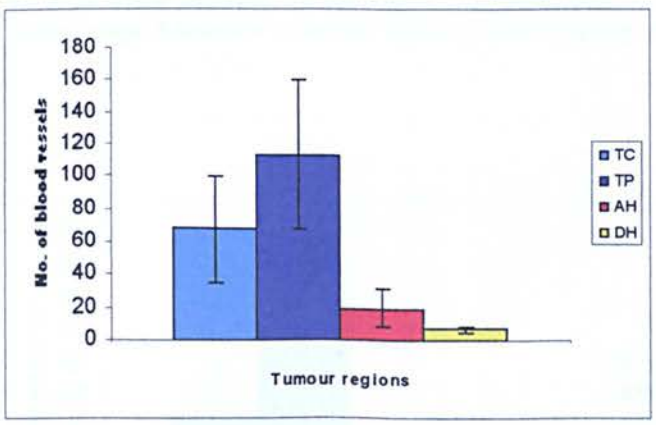


Fig 3.16 Graph showing the relationship between intramicro vascular density and tumour regions in colorectal tumours with extratumoral venous invasion (n=7).

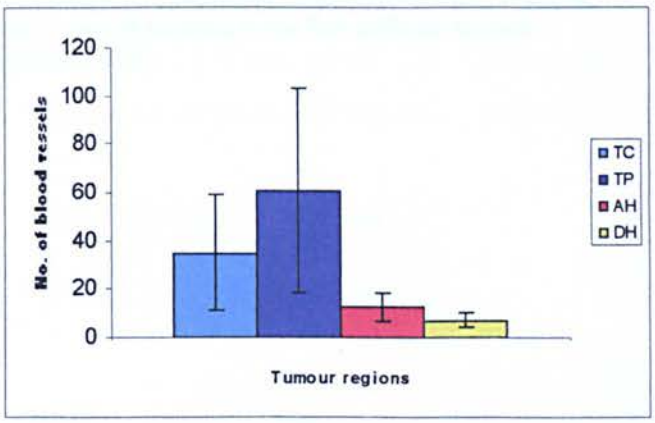


Fig 3.17 Graph showing the relationship between micro vascular density and tumour regions in colorectal tumours with no extratumoral lymphatic invasion (n=17).

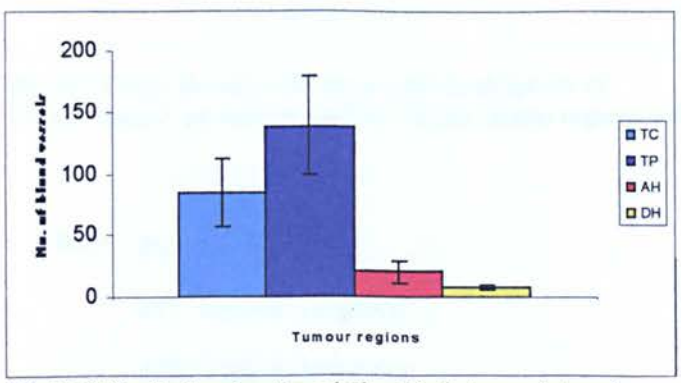


Fig 3.18 Graph showing the relationship between intramicro vascular density and tumour regions in colorectal tumours with extratumoral lymphatic invasion (n=12).

Key: TC- Tumour centre

TP- Tumour periphery

AH- Adjacent host tissue

DH- Distant host tissue

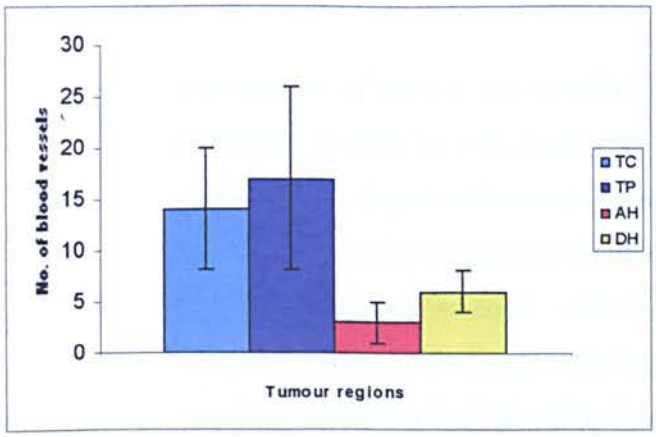


Fig 3.19 Graph showing the intramicrovessel density of Dukes' stage A tumours in the four distinct tumour regions (n=5).

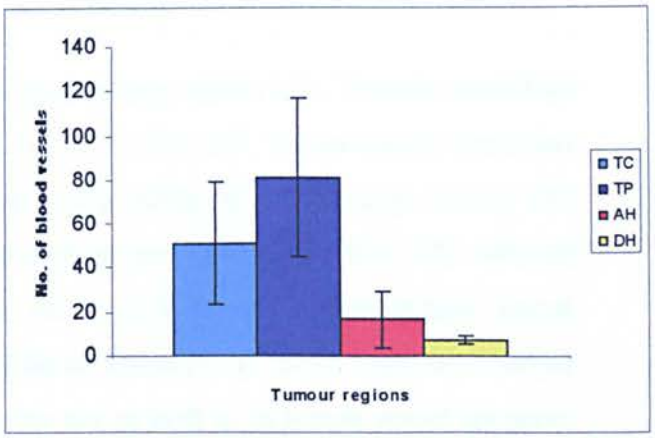


Fig 3.20 Graph showing the intramicrovascular density of Dukes' stage B tumours in the four different tumour regions (n=16).

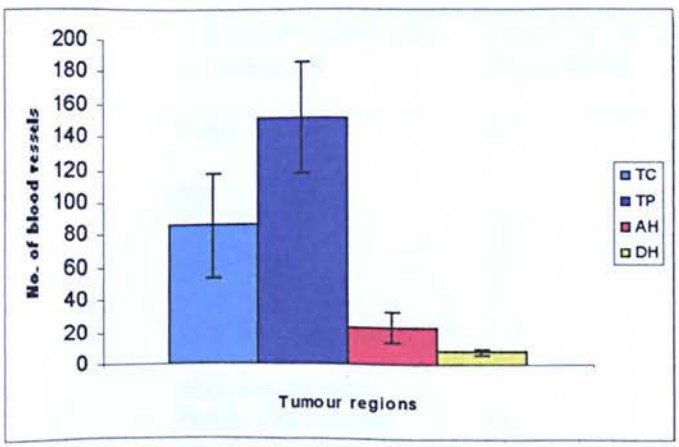


Fig 3.21 Graph showing the intramicrovessel density of Dukes' stage C tumours in the four distinct tumour regions (n=10).

Key: **TC**- Tumour center

TP- Tumour periphery

AH- Adjacent host tissue

DH - Distant host tissue

The number of vessels was noted to increase with tumour stage (table 3.1). Vessels quantified in Dukes' A were 31 ±13, Dukes' B 132 ±51 and Dukes' C 229 ±43. Vessel counts were also related to the degree of tumour differentiation with poorly differentiated tumours having 222 ±43 blood vessels, moderately differentiated tumours displayed a vessel count of 124 ±49 and well differentiated tumours with a vessel count of 31 ±13. Tumours with lymph vessel invasion contained a significantly high number of blood vessels (217 ±44). Tumours without lymphatic invasion had 96 ±59 vessels. Similarly, but not as high as in lymph vessel invasion, blood vessel invasion was associated with increased vessel numbers (177 ±63) when compared to non-invasion (42 ±28). Higher numbers of tumour blood vessels were associated with variables linked to poor prognosis for example poor histological differentiation, presence of lymph and blood vessel invasion and Dukes' C.

CLINICOPATHOLOGIC VARIABLES	NUMBER OF TUMOUR SPECIMENS	VESSEL COUNTS PER mm^2 AND MEAN ± S.D.	STATISTICAL SIGNIFICANCE
Total	31	-	-
Sex			
Female	13	161 (68)	N.S.
Male	18	136 (89)	
Histological grade of colorectal adenocarcinomas			
Poorly differentiated	12	222 (43)	
Moderately differentiated	14	124 (49)	p<0.001
Well differentiated	5	31 (13)	
Venous vessel invasion			
Absent	24	42 (28)	P<0.001
Present	7	177 (63)	
Lymph vessel invasion			
Absent	17	96 (59)	P<0.001
Present	12	217 (44)	
Dukes' stage			
A	5	31 (13)	
B	16	132 (51)	P<0.001
C	10	229 (43)	

S.D : standard deviation
N.S.: not significant
note only significant p values are described.

Table 3.1 Blood vessel counts of colorectal carcinomas and their correlation to clinicopathologic variables.

The presence of lymph vessels in colorectal carcinomas was only noted in Dukes' stage C tumours (table 3.2). Eight vessels were quantified (median 7, inter-quartile range 13) where as no vessels were observed in Dukes' A and B. Tumours from female patients presented with an average of 2 tumour lymph vessels (median 0, inter-quartile range 7). Lymph vessels were also noted in poorly differentiated tumours (count 7, median 4, inter-quartile range 13) as well as in tumours from patients with venous invasion (count 3, median 0, inter-quartile range 7) and lymph vessels invasion (count 7, median 4, inter-quartile range 12). Tumours from other clinicipathological parameters presented with no lymph vessels (table 3.2).

CLINICOPATHOLOGIC VARIABLES	NUMBER OF TUMOUR SPECIMENS	VESSEL COUNTS PER mm^2 AND MEDIAN AND INTER-QUARTILE RANGE.	STATISTICAL SIGNIFICANCE
Total	31	-	-
Sex			
Female	13	2 (0,8)	N.S.
Male	18	0 (0,7)	
Histological grade of colorectal adenocarcinomas			
Poorly differentiated	12	7 (4,13)	
Moderately differentiated	14	0 (0,1)	p<0.001
Well differentiated	5	0 (0,0)	
Venous vessel invasion			
Absent	24	0 (0,0)	P<0.003
Present	7	3 (0,7)	
Lymph vessel invasion			
Absent	17	0 (0,1)	P<0.001
Present	12	7 (4,12)	
Dukes' stage			
A	5	0 (0,0)	
B	16	0 (0,2)	P<0.001
C	10	8 (7,13)	

N.S.: not significant
note only significant p values are described.

Table 3.2 Lymph vessel counts of colorectal carcinomas and their correlation to clinicopathologic variables.

Discussion

Post-operative prognosis of patient colorectal cancer, a common neoplasm with a relatively high cure rate, depends strongly on this tumour's ability to metastasize to regional lymph nodes, a key element for Dukes' staging (Lee *et al*, 2000). None-the-less, Dukes' stage fails to predict outcome in a large proportion of patients and therefore, additional markers of tumour aggressiveness are required to complement patients' prognosis (Georgiou *et al*, 2006).

Angiogenesis has emerged as a major prognostic factor in many human malignancies and it is a prospective target for cancer therapy (van Moorselaar and Voest, 2002). Since Weidner *et al*, (1991) demonstrated that intratumoral microvessel density (IMD), assessed by immunohistochemical staining for endothelial cells, was an independent prognostic marker for breast carcinoma, other studies have reported IMD to be a possible prognostic marker in colorectal cancers (Takei *et al*, 2000; Nakayama *et al*, 2001).

Increase in IMD has been associated with regional and distant tumour spread and many studies have correlated IMD to survival (Ushijima *et al*, 2001; Khan *et al*, 2002; Acikalin *et al*, 2005). Therefore, it was important to investigate tumour vascularity and assess the number of vessels that a tumour elicits over time. This experiment was carried out in a mouse tumour model, grown from a human colo26 cell line, as a 'tumour blood vessel growth verses time' study which would have been impossible to carry out in human resected tumours. The present study also set out to investigate the spatial distribution of these vessels to allow us to understand their growth patterns and how the positioning of these vessels influence tumour growth.

It is established that newly implanted tumours elicit neovasculature within three days and establish a blood flow (Bicknell *et al*, 1997). As the implantation time progresses new vessel are continuously generated to develop a network of vessels that sustain and aid the growth of a tumour (Folkman 1990). Parallels have been drawn in the blood vessel growth patterns of the colo26 mouse tumour model. Tumours excised after eight days demonstrated the presence of a small number of tumour vessels indicating that at this time point this tumour had already started to establish a network of blood vessels hence, tumour angiogenesis was taking place.

Tumours were also excised at time points of 16, 18, 21 and 22 days and the highest vessel counts were noted in tumours with a growth time of 21 days. Never-the-less a steady increase in vessel counts as the age of the tumour progressed was observed. This suggested that this murine tumour induces an angiogenic response to produce a network of tumour vessels, recruited from either of the two pathways: a) from pre-existing host vasculature or b) vessels resulting from the angiogenic response of host vessels to cancer cells (Folkman 1995), over time.

Hansen-Algenstaedt *et al*, (2005) analysed a tumour model grown in SCID (severe combined immunodificient) mice. Tumour cells were propagated from a bone marrow derived endothelial cell line. The group assessed tumour vascularity over particular time points spanning from day 1 to day 85. They observed a gradual increase in the number of tumour vessels up to day 37 and then a plateau phase was noted, this was due to the cessation of capillary sprouting.

This study also demonstrated a peak in vessel number at a time point of 21 days but then a decrease in vessel number was scored at day 22. This may be due to the termination of the production of the vascular branching in the tumour resulting in a lower vessel count at day 22 than in a 21 day old tumour. The fact that endothelial cells proliferate at a slower rate than tumour cells may be another factor influencing the reduction in vessel number (Jain 2005). Alternatively, the decrease in vasculature could have been due to the fact that the new vessels recorded at 21 days started to degenerate as the tumour became increasingly necrotic as it increased in age and size (Breward *et al*, 2004).

This characteristic, of the cessation of angiogenesis at a particular time point in transplanted tumours, can lead to deficiency of nutrients to the tumour cells and also the removal of waste diminishes with increasing distance between each capillary. These factors may contribute to a decrease in rate of tumour growth (Baluk *et al*, 2005).

Qualitative studies revealed distinctive patterns of blood vessel distribution in xenografts of human colon cancer. A large number of vessels, notably capillaries, were clustered in the peripheral zones of the tumour. These clusters of capillaries were situated adjacent to vessels present in the host.

This pattern of distribution suggested that there was evidence of angiogenic activity taking place with in the tumour and tumour vessels were being generated from existing host vasculature. Reports show that there is increase in vascular density in the host tissue, adjacent to the invasive tumour edge, in transplanted mouse carcinomas (Folkman 1990). This is thought to be due to the transplanted tumour eliciting an angiogenic response for example, the tumour initiates a cascade of angiogenic events leading to the release of angiogenic growth factors and as a consequence the migration and proliferation of host endothelial cells into the murine tumours takes place (Kwon and Libutti, 2006).

Peripheral regions of the colo26 tumour were notably more vascular than the central regions which in most cases were avascular and necrotic. This complies with numerous reports that transplanted carcinomas in rodents have spatial heterogeneities in blood vessel distribution (Folkman 1990; Bicknell *et al,* 1997; Tufto *et al,* 1998). Such regional differences arise due to rapid growth of transplanted tumours where endothelial cell proliferation lags behind tumour cell proliferation. As a result tumour regions tend to become necrotic due to the lack of a blood supply (Bussink *et al,* 1998).

Further studies on the spatial distribution of tumour vessels were carried out in human colorectal carcinomas. Tumour sections displayed a characteristic arrangement of vessels which mimicked that of the mouse tumour transplant (colo26). High numbers of vessels were noted in peripheral regions as well as the invasive fronts. Takei *et al,* (2000) and Khan *et al,* (2002) also established that human colon tumours express a high number of vessels in their invasive edges. However, in this study, no distinct clusters of vessels in the adjacent normal colon tissue were noted near to the tumour border.

Avascularity and necrosis were prominent in poorly differentiated tumours rather than in well or moderately differentiated tumours of the human colon. Poorly differentiated tumours are associated with metastatic cancers, therefore, as the tumours grow and become invasive the distances between tumour vessels become larger. At some critical distance hypoxia will develop, depending on the oxygen consumption by the surrounding cells. The larger the distance between vessels the more hypoxic cells become and as the tumour expands cells start to die from the lack of oxygen and nutrients and as a consequence leads to regional necrosis (Rijken *et al,* 2000; Hoskin *et al,* 2004).

Necrosis has an effect on structural viability in or near necrotic regions making it difficult to generate meaningful quantitative data whereas neovascularisation in peripheral regions of a tumour takes place without such deleterious effects (Breward *et al*, 2004). Therefore, other factors which are characteristic of tumours and play a key role in tumour angiogenesis should also be analysed to better understand the pathogenesis of cancer.

Nevertheless, quantifying tumour angiogenesis by means of counting intramicrovessel density (iMVD) has proved to be an invaluable prognostic marker especially in breast cancer. However, there has been considerable variability between iMVD counts which in turn have given variable results linking iMVD to survival rates (Offerson *et al*, 2003).

A standardised method of conducting the count of microvessels in the tumour remains to be established (Takei *et al*, 2000). 'High microvessel count' (HMC), a method taking into account an area with the largest number of vessels in a tumour, has been reported to be a reliable indicator in breast cancers by Martin *et al*, (1997) and Hansen *et al*, (1998). On the other hand, Kato *et al*, (1999) reported that taking the 'average microvessel count (AMC) in the invasive borders of the tumour was the best technique of assessing tumour angiogenesis.

Ahlgren *et al*, (2002) reported that there is marked intratumoral variation of neovascularisation and due to this they concluded that 'hot spot' MVD assessment is more influenced by sampling procedure than a true reflection of the angiogenic capacity of a tumour. 'Hot spot' evaluation only quantifies selected areas of intense vascularisation, depicted by the investigator, in various regions of the tumour (Sarioglu *et al*, 2000). This approach is prone to error due to sampling bias (Offerson *et al*, 2003). Therefore, this study set out to find a more methodological approach that would reduce the inconsistencies involved in quantifying tumour vessels. To lower the variability in tumour vessel numbers the study set out to count all the vessels in the cross-sectional area, in a series of eight sections, taken from each of the colorectal tumours examined in the study. This method may have influenced the positive correlation between tumour blood vessel numbers to the clinical prognosis of the colorectal cancer patients.

For consistent and reliable data every aspect to the experimental protocol needed to be assessed. Therefore, to enable accurate measurement of tumour vessel numbers, three of the most commonly used endothelial markers vWF, CD31 and CD34 (van Moorselaar and Voest 2002) were investigated for tumour vessel endothelium specificity.

Upon analysis it was found that both CD31 and CD34 provided more consistent results with intense and specific endothelial staining. The reasons for vWF's lack of specificity may be attributed to the fact that it is expressed by mature endothelium where as tumour vessel endothelium is newly formed and actively growing. CD31 and CD34 are known to be selective to endothelium in cancer tissue (Boyle *et al,* 2003), hence, optimal results were obtained in this study by the two endothelial markers. The superiority of the two markers is contested and certain groups have found that vWF is more specific in tumour tissue. The contrast in results may be due to the heterogeneity of vessel endothelium from different tumour tissue (Naresh *et al,* 2001).

Further to this, it was subsequently discovered that, after morphometrical analysis, CD34 was only positive in tumour blood vessel endothelium and that the vessels left unstained were actually tumour lymph vessels. CD31 highlighted both types of vessels and therefore, this antibody gave higher vessel numbers.

These results now directed the study in two directions: 1) to assess the correlation of tumour blood vessels with colorectal cancer prognosis and 2) to investigate the significance of tumour lymph vessels in human colorectal cancers and if they have any influence on patient prognosis.

The role of angiogenesis in the growth, invasion and metastasis of solid tumours is well established. It has been found to be a strong independent predictor of prognosis in tumours of the breast, prostate, lung, skin, larynx, oral cavity and the bladder. However, the role of angiogenesis in colorectal cancer is disputed and reported findings are conflicting (Acikalin *et al,* 2005).

The biological behaviour of colorectal carcinomas is related, to some extent, to the number of clinical and pathological parameters. Parameters which have been found to have prognostic value are: tumour stage, grade and the presence of vascular invasion (Lee *et al,* 2000). The

most important prognostic factor is the pathological extent of the disease, namely tumour stage and hematogenous and nodal metastasis. These are a major cause of death in patients with colorectal carcinoma (Mathur *et al*, 2001).

The present study set out to examine the relationship between the different clinicopathological variables and the extent of tumour vascularity in human colorectal carcinomas by using optimal methods previously assessed. Tumours with lymphatic and venous invasion presented with higher blood vessel counts. The value of the counts was almost double to that of non-vascular invasion. This suggests that angiogenesis increases the possibility of cell invasion and the subsequent metastasis (Mathur *et al*, 2001). Lymph node status is the most important determinant of adjuvant therapy. Greater percentage of patients with node-positive colon cancer experience recurrence of the cancer than those without. Therefore, another prognostic parameter such as tumour microvessel counts may help to define more accurate prognosis (Acikalin *et al*, 2005).

Poorly differentiated tumours are classed as high grade malignancies. Such tumours tend to be deeply infiltrating and, histologically, show a greater number of mitotic cells than the moderate or well differentiated tumours (Goligher 1984). In this study a high number of blood vessels were quantified from poorly graded tumours and the lowest number of vessels was observed in well differentiated ones. The results demonstrated that increased vascularity is associated with higher grade malignancies in human colorectal cancer.

Pathological staging by the Dukes' classification system is a traditional method to assess the clinical outcome of a cancer patient (Lee *et al*, 2000). The system categorises tumours according to the extent of the disease. The categories include grades from Dukes' A which encompasses adenomas and well differentiated tumours, with a high rate of survival, to Dukes' D which denotes metastatic disease with poor prognosis (Goligher 1984).

The present study assessed tumours from Dukes' A, B and C groups and verified that higher number of vessels were present in Dukes' stage C colorectal tumours. This clearly indicated that a greater number of tumour blood vessels were associated with histological characteristics linked to metastatic cancers.

Saclarides *et al*, (1994) found a positive correlation between microvessel counts and the staging of colorectal cancer. A strong association between high tumour vessel counts and tumour size, metastasis and poor survival was also demonstrated. Zheng *et al*, (2003) also found a significant correlation between microvessel density and tumour grades. Tanigawa *et al*, (1997) analysed human colorectal adenocarcinomas from 133 patients and reported a strong relationship between tumour vascularity and overall survival of the patient. They also described that MVD was closely linked to haematogenous metastasis. However, Lindmark *et al*, (1996) and Banner *et al*, (1998) found that high tumour microvessel counts were associated with patients with longer survival rates. Therefore, due to such conflicting data this study set out re-assess if tumour blood vessels have an influence on the prognosis of colorectal cancer by optimising experimental conditions to reduce variability in results.

As tumours disseminate with in the body by haematogenous as well as lymphatic spread clinical and pathological observations have long suggested that the initial dissemination of primary tumours is via lymphatic vessels (Sleeman 2000). Kishimoto *et al*, (2003) suggested that tumour lymphangiogenesis may play a vital role in lymph node metastasis particularly in the early stages of oral squamous cell cancer. However, no correlation between advanced stages of the cancer and tumour lymph vessel counts was found.

This study demonstrated that there was a correlation between blood and lymph vessel invasion and the number of tumour lymph vessels present in colorectal cancer. The study also presented a strong correlation between tumour lymphangiogenesis and Dukes' stage C tumours which is contradictory to the results obtained by Kishimoto *et al*, (2003).

It is now established that the angiogenic growth factor VEGF-C is known to specifically induce lymph vessel formation via the VEGF-R3 (a receptor restricted to the lymphatic endothelial cells) (Karkkainen and Alitalo, 2002). Therefore, studies have now confirmed the presence of tumour lymph vessels and are able to now investigate the significance of lymphangiogenic associated molecules. For instance, Akagi *et al*, (2002) demonstrated that VEGF-C protein and its mRNA do not correlate with colorectal tumour vascularity. However, increased expression of VEGF-R3 mRNA in human gastric carcinomas, has been observed, in comparison to the normal gastric mucosa (Amioka *et al*, 2002).

Histological grading, Dukes' staging, and blood and lymph vessel invasion are the main clinicopathological variables used to assess the extent of cancer spread (Acikalin *et al*, 2005). This study has demonstrated that the clinicopathological variables of colorectal tumour associated with poor prognosis bear greater numbers of blood and lymph vessels. These findings support the importance of tumour vessel density to the progression of colorectal cancer and suggest that tumour vessel counts may be another useful marker for the evaluation of patient survival.

Chapter 4

Cathepsin B and dipeptidyl peptidase IV (DPPIV) in colorectal cancer

4.1 Introduction

Proteases are enzymes which catalyse the hydrolysis of peptide bonds (Yousef *et al,* 2003). They are widely distributed in cells, and are either associated with plasma membranes and subcellular organelles or secreted from the cells to participate in local proteolysis. In 1960, Hartly proposed that proteases should be classified into four groups: serine, cysteine, aspartate or metallo- proteases (table 4.0). They are also classified as endopeptidases due to the type of amino acids present at the catalytic site, however, the term 'protease' is more suitable for the enzymes and will be used in this thesis (Nduwimana *et al*, 1995).

All proteases are intracellular up to a certain stage in their life and then they are either exported to the extracellular medium where they perform their biological functions or are synthesised and stored in granules before being secreted by the cells. Some proteases act on peptides and proteins which are localised inside a cell whilst others cleave extracellular proteins that are transported into the cell by endocytosis (Koblinski *et al*, 2000).
Regardless of the subcellular localisation of proteases, they are primarily responsible for the hydrolysis of peptide bonds within a peptide chain. For example, they are involved in the essential common step of converting precursors (pre- or proproteins) into biologically active proteins (Sloane *et al*, 2005).

Group	*Enzymes*	*Subcellular compartment*
Serine endopeptidases (SEP)	Prolyl endopeptidase	Cytosol
	ATP-dependent SEP	Mitochondria
	Processing proteases	Mitochondria and endoplasmic reticulum
	Trypsine-like SEP	Plasma membrane
	Chromatin proteases	Nuclei
	Cathepsin R	Endoplasmic reticulum
Cysteine endopeptidases (CEP)	Cathepsin B,H,L,M,N,S,T	Lysosomes
	Calpains	Cytosol
	ATP-dependant CEP	Cytosol
	Metal-dependant CEP	Cytosol
Aspartate endopeptidases	Cathepsin D,E	Lysosomes
Metallo-endopeptidases	Endopeptidases 24.11	Plasma membrane
	Meprin	Plasma membrane
	PABA-peptide hydrolase	Plasma membrane
	Procollagen C-peptidase	Plasma membrane
	Metallo-endopeptidase	Cytosol

Table 4.0 Proteases found in mammalian cells adapted from Nduwimana *et al*, 1995.

4.1.1 *Protease regulation*

Proteases must be regulated to avoid inappropriate and uncontrolled protein degradation. Their regulation depends on four main factors: 1) zymogen activation, 2) protease-protease inhibitor equilibrium, 3) genetic control of protease expression and 4) tissue/subcellular localisation (Nduwimana *et al*, 1995).

1) Proteases are synthesised as latent forms called zymogens which must be converted to mature, active enzymes (Koblinski *et al*, 2000). This takes place in several proteolytic stages for example, the activity of trypsin results from the action of enterokinase on trypsinogen. The process of blood coagulation is an example of proteolytic cascade activation (Nduwimana *et al*, 1995).

2) Protease-protease inhibitor equilibrium describes the event of stopping proteolytic activation. This prevents biological events such as the expansion of a clot outside a wound site, which would otherwise cause thromboses or emboli. Protease inhibitors are the molecules that modulate and regulate proteolytic activity (Talieri *et al*, 2004). For each group of proteases, polypeptide factors (proteolytic inhibitor involved in modulating and regulating proteolytic activity) have also been isolated in cells (Sloane *et al*, 2005).

For cysteine proteases, cystatins and calpastins have been identified as the key inhibitors. Serpins are a category of peptides isolated as inhibitors for serine proteases. The equilibrium between proteases and their inhibitors probably determines cell behaviour and indeed some pathological conditions can be explained by the disruption of this equilibrium for example, tumour angiogenesis (Nyberg *et al*, 2006).

3) In addition to the regulation of proteolytic activity by inhibitors, genetic control of protease expression also occurs. Gene expression of proteases is induced by other factors such as growth factors, cytokines, tumour promoters and oncogenes (Yousef *et al*, 2003).

4) The regulation of proteases can also be dependent on tissue/subcellular localisation of proteases. Protease expression has been shown to be tissue specific and differ in concentration within different cell types. This has been demonstrated by many studies using immunohistochemical methods (Herszényi *et al*, 2000 and Bleeker *et al*, 2000). Subcellular localisation is an important factor for proteases as the environment has an influence upon their stability and activity. For example the cathepsin proteases are localised in lysosomes due to the acidic pH of the environment which is essential for their activity (Koblinski *et al*, 2000).

4.1.2 *Protease functions*

The presence of intracellular proteolytic activity has been reported in the kidney, intestine, anterior pituitary, leucocytes, erythrocytes and spermatozoids. The extensive distribution of proteases in both organs and sub-cellular compartments suggests that they play a key role in physiological and pathological events (Nduwimana *et al*, 1995).

Proteolysis plays an important part in certain physiological processes such as the immune response. This requires the participation of the enzymes in complement activation, phagocytosis and antigen presentation. Leucocytes display abundant amounts of cytoplasmic granules, containing proteolytic enzymes, mainly when they are activated. Cytotoxic T lymphocytes and natural killer cells produce secretions (serine endopeptidases) from the cytoplasmic granules. They destroy infected or tumour cells, this demonstrates the importance of such enzymes in immunity (Nduwimana *et al*, 1995).

The involvement of proteases in the migration of activated immuno-competent cells to an inflammatory source was described by Simon *et al*, (1991). They discovered that the murine T cell-specific serine endopeptidase (MTSP-1) is involved in the migration of activated immuno-competent cells to the inflammatory source. It degrades the collagen IV of the vascular basement membranes, proteoglycans and fibronectin, which then allows the migration of lymph cells to the site of inflammation. The basement membrane degradation is attributed to metallo-endopeptidases which are released from neutrophils and macrophages (Karagiannis and Popel, 2006).

Proteases are also responsible for the maturation of growth factors, their receptors and cell adhesion molecules. They participate in cellular migration and fusion events. Embryonic development, post-natal growth, wound healing and tumour growth require neovascularisation induced by angiogenic factors, of which bFGF (basic fibroblast growth factor) is the most powerful. In fact, bFGF induces the proliferation and migration of endothelial cells; it stimulates the expression of plasminogen activators and collagenase in these cells, which enables channels for the neovessels to be formed in the basement membrane (Bremnes *et al*, 2005).

Endothelial cell migration out of the vascular system is associated with fragmentation and dissolution of the vascular basement membrane and adjacent matrices. These structures are of complex composition and contain proteoglycans, glycoproteins, different collagen types and elastin, and their degradation by the migrating cells would require a large number of proteases directed against this variety of proteins (Koblinski *et al*, 2000). Schmitt *et al*, (1992) showed that bovine aortic endothelial cells grown on these structures degraded all of the components of the matrices through the production of different proteases.

Several proteases are implicated in cell migration, such as the plasminogen activator of the urokinase type (uPA) and the metalloproteases, which include the metalloproteases, the collagenases, gelatinases and stromelysin. The regulation and control of these proteases is mediated by specific molecular interactions and by a controlled release of proteases and their inhibitors (Chin *et al*, 2005).

Angiogenesis necessitates the activation of a series of molecular events that are responsible for endothelial cell migration, proliferation and differentiation into capillary structures. Proteolytic activity is critical for migration and invasion of endothelial cells into neighbouring tissue (Im *et al*, 2005).
The best characterized protease system involved in angiogenesis is the plasminogen activator (PA) system. Here, uPA and tissue-type PA (tPA) convert plasminogen to plasmin. Plasmin has a wide spectrum of activities and can degrade fibrin as well as extracellular matrix (ECM) compounds such as laminin and fibronectin. Plasmin can also activate metalloproteases and elastases. uPA too, contributes to matrix degradation by activating other proteases such as collagenase (fig 4.0) and plays an important part in physiological and pathological tissue remodelling (Duffy and Duggan, 2004).

uPA is known to induce cell migration and invasion in endothelial cells. The mechanism by which migration is induced has been characterised in the epithelial cell line WISH by Busso *et al*, (1994). The study describes the cascade as starting from the activation of uPA receptors by pro-uPA. This induces migration and the phosphorylation of a protein on serine protease residues. uPA must be localized on the cell surface in order to promote migration. Growth factors such as FGF and VEGF stimulate uPA production and endothelial cell activity (Bremnes *et al*, 2005).

Aside from its role in promoting tumour angiogenesis, the PA system may control two inhibitory signals. The first is the generation of transforming growth factor β (TGF-β) and the second is the formation of a fragment of plasminogen. This fragment has recently been reported as having properties of a specific angiogenic inhibitor and is named angiostatin. The inhibitor was first localised in Lewis lung carcinomas and the investigation subsequently found that after the removal of the tumour a rapid onset of angiogenesis was noted. Therefore, this led to the conclusion that the tumour was the source of angiostatin (Li *et al*, 2001).

The inhibitory activity is specific for endothelial cells and blocks neovascularisation, therefore, plasminogen not only plays a part in matrix degradation but also generates an angiogenesis inhibitor (Duffy and Duggan, 2004).

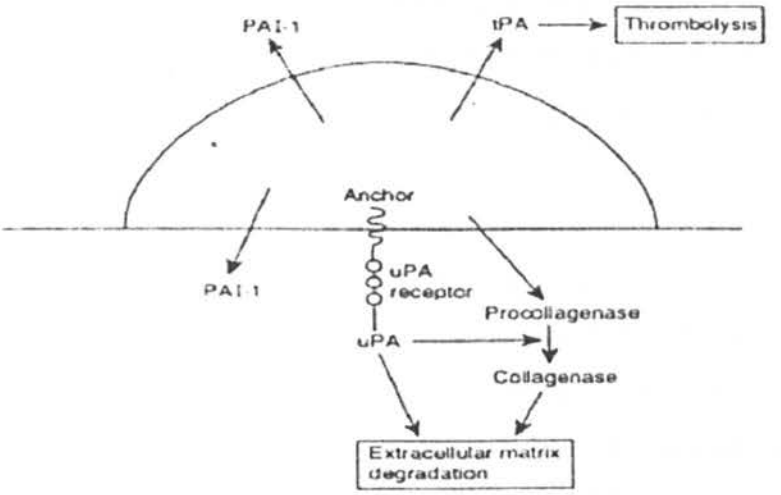


Figure 4.0 Diagram showing the sequence of events involving the protease uPA in the process of extracellular matrix degredation. uPA and latent collagenases are secreted are secreted to the basal pole of the endothelial cell. After secretion, uPA binds to a specific receptor expressed on the cell surface and participates in the activation of the procollagenases. uPA and collagenases are involved in extracellular matrix degradation. tPA is secreted in the luminal side of the endothelial cell and is involved in the fibrin clot degradation and consequently in thrombolysis (Menashi *et al*, 1993).

4.1.3 *Proteases and cancer spread*

Cell migration and extracellular degredation are two of the stages in the step-wise process of tumour progression. Tumour cell invasion involves the attachment of tumour cells to the underlying basement membrane, local proteolysis and migration of tumour cells through the proteolytically modified region (Nyberg *et al,* 2006).

Local proteolysis is facilitated by proteases outside the tumour cell, perhaps bound to the cell surface and/ or secreted from the tumour cell. Proteases inside the tumour cell also participate in local proteolysis by digesting phagocytosed extracellular matrix. In order for a cell to metastasis, it must be able to move into the vasculature, survive in the circulation, move out of the vasculature, invade into the surrounding tissue and grow (Van Damme *et al*, 2004).

Studies show that proteases are involved in tumour growth at both primary and metastatic sites. The cells involved in the process ie. tumour, stromal, lymphocytes, endothelial and the extracellular matrix cells express proteases that participate in many of these steps (Koblinski *et al*, 2000).

Many studies have also demonstrated their involvement in pathological conditions. Studies show that tumour invasion and metastasis are associated with elevated levels of proteases. Tissues of primary cancer and/or metastases of the breast, ovary, endometrium, cervix, uterine, bladder, kidney, prostate, liver, lung, and the gastrointestinal tract have been reported to contain high levels of proteases such as cathepsin B, D, L or uPA compared to benign tissue. Hence, evidence has now accumulated that the amount of protease correlates to the degree of malignancy therefore, they may have the potential to serve as prognostic factors in solid tumours (Schmitt *et al*, 1992 and Berdowska 2004).

uPA was the first protease shown to be a prognostic marker in human malignancy. It is an important prognostic marker for breast, colorectal and bladder cancers. The aspartyl protease cathepsin D has shown to also be a prognostic marker for breast cancer however, there is conflicting data showing its relevance in disease progression in subgroups of breast cancer patients (Herszényi *et al*, 2000).

Cathepsin L has shown to be a stronger marker for breast cancer than cathepsin B and matrix metalloprotease 2 (MMP-2) was significantly associated with the malignancy. High levels of MMP-2 have been found in patients with advanced gastric cancer and ones who died from the disease, therefore, MMP-2 is associated with poor prognosis (Egeblad and Werb 2002).

4.1.4 *Cathepsin B (CB)*

Cathepsin B is a lysosomal cystein protease of the papain family and can be found in the human liver. CB is a major lysosmal proteinase in that the enzyme can contribute up to 10% of the total lysosomal protein. At the mRNA level rat cathepsin B varies in various tissues with the highest levels in the kidney and progressively lower levels in spleen, lung, brain, heart, liver and thymus with no CB detected in whole pancreas. So far, there has not been a study to identify mRNA levels of the enzyme in normal human tissue. However, cathepsin B activity is highest in liver, thyroid, kidney and spleen (Berdowska 2004; Im *et al*, 2005).

Human pro-cathepsin B is synthesised from a single gene, located on chromosome 8p22 and comprises of at least twelve exons. However, there is more than one mRNA species, with the transcripts varying in size depending on the length of the un-translated regions (Mai *et al*, 2000). The mRNA is translated on membrane bound ribosomes, and the signal peptide is cleaved co-translationally. The newly synthesized pro-enzyme passes into the rough endoplasmic reticulum and then on to the Golgi apparatus. Here the enzyme is glycosylated and the membrane bound mannose-6-phosphate receptors in the trans-Golgi network bind the phosphorylated protein and is transported to lysosomes. The receptors transport the, now, pro-cathepsin-B to an acidic compartment where the enzyme is activated (fig. 4.1) (Berquin and Sloane 1996).

Under normal conditions of activation the pro-peptide is degraded once it is cleaved from the pro-enzyme. The body has an in-built mechanism to protect it from damage by inappropriate proteolysis. It does this providing a neutral to alkaline pH environment where the enzyme loses stability (Strojnik *et al*, 2005). However, under some circumstances, cathepsin B is secreted from the cell as a non-covalent complex between the cleaved propeptide and the active enzyme. This may represent a reservoir of potential extracellular proteolytic activity (Linebaugh *et al*, 1999).

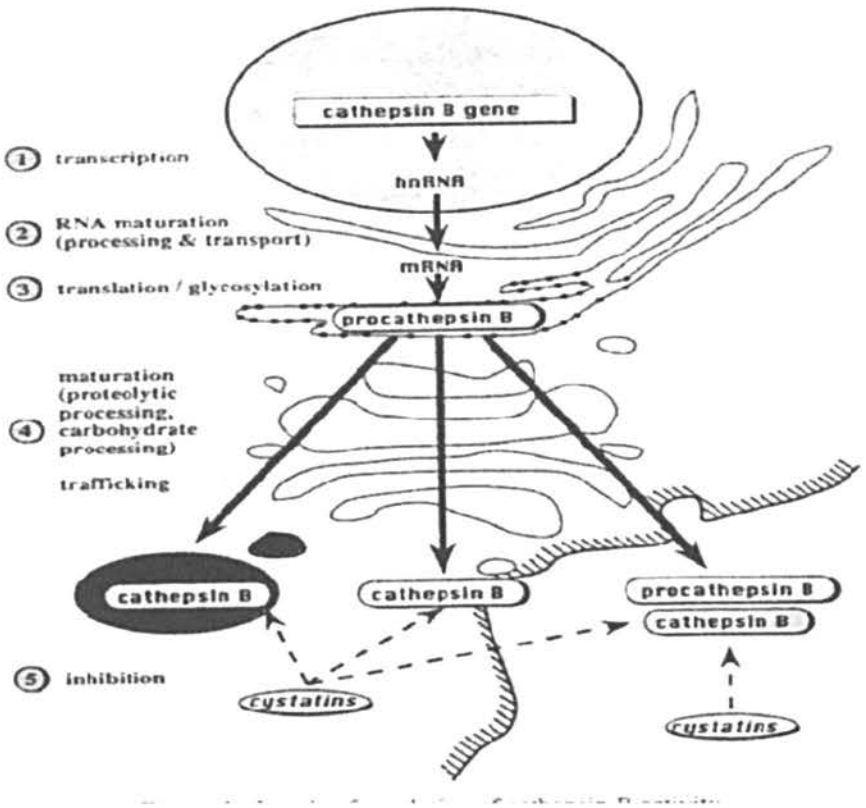


Figure 4.1 **The synthesis of the lysosmal protease cathepsin B (Berquin and Sloane, 1996).**

Evidence suggests that CB is also involved in tumour invasion and metastasis. Increases in expression at the mRNA and protein levels, increased protein levels, increased activity and altered trafficking (localisation and secretion) of CB have found to correlate with malignancy of murine and human tumours (Sloane *et al*, 2005).

Tumour progression is a multi-stage process which includes several steps that require proteolytic activity. The most representative are: local invasion, angiogenesis (necessary for tumour growth beyond a diameter of 2mm), intravasation (when tumour cells enter the vascular system), and extravasation (when tumour cells exit the vascular system at the metastatic site). Berquin and Sloane (1996) hypothesised that the alterations in expression and trafficking of CB protease, seen in malignant cells, is responsible in part for enhanced invasive capability of tumour cells.

CB degrades the extracellular matrix proteins: laminin, fibronectin and type VI collagen at neutral as well as acidic pH. These are the components of the basement membrane of blood vessels, a structure which forms a barrier to metastasizing tumour cells (Berndt *et al*, 2000 and Turk *et al*, 2004).

Several enzymes are secreted as inactive precursors which require activation by another protease ie. CB can activate prourokinase to urokinase. In turn, procathepsin B can be activated by serine proteases and urokinase. Thus, secreted CB might directly or indirectly facilitate the local invasion and distant metastasis of tumour cells (Koblinski 2000).

Of all cathepsins, CB has been the most extensively investigated due to its role in cancer. Accumulating studies have implicated CB in a variety of human tumours. High levels of enzyme activity have been found in breast, colon, cervix and ovary, stomach, and lung cancer. There is evidence that increased CB activity and protein concentration is associated with aggressive tumour behaviour, early relapse, and shorter survival as well as higher lymph node invasion (Troy *et al*, 2004).

Many studies have shown elevated activity of CB in human colon tumours in comparison to the normal human colon. Campo *et al*, (1994) showed that elevated activity and staining of the enzyme occurred in human colon tissue compared to normal tissue. A correlation between activity and clinical staging was also noted. Tumours staged for distant metastasis (Dukes' stage C and D) displayed higher CB activity.

Talieri *et al*, (2004) also discovered that increased levels of CB antigens were correlated with Dukes' stage of human colorectal adenomas and carcinomas. However, there was no correlation to the antigen level and lymph node status.
Satoh *et al*, (1996) observed that activity of this enzyme was higher in adenocarcinomas than compared to the adjacent normal tissue. They also noted that higher levels existed in tumours that had invaded the serosa and subserosa. Such studies support the fact that activity is related to the over expression of CB mRNA.

Nevertheless, Adenis *et al*, (1995) stated that, their results showed no correlation between CB activity and clinical stage. This shows conflicting results and as yet CB activity has not shown to be a reliable prognostic marker in human colon cancer.

4.1.5 *Dipeptidylpeptidase IV (DPPIV)*

The enzyme DPPIV was first discovered, in 1966, by Hopsu-Havu and Glenner in rat liver homogenates. Since then the enzyme has been extensively studied and investigators have realised that the enzyme has a multifunctional role. Not only does it execute proteolytic activity but also serves as an endothelial cell molecule known as CD26 (Dobers *et al*, 2000).

DPPIV is a cell surface protein that is expressed by many cell types. In humans, it is expressed on epithelial cells of liver, intestine, kidney and in a soluble form in the circulation however, its expression is regulated in T and B cells. These findings indicate the heterogeneity of the molecule (Ben-Shooshan *et al*, 2002).

The human CD26/DPPIVgene is located on the long arm of chromosome 2 and spans approximately 70kb. It is a serine protease that is able to degrade collagen, thereby facilitating trafficking of cells through the extracellular matrix. The enzyme has been reported to be one of the mediators of lymphocyte migration in the thymus from the cortical region to the medulla during maturation (Boonacker and Van Noorden, 2003).

Aside from proteolytic activity CD26/DPPIV also functions as a receptor. The human prostate tumour cell line 1-LN synthesizes and secretes large amounts of plasminogen activators and matrix metalloproteases (MMP) and it was demonstrated that in these cells the receptor for plasminogen type II is CD26/DPPIV (Boonacker and Van Noorden, 2003).

Clinical assessment supports data that indicates some cancers favour select secondary locations for metastasis. Prostatic carcinomas and small cell carcinomas of the lungs preferentially colonize bones and the brain respectively, whilst breast carcinomas most frequently metastasis to the lungs, but also to liver, bones, brain and adrenals (Cheng *et al*, 1998). CD26/DPPIV has been found to act as a cell adhesion molecule that mediates breast carcinoma cells to the lung. The enzyme has a high binding affinity for lung metastatic rat breast carcinoma cells. Its binding affinity parallels the propensity with which these tumour cells metastasise to the lungs.

Subsequent studies have confirmed that CD26/DPPIV is an endothelial cell adhesion molecule for rat breast cancer cells and mediates metastasis by these tumour cells. The CD26/DPPIV ligand is identified as tumour-cell associated fibronectin (Cheng *et al,* 1998).

Triggering of CD26/DPPIV can induce MMP-9 expression and when this occurs in cancer cells, it may facilitate metastasis. On the other hand, CD26/DPPIV also plays a role in the differentiation of cells, thereby, blocking the cells from conversion into a malignant phenotype (Kikkawa *et al,* 2005). Wesley *et al,* (1999) inserted a mutation in the enzyme molecule and discovered that the mutated CD26/DPPIV did not suppress tumourigenicity or anchorage-dependent growth of cancer cells, nor did it reverse the block in differentiation, showing that DPPIV activity is required in suppressing the malignant phenotype.

This has been demonstrated in the differentiation of gliomas which has shown to be related to CD26/DPPIV activity and its subcellular distribution pattern. Low activity is found in poorly differentiated gliomas and high activity in well differentiated ones. Decreased enzyme levels have also been observed in metastatic prostate tumours (Boonacker and Van Noorden 2003). Kikkawa *et al,* (2005) demonstrated that ovarian carcinoma cell lines with higher CD26/DPPIV expression showed less invasive potential. Down-regulation of the enzyme is also exhibited in endometrial adenocarcinomas with increasing grades (Khin *et al,* 2003).

DPPIV/CD26 has been shown to be expressed in some human colon cancers and human colon cancer cell lines (Darmoul *et al,* 1992). Cordero *et al,* (2000) investigated the diagnostic efficiency and prognostic value of CD26/DPPIV serum levels in colorectal cancer. There was a significant difference between CD26/DPPIV levels in healthy donors and cancer patients and showed that the diagnostic efficiency of the enzyme is higher in patients at the early stages of the disease. However, there was no correlation between CD26/DPPIV activity with Dukes' stage classification, age, gender, tumour location or degree of differentiation.

Data linking the metastatic potential of human colorectal carcinomas to the levels of CB and DPPIV enzyme activity is relatively lacking. The best characterised system involved in angiogenesis is the PA system (Chin *et al,* 2005), therefore, further studies to assess the significance of CB and DPPIV in this process would aid a better understanding of colorectal tumour angiogenesis.

4.2 Aim

The aim of this chapter was to assess the presence of protease enzymes CB and DPPIV in human colorectal carcinomas and assess their correlation to metastatic parameters associated with colon cancer progression. This was carried out by a) assessing the presence of the enzymes in human colon, rectal and endothelial cells. b) Once evident in these cell lines further studies of identification was conducted in human colorectal carcinoma tumour resections of varying grades and c) levels of CB and DPPIV enzyme protein levels as well as mRNA levels were analysed in colorectal tumours ranging from Dukes' stages A-C.

4.3 Materials and methods

4.3.1 *Cell Culture*

The endothelial cells (HUVEC's) were isolated from human umbilical cords by a modification of the method originally described by Jaffe et al. 1973. The human umbilical cords were obtained from the haematology department, St George's in Tooting under their ethical regulations.

The vein within the umbilical cord was gently flushed with HBSS medium (Life Technologies, U.K.) until the effluent ran clear. The vessel was then filled with collagenase (Boehringer Mannheim, U.K.). After 20 minutes of incubation the detached endothelial cells were flushed out using HBSS medium with fetal calf serum (Life Technologies, U.K.).

The cells were then centrifuged and re-suspended in DMEM (Life Technologies, U.K.) with 20% of fetal calf serum, 2% heparin and 3mg/ml of endothelial growth supplement (Sigma, U.K.), 50 U/ml of heparin (NHS. U.K) and grown until confluent in T25 culture flasks (Life Technologies, U.K.). HUVECs were used between passages 1 and 3 for all the following experiments. The cords were used within 24 hours of delivery.

The HT29 (human colon carcinoma) and HRT 18 (human rectal carcinoma) cells used in the experiment were obtained from the European Collection of cell cultures (ECACC) and cultured accordingly in T25 flasks.

Human liver cells (ECACC) were cultured to use as a positive control for cathepsin B and human thyroid follicular epithelial cells (ECACC) were used as a positive control for DPPIV.

For effective cell culture propagation HT29, HRT 18, HUVECS, human liver cells and human thyroid follicular cells were passaged every 3-4 days and incubated at 37°C in 5% CO_2. Cell culture media used for HT29 was MEM (Life Technologies, U.K.) with 50 mls of fetal calf serum, 50 mls of 2mM glutamine, 4.5 mls of penicillin, 4.5 mls of fungizone and 4.5 mls of non-essential amino acid (NEAA). The only difference with the HRT18 cell culture media RPMI (Life Technologies, U.K.) was that it did not contain NEAA (Life Technologies, U.K.).

Upon confluence the cells in T25 flasks were then passaged into chamber well slides (Fisher Scientific U.K.) and incubated until they also reached confluence in order to carry out immunocytochemistry.

4.3.2 *Immunocytochemistry*

Confluent cells grown in chamber slides (each slide consisted of two wells) were washed with phosphate buffer solution (PBS) (Life Technologies, U.K.) to clean cells of existing culture media. Cells were then fixed in equal volumes of acetone and methanol for 10 minutes. They were then washed with Tris buffer saline (TBS).

To target cathepsin B and DPPIV antigens the cells were incubated with the primary antibodies mouse anti-human CD26 (molecule also known as DPPIV) (Serotec Ltd. U.K.) and sheep anti-human cathepsin B (Serotec Ltd. U.K.). A chamber on each slide was investigated for either cathepsin B or DPPIV with the adjacent chamber used for a negative control for the antibody in question. This process was carried out on all cell lines: HT29, HRT 18, HUVECs as well as the positive control cell lines which were human liver cells (positive control for cathepsin B) and human thyroid follicular epithelial cells (positive control for DPPIV). (Note: the negative control was conducted to highlight non-specific bonding of the antibodies).

A universal secondary antibody raised in donkey: biotinylated secondary reagent conjugated to biotin (Serotec Ltd. U.K.), a reagent designed for the use in immnohistology to detect mouse, rabbit or sheep primary antibodies, was used as the secondary step of the immunocytochemistry process.

Initially, varying titrations of the antibodies were investigated to achieve an optimal working dilution. This was necessary as both the primary antibodies and the secondary reagent were not tested for use in immunocytochemistry. The primary antibodies were diluted in phosphate buffered saline (pH 7.4) and the secondary antibody in phosphate buffered saline (pH 7.2).

The optimal dilution for cathepsin B used was 1:50, DPPIV was 1:10 and the secondary reagent was 1:100. Cells were incubated at 37°C for 1hour in primary antibody and then washed in TBS carefully so as not to detach cells from the wells. They were then incubated at 37°C for half an hour in secondary antibody. The incubation period was also assessed for optimal times prior to the experiment. For negative controls the primary antibody was omitted from the procedure.

After incubation the cells were rinsed once again in TBS and the ABC (strept-avidin-biotin complex) reagent (Vector Laboratories U.K.) was added and incubated for half an hour at room temperature. The cells were then washed in TBS and stained with 3,3' - diaminobenzidine substrate (Dako, U.K.) for 2 to 12 minutes depending on how fast the stain takes to develop.

Finally, the cells were washed again with TBS and then the side panels on the slides that made up the chambers were removed. The slides with the stained cells were mounted with a coverslip using histomount and observed under a light microscope.

Tissue sections of human colon tumour tissue and normal human colon tissue were also investigated for the presence of the proteases DPPIV and cathepsin B by using immunohistochemical methods.

4.3.3 *Immunohistochemistry*

Tumour specimens from 31 patients were obtained from the St. George's Histopathology archives under their ethical approval guidelines. Clinical profiles of patients from which the tumour specimens were excised were also obtained. Details such as age, sex, tumour histology (venous and lymphatic invasion) and tumour grade (Dukes' stage) were noted (see table 3.1a for the number of tumours in each category). These were surgical resections from patients of varying histological grades of colorectal carcinomas. All specimens used in the study were from patients who had no prior chemo- or radio- therapy. Eight sections of tumour tissue were cut at 5um thickness and mounted on positively charged slides. These were incubated at 37°C before any staining procedure was performed. At least eight sections (approximately a total of 40um of the tumour) were cut from every patient sample for immunohistochemical analysis.

Sections were de-paraffinised in two changes of xylene (BDH, Poole, U.K.) and then rehydrated in absolute alcohol and then in 95% ethanol. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide solution for 30 minutes and then antigen retrieval was performed. This was done by placing slides in a closed container filled with citrate acid buffer and then microwaved at an optimal time of 5 minutes. Slides were then immersed in 0.1% bovine serum albumin/Tris-HCL for 20 mins to block the non-specific binding of antibodies.

Four sections from each tumour grade were incubated with sheep anti-human cathepsin B (Serotec Ltd. U.K.) at a dilution of 1/100 and at an optimal time of 1 hour. The rest of the sections were incubated with the primary antibody mouse anti-human CD26 (molecule also known as DPPIV) (Serotec Ltd. U.K.) at an optimal dilution and time of 1/50 and 1 hour respectively. All slides were then incubated with secondary antibody: biotinylated secondary reagent conjugated to biotin (Serotec Ltd. U.K.) at a dilution of 1/150 for 30 mins. The primary antibodies were diluted in phosphate buffer saline (pH 7.4) and the secondary antibody in phosphate buffer saline (pH 7.2) and the incubation chamber was set at 28°C.

Sections were then incubated with the avidin-biotin-peroxidase complex for 30 mins at room temperature. Staining was observed using 3,3'-diaminobenzidine (Sigma Fast™ Dorset, U.K.) until the reaction products were visible. Tissues were counterstained with hematoxylin (BDH, Poole, U.K.) before being dehydrated with 95% ethanol and then absolute alcohol. Sections were finally immersed in xylene before being mounted ready for analysis under a light microscope.

Positive controls for cathepsin B were performed using the above procedure on human liver tissue and for DPPIV human tonsil tissue. For negative controls the above immunohistochemical procedure was also carried out but omitting the addition of the primary antibody step. A negative and a positive control was performed along side each experiment.

All sections were assessed for the percentage of positively stained cells and the criteria for immunopositive tissue sections were used: greater than 500 cells positive for the enzyme under investigation were considered to be positively stained. Sections were further analysed for staining intensity.

4.3.4 *In situ hybridisation*

This procedure was conducted to identify the presence and the qualitative changes in the levels of cathepsin B and DPPIV mRNA in cells of human colorectal tissue as well as in adjacent normal human colon.

This was done by allowing the visualisation of labelled DNA probes which were hybridised with the complementary target RNA sequences in the cell.

The *In situ* Hybridization and Detection System (Life Technologies $_{TM}$) used in this experiment is designed for non-radioactive detection of subcellular mRNA in tissue preparations. In this experiment paraffin embedded human colorectal tumour tissue and normal human colon tissue was used.

Tissue sections

Ten slides from each tumour grade of Dukes' stages A, B, C were used. The tumour resections used consisted of normal human colon tissue adjacent to the tumour. Six slides of normal human colon tissue distant from the tumour were also used. Half of the slides from each group were used to detect cathepsin B and the other to detect DPPIV.
Sections from human thyroid and human liver were used as positive controls for DPPIV and cathepsin B respectively.

Precautions

Before the experiment was carried out samples were protected from RNAses which are ubiquitous enzymes that degrade RNA. Finger-prints are a common source therefore, gloves were worn at all times and glassware was baked at 200°C or disposable plastics were used where ever possible.

All solutions used in the experiment were treated with 0.1% diethylpyrocarbonate (DEPC) (Sigma U.K). This denatures RNAse that might have contaminated the distilled water used in making the solutions in the experiment. DEPC is added to the distilled water and then left at room temperature for 12 hours. The water is then autoclaved for 15 minutes to destroy the DEPC.

Pre-treatment

The procedure began with baking slides at 60°C and then the slides were de-paraffinized in two changes of xylene for 5 minutes. The slides were then immersed in two changes of absolute ethanol for 1 minute each and then air dried for 5 minutes.

Following de-paraffinization the slides were deproteinized to expose the nucleic acid target molecule mRNA. The slides were proteolytically digested with the enzyme proteinase K, diluted at a concentration of 40mg/ml in PBS pre-warmed at 37°C for 30 minutes, by immersing them in the solution for an optimal time of 12 minutes.

The slides were then immediately immersed in PBS to stop the digestive process and then placed in 10% buffered formalin at room temperature for 10 minutes. After which they were rinsed in PBS and then dehydrated through a graded series of alcohol dilutions of 50%, 70%, 90% and 100% for 3 minutes each.

Hybridization

Slides were air dried to prepare them for the hybridization process. Specific primers corresponding to positions 45 and 509 in the published sequence by Dunphy et al, 1999 for the DPPIV mRNA protein were used to highlight mRNA in the tissue sections. These were the oligonucleotide anti-sense probe for DPPIV [primer sequence 5'TTT GGA ATC TTC TCT TCT GTG AT-3' (Life Technologies $_{TM}$)] and the sense probe [5' CTG CGC TCA AGC TTC AGA GTT CA-3' (Life Technologies $_{TM}$)] was used in the negative control in tumour and normal human colon sections. Sections from human thyroid tissue were used for the positive identification of DPPIV mRNA (positive control).

An oligonucleotide anti-sense probe for cathepsin B [5' GGC CTT TTC TTG TCC AGA AG-3' (Life Technologies $_{TM}$)] was prepared based on the nucleotide sequence (570 to 594) reported by Hirai et al.1999. The negative control used the sense probe [5' TTC GAT GCA CGG GAA CAA TG-3' (Life Technologies $_{TM}$)]. Human liver tissue sections were used for the positive identification of cathepsin B mRNA (positive control).

Probes were prepared by centrifuging the viles to collect the RNA at the bottom before reconstituting them with 10μmolar of TE (10mM Tris-HCL, pH 8.0,
1mM EDTA). The solution was left for 2 minutes before being vortexed for 15 seconds and stored at -20°C.

For tissue application 8.6 μl of probe was diluted with 200 μl of 2X hybridization buffer (4X SSC, 0.2 M sodium phosphate (pH 6.5), 2X Denhardt's solution, 0.1 mg/ml sodium azide) makes a concentration of 0.5 μg/ml (*in Situ* Hybridization and Detection System Kit, Life Technologies). Prior to this 20 μl of vanadyl ribonucleoside complex (an RNase inhibitor) (Life Technologies) was added.

Equal amounts of probe solution and 20% dextran sulphate (*in Situ* Hybridization and Detection System Kit, Life Technologies) were mixed together and then the mixture was

pipetted onto the tissue sections and covered with a coverslips. Slides were incubated in a glass chamber at 42°C at an optimum time of 12 hrs.

After hybridization the slides were immersed in 0.2X SCC solution to allow the coverslips to float off. They were then put through 2 more washes 0.2X SSC for 15 minutes each.

Detection of mRNA

Tissues were covered with 200µl of blocking solution and then incubated at room temperature for 15 minutes. This solution was then removed by blotting the liquid without touching the tissue. A conjugate solution consisting of 10µl of streptavidin-alkaline phosphatase conjugate with 90µl of conjugate dilution buffer was added to each slide and then incubated for 15 minutes

The slides were rinsed twice in TBS for 15 minutes each and then in alkaline-substrate buffer for 5 minutes. They were then placed in pre-warmed alkaline substrate buffer at 37°C, which contained 200µl of NBT (nitroblue tetrezolium) and 166µl BCIP (4-bromo-5-chloro-3-indolyl-phosphate), for the optimum time of 30 minutes. This colour development was stopped by placing slides in several changes of de-ionized water. Sections were then counterstained with methyl green (Sigma U.K.) and then dehydrated through a series of graded alcohols (50%, 70%, 90%, 100%) for a minimum of 1 minute in each concentration.

Sections were then air dried, permanently mounted with a coverslip using histomount, and then observed under a Ziess light microscope.

4.4 Results

4.4.1 *Immunocytochemistry*

Cathepsin B (CB) was firstly, identified in positive control human liver cells (fig 4.2) and then a negative control (fig 4.3) was also conducted which produced no staining hence, the cathepsin B antibody is specific to highlighting the protease.

DPPIV was also highlighted in its positive control tissue which was human thyroid cells (fig 4.4). The negative controls showed no staining (fig 4.5).

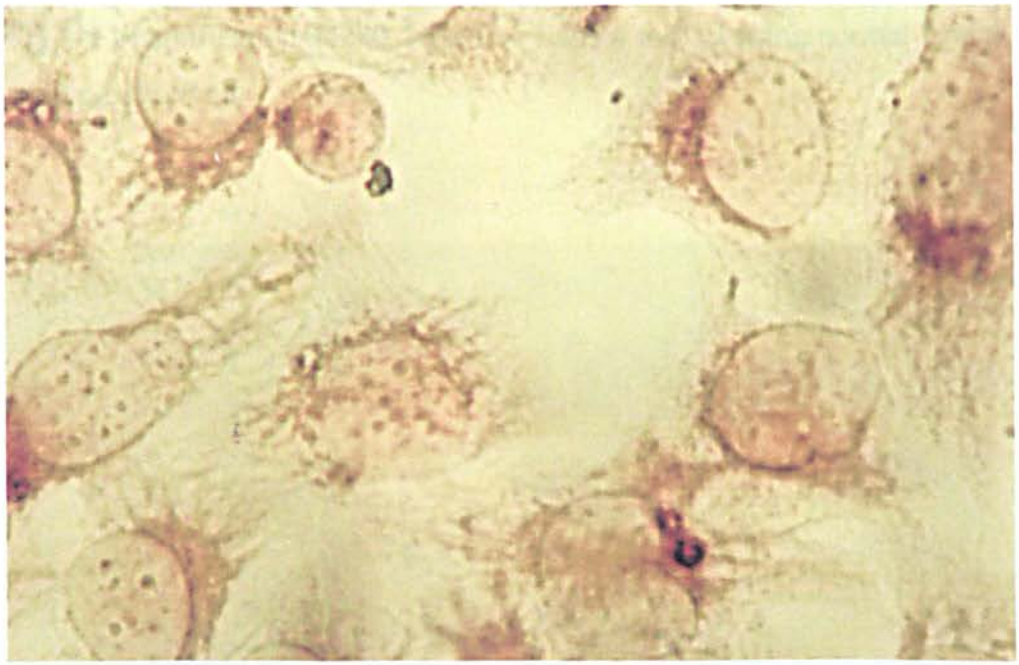


Fig 4.2 Magnification x4480. Positive control for CB using normal human liver cells. Cells have stained positively for CB.

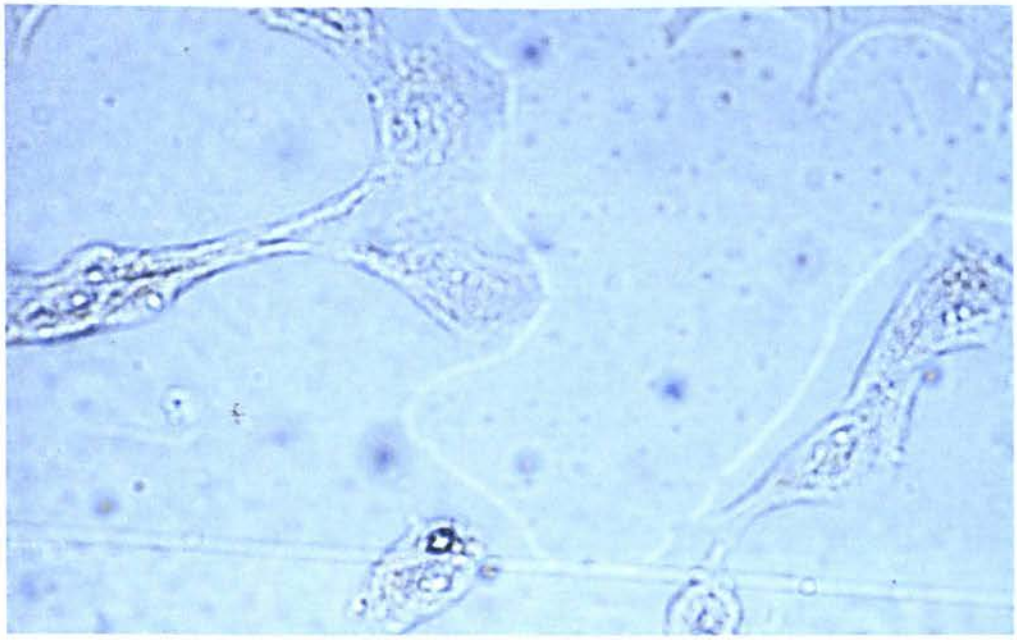


Fig 4.3 Magnification x4480. Negative control for CB using normal human liver cells. No staining observed.

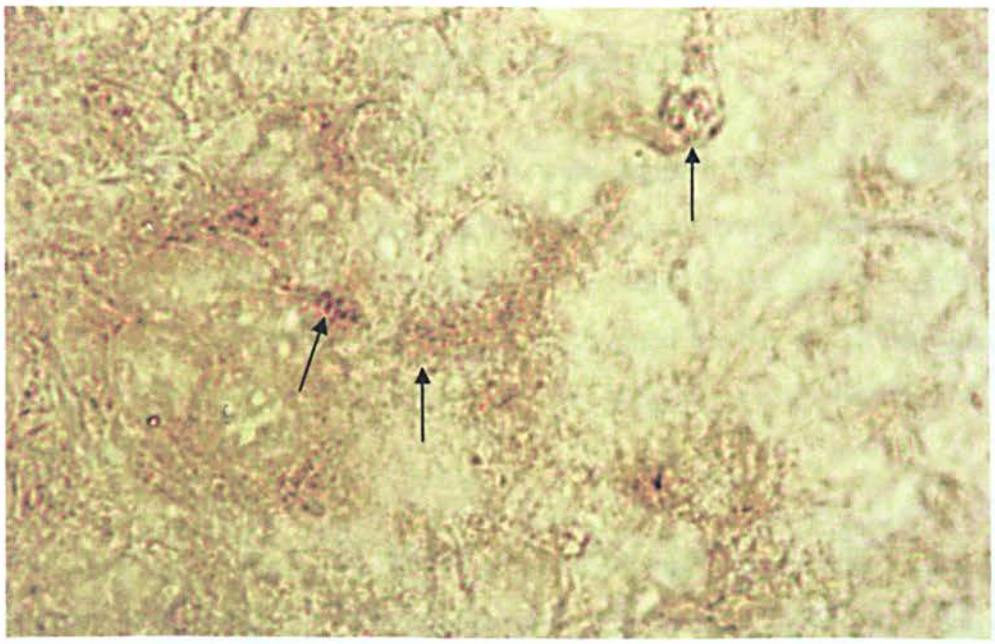


Fig 4.4 Magnification x4480. Positive control for DPPIV using normal human thyroid cells. DPPIV is shown to be expressed in these cells (arrows).

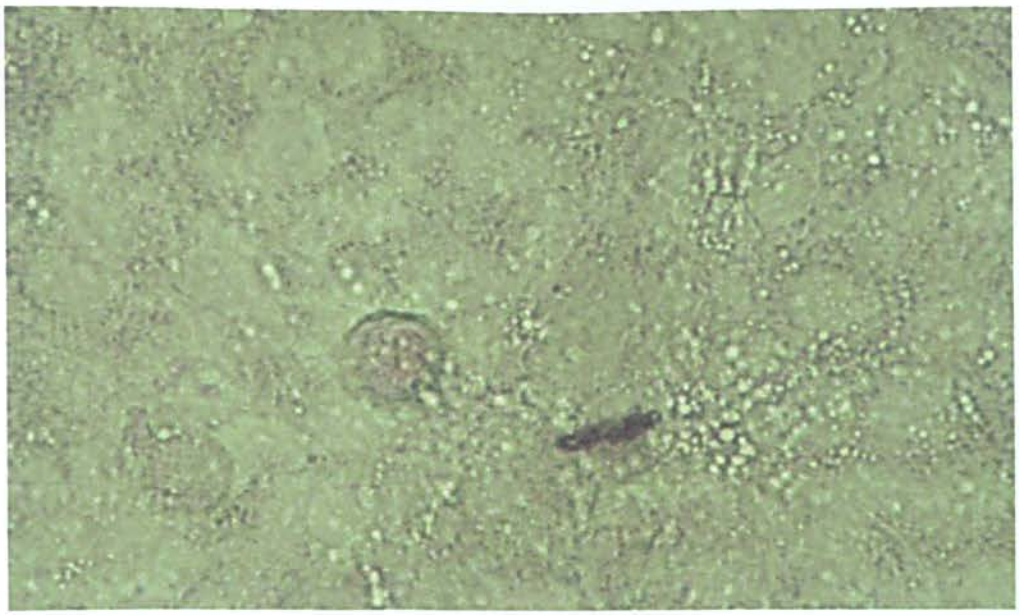


Fig 4.5 Magnification x4480. Negative control for DPPIV using normal human thyroid cells.

Human umbilical vascular endothelial cells (HUVECs)

Once grown to confluence HUVECs were stained for CB and DPPIV. The negative controls were performed first and no staining was observed (fig 4.6). The cells were highly positive for CB with cells showing intense staining in the cell membrane. This pattern of staining was observed in 95% of all slides stained for CB which demonstrates that CB is strongly expressed in HUVECs with the enzyme specifically located in the plasma membrane regions (fig 4.7).

The negative controls for DPPIV were conducted by omitting the primary antibodies showed no staining (fig 4.8). Confluent HUVECs stained for DPPIV also exhibited strong staining, however, the enzyme localisation was observed not only on the cell membrane but also in the cytoplasm (fig 4.9).

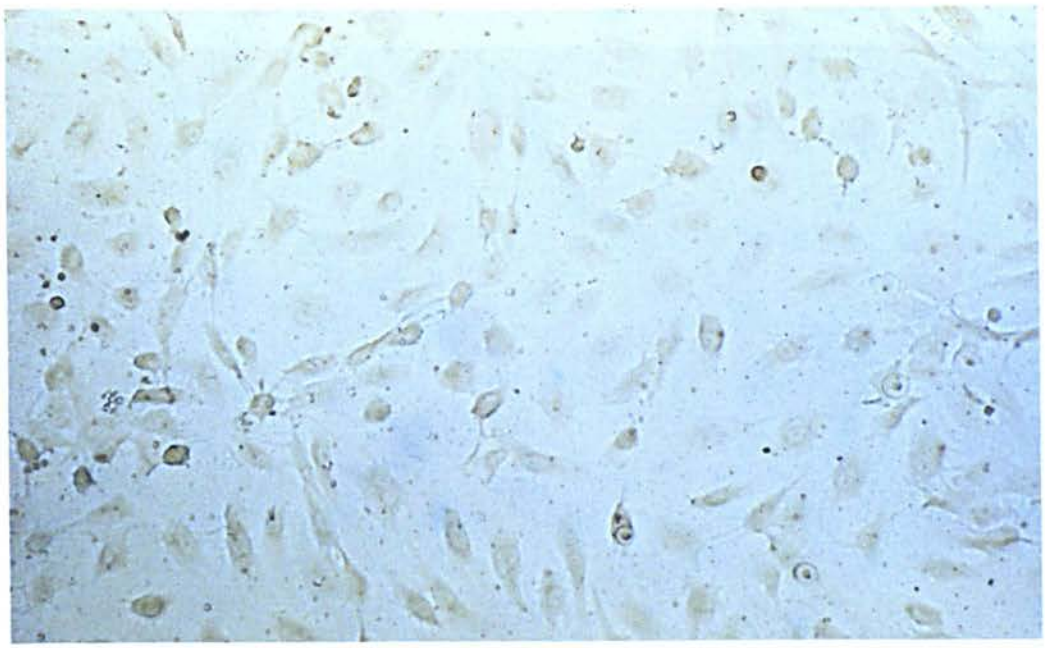


Fig 4.6 Magnification x2800 .Negative control for CB in HUVECs.

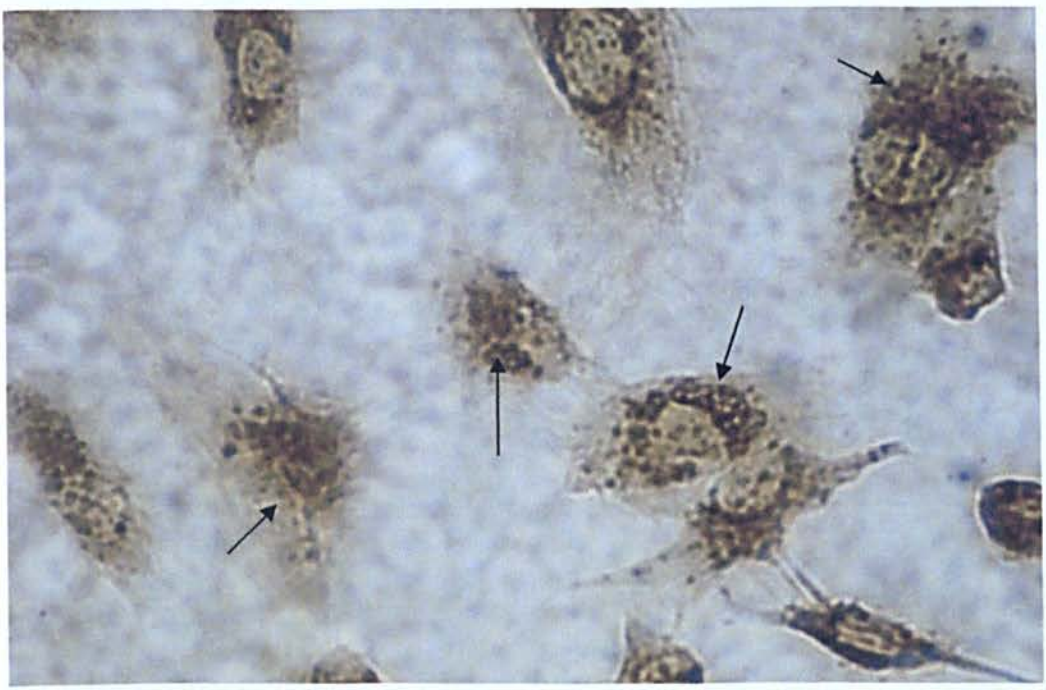


Fig 4.7 Magnification x3360. Demonstrates intense staining localised on the plasma membrane for CB in HUVECs (arrows).

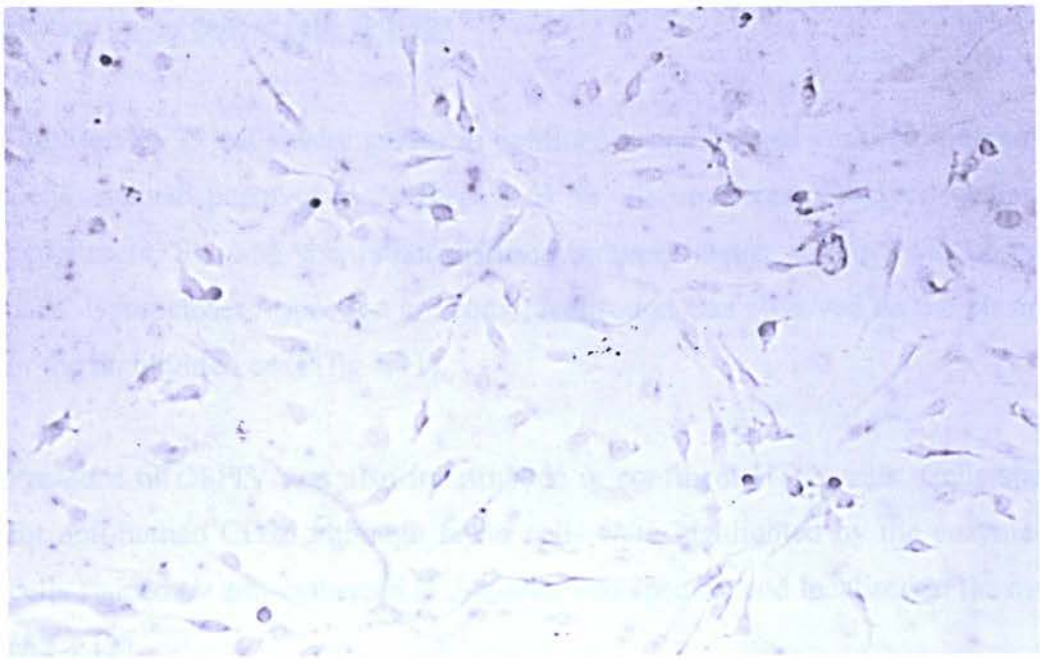


Fig 4.8 Magnification x2800. Negative control for DPPIV in HUVECs.

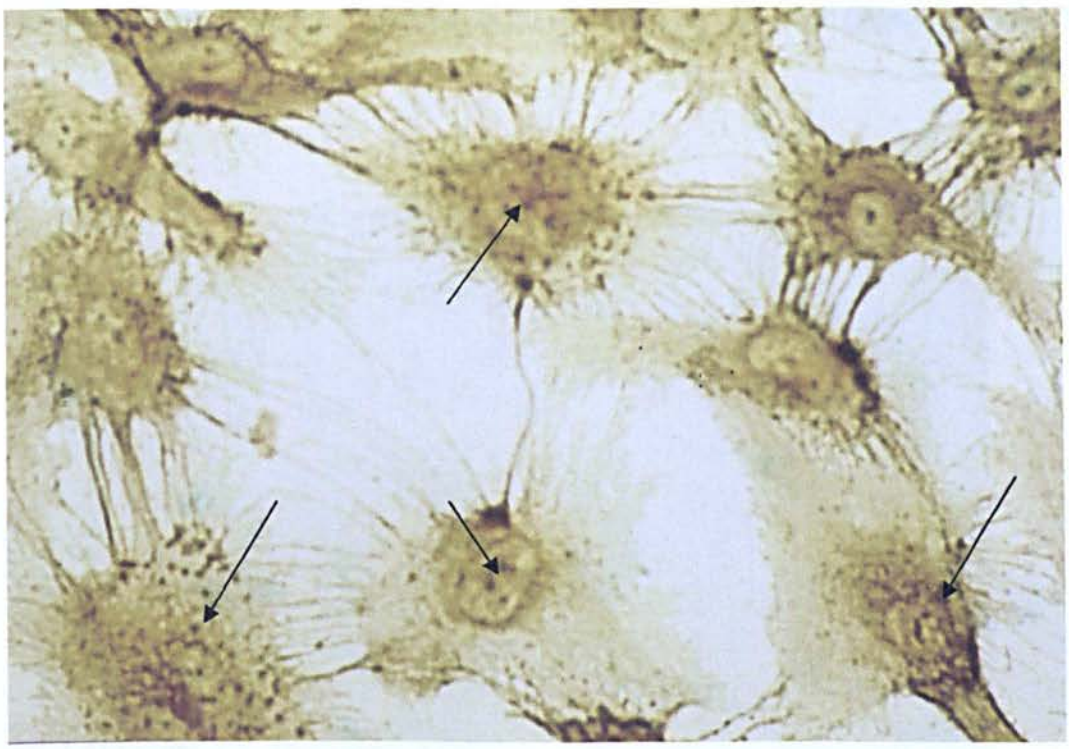


Fig 4.9 Magnification x3360. This photomicrograph demonstrates subcellular localisation of DPPIV in the cytoplasm as well as on the plasma membrane of endothelial cells. The cells have stained strongly with greater specificity to anti-CD26 (arrows).

Human colon cancer cells (HT29)

Cultured HT29 cells were grown to confluence and stained with anti-human cathepsin B. Cells stained positive for cathepsin B in all confluent chamber wells used in the experiment. Staining was rather diffuse however, intense staining was observed in some cells. Upon closer inspection antibody localisation was observed on the plasma membrane of the highlighted cells (fig 4.11).

Presence of DPPIV was also investigated in confluent HT29 cells. Cells stained positive for anti-human CD26 although fewer cells were highlighted by the enzyme marker than cells stained by anti-cathepsin B. Staining was specific and localised in the cytosol regions (fig 4.13).

The immunocytochemical method for the negative control, for both antibody markers, omitted the primary antibody. This was conducted to analyse whether the secondary antibody or the detection method gives rise to background staining. For all the controls performed along side the experiments no staining was observed (figs 4.10 and 4.12).

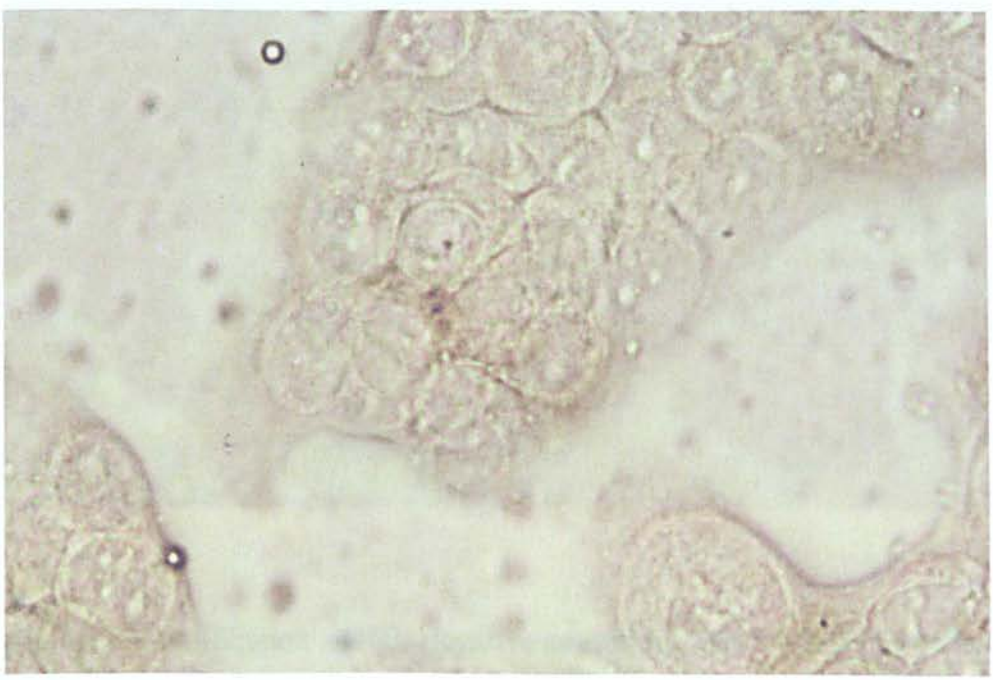


Fig 4.10 Magnification x4480. Negative control for CB in HT29 cells.

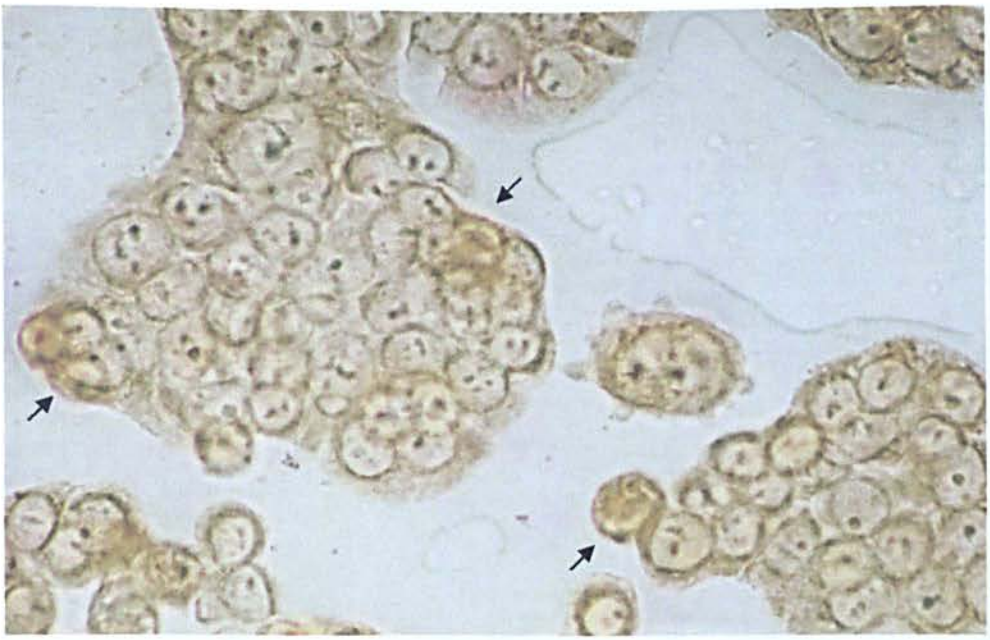


Fig 4.11 Magnification x 1400. HT29 cells stained positive for CB. Certain cells express CB which is highlighted by a dark brown stain 3,3'-diaminobenzidine (arrows). Some cells exhibit strong positive staining on their cellular membrane whereas other cells show granular staining in the cytoplasmic regions.

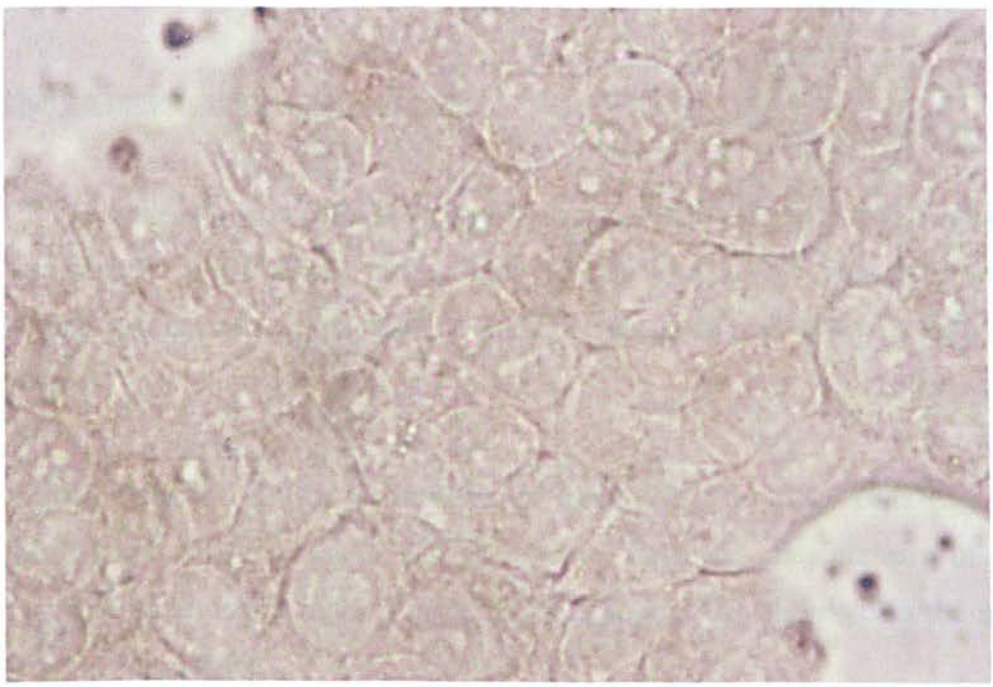


Fig 4.12 Magnification x4480. Negative control for DPPIV in HT29 cells.

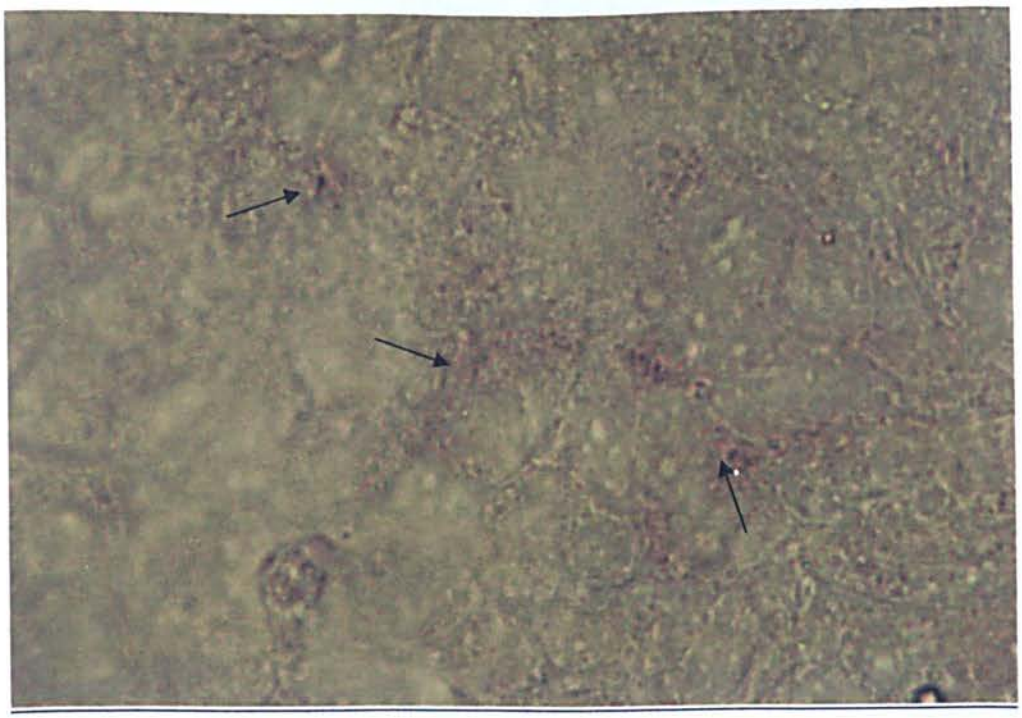


Fig 4.13 Magnification x 4480. A photomicrograph of HT29 cells stained for DPPIV. Staining is weaker for DPPIV than CB (fig 4.4) however, the enzyme, DPPIV, is highlighted on the plasma membrane (arrows).

Human rectal cancer cells (HRT18)

HRT18 cells stained positive for anti-human cathepsin B (fig 4.15). Antibody localisation was specific and detected in approximately 20% of all the cells observed. Staining was mainly present on cell membrane and low levels of cathepsin B were detected in cell cytosol.

Higher levels of DPPIV were detected using CD26 in HRT cells (fig 4.17). Antibody localisation was specific to cell membrane, however, the intensity of the stains were similar to that of CB in HRT18 cells. No staining was observed in the negative controls (figs 4.14 and 4.16).

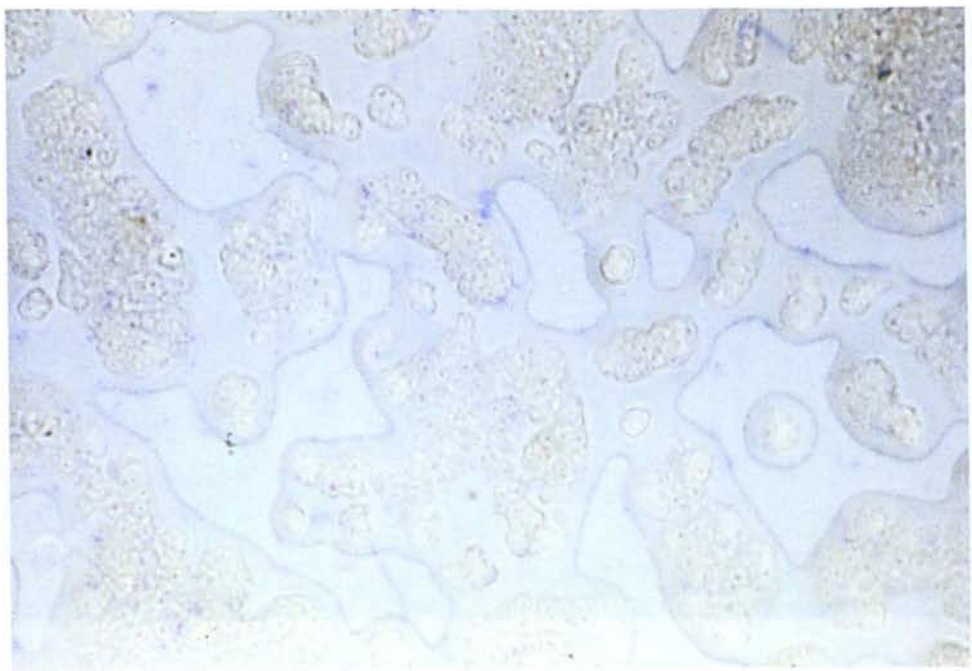


Fig 4.14 Magnification x840. Negative control for CB in HRT18 cells.

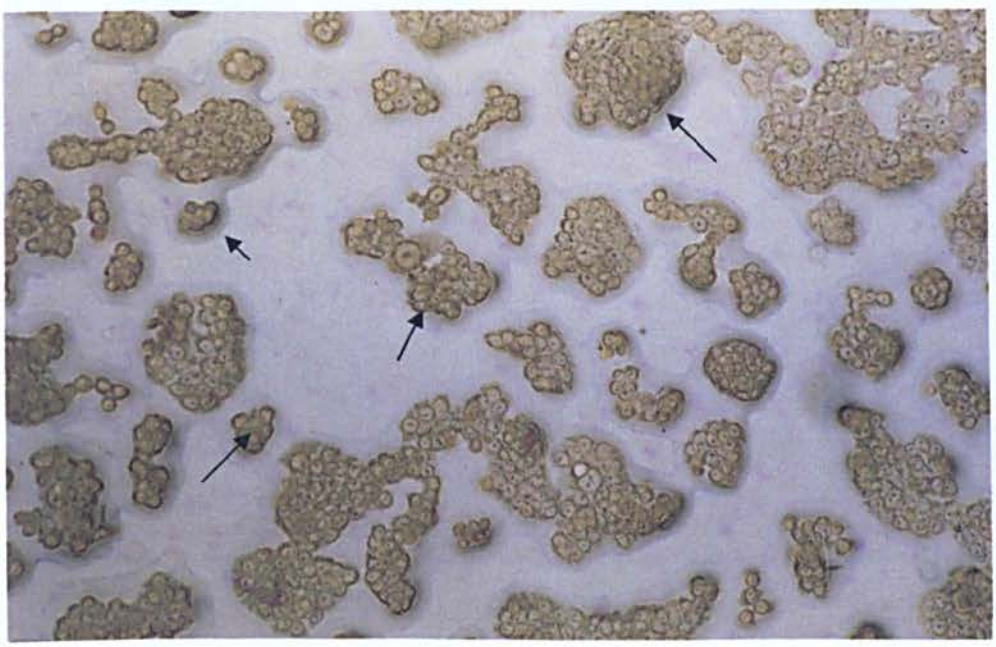


Fig 4.15 Magnification x 840. HRT18 cells stained for CB. Cells show a strong positive staining, predominantly on their cellular membrane, for CB (arrows).

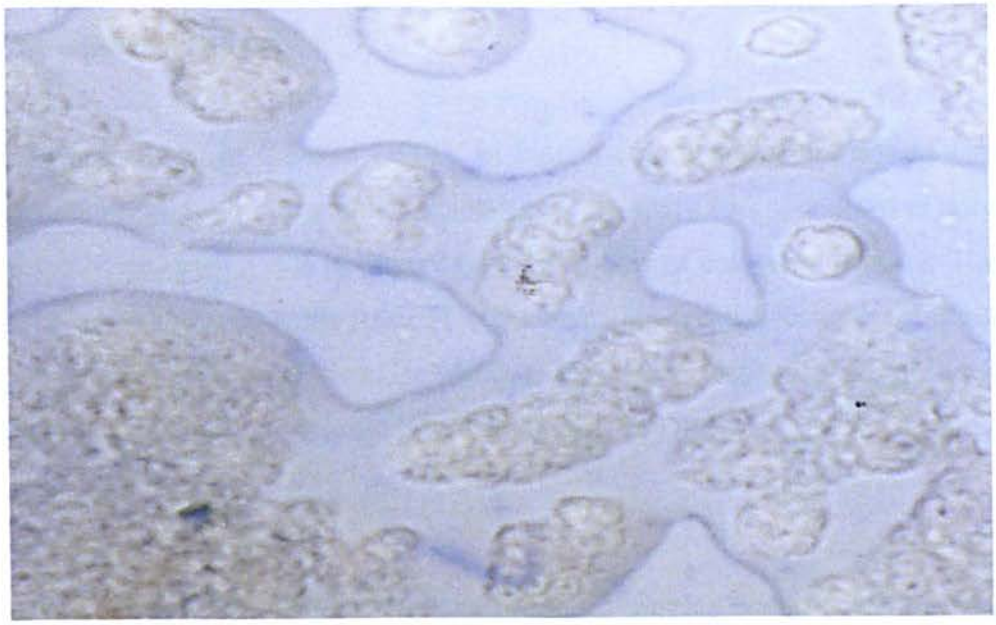


Fig 4.16 Magnification x1160. Negative control for DPPIV in HRT18 cells.

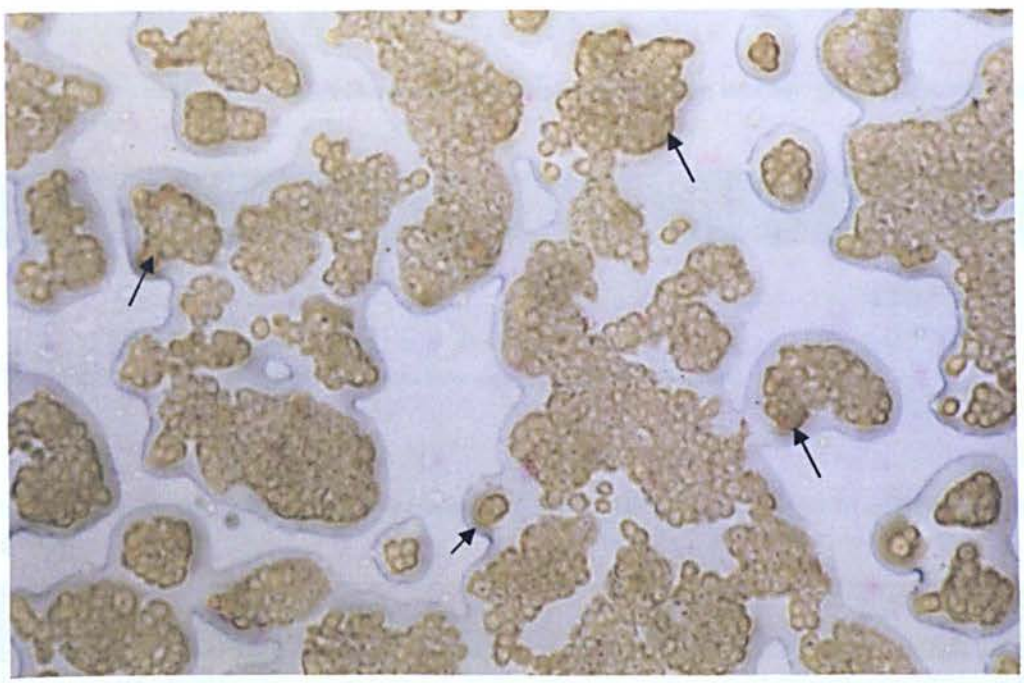


Fig 4.17 Magnification x 840. HRT 18 cells stained for DPPIV. The intensity of staining is slightly less here than for CB however, majority of the cells were positive for anti-CD26 (arrows).

Immunohistochemistry

The localisation of CB and DPPIV in human colorectal carcinomas of varying grades was carried out by using immunohistochemistry. The study was conducted in order to clarify if the two enzymes play a role in the progression of human colorectal cancers. The total number of samples investigated came from 31 cancer patients and the histological tumour grades within the sample group ranged from Dukes' A to C. Sixteen sections were cut from each tumour and half were stained for cathepsin B and the others for DPPIV.

Table 4.1 % cases of human colorectal carcinomas, of varying tumour grades, showing immunopositivity for CB

Histological type	No. of tumours	% of cases immuno-positive for CB
Dukes'A	**5**	60%*
Dukes' B	**16**	68%*
Dukes' C	10	80%*

*> than 500 cells stained positive for the enzyme were considered as positively stained.

Table 4.2 % cases of human colorectal carcinomas, of varying tumour grades, showing immunopositivity for DPPIV

Histological type	No. of tumours	% of cases immuno-positive for DPPIV
Dukes'A	**5**	0%*
Dukes' B	**16**	43.75%*
Dukes' C	10	50%*

*> than 500 cells stained positive for the enzyme were considered as positively stained.

Table 4.3 showing the staining intensities of CB and DPPIV in the human colorectal tumours of varying grades

Dukes' stage	Staining intensity of CB	Staining intensity of DPPIV
A (n=5)	±	-
B (n=16)	+	±
C *(n=10)*	+	±

Key: Staining patterns represented by – negative (positive cells <10%), ± weakly positive (positive cells between 10-50%), + positive (positive cells >50%).

Table 4.4 showing the staining intensities of CB and DPPIV in tumours of different histological types.

Histological type	CB	DPPIV
Vessel Invasion		
positive	+	±
negative	+	±
Lymph vessel Invasion		
positive	±	±
negative	±	-

Key: Staining patterns represented by – negative (positive cells <10%), ± weakly positive (positive cells between 10-50%), + positive (positive cells >50%).

Immunohistochemistry revealed CB immunopositivity in cancer cells and in the proximal epithelial cells adjacent to tumour sites. However, very little immunoreactivity was observed in stromal cells and connective tissue surrounding the tumours exhibited immunonegativity for CB. Non cancerous tissue distant from the tumours was negative for the enzyme.

The control tissue for CB was normal human liver. The positive control highlighted pockets of CB positive cells (fig 4.18) and the negative control showed no staining for the enzyme (fig 4.19). The control tissue for DPPIV was normal human thyroid. The tissue exhibited positive staining for DPPIV (fig 4.20) and the negative control displayed no staining (fig 4.21).

Dukes' stage A (fig 4.22) tumours exhibited a more granular staining pattern where as the higher grade tumours (Dukes'B and C) displayed more dense and diffuse staining. In 60% of samples from Dukes' B (fig 4.23) and Dukes' C, CB was highlighted in the vascular endothelium present within the tumour samples. Intense staining was also observed in tumour tissue surrounding the tumour vessels.

In the CB positive samples the highest intensity of immunoreactivity was observed in the advancing edges of the tumour and the surrounding non cancerous connective tissue was very weakly stained or negative to CB. This pattern of staining is prominent in more advancing cancers such as the Dukes' stage C samples. Therefore, Dukes' stage C cancer cells are highly positive for CB (fig 4.25), negative controls for CB in Dukes' C tumours showed no staining (fig 4.24).

The stromal cells in the mucosal regions of Dukes' stage A tumours show high positivity to CB. Regions within Dukes' stage A tumours displayed more granular pattern of reactivity and less intensity in staining. 60% of all tumours in this histological group were positive for CB (table 4.1) and the staining intensity was weakly positive (table 4.3). Strong immunoreactivity was noted in tumours with blood vessel invasion and this was also observed in tumours without blood vessel invasion. Cancer cells, in tumours with and tumours without lymph vessel invasion, expression for CB was weakly positive (table 4.4).

In Dukes' stage B tumours CB highlighted regions were more dense and diffuse and vascular endothelial cells also expressed CB. 68% of the samples from this category were immunopositive and the overall staining was classified as positive due to the fact that greater than 50% of the cells were highlighted for CB (table 4.1).

Dukes' stage C tumours displayed greater intensities of staining for CB with more defined parameters of CB positive cells. Clear differences in the localisation of the protease enzyme was noted with distinct immunopositive tumour regions adjacent to non cancerous connective tissue negative for CB. 80% of tumour sections were positive for CB with intense staining (table 4.1 and 4.3).

Fig 4.26 demonstrated no staining in the negative control for DPPIV in Dukes' grade A human colorectal tumour. The cancer cells expression for the enzyme protease DPPIV (fig 4.27) was less intense than CB. In comparison, Dukes' A sections were immunonegative to DPPIV whereas 60% of Dukes' A tumour sections expressed CB. However, a marked increase in the number of tumour cells positive for DPPIV was noted in Dukes' B (fig 4.29) (43.75%) and in Dukes' C (fig 4.31) (50%) although, the expression of CB within theses tumour samples was greater (Table 4.1 and 4.2). Negative controls for DPPIV in Dukes' grade B and C tumours showed no staining (figs 4.28 and 4.30).

The DPPIV signal was weaker than CB within the tumour sections and the staining was granular and mainly cytoplasmic. The immunoreactivity of the enzyme was specific with normal colon connective tissue cells, adjacent to tumour tissue, negative to DPPIV.

A greater number of cells were positive for DPPIV in Dukes' C tumours. Staining was dense (fig 4.31) which reflects the fact that there is greater area of tumour tissue, stretching from mucosal to submucosal regions, within this tumour grade.

Immunoreactivity for DPPIV in tumours with blood and lymph vessel invasion and blood vessel non invasion was weak and negative in tumours with lymph vessel non invasion (table 4.4).

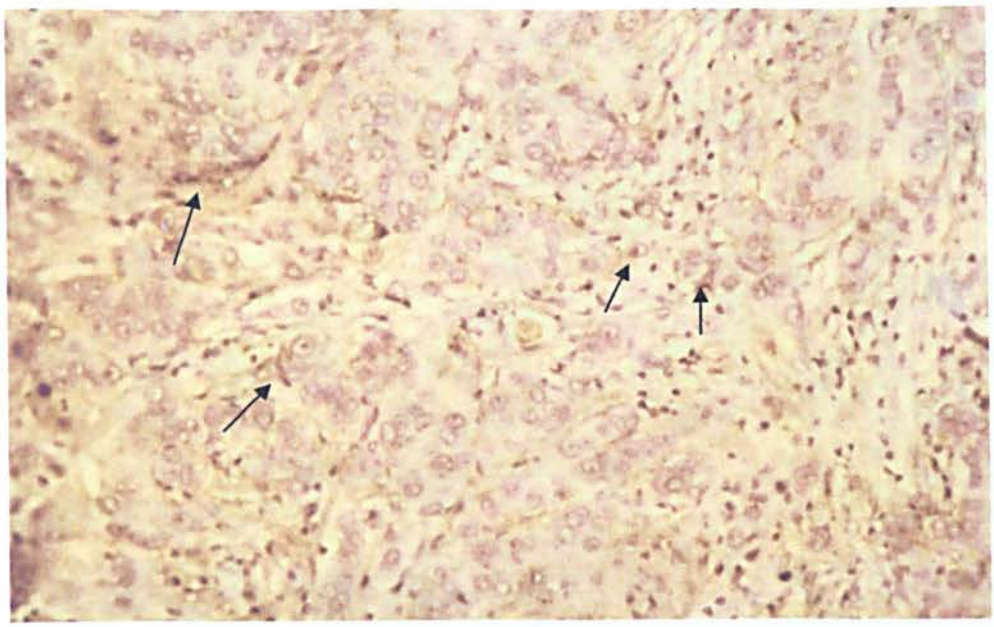


Fig 4.18 Magnification x1160. Positive control for CB. CB is highlighted in the control tissue normal human liver tissue (arrows).

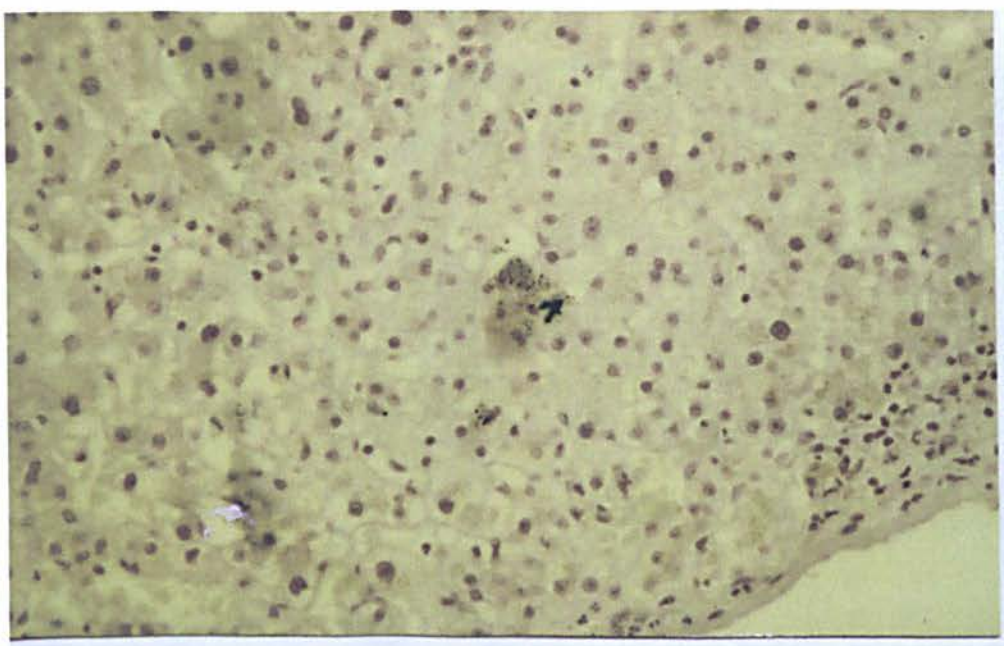


Fig 4.19 Magnification x1160. Negative control for CB using normal human liver tissue. No staining observed.

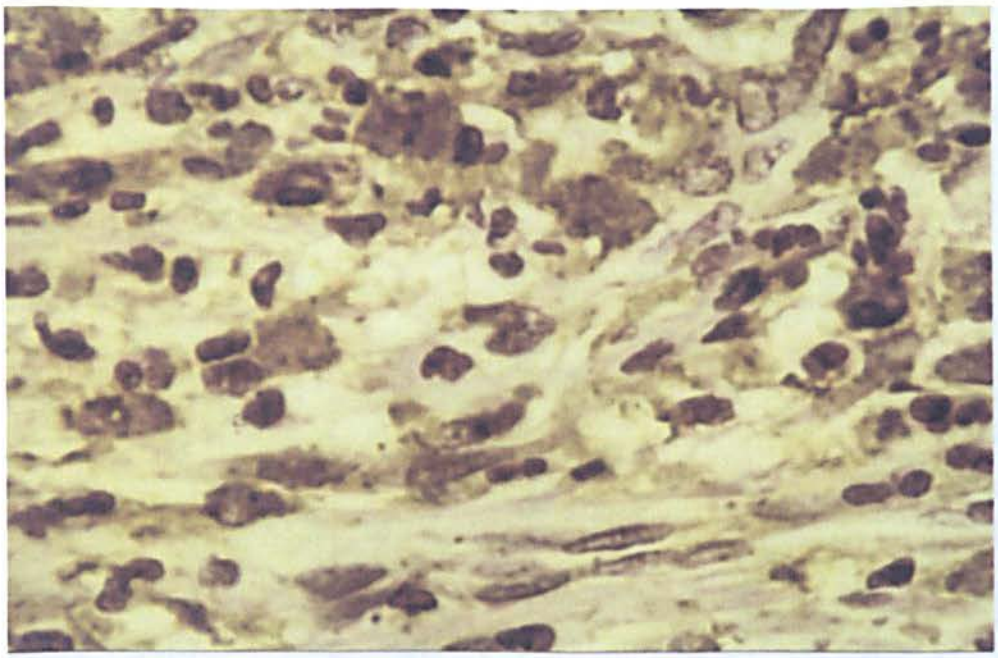


Fig 4.20 Magnification x2000. Normal human thyroid tissue used as positive control tissue for detection of DPPIV demonstrated subcellular localisation of the enzyme.

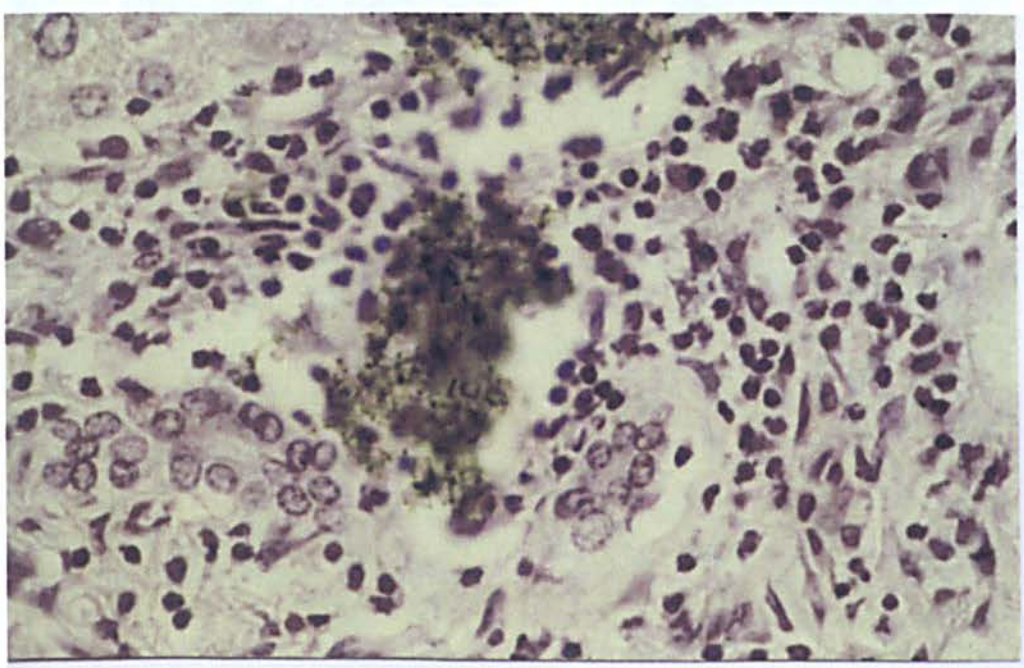


Fig 4.21 Magnification x2000. Negative control for DPPIV using normal human thyroid tissue. No staining observed.

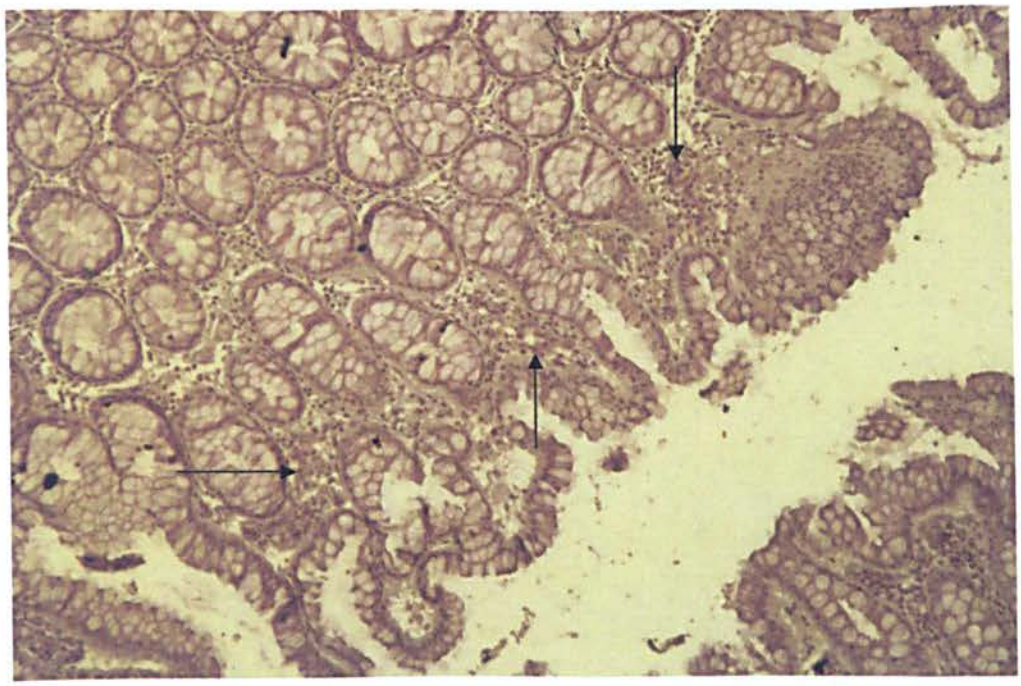


Fig 4.22 Magnification x 700. Highlights CB in Dukes' stage A tumour. Cancer cells surrounding neoplastic glands show localised presence of CB (arrows).

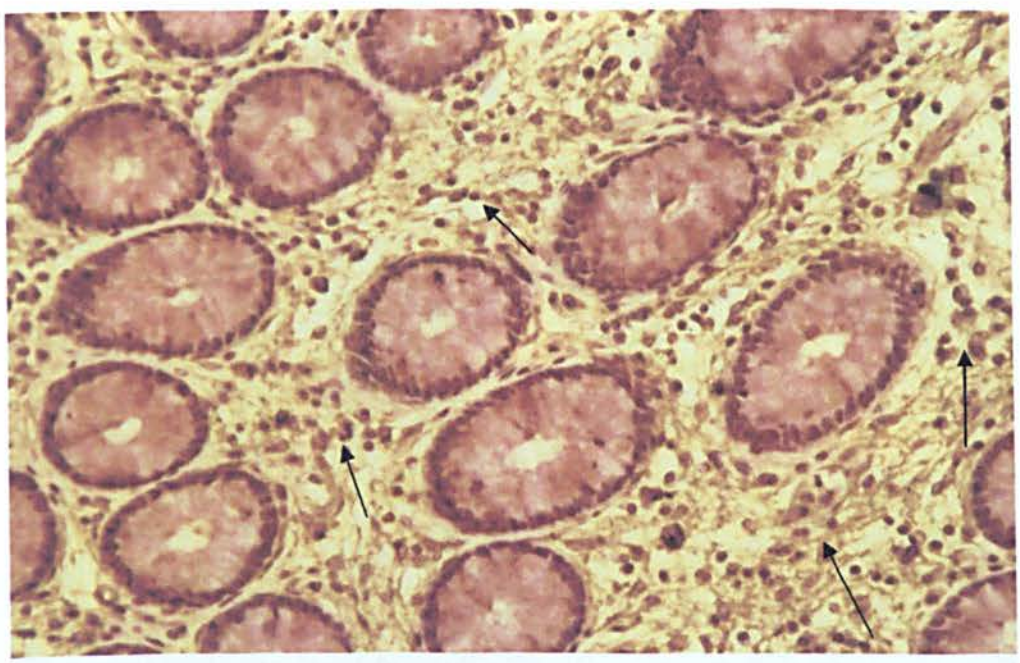


Fig 4.23 Magnification x 1120. CB staining of Dukes' B tumour tissue. Section shows the presence of CB in the mucosal region of the human colorectal carcinoma. Majority of the neoplastic cells in the region have stained for CB (arrows).

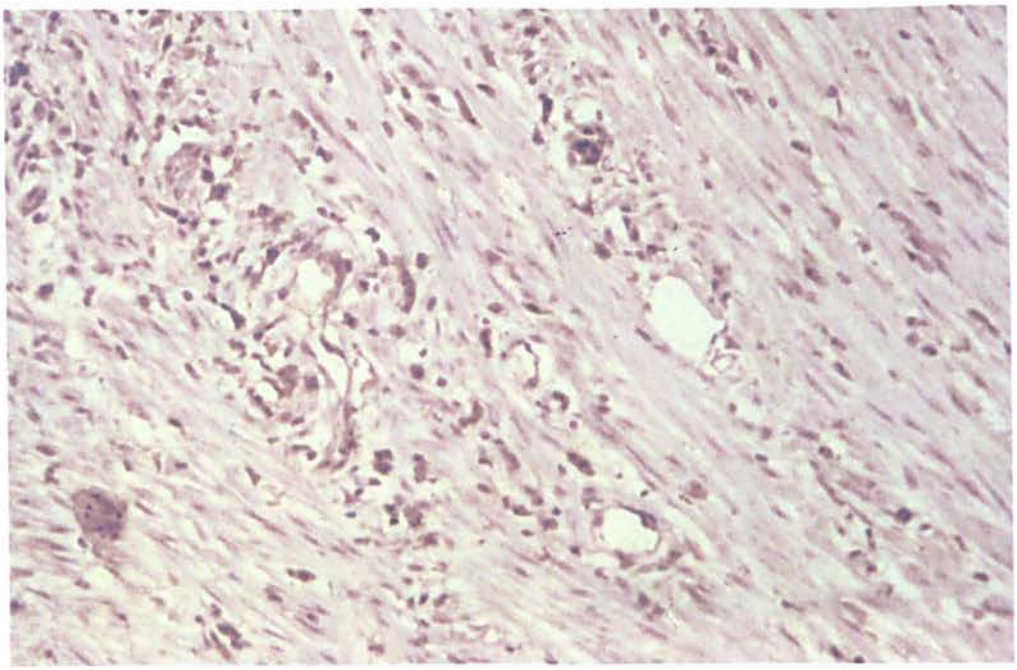


Fig 4.24 Magnification x1160. Negative control for CB in human colorectal tumour grade C. No staining observed.

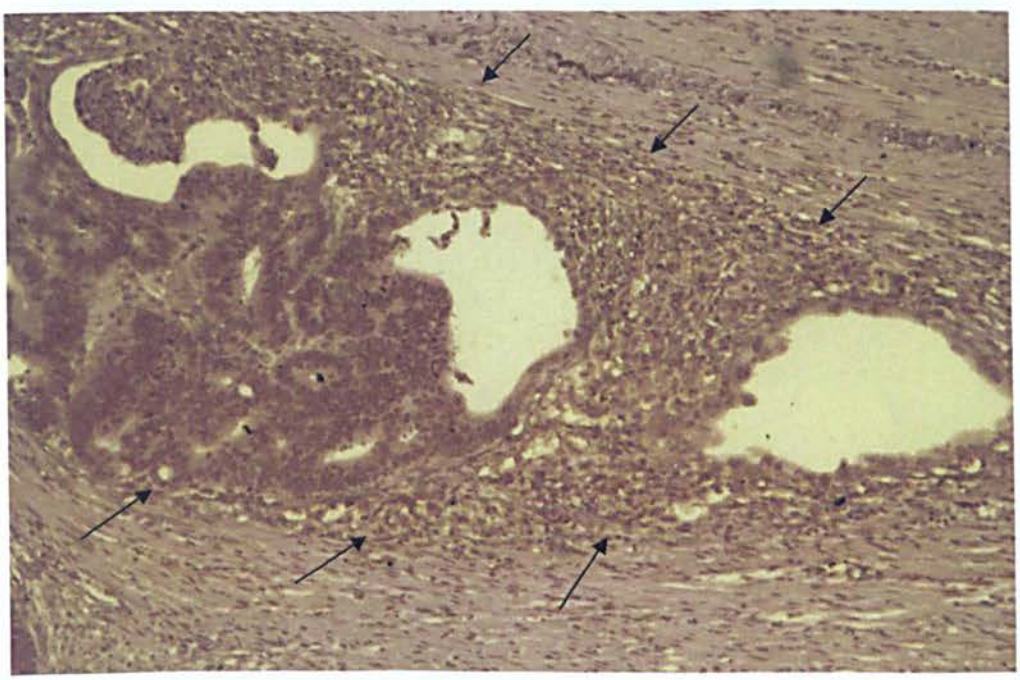


Fig 4.25 Magnification x 700. Staining of human colorectal tumour section for CB (tumour grade Dukes' C). Staining is specific where CB is localised in neoplastic tissue only (arrows).

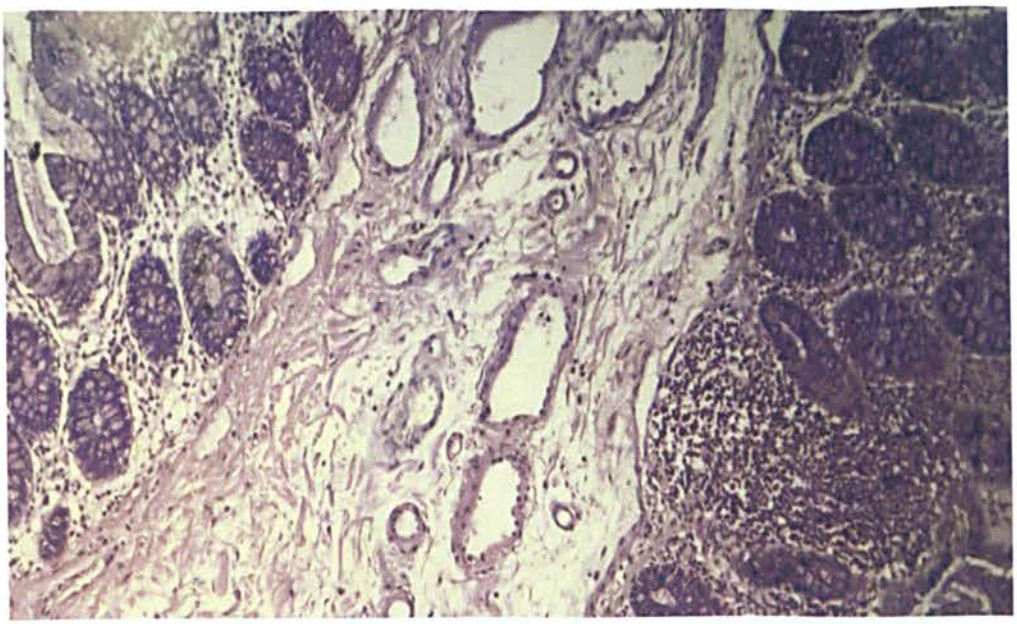


Fig 4.26 Magnification x560. Negative control for DPPIV in Dukes' grade A human colorectal tumour tissue. No staining observed.

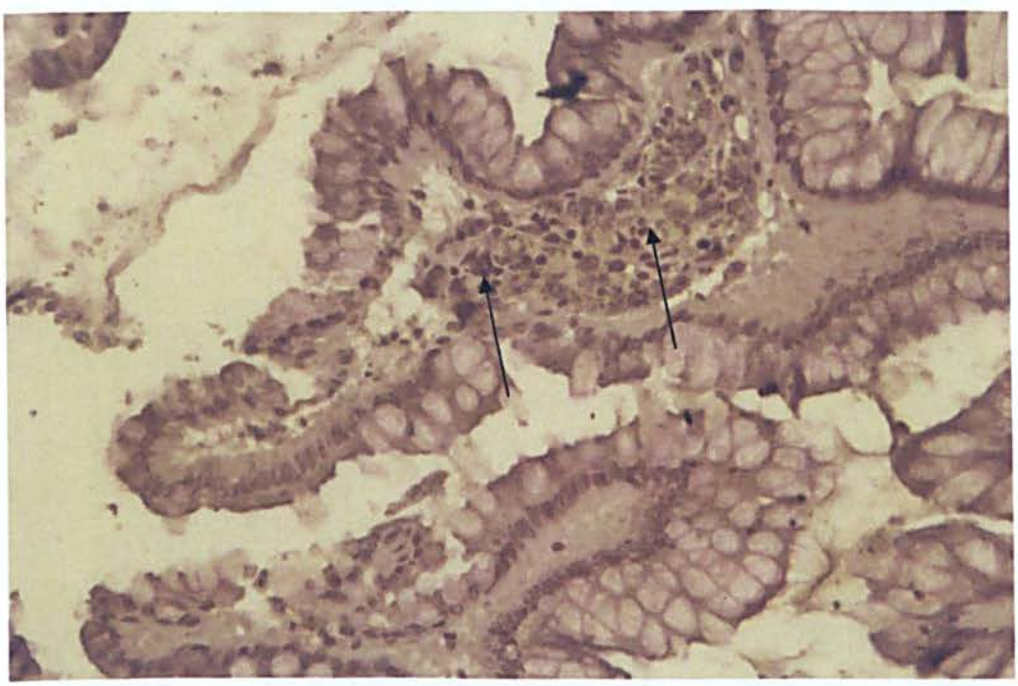


Fig 4.27 Magnification x 1140. DPPIV staining in colorectal tumour (Dukes' A). Staining is weak and minimal. CB is contained in small pockets of neoplastic cells in the mucosal area (arrows).

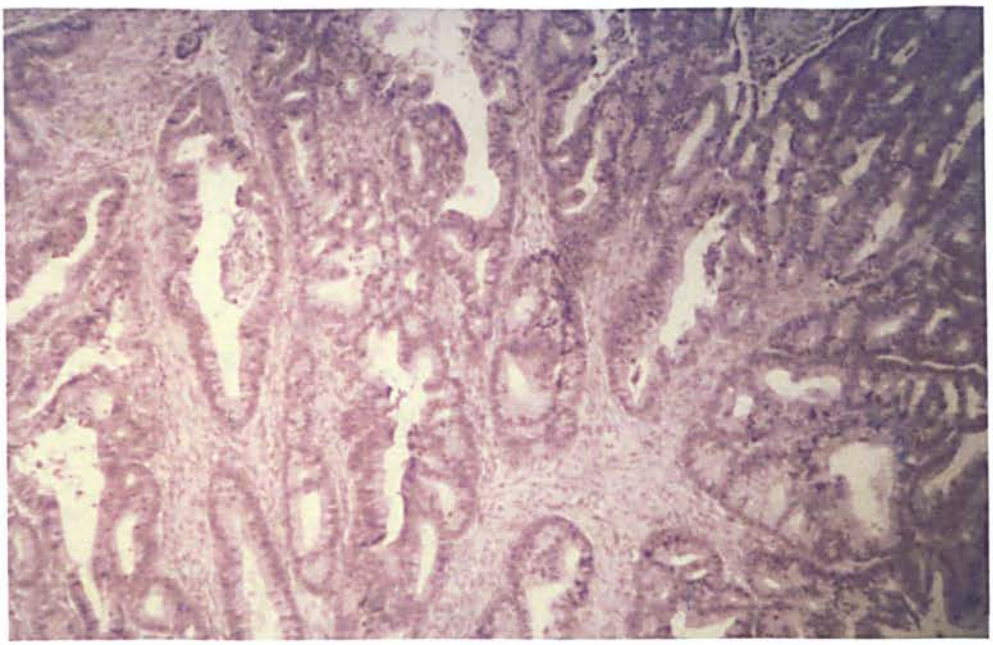


Fig 4.28 Magnification x 700. Negative control for DPPIV in Dukes' grade B tumour. No staining observed.

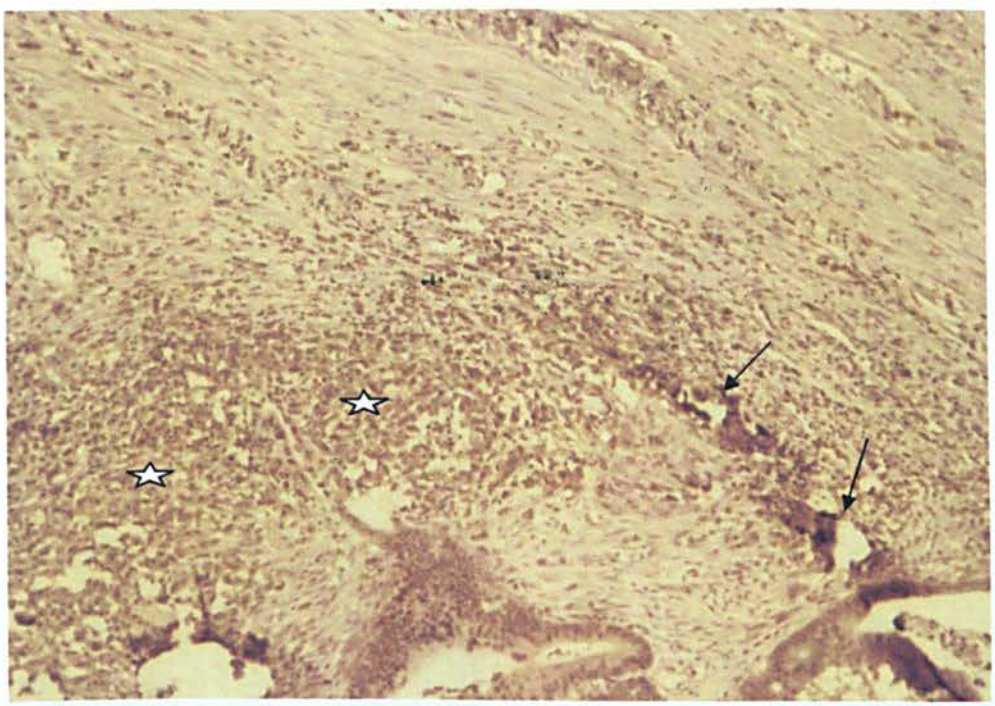


Fig 4.29 Magnification x 700. DPPIV staining in Dukes' B tumour. Cancer cells in the submucosal regions (stars) exhibit DPPIV staining. Intense staining is also observed around vessels (arrows).

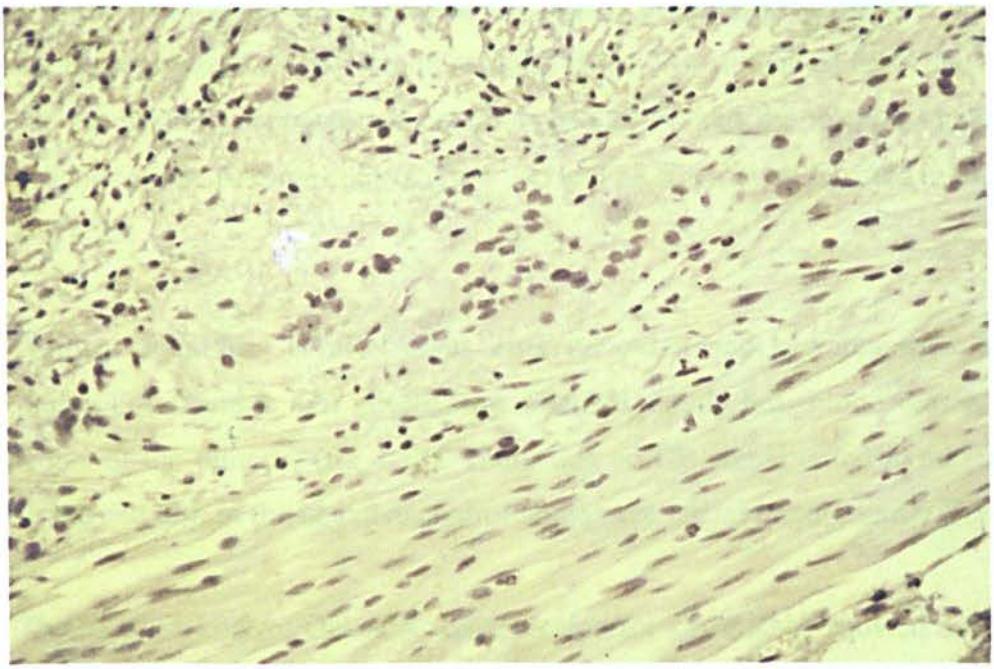


Fig 4.30 Magnification x1160. Negative control for DPPIV in Dukes' C tumour. No staining observed.

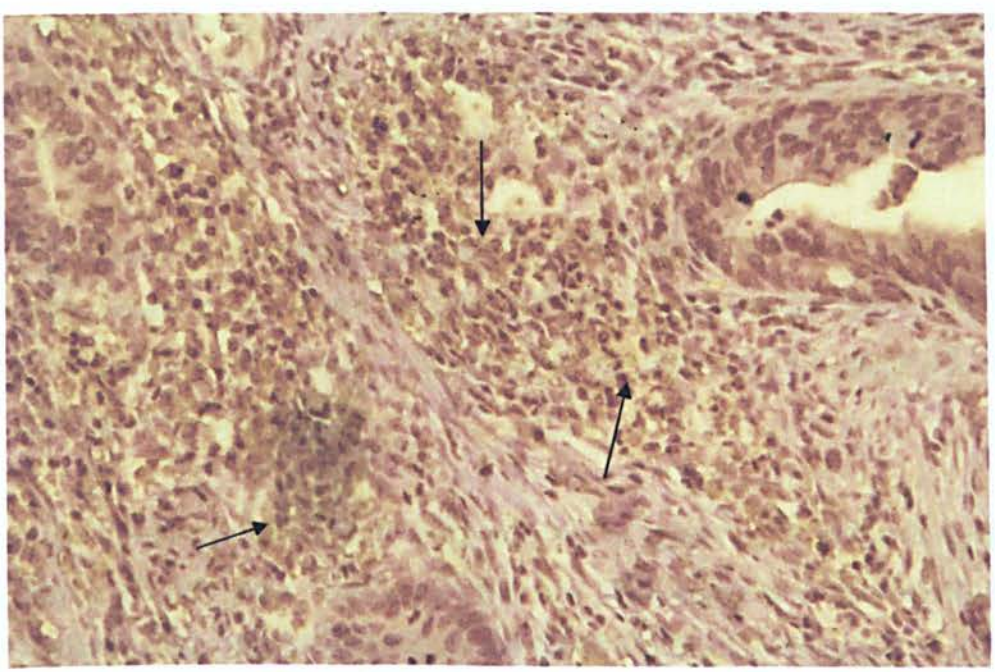


Fig 4.31 Magnification x 1120. DPPIV staining of Dukes' C tumour. A greater number of cancer cells show the presence of DPPIV (arrows). Staining is also greater in intensity for Dukes' C tumours.

Insitu Hybridisation

Positive controls for CB expressed CB signals in normal human liver tissue (fig 4.32) and the negative controls showed no staining (fig 4.33). Human tonsil tissue was used to carry out the positive and negative controls for DPPIV and the mRNA signal for DPPIV was detected in the positive control (fig 4.34) and no signal was detected in the negative control (fig 4.35).

A lower signal for CB mRNA was observed and distinct key areas were highlighted by the probe. For example, CB mRNA was detected mainly near blood vessels and the invasive fronts of the tumours (fig 4.37). There was no CB signal noted in adjacent normal colon tissue and also in the negative control human colorectal tumour tissue (fig 4.36).

The negative control Dukes'A tissue section for CB mRNA showed no staining (fig 4.38) and minimal Staining was observed in the Dukes' A section for CB demonstrating low levels of CB mRNA in this tissue grade (fig 4.39).

Dukes' B (fig 4.41) and C (fig 4.43) samples displayed greater number of cells positive for DPPIV mRNA than in Dukes' A (fig 4.45). No difference in mRNA expression for CB was noted between the histological stages of the colorectal tumours. It was noted that cancer cells displayed the mRNA signals for the two proteases and not the adjacent normal colon tissue. Negative controls showed no staining (figs 4.40, 4.42 and 4.44).

The method gave a strong and distinct signal for both CB and DPPIV mRNA in the cancer cells. However, the signal for DPPIV mRNA was stronger (fig 4.47). Negative control sections hybridised with a sense probe did not detect mRNA signals for both DPPIV and CB (figs 4.46 and 4.48).

An increase in mRNA levels were detected in cancer cells surrounding the secretory cells within the colorectal tumour sections and, also, adjacent to tumour blood vessels (4.49). However, the lack of expression for DPPIV mRNA was noted in surrounding normal tissue.

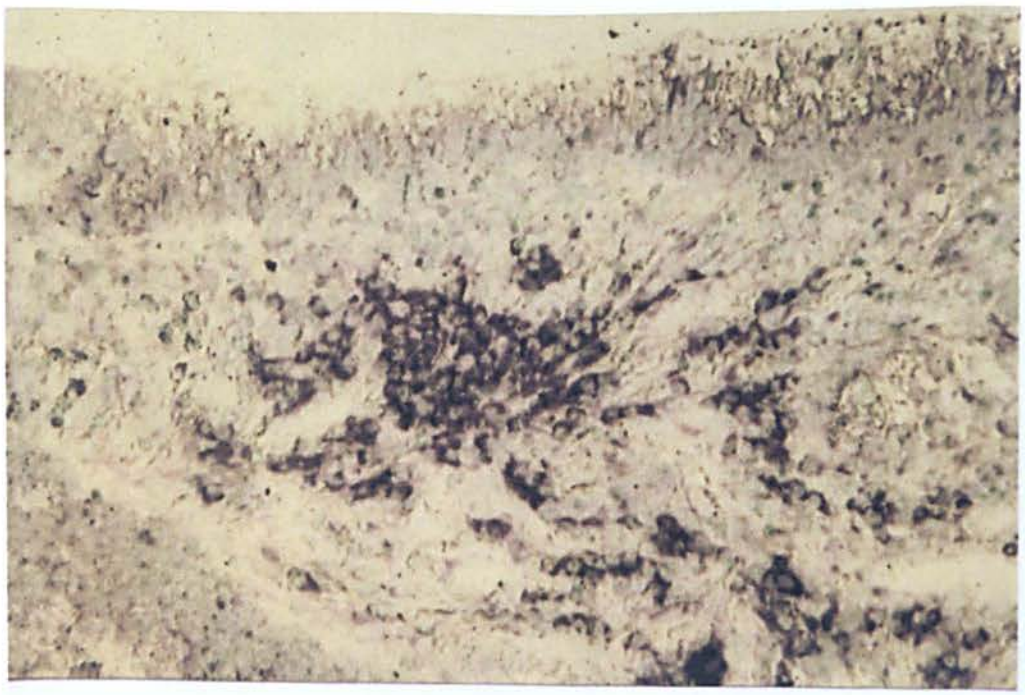


Fig 4.32 Magnification x1120. Positive control for CB mRNA in normal human liver tissue.

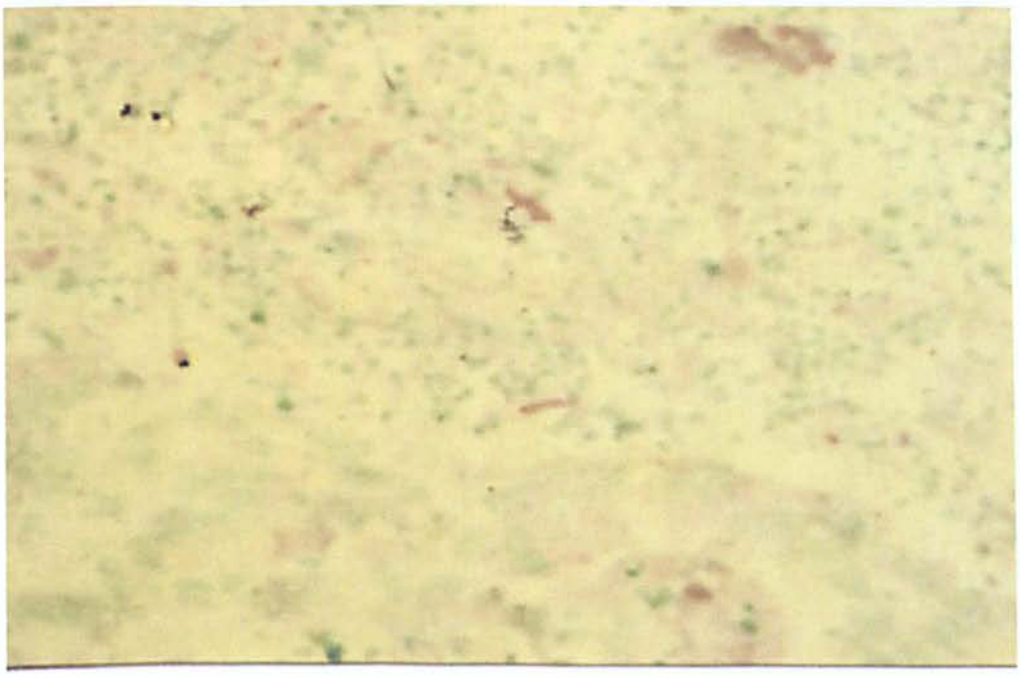


Fig 4.33 Magnification x1160. Negative control for CB mRNA in normal human liver tissue.

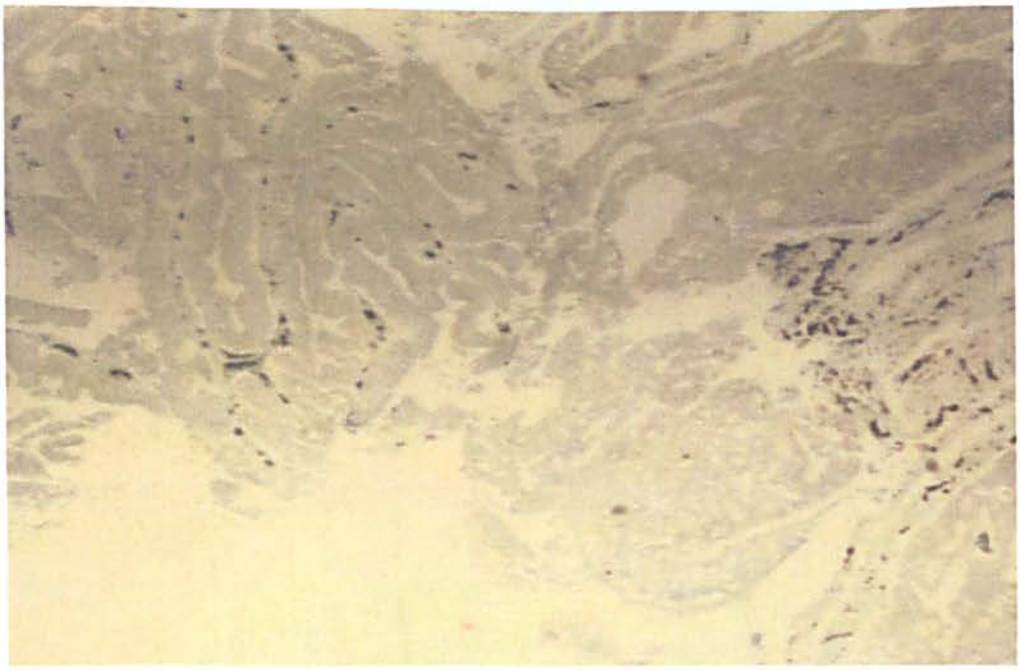


Fig 4.34 Magnification x700. Positive control for DPPIV mRNA in normal human tonsil tissue.

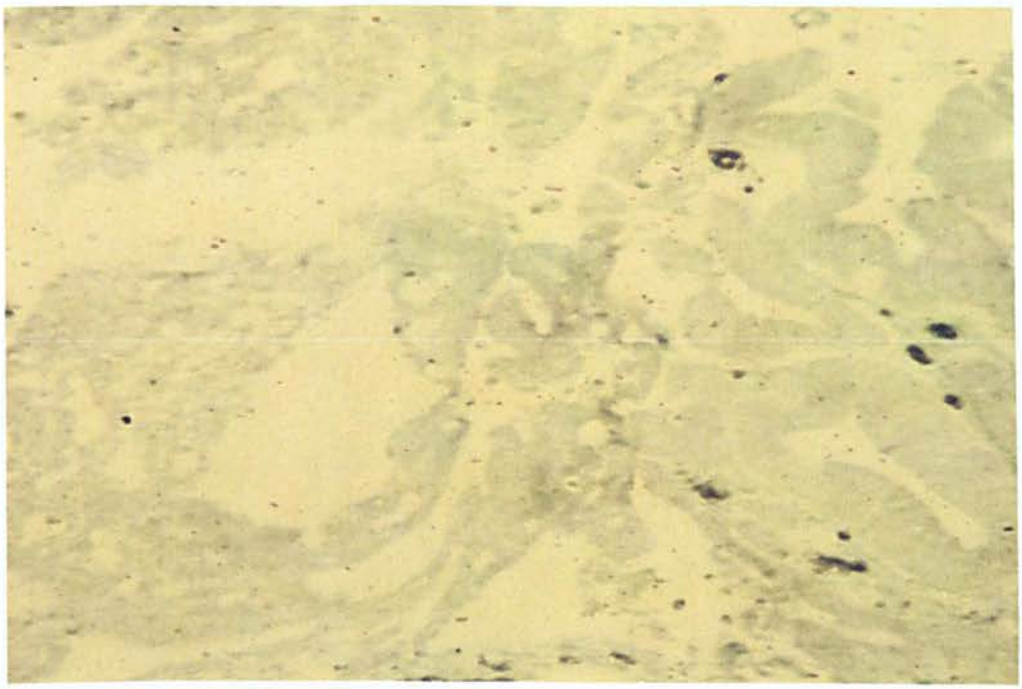


Fig 4.35 Magnification x700. Negative control for DPPIV mRNA in normal human tonsil tissue.

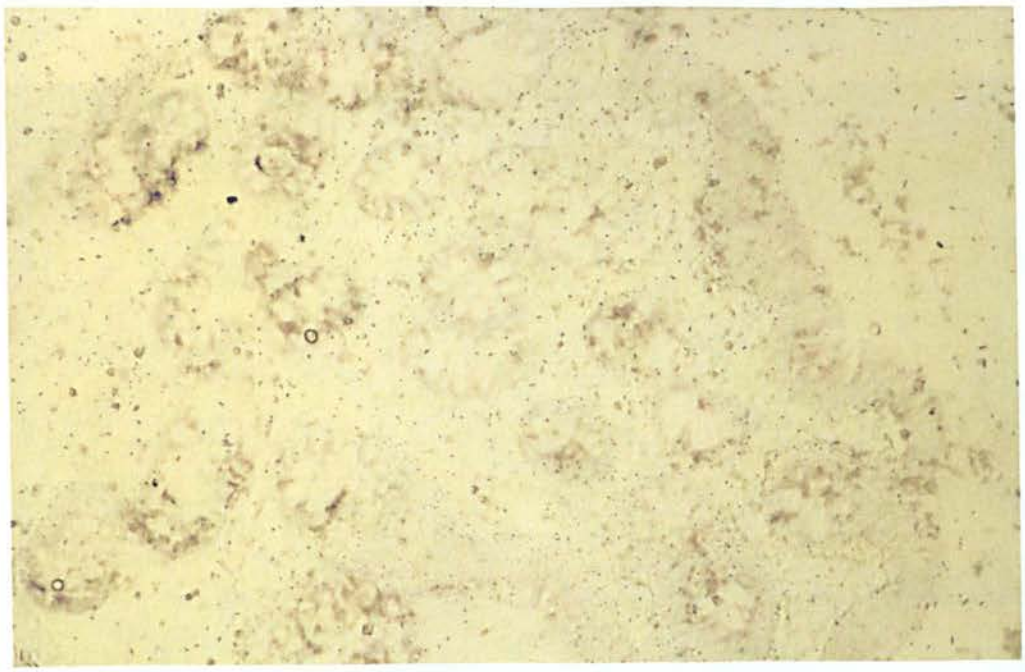


Fig 4.36 Magnification x840. Negative control for CB mRNA in human colorectal carcinoma tissue.

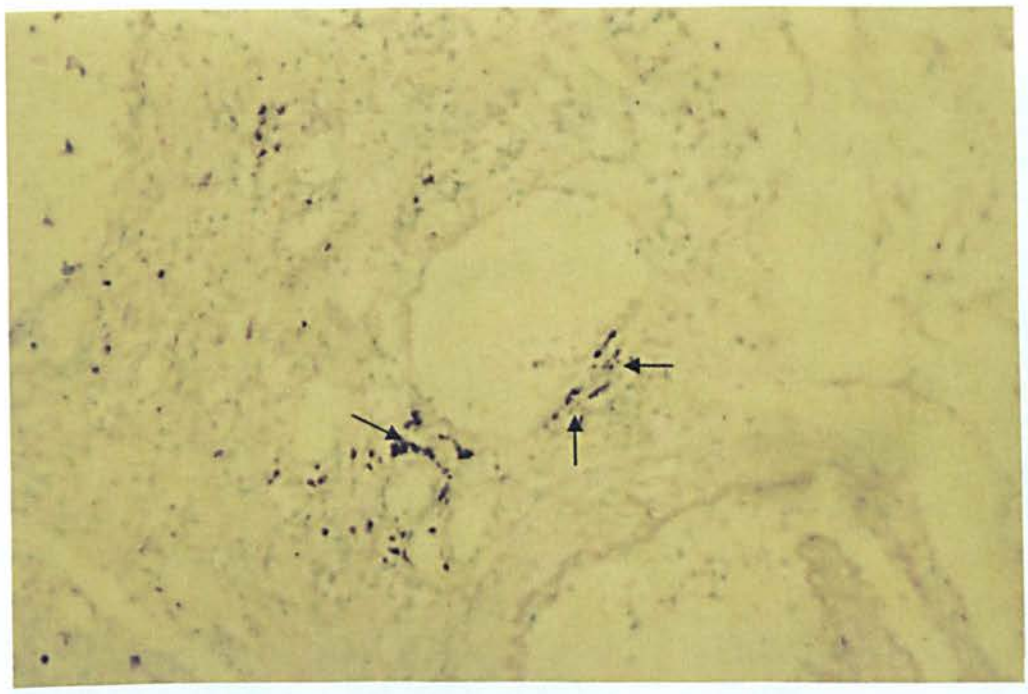


Fig 4.37 Magnification x 700. CB mRNA signal in colorectal carcinoma. CB mRNA is localised around tumour vessels. Intense CB mRNA signal is observed in the vascular endothelium (arrows).

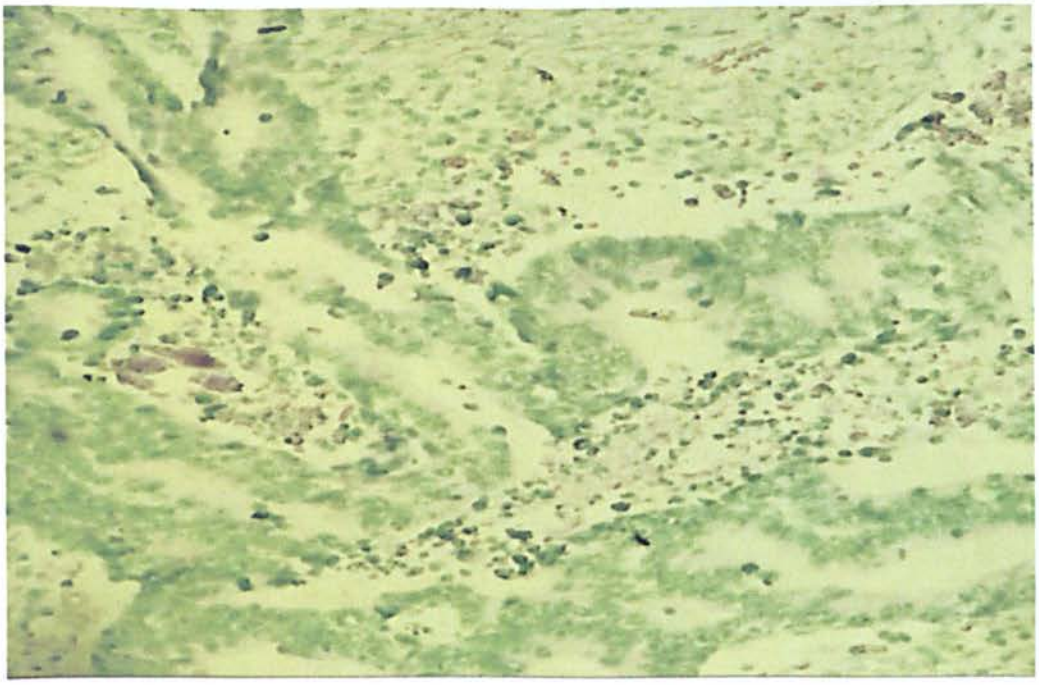


Fig 4.38 Magnification x 840. Negative control for CB mRNA in Dukes' stage A tumour. No signal detected.

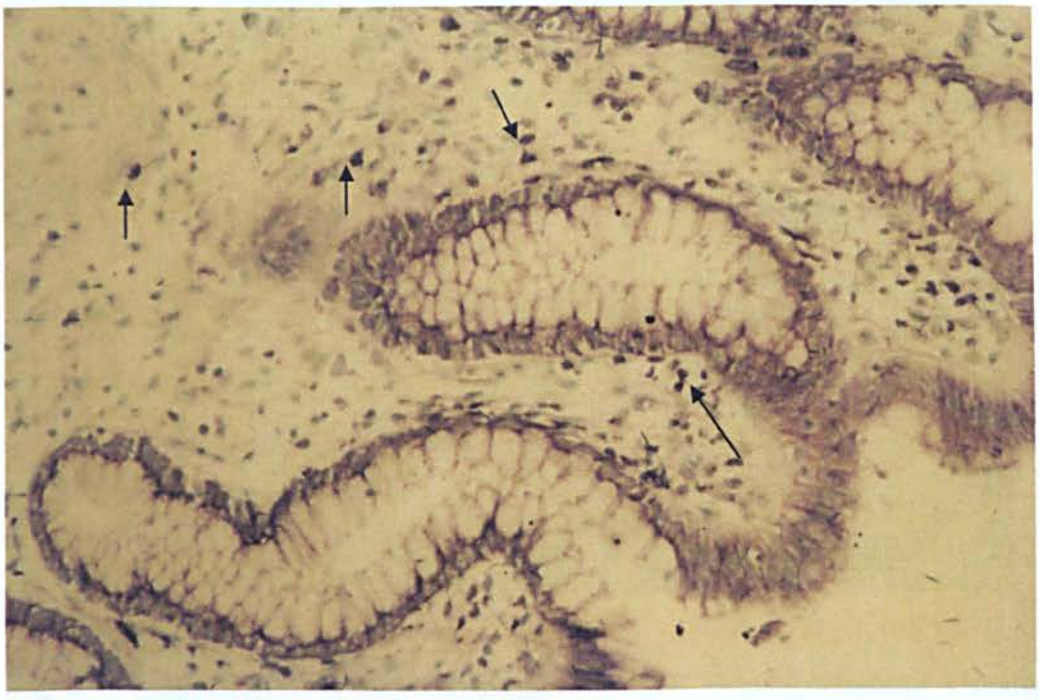


Fig 4.39 Magnification x 1120. CB signal in Dukes' stage A tumour. Certain cancer cells (arrows) exhibit CB mRNA however, expression is low.

Fig 4.40 Magnification x 700. Negative control CB mRNA in Dukes' grade B tumour.

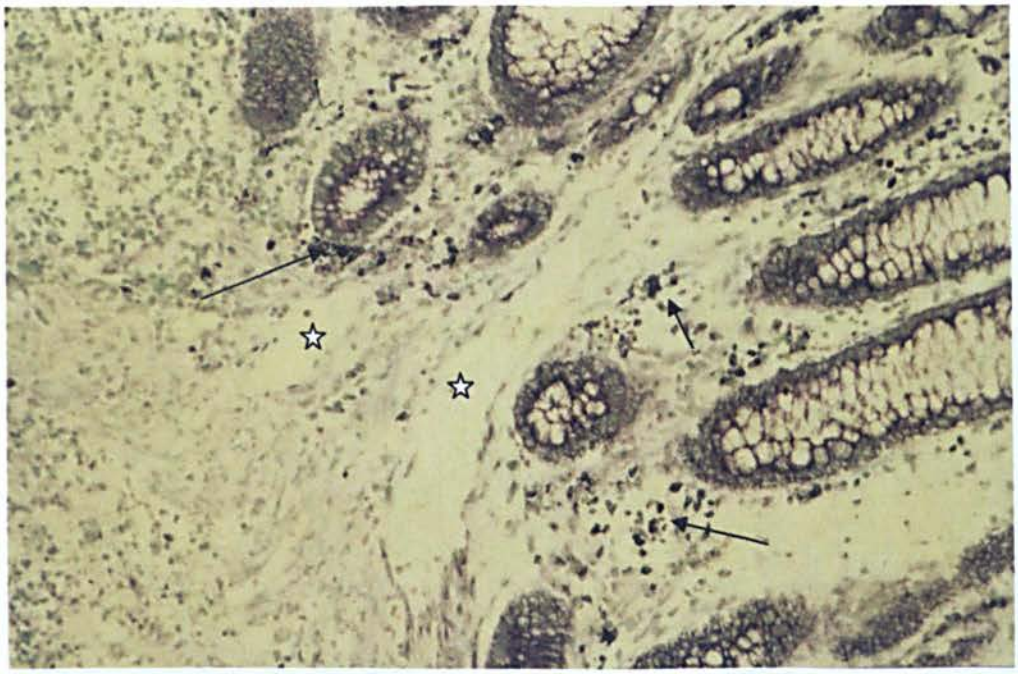


Fig 4.41 Magnification x 700. CB signal in Dukes' B graded tumour. Greater intensity of staining for CB mRNA is shown in the cancer cells surrounding the neoplastic glands in the tumour (arrows) and greater expression is also noted around tumour vessels (stars).

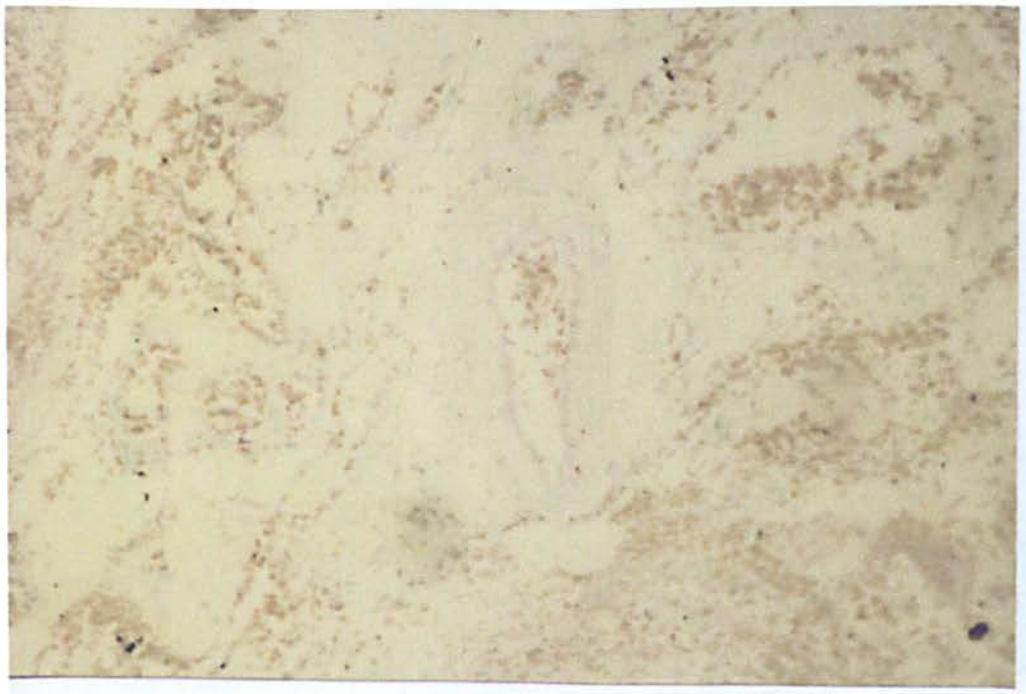


Fig 4.42 Magnification x700. Negative control for CB mRNA in Dukes' stage C tumour.

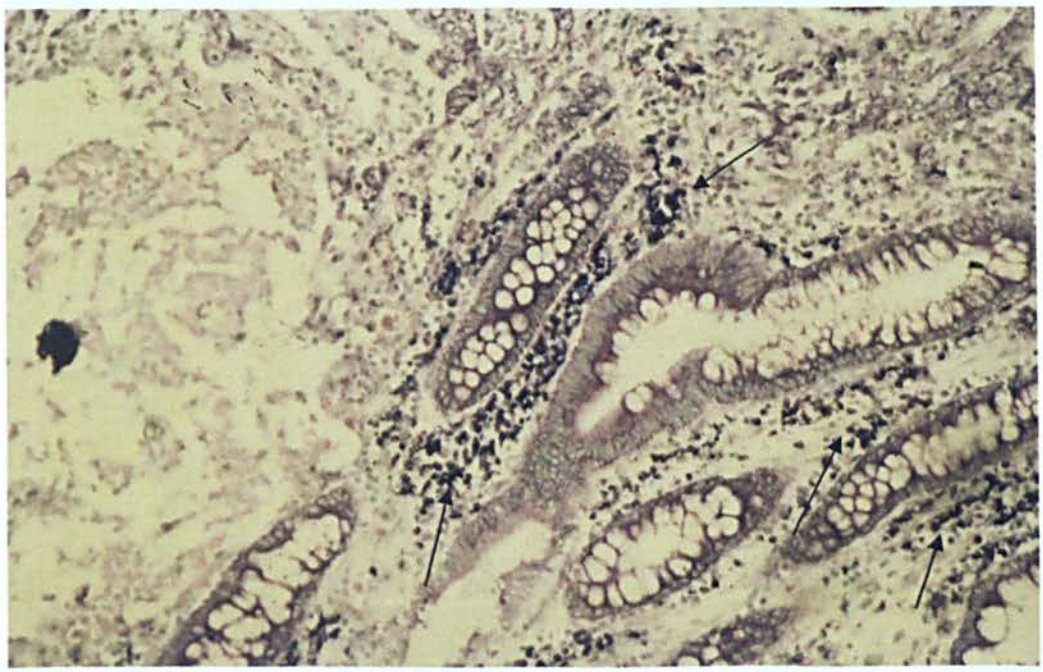


Fig 4.43 Magnification x 560. CB signal in Dukes' stage C tumour. The grade C tumour shows the greatest intensity and a larger number of cancer cells expressing CB mRNA levels (arrows).

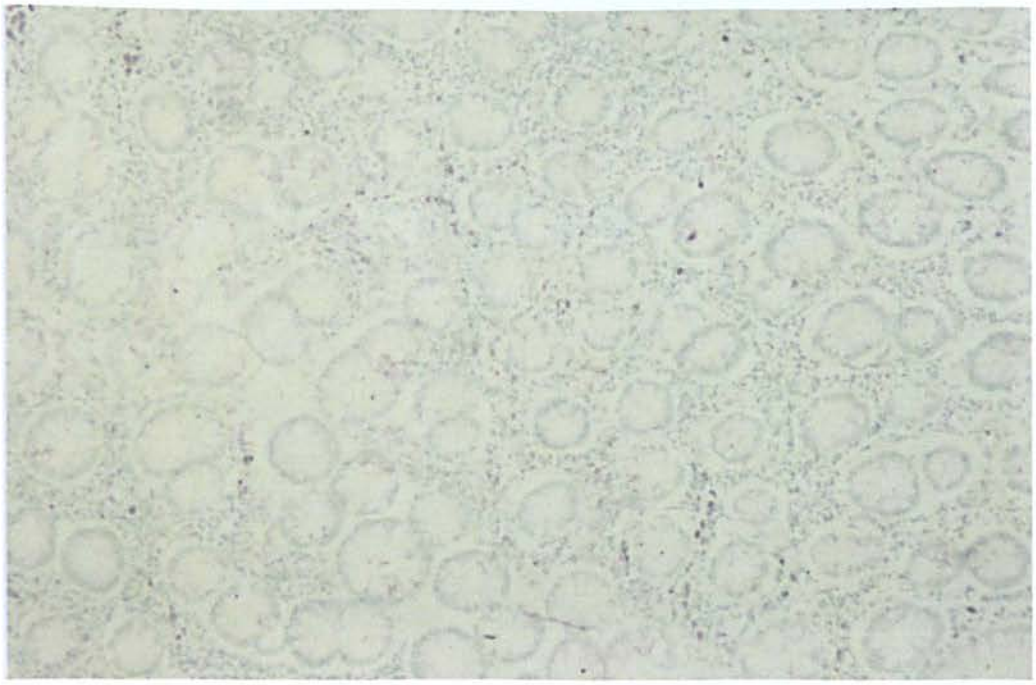


Fig 4.44 Magnification x560. Negative control for DPPIV mRNA in Dukes' stage A tumour.

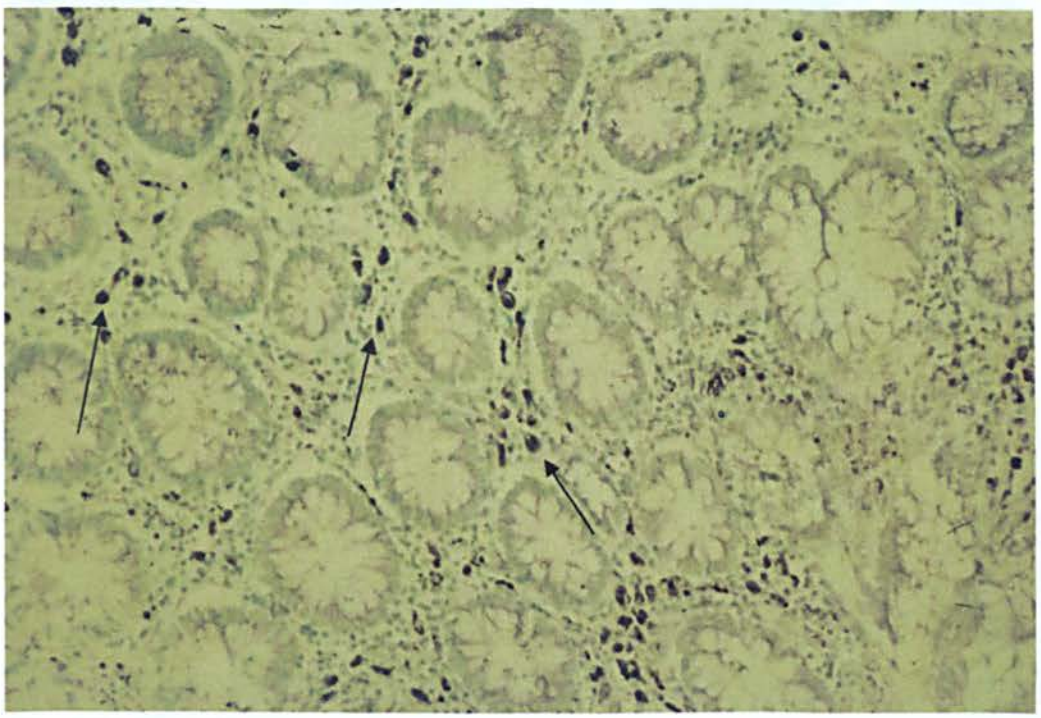


Fig 4.45 Magnification x 840. DPPIV staining in Dukes' stage A tumour. DPPIV mRNA expression is localised in cancer cells surrounding neoplastic glands of the colorectal tumour (arrows).

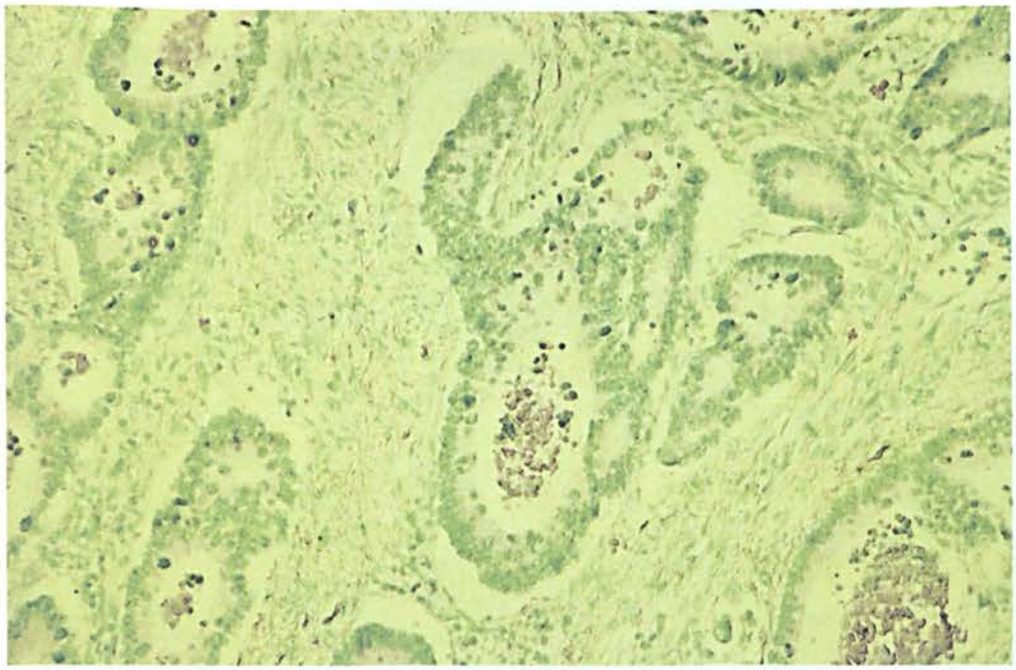


Fig 4.46 Magnification x840. Negative control for DPPIV mRNA in Dukes' B graded tumour.

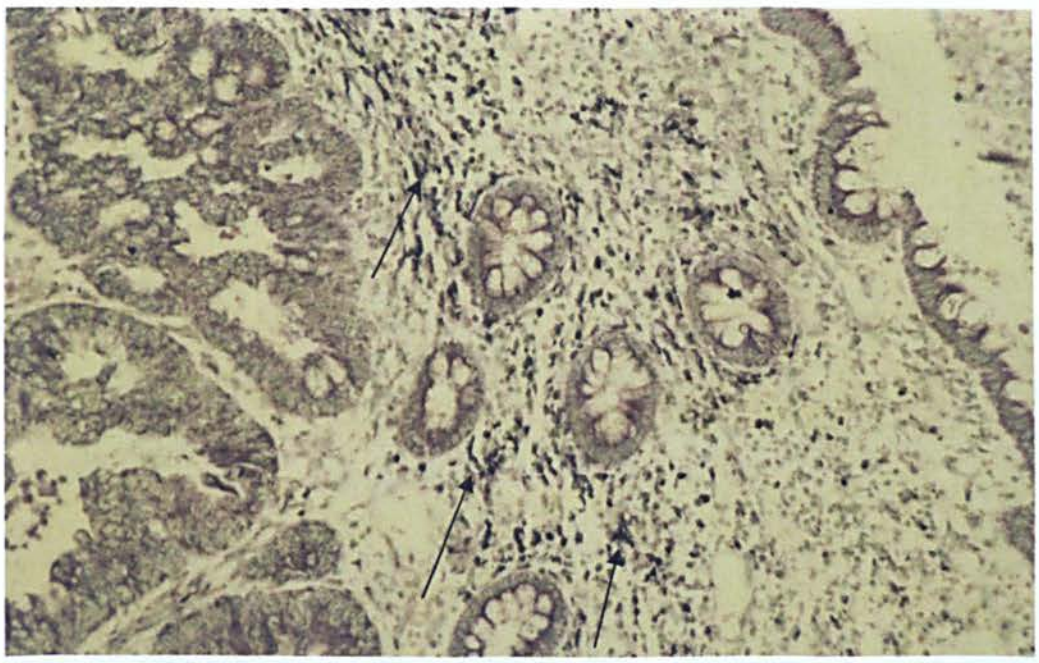


Fig 4.47 Magnification x 700. DPPIV signal detected in Dukes' B tumour. A greater number of neoplastic cells exhibit DPPIV mRNA in this tumour grade (arrows).

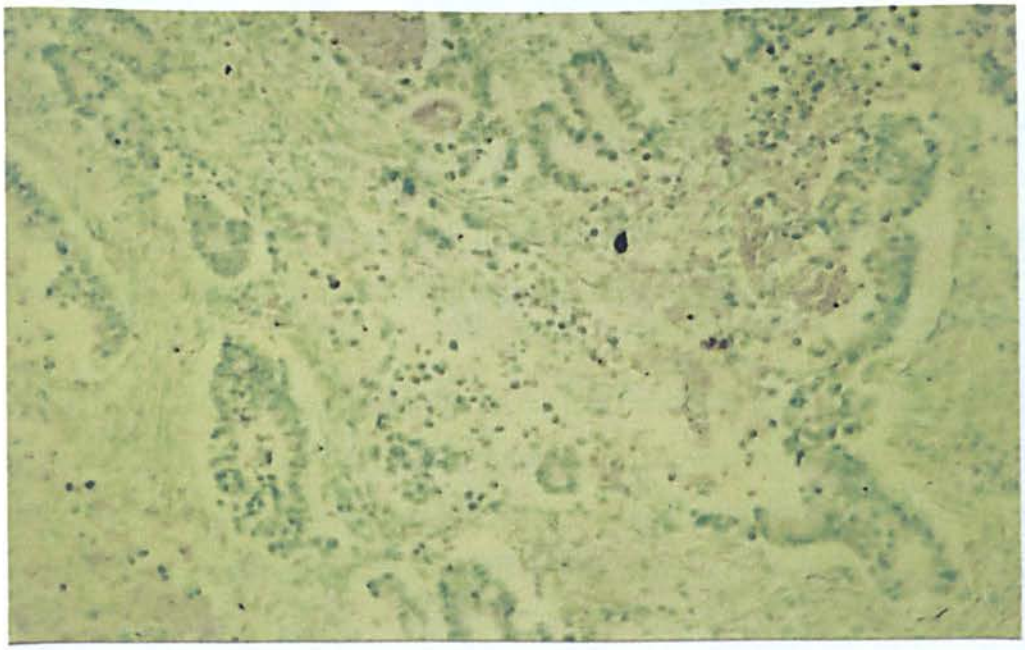


Fig 4.48 Magnification x700. Negative control for DPPIV mRNA in Dukes' grade C tumour.

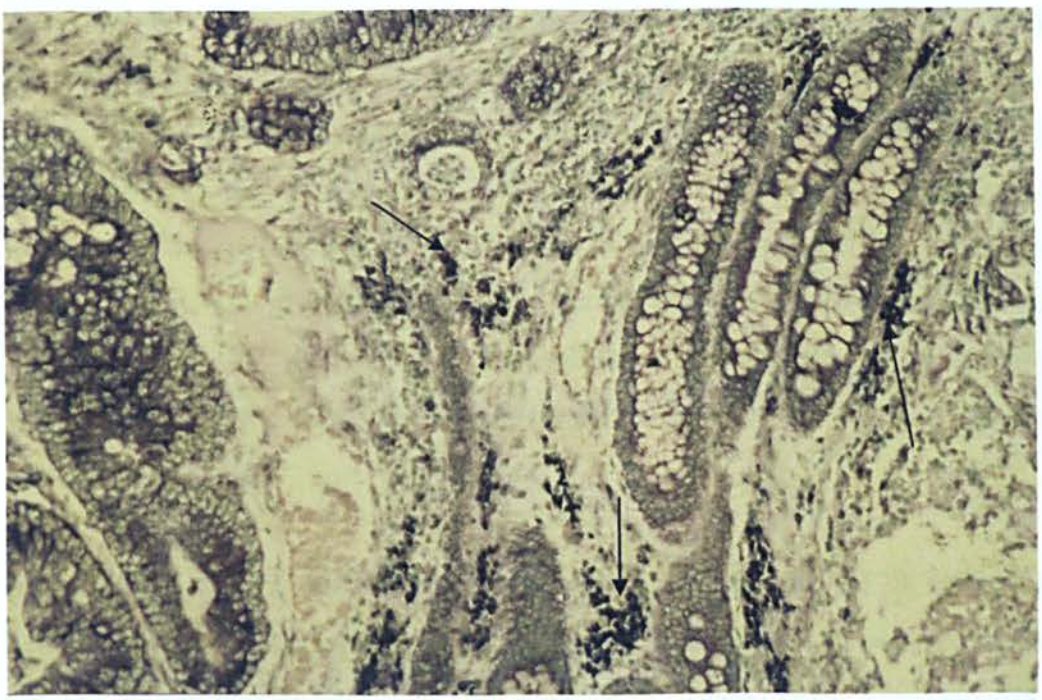


Fig 4.49 Magnification x 560. DPPIV signal in Dukes' C tumour. Highest level of DPPIV mRNA signal is displayed by grade C tumours.

4.5 Discussion

The metastatic potential of a tumour is its ability to invade adjacent tissue and spread to distant sites (Duffy 1996). For a cancer to metastasise, the natural barriers such as interstitial connective tissue and the basement membrane have to be degraded. This process is facilitated by proteolytic enzymes, released by the primary tumour.

Evidence implicating proteases in cancers and cancer spread have been described by many groups. Different proteases have been shown to be involved in the process of interstitial connective tissue and the basement membrane breakdown including cathepsin B (CB) and dipeptidyl peptidase IV (DPPIV) (Koblinski *et al*, 2000; Kelly 2005).

Several protease systems have been identified as being involved in tumour angiogenesis including the PA and the matrix mettalloprotease systems. However, a direct correlation of the levels of the cystein protease CB and serine protease DPPIV to the angiogenic potential of a tumour show conflicting results.

Therefore, this study aimed to measure the activity of CB and DPPIV, two enzymes known to possibly play a crucial role in tumour progression, in human cancer cell lines HT29 (colon cancer cells), HRT18 (rectal cancer cells) and in normal human endothelial cells (HUVECs). Also, the presence of CB and DPPIV was studied *in vivo* by examining the levels of protein expression of the enzymes in varying grades of human colorectal tumour and normal human colorectal sections. A further analysis to detect possible mRNA signals of DPPIV and CB in colorectal cancer cells was performed by *insitu* hybridisation. This was carried out to assess where and in which cells of the tumour/ non-tumour tissue protease production actually takes place.

Immunocytochemical methods conducted on cancer cell lines HT29, HRT18 and HUVEC cells highlighted both proteolytic enzymes CB and DPPIV. It was, however, noted that staining was more intense in HUVECs than in the cancer cells. This suggests that CB and DPPIV expression is greater in endothelial cells and that these cells probably display greater number of antibody receptor sites for CB and DPPIV than the cancer cells.

Cathepsins are known to be overexpressed in malignant cells (Garcea *et al*, 2005). It is found associated with the plasma membrane of tumour cells. This plasma membrane association of CB correlates with the metastatic potential of cells (Linebaugh *et al*, 1999).

Krueger *et al,* (2005) reported substantially stronger CB expression in human colon cells (HT29) indicating the proteolytic or invasive potential of a tumour mass.

CB is secreted from tumours and cancer cell lines, it is initially synthesised as a pre-pro-enzyme and is activated in pre-lysosomal acidic vesicles prior to its delivery to lysosomes. CB normally localises in lysosomes serving as a housekeeper enzyme. Increased secretion of pro-cathepsin B or mature CB by human colorectal cell lines has been reported (Mai *et al,* 2000). The present study shows increased expression of CB in HUVEC cells than in the cancer cells, which contradicts reports by Poole *et al,* (1980). They stated that CB secretion is higher in malignant cells than in non-malignant cells.

The elevation of DPPIV in HUVECs was demonstrated by Ikushima *et al,* (2002). They state that CD26/DPPIV enhances transendothelial migration of T cells following its binding to endothelial cells via its specific interaction with endothelial receptor M6P/IGFIIR. Therefore, this receptor is now known as the putative receptor for CD26/DPPIV in HUVEC cells.

Ghersi *et al,* (2001) also reported that a new sympathetic vasoconstrictor peptide NPY (neuropeptide Y) is expressed in HUVEC cells and that DPPIV, also expressed by endothelial cells, may function as a switch converting NPY as a vasoconstrictor to angiogenic.

However, DPPIV has also been shown to be expressed on Caco-2 cells which are derived from human colon adenocarcinomas (Erickson *et al,* 2000). Although, Komatsu *et al,* (1987) demonstrated that DPPIV activity in tumour cells of patients decreased in activity. They showed that gastric carcinoma tissue had lower DPPIV activity than normal gastric tissue from the same patient. This reduced DPPIV activity has also been reported in human colon cancer. Kikkawa *et al,* (2005) also reported that DPPIV has no effect on carcinoma cell growth and enzyme activity is related to anti-invasiveness. This supports the fact that DPPIV expression was greater in HUVECs cells than in HT29 and HRT18 cells.

So far, contradictory results have been obtained from investigations assessing the expression and functional significance of CB in colon cancers, therefore, this study set out to investigate CB and DPPIV levels of expression in colorectal carcinomas of varying grades. Tumour sections, obtained from tumours with grades ranging from Dukes' A to C,

from colorectal cancer patients were analysed by localising CB and DPPIV enzyme protein by immunohistochemistry.

Intense staining for CB was observed and intensity increased with higher tumour grades. Staining with CB was greater in tumour tissue than staining with DPPIV. Cancer cells, epithelial cells near tumour sites, stromal cells and connective tissue around the tumour were all immunopositve for CB. However, non-cancerous tissue adjacent to the tumour was negative for the enzyme. Distinct increase in CB staining was observed in the invasive edge and the vascular endothelium of the tumours.

These staining patterns typically suggest that CB is actively expressed in tumours especially invasive cancers and that it contributes to the progression of colorectal cancers by tumour angiogenesis and growth. Talieri *et al,* (2004) reported that CB expression is significantly higher in colon cancer tissue as that compared to normal mucosa of the colon.

Talieri *et al,* (2004) and Campo *et al,* (1994) demonstrated that the epithelial cells of the normal mucosa were negative for CB and that there was increased expression of the enzyme in the invasive fronts of the colon carcinomas. Adenis *et al,* (1995) also demonstrated very low CB activity in normal colorectal tissue compared to tumour tissue.

Lah *et al,* (2000) stated that cystein proteases are more widely distributed in stromal cells such as fibroblasts and endothelial cells which matches with our findings of the presence of CB in stromal cells and in particular tumour endothelial cells.

Immunostaining for CB in sections from varying tumour grades was found to be significantly higher in Dukes' stage B and C. Repeated staining patterns in the carcinomas suggested that increased tumour cell staining correlated with the stage of the disease. These results follow the ones obtained by Talieri *et al,* (2004).

Murnane (1991) postulated that CB mRNA expression was 4-fold greater in Dukes' stage A and B than in Dukes' stages C and D. Other contradictory results have also been obtained by Hazen *et al,* (2000) who reported an inverse relationship between the differentiation of the tumour and CB activity. Troy *et al,* (2004) found that CB activity of tumour to normal ratios were high in early stage disease and declined steadily with advancing tumour stage.

None-the-less, majority of the investigating groups demonstrated that immunoreactivity for CB was observed in fibroblasts of the tumour stroma and macrophage cell clusters at the invasive fronts (Krueger *et al*, 2005). Mckerrow *et al*, (2000) showed that CB was especially prominent extracellularly at the interface between the invading tumour and normal tissue. Bleeker *et al*, (2000) describes that Hazen *et al*, (2000) found CB activity localised in the invasive fronts and also in areas of angiogenesis and inflammation in colon biopsies. This also coincides with our findings.

CB expression was also positive in carcinoma samples that came from tissue with lymph or blood vessel invasion. CB expression was positive in all cases suggesting that CB may not only play a role in local tumour invasion but also in distant metastasis.

In tumours stained with DPPIV cells were positive for the enzyme however, intensity of staining was lower compared to CB immunoreactivity. Analysis of DPPIV expression, in the varying tumour grades, exhibited weak positive staining in Dukes' stage B and C cancers. However, Dukes' stage A tumours were negative for the enzyme.

No staining was observed adjacent to the tumour site. Weak staining was observed in tumours from tissue with blood vessel and lymph vessel invasion and also in tissue without blood vessel invasion. No staining was observed in tumours from tissue without lymph vessel invasion.

The expression of the membrane-bound enzyme, DPPIV, has been considered as a possible tumour marker (Komatsu *et al*, 1987). However, even though an up-regulation of DPPIV is exhibited by tumours with increasing tumour grades, their expression is relatively weak. This may be due to the fact that DPPIV is not actively expressed in human colon cancers, contradictory to other findings.

Darmoul *et al*, (1992) reported that DPPIV is expressed in human colon cancers. Boonacker and van Noorden, (2003) stated that CD26/DPPIV expression in cancer cells seems to down-regulate cancer progression because it induces differentiation. On the other hand it plays a direct role in tumour invasion and metastasis.

Iwata and Morimoto, (1999) stated that CD26/DPPIV expression may be a powerful tool for diagnosis in thyroid carcinomas. It is also known that DPPIV activity is high in basal cell carcinomas and in breast cancers.

Inverse immunoreactivity was reported in endometrial adenocarcinomas. DPPIV staining was also reported to be stronger in well differentiated tumours as compared to the weak staining observed in poorly differentiated ovarian carcinomas (Kikkawa *et al,* 2005). These studies suggest that, although, the expression of DPPIV seems to vary from type to type of carcinoma, this protease may be negatively related to differentiation.

However, results from this study are contradictory to the above studies. This may probably be due to the use of different tumour types and different methods which, in turn, makes comparisons of the results difficult.

In situ hybridisation revealed that CB mRNA signal was weaker than the signal for DPPIV. However, distinct areas of CB mRNA were located such as invasive fronts and in areas close proximity to the tumour vessels. Normal tissue, adjacent to tumour tissue, was negative for CB and there was no difference in the enzyme expression between tumour grades. In comparison, the signal for DPPIV mRNA was strong in cancer cells and also noted to be elevated in Dukes' stage B and C. No staining was observed in adjacent normal tumour tissue.

The increase in CB mRNA has been linked to tumour progression. In colon carcinomas the mRNA levels are higher than in normal colon tissue (Mai *et al,* 2000). This opposes our findings as no distinct correlation was observed between the mRNA signal for CB and the Dukes stages; taking into consideration that this study has only made qualitative comparisons.

The difference in results from protein expression of CB to its mRNA expression may be due to the fact that CB can occur from cells that do not exhibit an increase in mRNA levels (Sloane *et al,* 1994 and Mai *et al,* 2000). Mackenzie *et al,* (2001) showed that the increase in CB mRNA is characteristic in metastatic colorectal tumours. Strojnik *et al,* (2005) described the association between CB mRNA levels and malignant progression of gliomas. They also found high CB expression in endothelial cells of new vasculature within tumours.

An elevated signal of mRNA for CB was observed near tumour vessels in this study. This suggests that colorectal cancer cells near tumour blood vessels have a high potential for CB release which strongly implicates proteases in the process of tumour vessel formation.

This chapter demonstrated that there was a stronger DPPIV mRNA signal than CB in human colorectal cancer tissue. Although, DPPIV expression in cultured cancer and endothelial cells and in human colorectal tissue from this study, alongside results obtained by other groups all propose that elevated DPPIV expression is correlated to decreased metastatic potential (Boonacker and van Noorden, 2003). This indicates that colon cancer cells may exhibit a strong potential for the DPPIV enzyme by expressing the DPPIV mRNA signal. As colorectal cancers progress the release of the active DPPIV form may decrease as tumorigenicity increases.

This study suggests that the proteases CB and DPPIV may have the potential to be possible biological markers for the progression of colon cancers. However, many more experiments assessing the correlation to tumour progression and angiogenesis to the levels of the protease expression and activity need to be carried out as little is known of the exact roles of CB and DPPIV in tumorigenicity.

Chapter 5

Determination of the enzyme proteases cathepsin B and dipeptidyl peptidase IV activity and presence under the influence of the angiogenic proteins VEGF and bFGF in HT29, HRT18 and HUVEC cells.

5.1 Introduction

The development of the vascular supply is a fundamental requirement for organ development. Blood vessel formation is also necessary for tissue repair and reproductive functions in adults and substantial experimental evidence also implicates angiogenesis in the pathogenesis of a variety of disorders such as tumour formation (Ferrara 2004).

Tumour neovascularisation aids tumour growth by providing nourishment hence, studies have aimed to correlate metastatic behaviour with tumour vascularity. Many studies correlate cancers of the breast, colon, prostate, bladder and uterus with tumour microvessel density, therefore, the extent of angiogenesis may be considered as an important prognostic marker (Bamias and Dimopoulos, 2003).

There are three fundamental steps in the cascade of events in angiogenesis and they are:
i) degradation of the extracellular matrix of an existing vessel, ii) chemotaxis of endothelial cells towards an angiogenic stimulus, and iii) proliferation of endothelial cells (Takahashi et al, 2005). These steps are tightly regulated by a complex of different angiogenic growth factors such as acid fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), and tumour necrosis factor (TNF)α. These are established potent angiogenic growth factors which promote the proliferation of vascular endothelial cells *in vitro* and elicit neovascularisation *in vivo* (Takahashi *et al*, 2005).

Two of the most extensively studied and also the most powerful mediators of neovascularisation: vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Bremnes *et al*, 2005) (fig 5.0), will be examined in this chapter.

5.1.2 *VEGF*

VEGFs are, apparently, the most important angiogenic factors and are the prime regulators of both physiological and pathological angiogenesis in malignancy (Veikkola and Alitalo, 1999). It is a 45kDa glycoprotein encoded for by a gene located at chromosome 6 (6p21), is able to bind to heparin, and has 18% homology with platelet derived growth factor (PDGF).

There are five groups of VEGF which are VEGF-A, -B, -C, -D and –E where VEGF-A is of prime importance for angiogenesis (Onogawa *et al*, 2005). VEGF-A consists of further subgroups which are its isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$. *In vivo*, only three secreted isoforms $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$ can induce angiogenesis with $VEGF_{165}$ being the most predominant isoform secreted by benign and malignant cells (Milkiewicz *et al*, 2006).

VEGFs bind to receptors which exhibit tyrosine kinase (TK) activity. These receptors are permanently expressed independent of proliferation or quiescence state and are distributed in the total vasculature (Jakeman *et al*, 1992). Two TK receptors have been identified as VEGF receptors and they are VEGFR1 (also known as fms-like-tyrosine kinase (flt-1) and VEGFR2 (also named fetal liver kinase-1(flk-1) or kinase domain region (KDR)) (Boocock *et al*, 1995).

These have a very high binding affinity to VEGF and studies have shown that these receptors are essential for normal embryonic and postnatal development of the cardiovascular system. The two receptors have different signal transduction functions: VEGFR2 is a major mediator of the mitogenic, angiogenic and permeability-enhancing effect of VEGF. VEGFR1 is primarily involved in vessel differentiation /architectural organisation, haematopoiesis, and induction of the metalloproteases (Gasparini 2000).

Not only do endothelial cells secrete VEGF but so do other cells such as macrophages, stromal cells, epithelial cells of the retina and malignant cells. The target cells of VEGF are, primarily, vascular endothelial cells which regulate both normal physiological growth and pathological growth of any type of blood vessel through angiogenic and vasculogenic processes (Nagy and Senger, 2006).

The molecule is regarded as an endothelial cell specific mitogen and is able to induce a marked angiogenic response in a variety of *in vivo* models including the chick chorioalantoic membrane and the rabbit cornea. VEGF is also reported to influence the tubule formation of vessels (Bicknell *et al*, 1997).

It has been proposed that an increase in microvascular permeability is a crucial step in angiogenesis associated with tumours and wounds. Hence, the major function of VEGF is to induce plasma protein leakage. Other characteristics include: VEGF induces tissue factor expression in cultured endothelial cells. It has been proposed that the induction of such a procoagulant protein may contribute to the abnormal coagulative properties of tumour vessels in response to TNFα (Honorati *et al*, 2004).
It has also been shown to induce vasodilation which is termed as transient hypotension *in vivo*. The process is mediated by endothelial cell derived nitric oxide (Akbulut *et al*, 2002).

Oxygen tension plays an important regulatory role for VEGF both *in vitro* and *in vivo*. Studies have shown that VEGF mRNA is rapidly induced by exposure to low PO_2 levels in cultured tumour cells. In solid tumours elevated expression of VEGF mRNA was noted in ischaemic tumour cells adjacent to areas of necrosis. As tumours develop, malignant cells are increasingly deprived of oxygen due to their distance from the nearest blood vessels. Hypoxia in these cells induces the production of VEGF by increasing the transcription rate and stability of the VEGF mRNA (Bremnes *et al*, 2005).

Other factors also induce VEGF production. Reports state that the increase in VEGF gene expression is noted after inactivation of p53 and after the expression of the oncogene *Src* (Gasparini 2000). Several cytokines have been shown to up-regulate the expression of VEGF mRNA or induce release of VEGF protein. Factors such as endothelial growth factor (EGF), tumour growth factor-β (TGF-β) and keratinocyte growth factor help this process which suggests that VEGF functions as a paracrine mediator for indirect- acting angiogenic agents (fig.5.0) (Garcea *et al*, 2005).

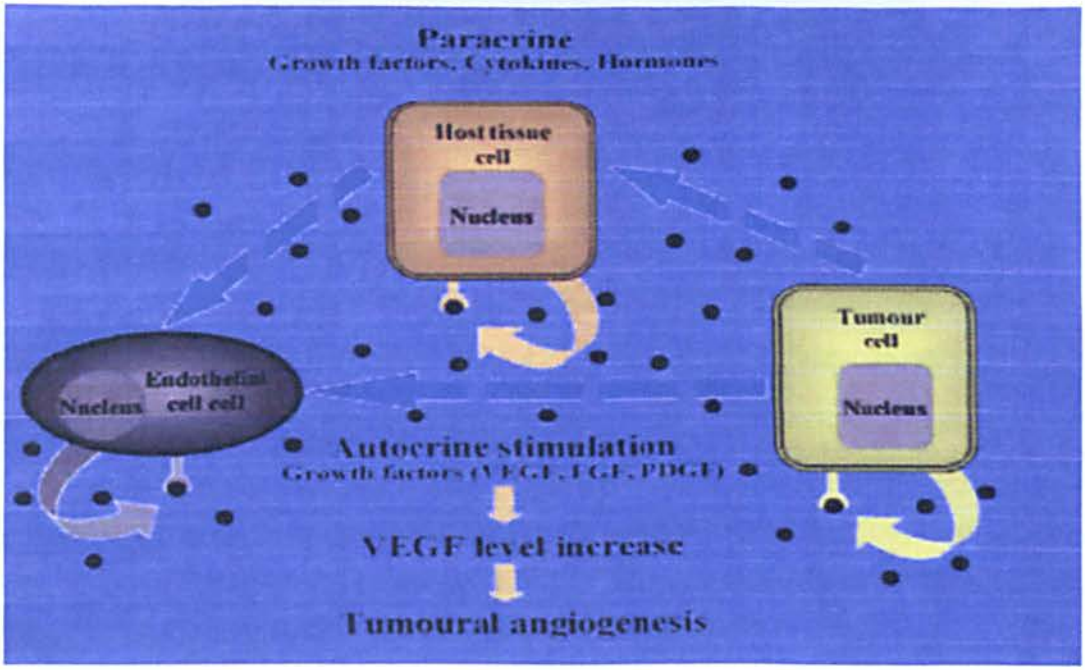


Fig 5.0 Paracrine and autocrine stimulation of tumour angiogenesis. In addition to tumour cells, tissue stroma and blood cells play a role (Bremnes *et al*, 2005).

In situ hybridisation studies have shown that VEGF mRNA is markedly increased in tumours of the kidney, bladder, breast, ovary, and the gastrointestinal tract. In all of these cases the mRNA is expressed in tumour cells and not in endothelial cells; this is consistent with the fact that VEGF is purely a paracrine mediator (Aonuma *et al*,1999; Lauer *et al*, 2000).

Immunohistochemical methods have localised the VEGF protein not only in tumour cells but also in tumour vasculature. This suggests that tumour-secreted VEGF accumulates in the target cells. There is a strong correlation between the degree of vascularisation in a tumour and its VEGF mRNA levels, in addition, VEGF receptors flt-1 and KDR are up-regulated in tumour associated endothelial cells compared to normal tissue (Lee *et al*, 2000; Onogawa *et al*, 2005; Duff *et al*, 2006).

Warren *et al*, (1995) demonstrated that VEGF is a major mediator of the *in vivo* growth of human colon carcinoma cells in a nude mouse model of liver metastasis where the tumour cells were injected into the spleen.

Lee *et al*, (2000) found that VEGF expression was positively correlated with the progression of human colorectal cancer however, their further evaluation of VEGF phenotypes in colorectal cancer patients did not provide additional prognostic value.

Duff *et al*, (2006) summarised that immunohistochemical expression increased in advanced stages of the cancer and therefore, was an independent prognostic factor for colorectal cancer patients.

Measurements of VEGF in patient sera have also been carried out to evaluate the prognostic significance of this growth factor in colorectal cancer patients (Aonuma *et al*, 1999; Duff *et al*, 2005). Werther *et al*, (2000) presented a study which indicated that high pre-operative VEGF values were associated with a poor patient outcome. However, Duff *et al*, (2006) found that VEGF levels in colorectal cancer patients were no different from the control patients and hence, could not link serum levels with clinicopathological variables. The present study set out to investigate the influence of circulating VEGF-A, VEGF-C and VEGF-D in pre-operative colorectal cancer patients.

5.1.3 *bFGF*

Basic fibroblast growth factor (bFGF), also classified as FGF-2, is a member of a group of heparin binding multifunctional polypeptides and is also a potent angiogenic factor. There are nine members of the FGF family labelled FGF1-9 (all FGFs are 18-30 kDa proteins) and their genes are structured in three exons separated by two introns exhibiting dramatically different lengths within the family members (Bicknell *et al*, 1997).

bFGF is a 18 kDa, 155 amino acid protein that was first purified from bovine pituitary. bFGF is found to be localised in the cytosol. However, higher molecular weight isoforms are predominantly associated with the nucleus. It is found in many cultured cell types including fibroblasts, endothelial cells, glial cells, and smooth muscle cells. Many tumours and tumour cell lines also express bFGF as an autocrine regulatory factor. This protein stimulates DNA synthesis, cell proliferation and migration hence, plays a key role in haematopoiesis and angiogenesis (Bremnes *et al*, 2005).

Two types of FGF-binding sites have been identified on a large number of tissues and cell types. High affinity receptors of FGF represent a low number but high-affinity binding sites. These receptors are members of the transmembrane tyrosine kinase family of receptors. The low-affinity receptors for FGF are predominantly proteoglycans carrying heparin sulphate side chains which present the FGF molecule to the high-affinity receptor

sites. Amongst the high-affinity fibroblast growth receptors FGF receptor 1 is shown to bind to bFGF (Bicknell *et al,* 1997; Takahashi *et al,* 2005).

Upon binding to their receptors FGF is rapidly internalised which is a process by which the molecule is stabilised and then accumulated within the cell, unlike other growth factors which are degraded through the lysosomal pathway (Bicknell *et al,* 1997).

Based on their pattern of expression and their biological activities they are thought to be involved in many physiological and pathological processes such as embryonic development, differentiation, cell survival, angiogenesis, oncogenic transformation and tumour cell proliferation (Bremnes *et al,* 2005).

Extensive studies on the expression of bFGF in tumour biopsies and functional studies on tumour cell proliferation provide ample evidence of its involvement in tumour development. Elevated expression of bFGF, in some studies, correlates significantly to advanced stages of malignancy ie. studies have been carried out in: human bladder, pancreatic, ovarian and gastric carcinomas, pituitary and renal tumours, mammary carcinoma cell lines, hepatoma cell lines, prostate carcinoma cell lines and colon carcinoma cell lines (Poon *et al,* 2001).

The role of intracellular bFGF in tumour growth, however, has not been elucidated. In human colorectal carcinoma cells bFGF promotes the process of tumour cell invasion (Akbulut *et al,* 2002). Studies on bFGF production and tumour cell growth have revealed that levels of the growth factor production are correlated to tumour cell proliferation (Kuhn *et al,* 2004). This confirms the results reported by Berger *et al,* (1999) who showed that intracellular concentration of bFGF was elevated in faster growing cells compared with cells exhibiting a low proliferation rate. The correlation of bFGF with tumour cell growth was also observed in human colon carcinoma and rat glioma cell lines by Gross *et al,* (1993).

Expression of FGF receptors is also shown to be elevated in a variety of cancer types such as breast cancer (Kuhn *et al,* 2004). Here, the amplification of FGF receptors is significantly correlated to the amplification of the gene *c-myc* (Bicknell *et al,*1997).

bFGF, as well as VEGF, is also known to induce tumour angiogenesis and in most cases bFGF expression correlates the degree of vascularity of the tumours. In order to induce tumour angiogenesis, FGFs produced by tumour cells, need to be released to bind FGF receptors on the surface of endothelial cells. However, bFGF lacks a signal sequence for secretion, and therefore usually found cell associated, and although the mechanism of its release from cells is known its export pathway has not been elucidated (Gontero *et al*, 2004).

It has also been suggested that endothelial cells within tumour vessels are also a source of bFGF production as well as tumour cells. Other sources include macrophages, which are recruited by the tumour, which induces them to produce bFGF. Furthermore, mast cells may also be taken up by tumours. The cells contain heparin which might be released in the tumour and hence, amplify the effects of bFGF (Ogawa *et al*, 2000).

In many tumours that have been studied the extent of vascularisation correlates with the expression of FGF (Shintani *et al*, 2005). bFGF have been found to be highly expressed in Kaposi sarcoma lesions of AIDS patients (Bicknell *et al*, 1997). Several authors have reported a correlation of bFGF expression as well as bFGF serum levels with the progression and prognosis of non-small cell lung cancer NSCLC (Shou *et al*, 2001). However, some investigators have not found any links between the two variables (Ueno *et al*, 2001) whereas Guddo *et al*, (1999) found that bFGF expression was inversely correlated with NSCLC tumour progression.

There has been growing interest in the study of circulating angiogenic factors in relation to cancer progression and prognosis. Elevated urine or serum bFGF levels have been found in patients with a wide variety of cancers. A study of colorectal cancer demonstrated that the levels of bFGF in the blood taken from the mesenteric vein draining the tumours were more than fourfold higher than in the peripheral blood in the same patients, implying that a large proportion of circulating bFGF was released from the tumours (Poon *et al*, 2001).
High serum levels of bFGF have been associated with advanced disease and poor progosis in colorectal cancer (Dirix *et al*, 1997), prostatic cancer, renal cancer, ovarian cancer and lymphoma (Poon *et al*, 2001).

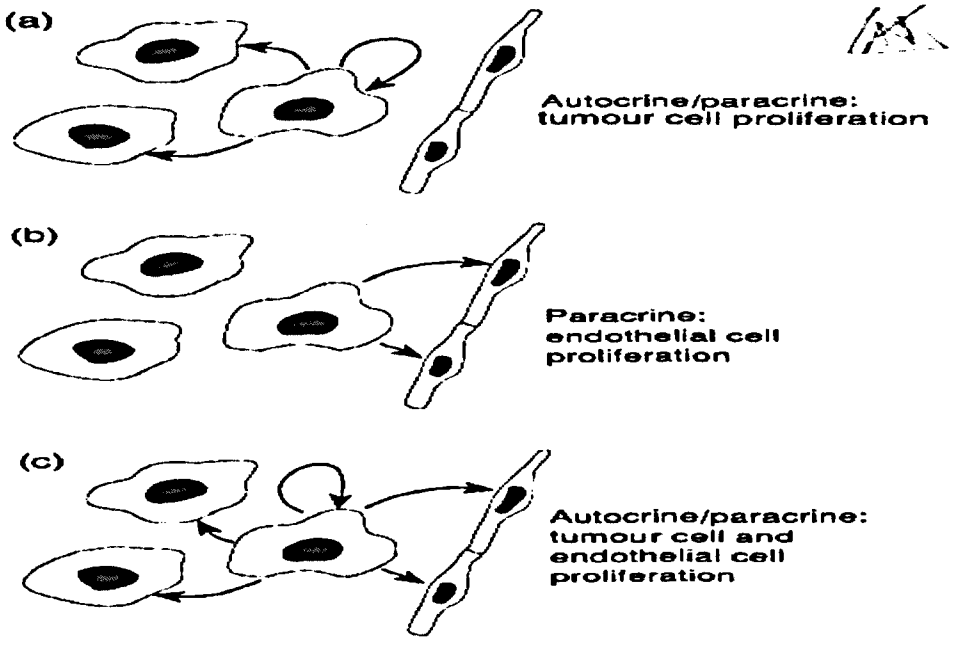
The experimental data on the role of FGFs in tumour angiogenesis are still circumstantial and indirect. It remains to be demonstrated that FGFs are actually required for neovascularisation during tumour progression. In addition to their angiogenic activity,

FGFs as autocrine growth factors are frequently responsible for tumour cell proliferation. Based on these diverse functions, three alternate pathways of FGF activity can be hypothesised (Bremnes *et al*, 2005) (fig 5.1):

Fig 5.1 The potential mitogenic pathways of FGFs during tumorigenesis.

a) Tumour cells that produce FGF may mitogenically stimulate themselves in an autocrine or paracrine manor.
b) FGF that is released by tumour cells exclusively stimulates proliferation of endothelial cells in a paracrine fashion.
c) FGF released by tumour cells induces proliferation of both tumour cells and endothelial cells. Other cell types such as stromal fibroblast may respond to FGF release.

(Diagram taken from 'Tumour angiogenesis' edited by Bicknell, Lewis and Ferrara, Oxford university press published in 1997).



5.1.4 *Association of proteases with bFGF and VEGF*

Proteolytic activity is critical for the migration and invasion of endothelial cells into neighbouring tissue. Several protease systems have been identified as being involved in this process ie. the plasminogen activator (PA) system, and the matrix metalloprotease (MMP) system. These protease systems are controlled by a variety of soluble angiogenic factors including the FGFs and VEGF (Herzényi *et al*, 2000; Lo and Hurta, 2002).

However, the influence of these growth factors on the cystein protease cathepsin B and the serine protease dipeptidyl peptidase IV has not been investigated.

bFGF induces endothelial cells to produce proteases that are capable of degrading basement membranes. The protein stimulates endothelial cell migration and causes the cells to form capillary-like tubes. Endothelial cells derived from large or small vessels are not only the targets for bFGF but also synthesize high amounts of the growth factor. Most of this protein, produced by the cells, is found associated with extracellular matrix and the basement membrane (Gontero *et al*, 2004).

The interaction of bFGF with protease systems has been characterised extensively. It is commonly known to stimulate protease production in endothelial cells. Urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1) are also modulated by bFGF. PAI-1 is expressed by endothelial cells and plays an important role in the turnover of uPA (Bicknell *et al*, 1997). bFGF also regulates uPA receptor levels; Mignatti *et al*, (1991) demonstrated that bFGF up-regulates uPA receptor levels in HUVEC cells.

bFGF is concentrated in the extracellular matrix (ECM) because of its high affinity for heparin sulphate proteoglycans. They both protect bFGF from proteolytic degredation. Plasmin and PAI-1 are also present in the ECM hence, bFGF and components of the PA system are located in the ECM. The protease plasmin helps to release the growth factor from the ECM by increasing its activity. Conversely, when PA activity is decreased bFGF is mainly associated with the ECM (Bremnes *et al*, 2005).

Activated polymorphonuclear cells were also found to release FGF-like molecules from cultured endothelial cells. The proteases elastase and cathepsin G seem to be responsible for this activity. Thus, proteases and heparanases possess a regulatory system that allows the mobilisation of cell surface or ECM bound bFGF (Bremnes *et al*, 2005).

VEGF stimulates the secretion and activation of proteolytic enzymes (MMP and PA), and induces the degradation of the ECM favouring the proliferation and migration of the endothelial cells (Lauer *et al*, 2000).

Pepper *et al*, (1991) reported that VEGF stimulates uPA, tPA (tissue-type plasminogen activator) and PAI-1 expression in cultures of bovine capillary endothelial cells. However,

PA production was not stimulated in HUVEC cells by VEGF which may be due to the fact that clonal heterogeneity was exhibited by the endothelial cells.

It has been demonstrated that plasmin cleaves the large form of VEGF and releases it from its cell surface binding site. This activates the molecule which then interacts with neighbouring endothelial cells and stimulates angiogenesis (Sandström *et al*, 1999).

5.2 Aim

MMP and PA, the most extensively studied proteases, are known to be stimulated by two of the most important growth factors in tumour angiogenesis: bFGF and VEGF. Therefore, this study set out to investigate whether these growth factors have any influence in the expression of the protease enzymes cathepsin B and DPPIV in three cell lines: HT29 (colon cancer cells), HRT18 (rectal cancer cells) and HUVECs (human umbilical vein endothelial cells). As yet there is no existing data that assesses the effects of bFGF and VEGF on these enzymes due to the fact that more knowledge is still being gathered about them.

This study measured the activity of the proteases in the presence of the growth factors by carrying out activity assays as well as highlighting the expression of the enzymes under such conditions by immunocytochemistry.

5.3 Materials and methods

5.3.1 *Cell Culture*

The effects of two of the most prominent growth factors VEGF and bFGF, in tumour angiogenesis, on the proteolytic enzymes CB and DPPIV was assessed in three cell lines HT29 (colon cancer cells), HRT18 (rectal cancer cells) and HUVECs (human umbilical endothelial cells). This was carried by propagating the cells and stimulating them with culture media containing the growth factors and then highlighting the enzymes by immunocytochemistry.

The endothelial cells (HUVEC's) were isolated from human umbilical cords by a modification of the method originally described by Jaffe *et al*, (1973). The vein within a cord was gently flushed with HBSS medium (Life Technologies, U.K.) until the effluent ran clear. The vessel was then filled with collagenase (Boehringer Mannheim, U.K.). After 20 minutes of incubation the detached endothelial cells were flushed out using HBSS medium with fetal calf serum (Life Technologies, U.K.).

The cells were then centrifuged and resuspended in DMEM (Life Technologies, U.K.) with 20% of fetal calf serum, 2% heparin and 3mg/ml of endothelial growth supplement (Sigma, U.K.), 50 U/ml of heparin (NHS. U.K) and grown until confluent in T25 culture flasks (Life Technologies, U.K.). HUVECs were used between passages 1 and 3 for all the proceeding experiments. The cords were used within 24 hours of delivery.

The HT29 (human colon carcinoma) and HRT 18 (human rectal carcinoma) cells used in the experiment were obtained from the European Collection of cell cultures (ECACC) and cultured accordingly in T25 flasks. Human liver cells (ECACC) were cultured to use as a positive control for Cathepsin B and human thyroid follicular epithelial cells (ECACC) were used as a positive control for DPPIV.

For effective cell culture propagation HT29, HRT 18, HUVECS, human liver cells and human thyroid follicular cells were passaged every 3-4 days and incubated at 37°C in 5% CO_2.

Cell culture media used for HT29 was MEM (Life Technologies, U.K.) with 50 mls of fetal calf serum, 50 mls of 2mM glutamine, 4.5 mls of penicillin, 4.5 mls of fungizone and 4.5 mls of non-essential amino acid (NEAA). The only difference with the HRT18 cell culture media RPMI (Life Technologies, U.K.) was that it did not contain NEAA (Life Technologies, U.K.). Upon confluence the cells in T25 flasks were then passaged into chamber well slides (Fisher Scientific U.K.) and incubated until they also reached confluence.

Once confluent the cells growth media was removed and fresh media was added with the addition of the growth factors under investigation. The slide chambers consisted of two compartments containing the cells and in each experiment one compartment was incubated with the original media, according to the cell line, as mentioned above and the other with the appropriate culture media containing either 50ng/ml of VEGF or 100 ng/ml of bFGF. Varying concentrations were initially assessed and the optimal doses of the growth factors were used in the study.

The stimulation times for the angiogenic growth factors on CB and DPPIV were 18 hours for CB and 42 hours for DPPIV in HT29 cells. Incubation period for HRT18 cells were 30 hours for CB and 12 hours for DPPIV and in HUVEC cells 12 hours for CB and 48hrs for DPPIV (table 5.1).
These incubation periods were noted after assessing optimal culture conditions for the proteolytic activity assays (see following study).

5.3.2 *Activity assays*

Time-course response

The cell lines HT29, HRT18 and HUVEC cells were grown until confluent in 96-well plates (Costar, U.K.). Once confluent optimal conditions for enzyme activity were investigated ie. the best culture media and optimal time for enzyme activity. Therefore, the cells were, initially, incubated for 48 hours in 6 different mediums: PBS, isotonic solution, Hanks, MEM, DMEM, and RPMI (Life technologies, U.K) supplemented with 0.3% BSA (Sigma, U.K). Then the optimal media was chosen for each cell line and enzyme as described in table 5.0.

Then intracellular and extracellular activities of CB and DPPIV were measured every 6 hours by adding 10µl of fluorescent synthetic substrate Na-CBZ-Arg-Arg-7-Amido 4-Methylcoumarin Hydrochloride (Sigma U.K.) for CB and Gly-Pro-AMC for DPPIV.

The cells were then incubated in the culture media that produced the highest enzyme activity and levels were measured every 6 hours in arbitrary units and therefore, optimal times for each enzyme in each cell line were recorded (table 5.1).

Table 5.0

	HT29	**HRT18**	**HUVECs**
CB	MEM	RPMI	MEM
DPPIV	MEM	RPMI	MEM

Table 5.1

	HT29	**HRT18**	**HUVECs**
CB	18 hours	30 hours	12 hours
DPPIV	42 hours	12 hours	48 hours

After assessing optimum conditions a new batch of 96-well plates were seeded with HT29, HRT18 or HUVEC cells and grown in the manufacturer's recommended medium. When confluent the media was extracted and 200µl of optimal media was added to the cells in the microwell plates.

Dose response

A dose response method was subsequently repeated on the cells at the optimally defined conditions in the presence of VEGF (Chemicon International, Inc., U.K.) and bFGF (Serotec, U.K.) at concentrations of: 5ng/ml, 10ng/ml, 25ng/ml, 50ng/ml, 75ng/ml, 100ng/ml. The varying concentrations of the growth factors were added to cells in the microwell plates and incubated for the recommended optimal time (table 5.1). Note: the stimulations were carried out in triplicate.

The intracellular and extracellular activity of both CB and DPPIV was subsequently measured using the synthetic substrate Na-CBZ-Arg-Arg-7-Amido 4-Methylcoumarin Hydrochloride (Sigma U.K.) for CB and Gly-Pro-AMC for DPPIV (Sigma U.K.).

Extracellular activity was measured by removing 50ul of the media plus growth factor from the cells grown on the 96-well plates after the optimal incubation period and placed in an empty well plate. To this 10ul of substrate was added and 140ul of Tris-HCL buffer. The plate was then left to stand for 1 and ½ hours before reading the enzyme activity in the cytofluor machine (activity was measured in arbitrary units).

Intracellular enzyme activity was measured from the cells in the well plates by discarding the remaining media. To the cells 10ul of substrate plus 190 ul of Tris-HCL buffer was added and again left to stand for 1 and ½ hours before reading the enzyme activity.
(Note all experiments in this study were repeated 8 times).

Statistical analysis

The mean and ± S.D. activity at each concentration of growth factors bFGF and VEGF influencing proteases CB and DPPIV in the three cell lines HUVECs, HT29 and HRT 18 were recorded. Spearman's rank correlation coefficient test was used to examine the association between the concentration of growth factors and the activity of the proteolytic enzymes. The data collated was not normally distributed therefore, a non-parametric test such as Spearman's rank correlation coefficient was used.

5.3.4 *Immunolocalisation*

Confluent cells grown in chamber slides (each slide consisted of two wells) were washed with phosphate buffer solution (PBS) (Life Technologies, U.K.) to clean cells of existing culture media. Cells were then fixed in equal volumes of acetone and methanol for 10 minutes. They were then washed with Tris buffer saline (TBS).

To target Cathepsin B and DPPIV antigens the cells were incubated with the primary antibodies mouse anti-human CD26 (molecule also known as DPPIV) (Serotec Ltd. U.K.) and sheep anti-human Cathepsin B (Serotec Ltd. U.K.). A chamber on each slide was investigated for either Cathepsin B or DPPIV with the adjacent chamber used for a negative control for the antibody in question. This process was carried out on all cell lines: HT29, HRT 18, HUVECs as well as the positive control cell lines which were human liver cells (positive control for cathepsin B) and human thyroid follicular epithelial cells (positive control for DPPIV).
(Note: the negative control was conducted to highlight non-specific bonding of the antibodies).

A secondary antibody: biotinylated secondary reagent conjugated to biotin (Serotec Ltd. U.K.), a reagent designed for the use in immunohistochemistry to detect mouse, rat, rabbit or sheep primary antibodies, was used as the secondary step of the immunocytochemistry process.

Initially, varying titrations of the antibodies were investigated to achieve an optimal working dilution. This was necessary as both the primary antibodies and the secondary reagent were not tested for use in immunocytochemistry.
The primary antibodies were diluted in phosphate buffer saline (pH 7.4) and the secondary antibody in phosphate buffer saline (pH 7.2).

The optimal dilution for Cathepsin B used was 1:50, DPPIV was 1:10 and the secondary reagent was 1:100. Cells were incubated at 37°C for 1hour in primary antibody and then washed in TBS carefully so as not to detach cells from the wells. They were then incubated at 37°C for half an hour in secondary antibody. The incubation period was also assessed for optimal times prior to the experiment. For negative controls the primary antibody as omitted from the procedure.

After incubation the cells were rinsed once again in TBS and the ABC (strept-avidin-biotin complex) reagent (Vector Laboratories U.K.) was added and incubated for half an hour at room temperature. The cells were then washed in TBS and stained with 3,' – diaminobenzidine substrate (Dako, U.K.) for 2 to 12 minutes depending on how fast the stain takes to develop.

Finally, the cells were washed again with TBS and then the side panels on the slides that made up the chambers were removed. The slides with the stained cells were mounted with a coverslip using histomount and observed under a light microscope.

A negative and a positive control was performed along side each experiment.

Slides were analysed for staining and a comparison of the intensity of staining between cells grown in culture media only and cells with the addition of the growth factors was recorded.

5.4 Results

5.4.1 *Measurement of enzyme activity using synthetic substrates*

5.4.2 *CB activity*

The incubation of HT29, HRT 18 and HUVEC cells in VEGF and bFGF resulted in the up-regulation of extracellular activity of CB. However, the response of all three cell lines to bFGF was greater.
CB activity was increased by 49.6% in bFGF stimulated HT29 cells (Fig 5.2). HUVEC cells showed an increase by 18% (Fig 5.3) after incubation with bFGF and HRT18 cells showed an up-regulation of CB activity by 31.73% (Fig 5.4). Maximum extracellular activity was reached at 50 ng/ml of bFGF in HT29 cells, 25ng/ml in HRT18 cells and 75ng/ml in the HUVEC cells.

The greatest extracellular down- regulation of bFGF in HT29 cells was noted at 75ng/ml (Fig 5.2) and 10ng/ml in HUVECs (Fig 5.3). Activity was down-regulated by 60% in the HT29 cells and 44% in HUVEC cells. However, all of the concentrations used in the study, of bFGF influenced the up-regulation of CB activity in HRT18 cells with the highest rise in activity by 35.29% at 25ng/ml and the lowest increment of 15.38% at 75ng/ml (Fig 5.4).

A dose dependant increase in extracellular activity of CB in the presence of VEGF was noted with 2.8% in HT29 cells (Fig 5.5), 13.3% increase in HUVEC cells at a concentration of 100ng/ml (Fig 5.6) and 17.24% in HRT18 cells (Fig 5.7) of the growth factor. The maximum down-regulation of the enzyme in HT29 cells with VEGF was noted at 10ng/ml with an 83% reduction. In HRT18 cells a reduction of 5.95% at a concentration of 50ng/ml and 20.2% decline in activity in HUVECs at a concentration of 25ng/ml of VEGF.

A down-regulation of intracellular activity of CB under the influence of bFGF and VEGF was noted in HT29 and in the HUVEC cells. In HRT18 cells bFGF lowered intracellular activity of CB (Fig 5.4) however, VEGF raised enzyme activity (Fig 5.7).

For HT29 cells incubated with bFGF the maximum down-regulation CB was 65% at 25ng/ml, for HUVECs the reduction was 62% at 100ng/ml, and for HRT18 cells there was an 11.1% down-regulation also at 100ng/ml.

HT29 cells with VEGF displayed a 61% lowering in intracellular enzyme activity at 75ng/ml of the growth factor. HUVEC cells activity was reduced by 62% at 50ng/ml, although, increase in activity by 12.25% was noted in the HRT18 cells at a concentration of 100ng/ml of VEGF.

Statistical analysis of CB activity

There was significant association between the extracellular activity of CB in HUVEC cells with the increase in concentration of both bFGF ($p< 0.001$) and VEGF ($p<0.001$). This implies that as the concentration of the growth factors increased the activity of CB also increased.

There was evidence of significant negative correlation between intracellular CB activity and bFGF ($p = 0.003$) concentrations for example, as concentrations of bFGF increased the activity of CB decreased. There was no correlation between intracellular activity of CB with an increase in VEGF concentrations ($p = 0.56$).

In HT29 cells there was no association between CB activity and increase in bFGF concentrations both extracellularly ($p = 0.3$) and intracellularly ($p = 0.78$). There was a significant positive correlation between the concentration of VEGF and extracellular CB activity ($p <0.001$) (ie. as concentration of VEGF increased the activity of CB increased). Also noted was the significant negative correlation between VEGF concentrations and intracellular CB activity ($p < 0.001$) (ie. as the concentration of growth factor VEGF increases then intracellular activity of CB decreases).

There was no evidence of a significant correlation between VEGF concentrations and both extracellular ($p = 0.6$) and intracellular ($p = 0.11$) CB activity in HRT18 cells. This was also demonstrated extracellularly ($p = 0.48$) and intracellularly ($p = 0.42$) for CB activity under different bFGF concentrations.

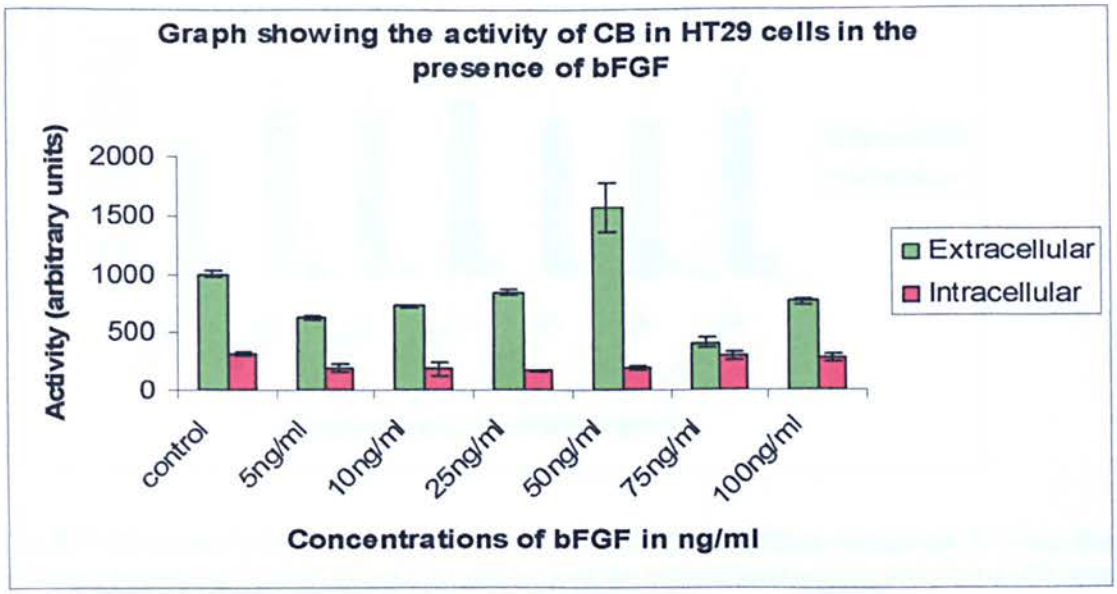


Fig. 5.2 The extracellular and intracellular activity of CB in HT29 cells stimulated by varying concentrations of bFGF for 18 hours. Cells were grown to confluence in MEM medium. Results are expressed as mean and ± S.D. of activity with p= 0.3 for extracelllular activity and p= 0.78 for intracellular activity (n=8).

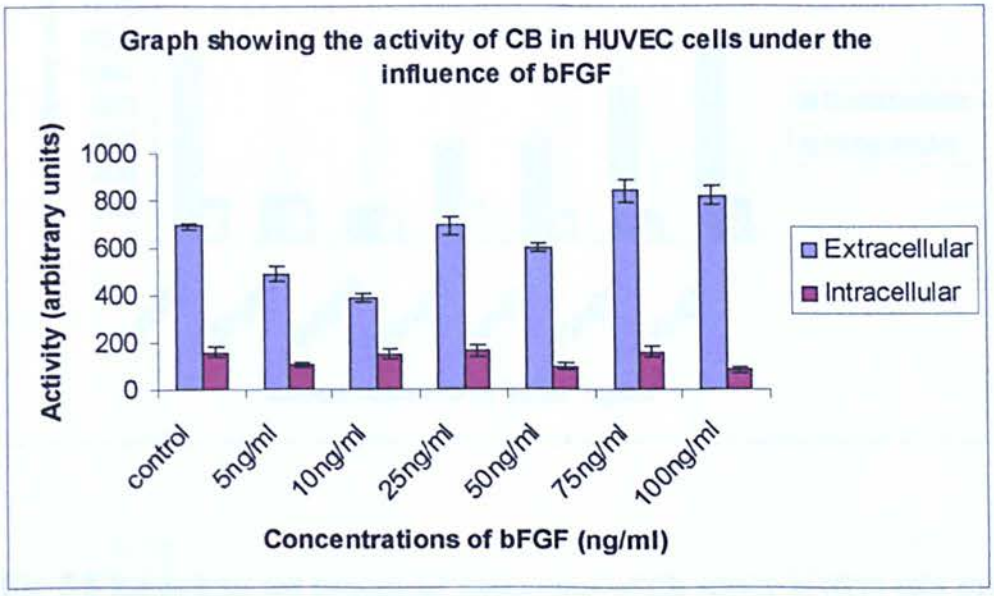


Fig 5.3 Extracellular and intracellular measurement of CB activity in HUVEC cells stimulated by bFGF. Stimulation period was 12 hours and cells were grown to confluence in MEM medium. Results are expressed as mean and ± S.D. of activity with p<0.001 for extracelllular activity and p< 0.003 for intracellular activity (n=8).

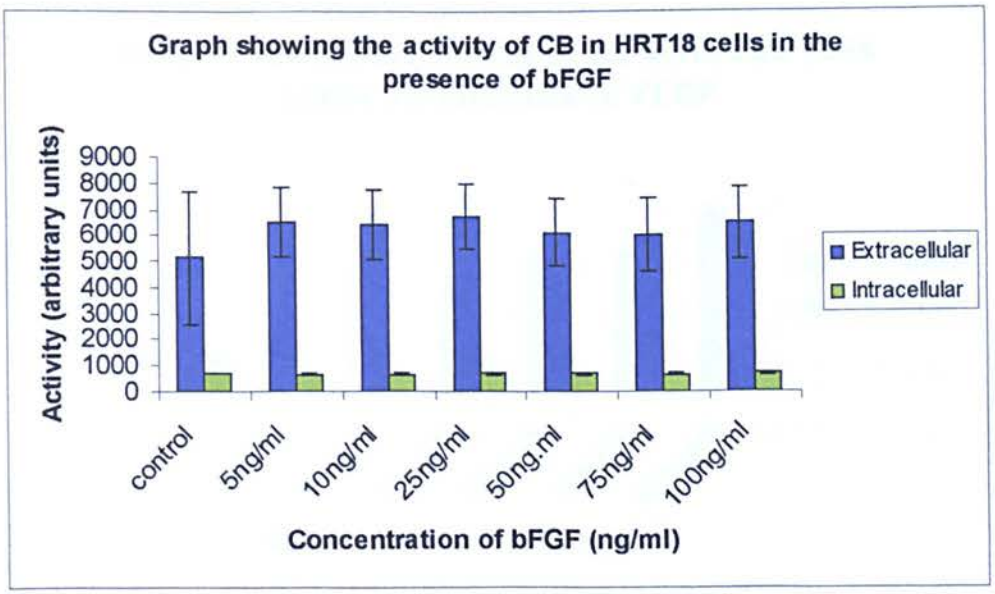


Fig 5.4 CB activity in HRT 18 cells after incubation with bFGF in RPMI medium for 30 hours. Results are expressed as mean and ± S.D. of activity with p= 0.48 for extracelllular activity and p= 0.42 for intracellular activity (n=8).

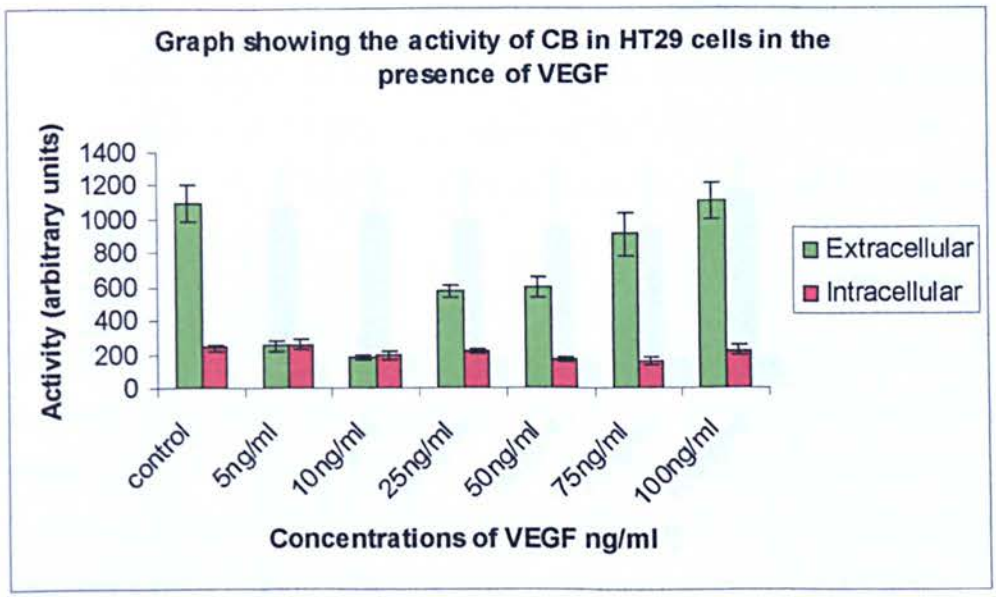


Fig 5.5 Extracellular and intracellular measurement of CB activity in HT29 cells stimulated by VEGF. Stimulation period was 18 hours and cells were grown to confluence in MEM medium. Results are expressed as mean and ± S.D. of activity with p<0.001 for extracelllular activity and p<0.001 for intracellular activity (n=8).

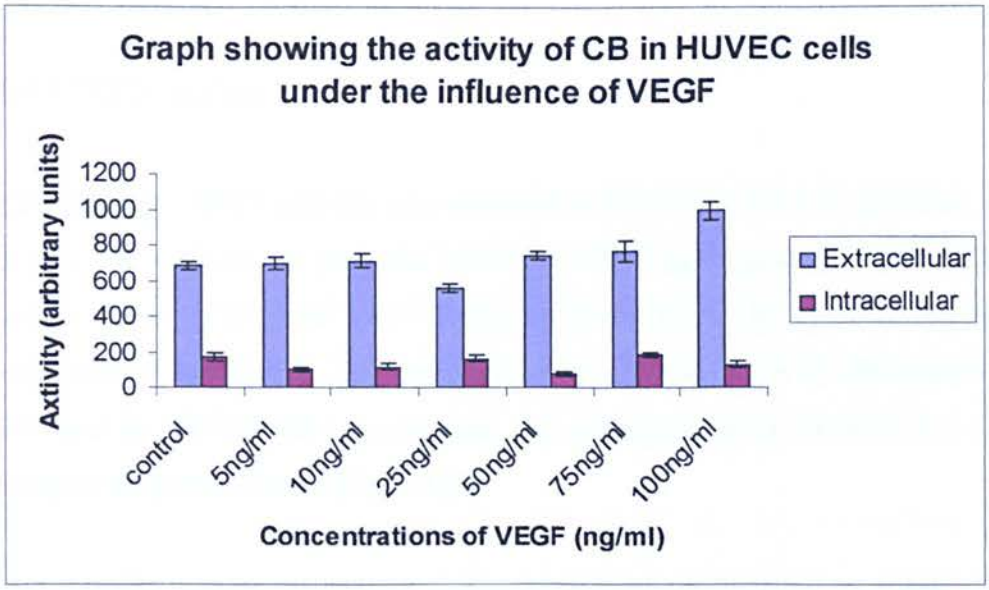


Fig 5.6 Extracellular and intracellular measurement of CB activity in HUVEC cells stimulated by VEGF. Stimulation period was 12 hours and cells were grown to confluence in MEM medium. Results are expressed as mean and ± S.D. of activity with $p<0.001$ for extracelllular activity and $p=0.56$ for intracellular activity ($n=8$).

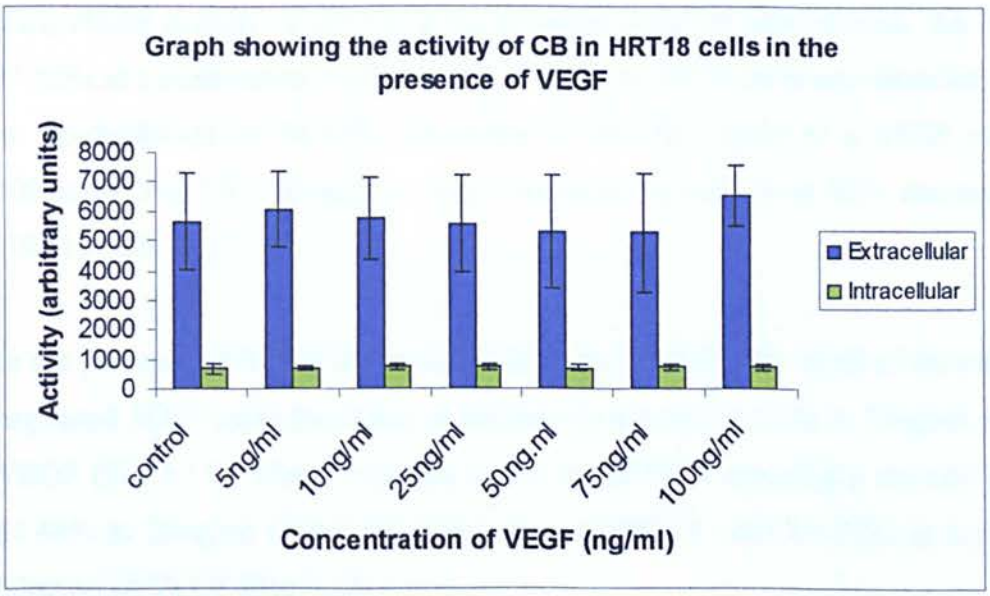


Fig 5.7 Extracellular and intracellular measurement of CB activity in HRT18 cells stimulated by VEGF. Stimulation period was 30 hours and cells were grown to confluence in RPMI medium. Results are expressed as mean and ± S.D. of activity with $p=0.60$ for extracelllular activity and $p=0.11$ for intracellular activity ($n=8$).

5.4.3 *DPPIV activity*

Extracellular DPPIV activity was elevated in HT29 and HRT18 cells but, down-regulated in HUVEC cells in the presence of bFGF. bFGF up-regulated DPPIV in HT29 cells at a concentration of 10ng/ml by 37% extracellularly (Fig 5.8). A down-regulation of DPPIV was noted in the HUVEC cells with the greatest reduction of 15.38% seen at 100ng/ml (Fig 5.9) and in HRT18 cells the enzyme was up-regulated by 48.64% at a concentration of 10ng/ml of growth factor (Fig 5.10).

Extracellular activity of DPPIV, under the influence of VEGF, in HT29 cells exhibited an immense up-regulation of activity by 162.16% at 50ng/ml (Fig 5.11). Great increases in activity were also noted in HUVEC cells with an up-regulation of 114.63% at 10ng/ml concentration of VEGF (Fig 5.12). A slight elevation of activity was noted in HRT18 cells with an up-regulation of 5.76% at 25ng/ml (Fig 5.13).

Intracellular activity of DPPIV in the presence of bFGF was variable. An up-regulation of 15.62% at a concentration of 10ng/ml of bFGF in HT29 cells was observed (Fig 5.8). Also an up-regulation of 16.95% was noted in HUVEC cells at a bFGF concentration of 100ng/ml (Fig 5.9), although, a major reduction in activity of 50% was seen at 5ng/ml in HRT18 cells (Fig 5.10).

In the presence of VEGF the intracellular activity of DPPIV in all of the cell lines was up-regulated. HT29 cells displayed an increase in activity by 21% at 75ng/ml concentration of VEGF (Fig 5.11). There was also a rise in DPPIV intracellular activity in HUVECs of 31.44% at 25ng/ml (Fig 5.12) and a rise in HRT18 cells by 25% at a concentration of 50ng/ml of VEGF (Fig 5.13).

There was a significant positive correlation between extracellular activity of DPPIV and increase in VEGF concentrations in HUVEC cells ($p < 0.001$). However, a significant negative correlation for DPPIV extracellular activity under increasing concentrations of bFGF ($p < 0.001$) was noted. There was no association between DPPIV activity and concentrations of both VEGF ($p = 0.46$) and bFGF ($p = 0.08$) intracellularly.

In HT29 cells there was no significant correlation between intracellular ($p = 0.48$) and extracellular ($p = 0.58$) DPPIV activity in growth factor VEGF. This was also established for the intracellular DPPIV activity under the influence of bFGF ($p = 0.03$). However, there was a strong negative correlation with DPPIV extracellular activity and increasing concentration of bFGF growth factor ($p < 0.001$).

There was no evidence of a significant association with intracellular ($p = 0.09$) and extracellular ($p = 0.52$) DPPIV activity in HRT 18 cells with increasing concentrations of bFGF. There was, also, no correlation between intracellular ($p = 0.54$) and extracellular ($p = 0.20$) DPPIV activity and increasing concentrations of VEGF.

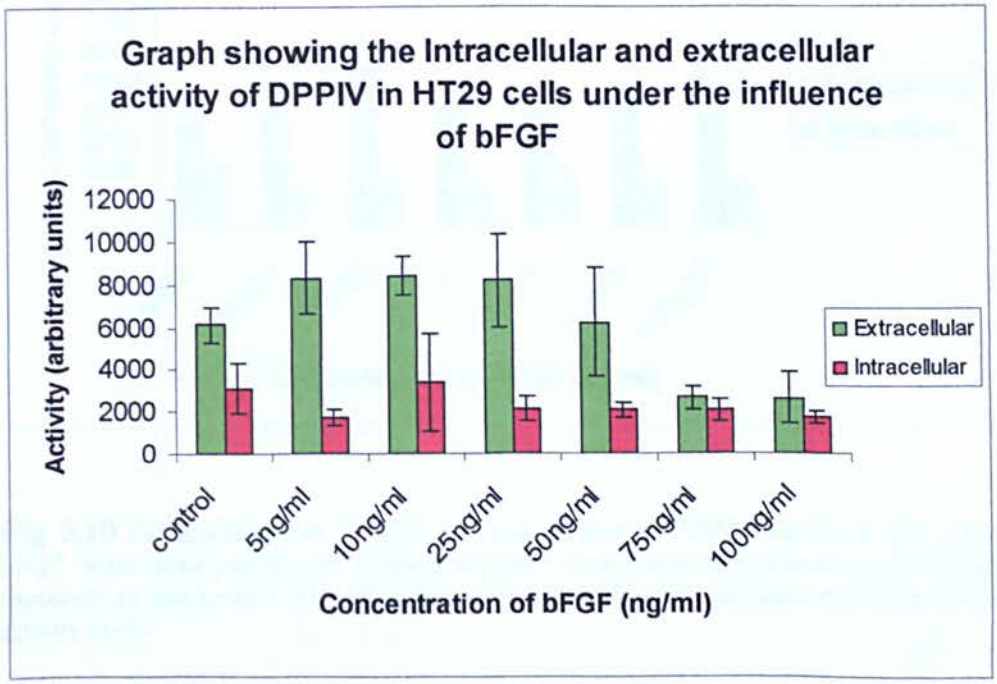


Fig 5.8 Extracellular and intracellular measurement of DPPIV activity in HT29 cells stimulated by bFGF. Stimulation period was 42 hours and cells were grown to confluence in MEM medium. Results are expressed as mean and ± S.D. of activity with $p<0.001$ for extracelllular activity and $p=0.03$ for intracellular activity ($n=8$).

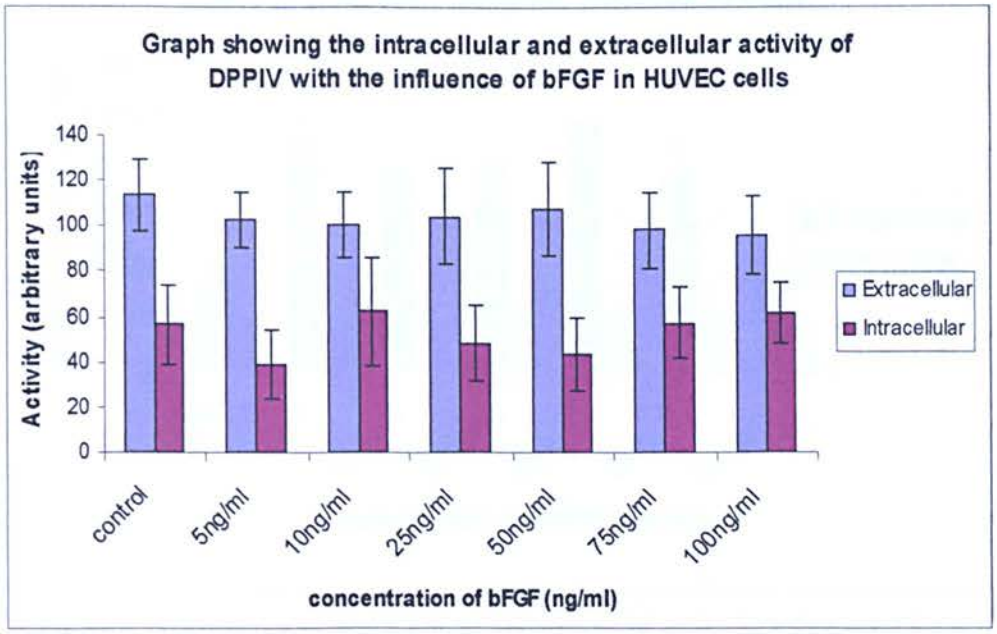


Fig 5.9 Extracellular and intracellular measurement of DPPIV activity in HUVEC cells stimulated by bFGF. Stimulation period was 48 hours and cells were grown to confluence in MEM medium. Results are expressed as mean and ± S.D. of activity with $p<0.001$ for extracelllular activity and $p=0.08$ for intracellular activity (n=8).

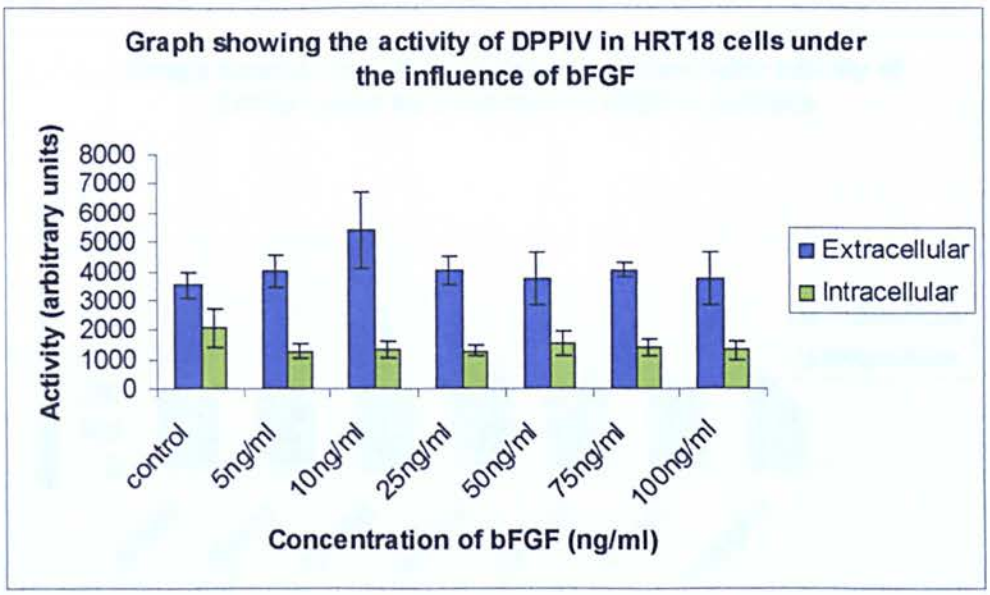


Fig 5.10 Extracellular and intracellular measurement of DPPIV activity in HRT18 cells stimulated by bFGF. Stimulation period was 12 hours and cells were grown to confluence in RPMI medium. Results are expressed as mean and ± S.D. of activity with $p=0.52$ for extracelllular activity and $p=0.09$ for intracellular activity (n=8).

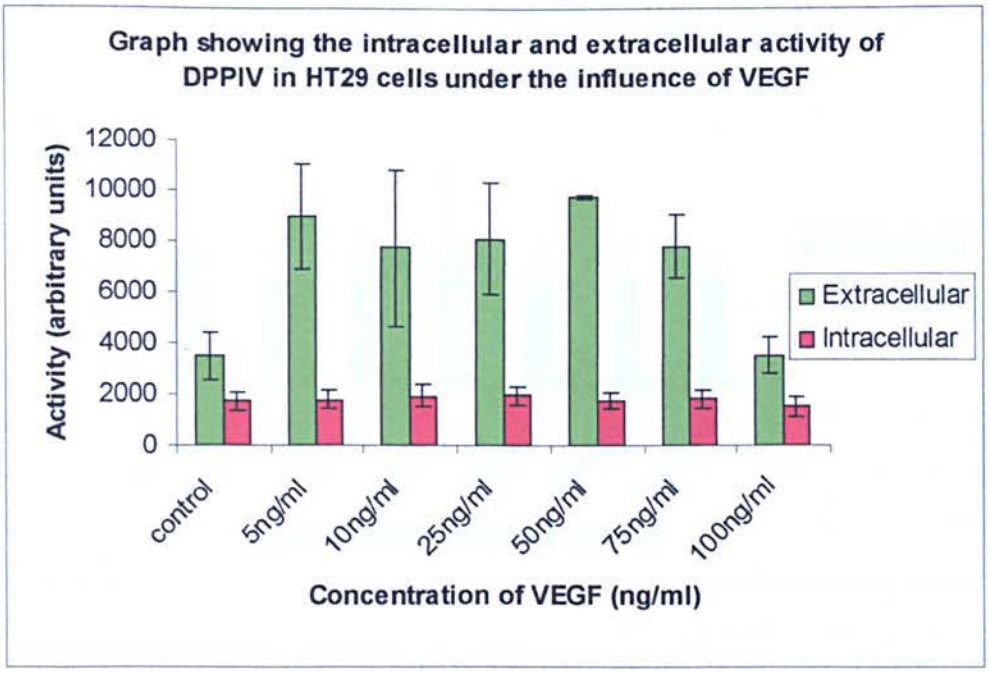


Fig 5.11 Extracellular and intracellular measurement of DPPIV activity in HT29 cells stimulated by VEGF. Stimulation period was 42 hours and cells were grown to confluence in MEM medium. Results are expressed as mean and ± S.D. of activity with p=0.58 for extracelllular activity and p=0.48 for intracellular activity (n=8).

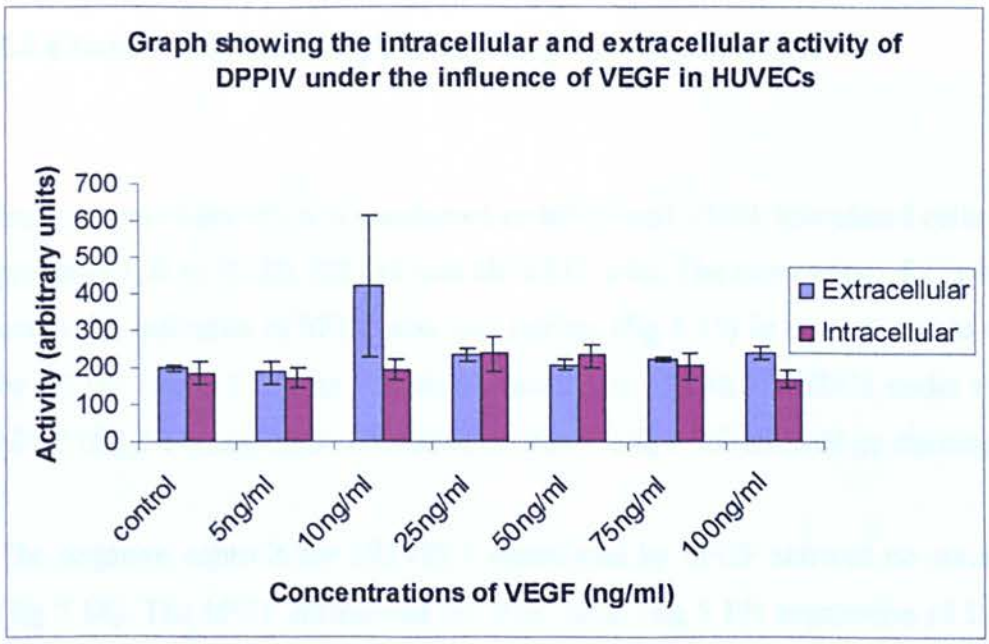


Fig 5.12 Extracellular and intracellular measurement of DPPIV activity in HUVEC cells stimulated by VEGF. Stimulation period was 48 hours and cells were grown to confluence in MEM medium. Results are expressed as mean and ± S.D. of activity with p<0.001 for extracelllular activity and p=0.46 for intracellular activity (n=8).

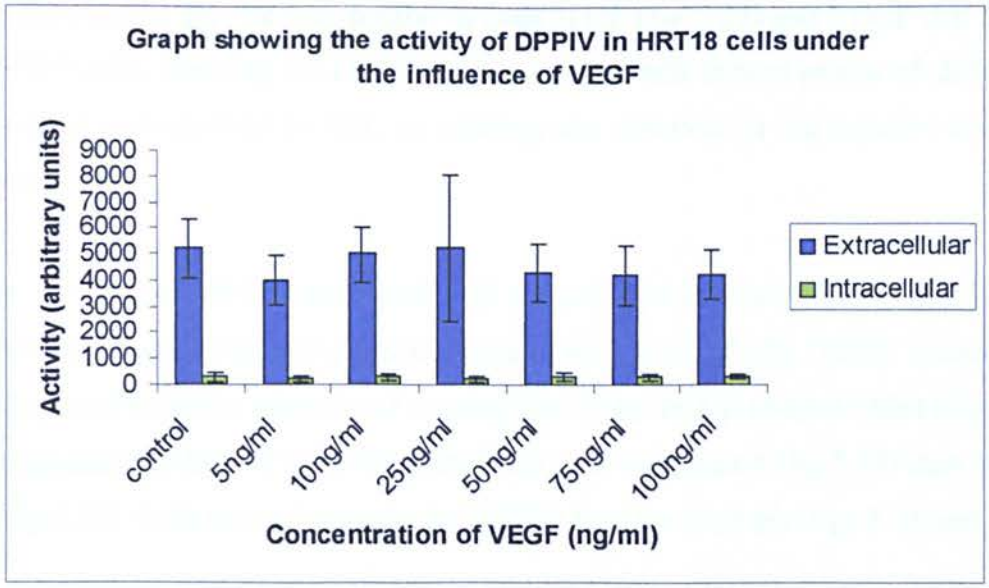


Fig 5.13 Extracellular and intracellular measurement of DPPIV activity in HRT18 cells stimulated by VEGF. Stimulation period was 12 hours and cells were grown to confluence in RPMI medium. Results are expressed as mean and ± S.D. of activity with p=0.20 for extracelllular activity and p=0.54 for intracellular activity (n=8).

5.4.4 *Immunolocalisation of protease enzymes CB and DPPIV*

Immunocytochemistry was conducted on bFGF and VEGF stimulated cells to highlight the proteases CB in HT29, HRT18 and HUVEC cells. The expression of CB in HUVEC cells under the influence of bFGF was less intense (fig 5.15) in comparison to cells stimulated by VEGF (fig 5.17). The negative controls for CB in HUVECs under the influence of bFGF (fig 5.14) and cells stimulated by VEGF (fig 5.16) showed no staining.

The negative controls for HUVECs stimulated by bFGF showed no staining for DPPIV (fig 5.18). The bFGF influenced HUVEC cells (fig 5.19) expression of DPPIV was very intense however, VEGF stimulated HUVEC cells (fig 5.21) expressed equal intensity of staining for DPPIV, no staining was observed in its negative control (fig 5.20).

Staining of CB in HT29 cells stimulated by bFGF was greater (fig 5.23) than in HT29 cells stimulated by VEGF (fig 5.25). None-the-less staining for CB in both experimental groups was more intense than in slides stained for CB without the influence of growth factors, which was used as the control for the experiments (figs 5.22 and 5.24). The level of

expression of DPPIV was similar in both bFGF (fig 5.27) and VEGF (fig 5.29) stimulated HT29 cells. Staining for DPPIV under both growth factors produced diffuse and slightly weaker staining than for CB, no staining was observed in the negative controls (figs 5.26 and 5.28).

In HRT18 cells bFGF stimulated cells stained more intensely for CB (fig 5.31) than VEGF stimulated cells (fig 5.33) and the control for CB (fig 5.30). VEGF induced HRT18 cells displayed a similar intensity of staining for CB to the CB control slides (fig 5.32).
Expression of DPPIV in bFGF stimulated cells was greater (fig 5.35) than in cells in VEGF (fig 5.37). Both stained strongly for DPPIV than the controls (figs 5.34 and 5.36).

Cathepsin B (CB) was firstly, identified in positive control human liver cells and then a negative control was also conducted which produced no staining hence, the cathepsin B antibody is specific to highlighting the protease.

DPPIV was also highlighted in its positive control tissue which was human thyroid cells. The negative controls showed no staining (see section 4.4).

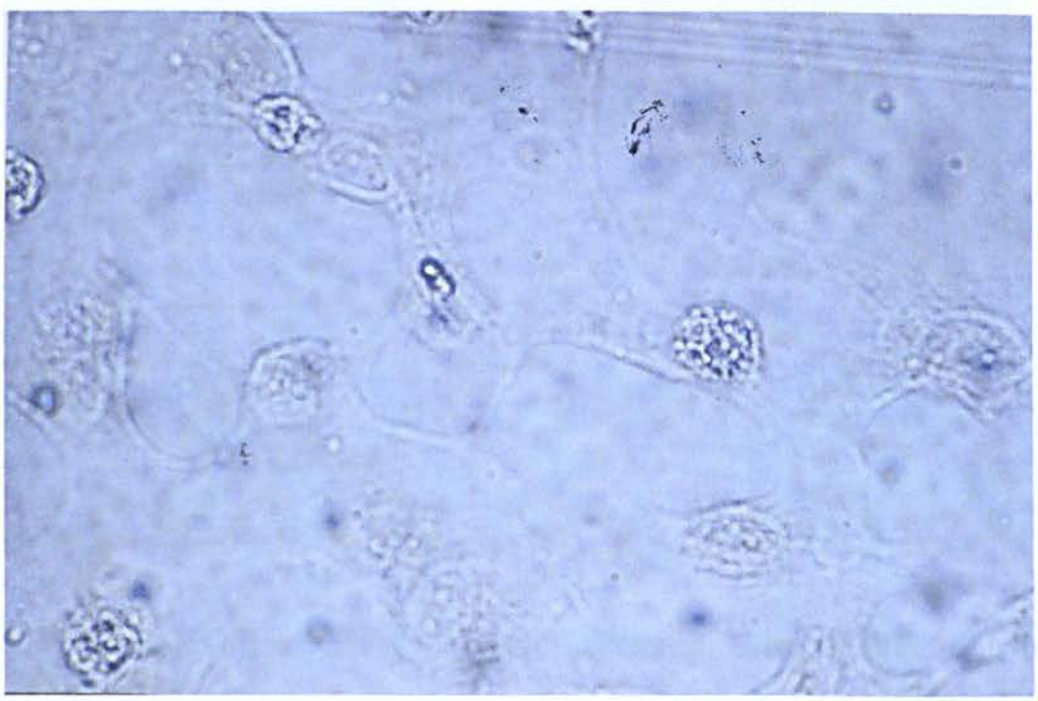


Fig 5.14 Magnification x 3360. Negative control for CB in HUVEC cells stimulated by bFGF.

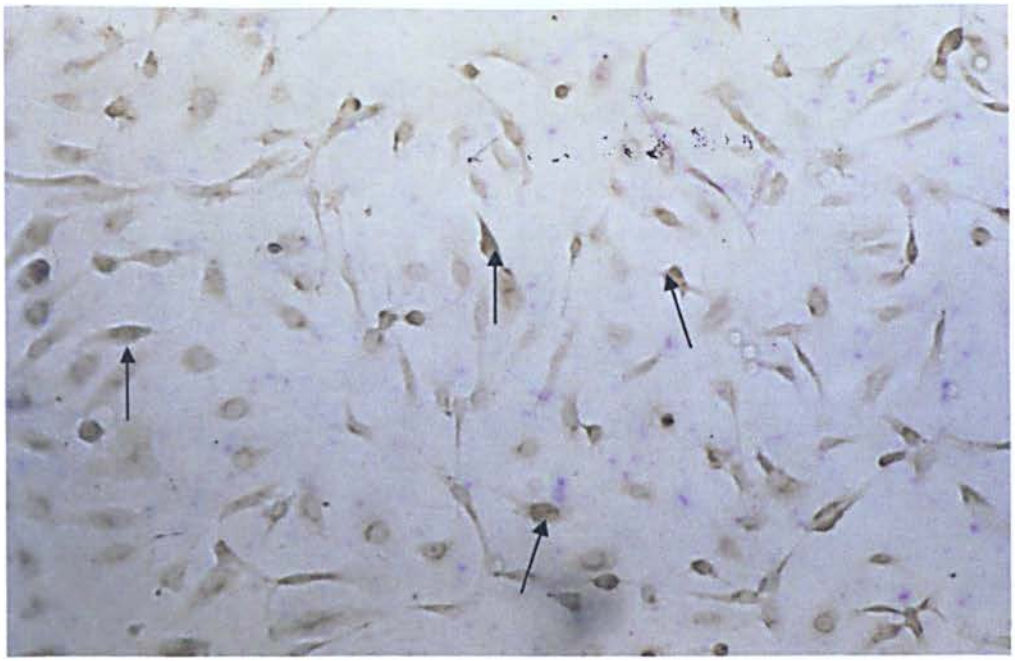


Fig 5.15 Magnification x 1160. CB highlighted in HUVECs cells after being stimulated with bFGF. Staining is weak in HUVEC's under the influence of bFGF, all cells express very low levels of CB (arrows).

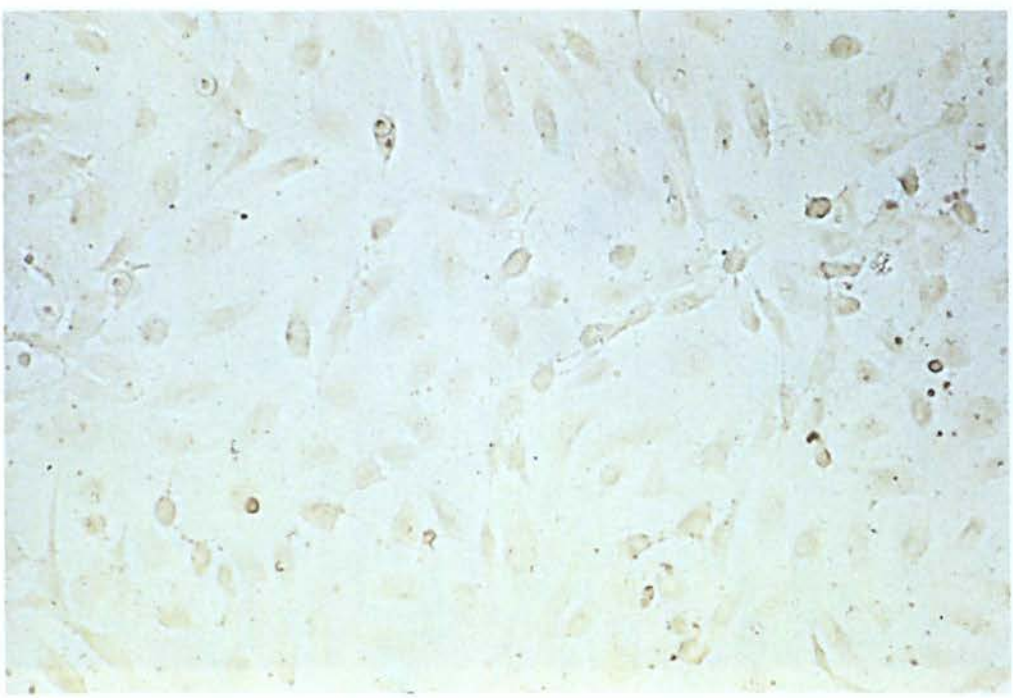


Fig 5.16 Magnification x 2800. Negative control for CB in HUVEC cells stimulated by VEGF.

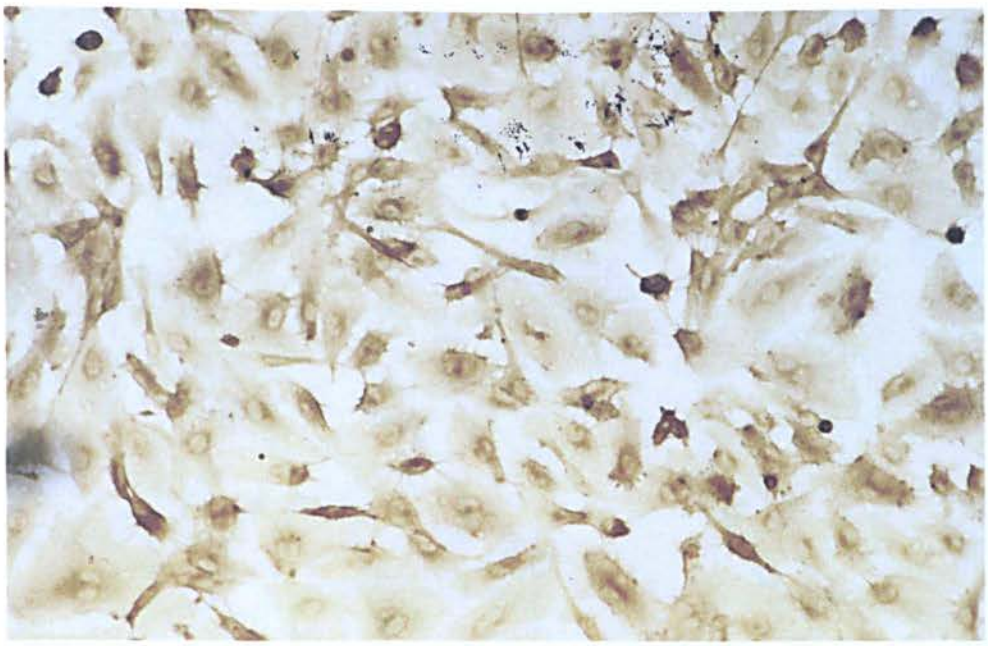


Fig 5.17 Magnification x 2800. CB highlighted in HUVECs cells after being stimulated with VEGF. Staining intensity for CB under the influence of VEGF is very strong in these cells.

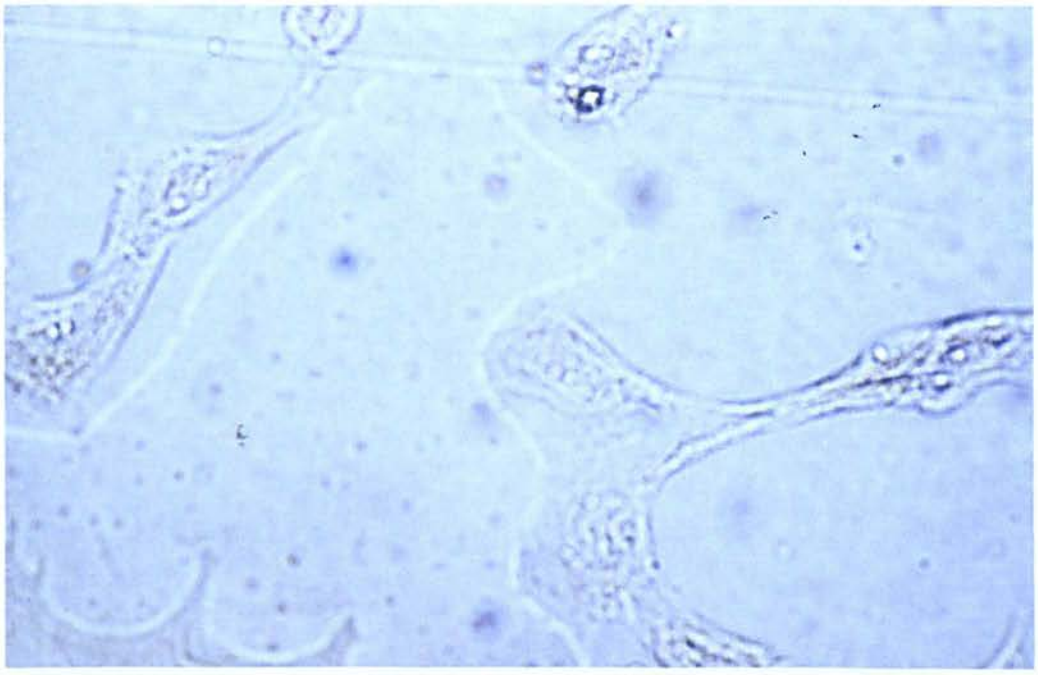


Fig 5.18 Magnification x3360. Negative control for DPPIV in HUVECs stimulated by bFGF.

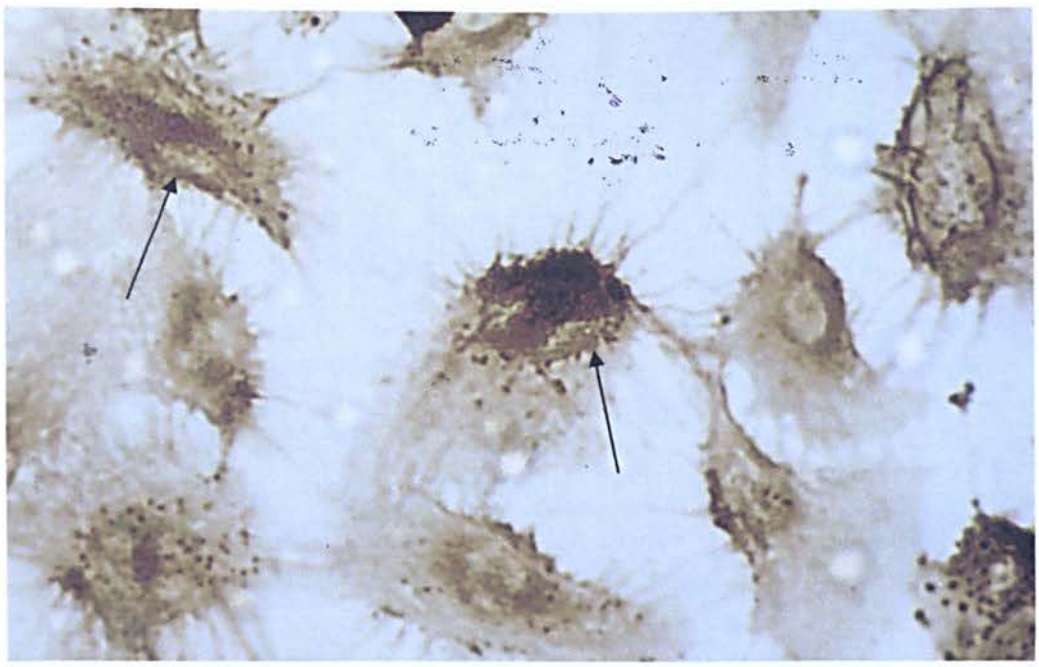


Fig 5.19 Magnification x 3360. DPPIV highlighted in HUVECs cells after being stimulated with bFGF (arrows). Increased levels of DPPIV are demonstrated in HUVEC's.

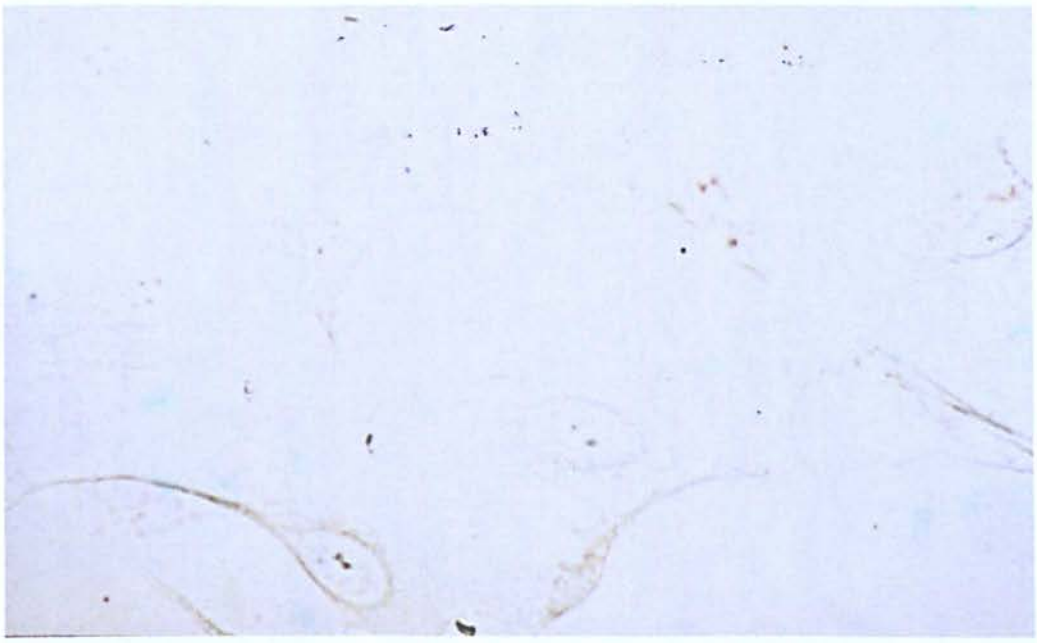


Fig 5.20 Magnification x 3360. Negative control for DPPIV in HUVECs stimulated by VEGF.

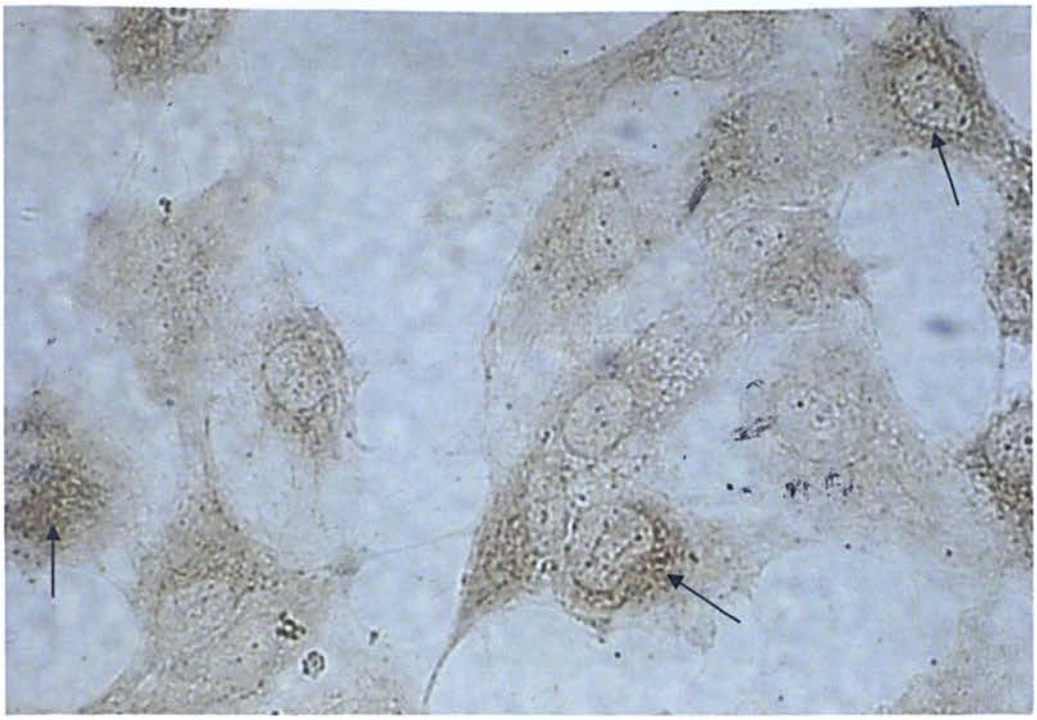


Fig 5.21 Magnification x 3360. DPPIV highlighted in HUVECs cells after being stimulated with VEGF (arrows). Staining is weaker for DPPIV under the presence of VEGF.

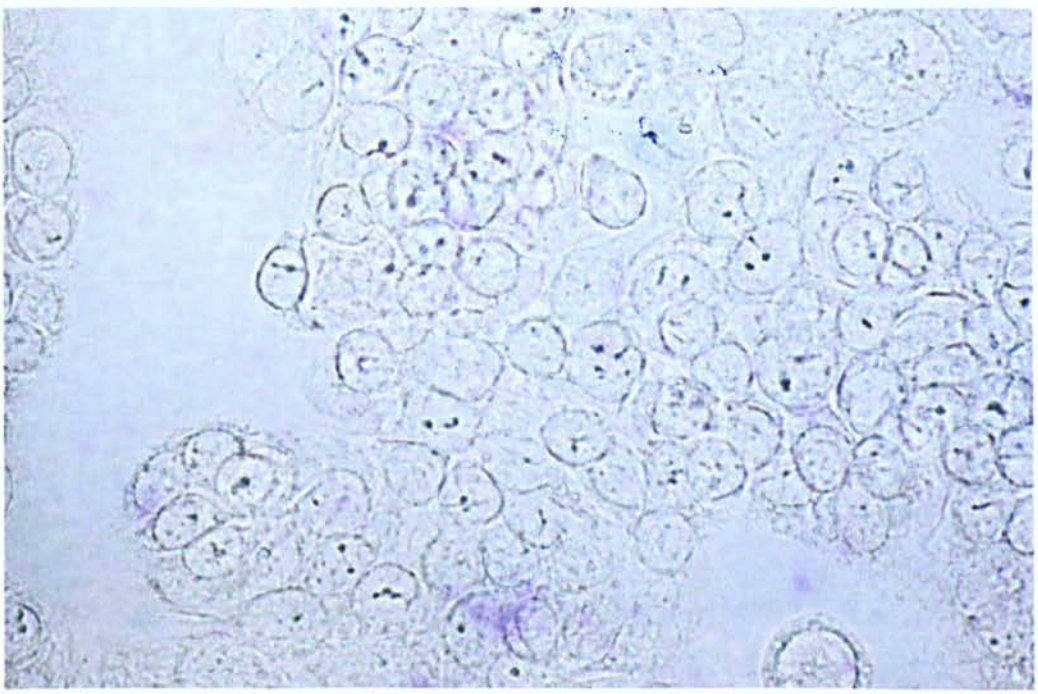


Fig 5.22 Magnification x1400. Negative control for CB in HT29 cells stimulated by bFGF.

Fig 5.23 Magnification x 2800. CB highlighted in HT29 cells after being stimulated with bFGF (arrows).

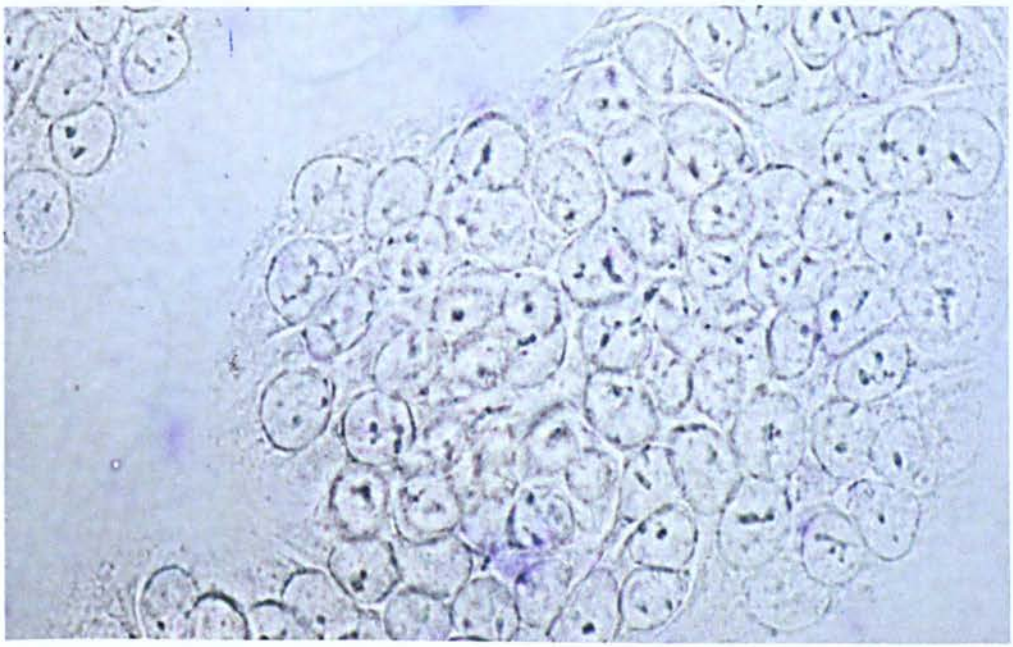


Fig 5.24 Magnification x2800. Negative control for CB in HT29 cells stimulated by VEGF.

Fig 5.25 Magnification x 2800. CB highlighted in HT29 cells after being stimulated with VEGF (arrows).

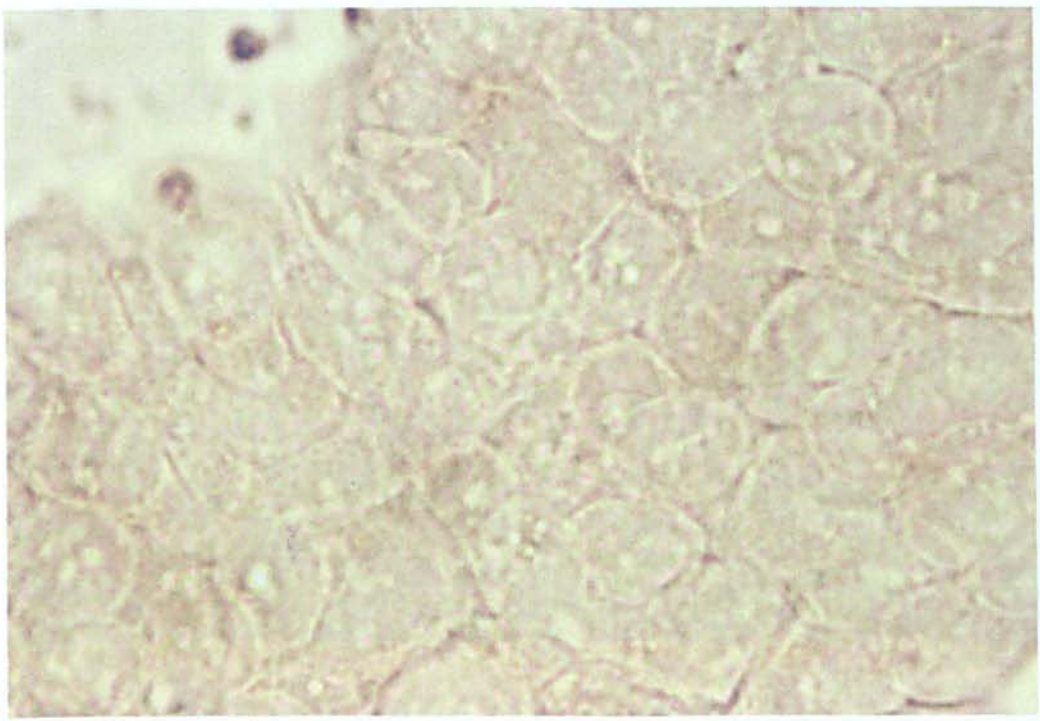


Fig 5.26 Magnification x3360. Negative control for DPPIV in HT29 cells stimulated by bFGF.

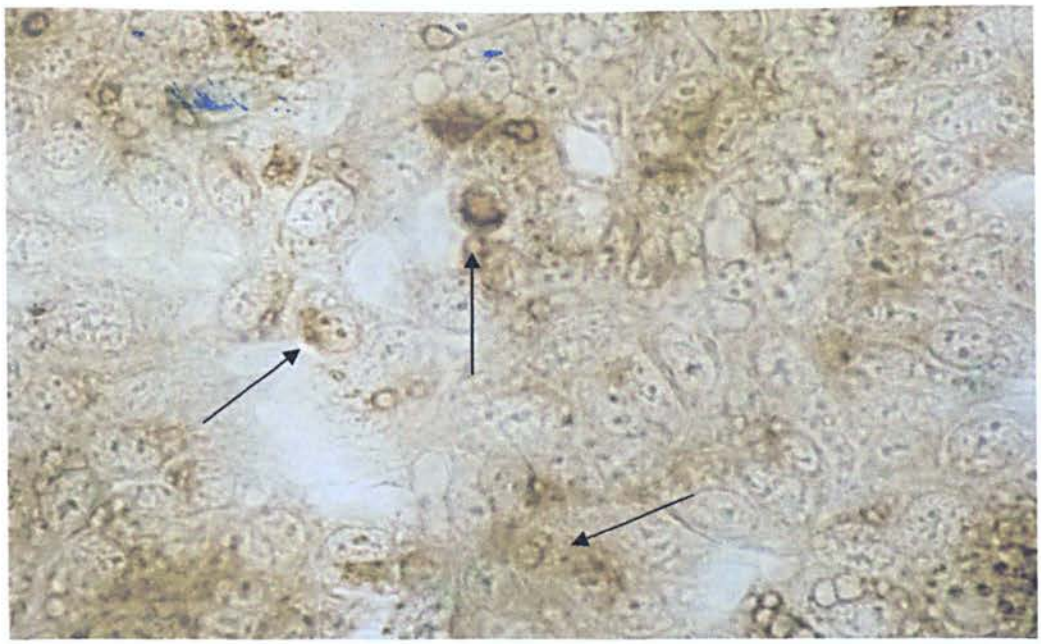


Fig 5.27 Magnification x 1200. The expression of DPPIV highlighted in HT29 cells after being stimulated with bFGF (arrows). Staining is diffuse and weak.

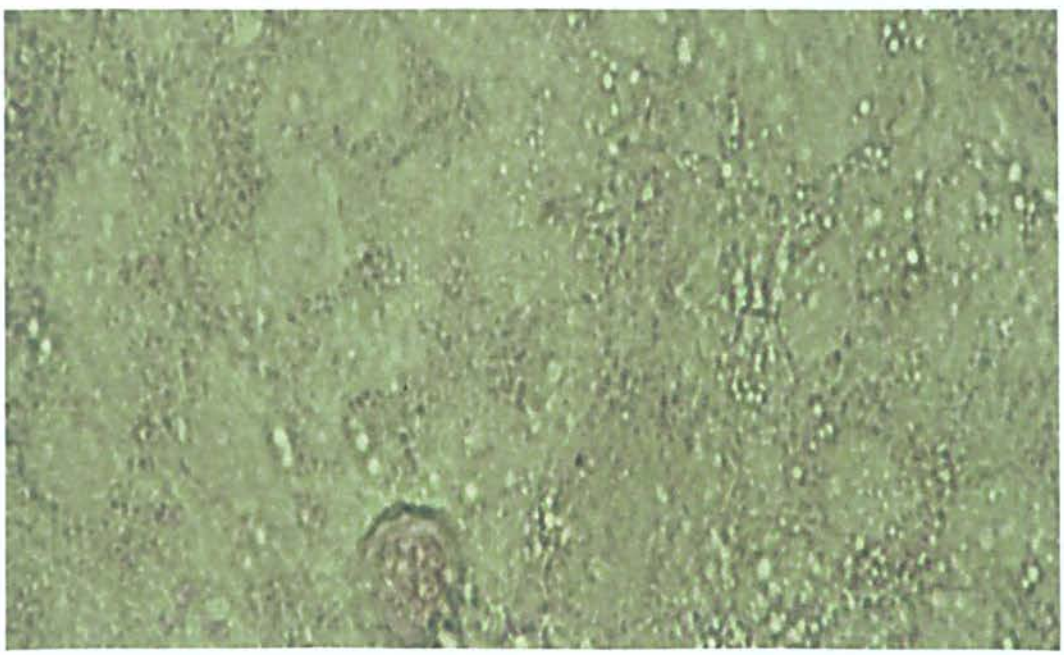


Fig 5.28 Magnification x3360. Negative control for DPPIV in HT29 cells stimulated with VEGF.

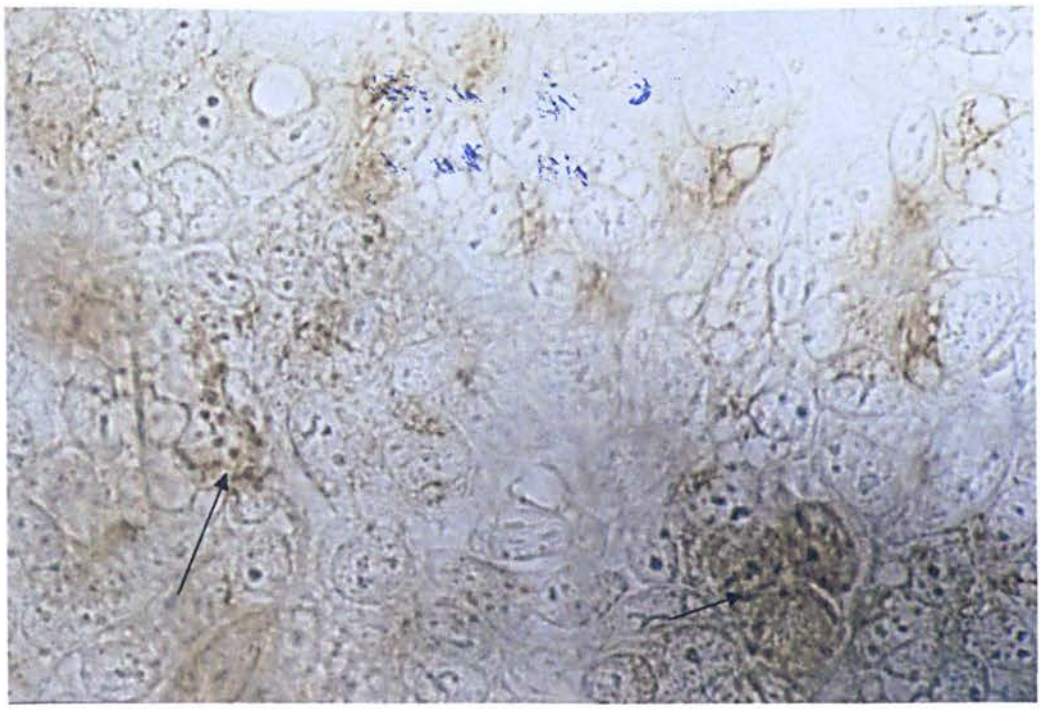


Fig 5.29 Magnification x 1200. DPPIV highlighted in HT29 cells after being stimulated with VEGF (arrows). Staining is diffuse and slightly weaker than bFGF stimulated HT29 cells, as shown in fig 5.27.

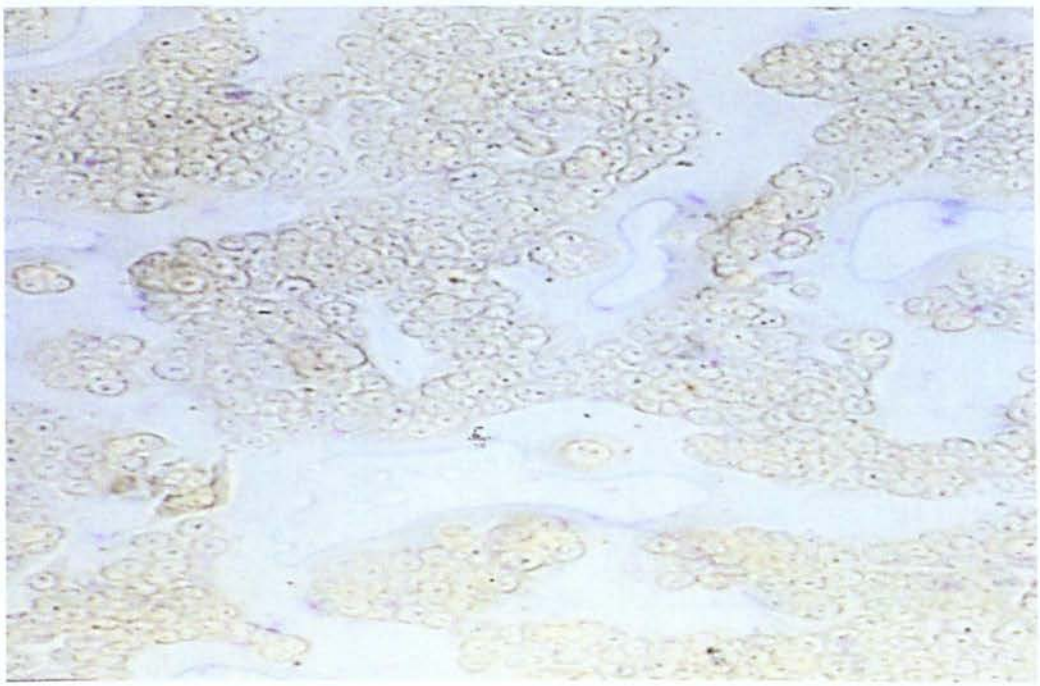


Fig 5.30 Magnification x 840. Negative control for CB in HRT18 cells stimulated by bFGF.

Fig 5.31 Magnification x 1400. CB highlighted in HRT18 cells after being stimulated with bFGF (arrows). Staining is localised and intense.

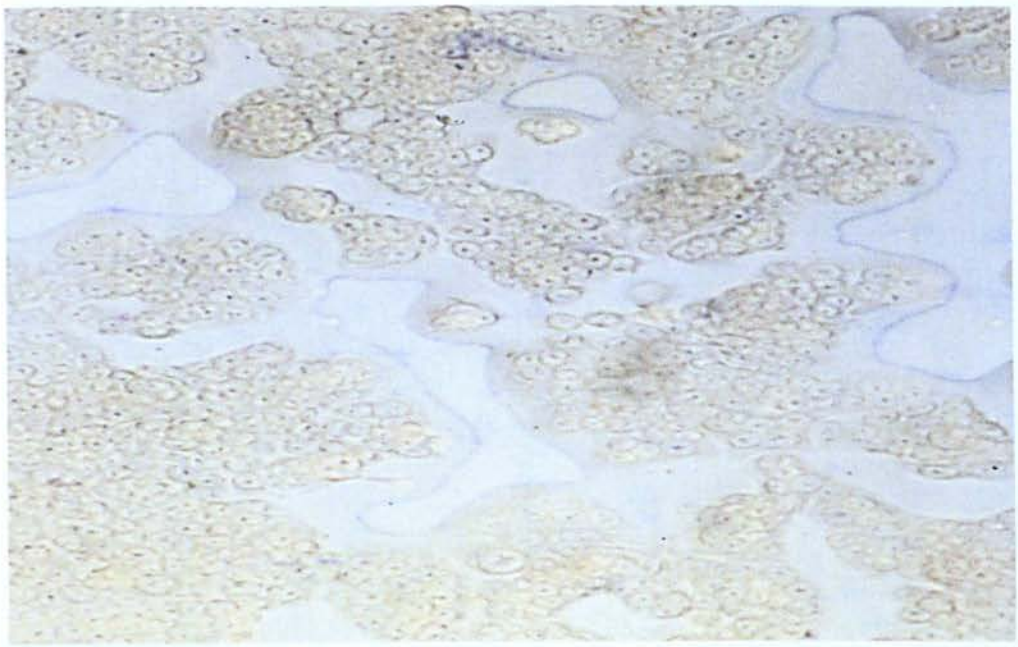


Fig 5.32 Magnification x840. Negative control for CB in HRT18 cells stimulated by VEGF.

Fig 5.33 Magnification x 2800. CB highlighted in HRT18 cells after being stimulated with VEGF (arrows). Staining is focal yet weaker than in bFGF stimulated HRT18 cells shown in fig 5.31.

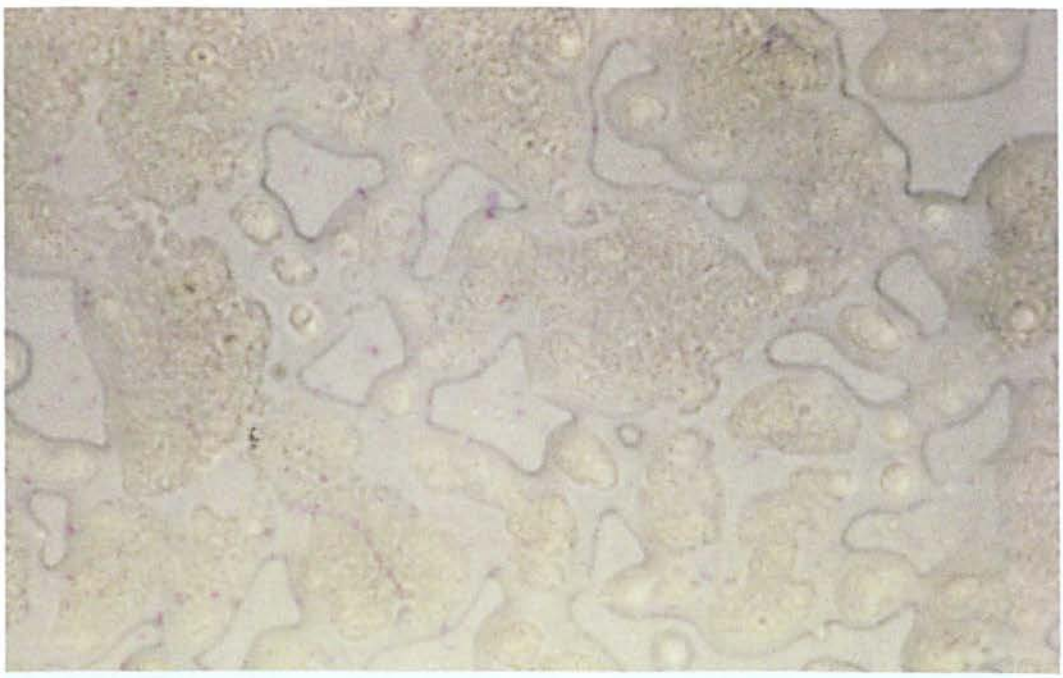


Fig 5.34 Magnification x 840. Negative control for DPPIV in HRT18 cells stimulated with bFGF.

Fig 5.35 Magnification x 2800. DPPIV highlighted in HRT18 cells after being stimulated with bFGF. Staining intensity for DPPIV was greater than for CB (fig 5.31). The presence of DPPIV is clearly exhibited in the plasma membrane of HRT18 cells (arrows).

Fig 5.36 Magnification x 3360. Negative control for DPPIV in HRT18 stimulated with VEGF.

Fig 5.37 Magnification x 3360. DPPIV highlighted in HRT18 cells after being stimulated with VEGF. Staining for DPPIV is weaker than bFGF stimulated HRT18 cells (arrows).

5.5 Discussion

Proteases are known to activate growth factors in angiogenesis. There are many supporting data indicating a central role for the plasminogen activator (PA) system in blood vessel formation. The PA system modulates angiogenesis by acting on migration, invasion and possibly lumen formation (Herszényi *et al*, 2000).

The factors regulating PA or its inhibitor expression by endothelial cells include FGFs and VEGF (Bicknell *et al*, 1997). bFGF is responsible for migration and induction of PA whereas, high molecular weight bFGF is responsible for cell proliferation. FGF receptors are up-regulated in tumour cells concomitant with the expression of FGF ligands, suggesting that tumour cells use both FGF receptors and ligands in tumour transformation and proliferation (Petrova *et al*, 1999).

VEGF is liberated from the endothelial cell matrix and activated by plasmin. Once activated VEGF stimulates endothelial cell proliferation by a paracrine mechanism (Bremnes *et al*, 2005). This growth factor is a key mediator for angiogenesis associated with malignancy (Bamias and Dimopoulos, 2003).

There is increased knowledge of proteases and other groups of enzymes such as cystein and serine proteases have been described in tumour progression. There are significant data implicating the role of cathepsin B (CB) (cystein protease) and dipeptidyl peptidase IV (DPPIV) (serine protease) in tumour progression (Boonacker and van Noorden, 2003; Krueger *et al*, 2005). However, there are no studies that describe the effects of the growth factors: bFGF and VEGF on them.

This study analysed the effects of the potent angiogenic growth factors on the activity and expression of CB and DPPIV. The first study assessed the effects of bFGF and VEGF on the intracellular and extracellular activity of the proteases in human colon cells (HT29), human rectal cells (HRT18) and human umbilical vein endothelial cells (HUVEC).

The increase in extracellular activity of CB suggests that these growth factors induce the release of CB from all three cell types. The observation of greater activity with bFGF suggests that this growth factor may have major influence in the activation of this enzyme, during tumour progression and angiogenesis, over VEGF.

The study showed that CB extracellular activity in the presence of bFGF was not as stimulatory on HUVECs as the others and CB activity under VEGF in HT29 cells was also down-regulated. These results are reflected in the immunocytochemistry experiments which demonstrated increased expression of CB in cells stimulated by bFGF and VEGF. Staining for CB in cells under the influence of bFGF was greater than staining for CB in cells in VEGF.
It was noted that staining for CB in VEGF stimulated HUVEC cells was far more intense than CB staining in bFGF stimulated HUVECs.

HT29 and HRT18 cells stimulated by bFGF showed an increase in DPPIV enzyme activity, however, there was a decrease in activity in HUVEC cells compared to the control experiment. In VEGF stimulated cells an up-regulation of DPPIV was noted in all three cell lines.

Activity assays for DPPIV directly reflected the results obtained from immunocytochemical staining for the enzyme. In HT29 cells the DPPIV activity was up-regulated and the staining was weak in bFGF and VEGF stimulated cells but still more intense than in the control. DPPIV activity in HRT18 was also up-regulated and staining under both conditions was very strong. However, in HUVECs activity of DPPIV under bFGF was down regulated and very weak to negative staining was observed. DPPIV staining under VEGF was up-regulated and weak staining was noted.

It is known that bFGF expression is amplified in a number of human tumours *in vivo* (Lo and Hurta, 2002). Elevated bFGF expression has also been demonstrated in tumour cell lines *in vitro* such as the colon carcinoma cell line. Further studies have defined these findings by reporting the up-regulation of FGF receptors in tumour cells, therefore, bFGF is identified as an inducer of tumour cell proliferation (Bicknell *et al*, 1997).

At the same time, bFGF is also mitogenic for endothelial cells and stimulates endothelial cell production of proteases. In vivo bFGF causes endothelial cells to migrate and form capillary-like tubes (Bremnes *et al*, 2005). Although, Koura *et al*, (1996) reported that bFGF was down-regulated in confluent tumour cells. However, the reason for the decreased expression in HUVEC of both CB and DPPIV compared to the tumour cells may be due to the fact that lower amounts of bFGF is produced by non-tumourigenic cells

(Aonuma *et al,* 1999). Hence, studies show that reduction in the production of bFGF is associated in the weak induction of angiogenesis (Aonuma *et al,* 1999).

VEGF acts preferentially on endothelial cells through two high affinity tyrosine kinase receptors flt-1 and KDR. It is a potent regulator of angiogenesis, proliferation and migration of endothelial cells, expression of adhesion molecules and vascular permeability (Chang *et al,* 2000). Hippenstiel *et al,* (1998) reported significant increases in cell number in HUVEC monolayers in response to doses of VEGF.

VEGF is also expressed by tumour cells and it has been reported that VEGF is an important angiogenic factor associated with metastasis of human colon carcinomas (Milkiewicz *et al,* 2006). Hence, its role in metastatic neoplasia is dedicated to inducing angiogenesis (Ferrara 2004). In this study an up-regulation of enzyme activity in HUVEC cells under the influence of VEGF is observed confirming the results obtained from Milkiewicz *et al,* (2006) and Ferrara (2004).

Reduction in the levels of CB activity was noted in HT29 cells stimulated by VEGF. This may be due to the fact that most tumour cells do not express VEGF specific receptors (Tischer *et al,* 1991). The reduction of enzyme activity under the influence of bFGF and VEGF in the tumour cells may also be attributed to the dual role that these growth factors play in promoting and inhibiting tumour growth (Bicknell *et al,* 1997).

Intracellular activity in all cases was lower, this may be due to the fact that intracellular enzyme of CB and DPPIV levels were lower due to their extracellular release. Never-the-less cells stimulated by the growth factors showed lower activity than the control samples, which were not stimulated. This suggests that bFGF and VEGF might have a negative response on intracellular CB and DPPIV activity in tumour and endothelial cells.

This study demonstrates that proteases are influenced by angiogenic growth factors (Lauer *et al,* 2000). Sandström *et al,* (1999) reported that the expression of the protease uPA, tPA and PAI-1 and VEGF were concentrated in the invasive border of the tumour. VEGF induced angiogenesis is accompanied by the increase in uPA rceptors on endothelial cells. In endothelial cells VEGF has been demonstrated to induce expression of tPA and uPA (Kolch *et al,* 1995).

Clinical studies show that there is a positive correlation between mRNA expression of VEGF and to the receptors for uPA in colorectal cancer tissue. This suggests that VEGF and proteases work together in tumour growth and progression (Sandström *et al*, 1999).

Investigations have also demonstrated that bFGF alters the expression profile of the proteolytic enzymes matrixmetalloproteases (MMP) (also known to be expressed in tumourproliferation and angiogenesis). Increased MMP altered expression increases with metastatic potential of cells. MMP-2 and MMP-9 are known to determine the ability of bFGF expressing cells to invade (Nishizuka *et al*, 2001).

This chapter highlights the importance of these angiogenic proteins in the regulation of proteolytic activity and lends further support to the view that proteases influence the angiogenic growth factors bFGF and VEGF.
This study demonstrates that CB and DPPIV (both implicated in tumour progression) activity and expression is directly influenced by the two growth factors. This suggests that VEGF and bFGF may stimulate the production of the proteases CB and DPPIV in human colorectal carcinomas.

Chapter 6

6.1 General Discussion

The aim of this investigation was to establish a method for vessel quantification, assess the spatial distribution of tumour vessels and investigate if vessel numbers in colorectal tumours of varying Dukes' grade are correlated to metastasis. As proteases play a role in tumour angiogenesis (Hornebeck *et al,* 2002) the study set out to define the roles of two proteolytic enzymes cathepsin B (from the cysteine family) and dipeptidyl peptidase IV (from the serine family) in colorectal cancer and if their enzyme protein or mRNA expression is associated with factors relating to cancer progression.

Growth factors VEGF and bFGF have been implicated in the initiation of tumour angiogenesis and vessel proliferation. Studies show that protease production may be switched 'on or off' by protease dependant release or activation of these growth factors (Bikfalvi *et al,* 1995). Therefore, this study led us further to investigate the influence of these growth factors on the two proteases in question in colon, rectal and endothelial cells. Therefore, assessment of these angiogenic factors and their possible relation to cancer spread may have prognostic significance and therefore, have the potential to be a marker for colorectal cancer metastasis (de Jong *et al,* 2006).

Pre-clinical tumour models such as sub-cutaneously grown human tumours in mice are commonly used in the assessment of inhibitors of angiogenesis (van Kempen and Leenders, 2006). *In vivo* bioassays of angiogenesis have also enabled investigators to progress in elucidating the mechanism of action of a variety of angiogenic factors (Bicknell *et al,* 1997). Hence, the initial pre-clinical assessment of tumour vascularisation was conducted in the mouse tumour model colo 26. Step by step analysis of the spatial distribution of tumour vessels has allowed this study to investigate how tumour vessels are distributed and whether they reflect the hypothesis of angiogenesis.

The study aimed to assess firstly, the growth of tumour vessels over time (8 to 22 days) and then to assess the spatial distribution of these vessels. Colo 26 tumours displayed low vascular counts until a time point of 21 days where a rapid increase in vessel number was noted. Never-the-less, the tumour displayed a steady increase in the number of vessels as

time progressed. The results proved the hypothesis that tumours from a possible pre-vascular dormant stage progress on to a vascular stage correlated with rapid tumour growth and a potential for metastasis (Folkman *et al,* 1990). The initial vascular phase and the degree of vascularisation is dependant upon the tumour cells and host endothelial cells switching to an angiogenic phenotype in the dormant stage. Further development of vascularity is then dependant on various tumour-host interactions (Bian *et al*, 2006).

The observed increase in vessel counts in the host murine tissue adjacent to the tumour coincides with the Folkman *et al,* (1990) study. The study concludes that tumours elicit new vasculature from existing host vessels by releasing growth factors to encourage proliferation and vessel formation of endothelial cells in the host tissue which then migrate to the tumour.

The need for the colo 26 tumour to induce tumour angiogenesis strongly suggests that its growth and survival is dependant on the supply of nutrients and the clearance of waste products which is provided by an established network of vessels (van Kempen and Leeders, 2006). Furthermore, the increased number of vessels in the periphery and invasive fronts, noted in both transplanted, as well as human colorectal tumours, indicate that tumour angiogenesis is active in areas where neoplasia is progressive (Talieri *et al*, 2004).

The assessment of angiogenic activity, in human tumours, has been recognised as an important parameter both as a predictive factor for metastasis and to study the effects of anti-angiogenic treatment of malignant tumours (Offersen *et al,* 2003). Therefore, it is extremely important to be able to obtain reliable results from reproducible methods (de Jong *et al*, 2006). Quantification of tumour vascular density has been performed in several types of tumours using various methods to estimate angiogenic activity, however, conflicting data on the significance of colorectal tumour vascularity on patient prognosis still exists (Georgiou *et al,* 2006).

Methods used to assess tumour vessels include the use of an endothelial cell specific marker to highlight tumour vessels that are then quantified by using an objective method to obtain vessel counts (Acikalin *et al*, 2005). However, studies investigating the correlation between tumour vascularity and factors influencing prognosis have used various methods to establish such results (Müller *et al*, 2002; El-Gazzar et al, 2005; Viera et al, 2005). This may play a role in the contradictory reports, hence, this study set out establish an endothelial marker that would produce maximum results.

As endothelial cells are highly heterogenous in different organs and tumour endothelial cells are constantly proliferating (Lee *et al*, 2000) it was imperative that this study investigated the presence of tumour endothelial cell specific antigens in colorectal cancer. From a panel of established endothelial markers CD31 was highly specific. However, analysing the staining specificity of CD34 resulted in a highly specific yet distinct immunohistochemical staining. After further morphological examination, it was evident that this marker was only specific to tumour blood vessel endothelium and not tumour lymph vessel endothelium. The finding enabled this study, with the use of CD34, to assess the significance of tumour blood vessel counts as well as tumour lymph vessel counts on clinicopathological variables of colorectal cancer metastasis.

The criteria for quantifying tumour vessels were based on those suggested by Weidner *et al*, (1993) (see chapter 3, methods). However, as well as to enable reproducible results and lower inter-observer variablility this study considered the possibility that counting vessels in all of the given cross-sectional area of the colorectal tumour would reflect the true angiogenic state of the cancer.

As a result, this study found a positive correlation between tumour blood vessel counts and pathological factors associated with metastasis which parallels the work performed by Saclarides *et al*, (1994); Frank *et al*, (1995); Engel *et al*, (1996); Tanigawa *et al*, (1997) and Zheng *et al*, (2003). Also discovered in this study was the association of increased number of lymph vessels to factors relating to metastasis in colorectal carcinomas. This suggests that tumour lymph vessels may serve as a route for cancer cells to spread to regional lymph nodes hence, increasing the chance of metastasis (He *et al*, 2004).

Therefore, it may be postulated that quantification of tumour blood and lymph vessels and the assessment of the distribution of colorectal tumour vessels provides crucial information on the metastatic behaviour of this cancer. The positive correlation of this study to the clinicopathological variables gives strength to the suggestion that colorectal tumour vasculature can be used as an additional marker for prognosis.

This present work defines the key importance of tumour vasculature in the progression of colorectal cancer, therefore, the understanding of the involvement of factors influencing the generation of these vessels is vital (Milkiewicz *et al*, 2006).

Proteolytic enzymes, strategic components of tumour angiogenesis, aid cancer cells and endothelial cells to invade the host extracellular membrane. This step is crucial for the growth and development of tumours and their vessels. Hence, the extent of their role in this process may provide important information on the regulation of tumour angiogenesis as well as their influence on colorectal cancer progression (Auguste *et al*, 2005).

Two proteases, each from a major class of proteolytic enzymes, cathepsin B (cysteine protease) and dipeptidyl peptidase IV (serine protease) were investigated for expression in human colorectal tumours. Results showed that levels of enzyme expression increased with tumour grade and tumours from patients with blood and lymph vessel invasion were also positive for the proteases. This denotes that cathepsin B and dipeptidyl peptidase IV may not only play a role in local tumour invasion but also in distant metastasis (Troy *et al*, 2004; Kikkawa *et al*, 2005).

The presence of cathepsin B protein, as well as mRNA near tumour vessels suggests that this protease is involved in tumour angiogenesis by promoting the breakdown of the extracellular matrix to permit neovascularisation (Im *et al*, 2005). This coincides with the study conducted by Kruszewski *et al*, (2004) who established that over expression of cathepsin B increases the intensity of angiogenesis in human colon adenocarcinomas.

Dipeptidyl peptidase IV expression has been reported in several kinds of human carcinomas such as endometrial, thyroid and prostate. Recent studies have provided evidence that this enzyme may play a role in tumour progression (Kikkawa *et al*, 2005). Komatsu *et al*, (1987) suggested that dipeptidyl peptidase IV expression may have possible use as a tumour marker, which led this study to investigate such a potential in human colorectal cancer which has not been assessed before.

Although increased protein expression of dipeptidyl peptidase IV in higher tumour grades were noted, the mRNA signal for the enzyme was greater suggesting that human colorectal cancer cells possess a high potential for dipeptidyl peptidase IV expression.

Nevertheless, this study is a qualitative approach and further studies involving direct measurements of the levels of enzymes in varying tumour grades should be carried out. A possible relationship between tumour vessel counts and enzyme levels would also directly implicate these proteases in modulating tumour angiogenesis and further to these results may allude to enzyme levels as potential biological markers.

With the knowledge that growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are potent angiogenic regulators (Javerzat *et al*, 2002; Nagy and Senger, 2006) it was imperative that the influence of these factors on the proteolytic enzymes was investigated. The biological role of proteases in the presence of either growth factor was assessed in human colon and rectal tumour cell lines and in human umbilical vein endothelial cells.

Results showed that both growth factors have the ability to increase the release of the proteases cathepsin B and dipeptidyl peptidase IV in both tumour tissue and endothelial cells. This lends support to the fact that VEGF and bFGF directly influence the release of proteases (Kuhn *et al*, 2004) and thus delineates the role of these enzymes in tumour metastasis and angiogenesis in human colorectal cancer.

The overall conclusion from these studies leads to the proposition that tumour angiogenesis and the angiogenic factors, involved in this process, may provide essential information on colorectal cancer progression. Factors such as tumour blood and lymph vessels and proteolytic activity may be recognised as effective biological markers for metastasis when used alongside traditional clinicopathological variables. However, these studies can be extended further to assess the relationship of these parameters with survival rates. Patient follow up data after tumour resection need to be collated for such a study.

6.2 *Conclusion*

This study aimed to assess the influence of tumour angiogenesis on the metastatic potential of colorectal carcinomas. The work has demonstrated that:

1) The extent of tumour vascularity can be reliably assessed by immunolabelling tumour endothelial cells by CD31 in order to allow defined quantification of all vessels within cross-sections of colorectal tumours.

2) Lymph vessels are present in colorectal carcinomas and can be highlighted by a differential staining method using endothelial markers CD31 and CD34 on alternate tumour tissue sections.

3) Increased number of blood and lymph vessels is positively correlated to clinicopathological variables associated with metastasis such as: histological profiles of moderate and poorly differentiated colorectal tumours, tumours excised from patients with blood and lymphatic vessel invasion of the cancer and tumours of Dukes' stages B and C. Therefore, vessel counts from colorectal tumours may demonstrate the possibility of being effective biological markers for disease progression.

4) The pattern of spatial distribution of vessels within the tumour sections examined, including the invasive fronts and normal host tissue. This indicates that tumours elicit an angiogenic response and generate their own blood supply from host vessels for growth and metastasis.

5) The increase in cathepsin B (CB) and dipeptidyl peptidase IV (DPPIV) enzyme protein and mRNA expression in sections of human colorectal carcinomas is correlated to tumours with metastatic potential. Elevated enzyme levels observed adjacent to tumour vessels as well as in tumour invasive fronts indicate that these proteases may play a significant role in both colorectal tumour angiogenesis and tumour progression. Hence, these enzyme proteases have the potential to be possible biological markers for the advancement of colorectal cancers.

6) This study shows the increase in cathepsin B (CB) and dipeptidyl peptidase IV (DPPIV) activity and expression to be directly influenced by two potent angiogenic growth factors basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in both colon and rectal cancer and normal endothelial cells. Hence, it may be concluded that VEGF and bFGF stimulates the production of CB and DPPIV in human colorectal carcinomas and may also play a role in inducing colorectal tumour vessels by increasing the release of the enzymes.

This thesis demonstrates that colorectal tumours undergo angiogenesis as well as lymphangiogenesis in order to grow and metastasise. The association of increased number of blood and lymph vessels as well as that of the increased expression of proteases to clinicopathological variables linked to poor prognosis supports the fact that colorectal tumour angiogenesis is an important parameter in assessing the extent of the disease. Therefore, it is a very strong contender to be an independent prognostic marker for colorectal cancer.

6.3 *Future studies*

In many tumour types, marked features of neoangiogenesis have been found to correlate with a high rate of metastasis. Established endothelial markers such as CD31, CD34 and vWF have not evolved as effective highlighters of tumour vessels for many groups. This may be a cause for the conflicting data that has been obtained from studies analysing the relationship between tumour vascularity and patient prognosis (Tjalma *et al*, 1998; Di Leo *et al*, 1998; Maurer *et al*, 1998; El-Gazzar *et al*, 2005).

Current opinions suggest that detection of activated tumour endothelial cells better reflects a tumour's capability to produce new vessels and hence determine a more accurate deduction of the effect of tumour vessels on tumour metastasis. This proposal may have superiority over traditional methods as it is based upon the fact that tumour endothelial cells are constantly proliferating to establish a network of vessels to sustain tumour growth and facilitate blood-borne metastasis (Cao *et al*, 2005).

Adam *et al*, (2003) and Dales *et al*, (2004) demonstrated that CD105 (endoglin) detects proliferating endothelial cells and found that it was a superior marker to CD34 in human tumours of the lung and breast. Tie2 is an endothelium specific receptor and its mRNA is abundantly expressed in human metastatic melanoma and endothelial cells undergoing angiogenesis. Its expression has also been correlated to the extent of vascularisation in tumours of the breast and brain and is regarded as a good marker for assessing activated endothelial cells.

These two endothelial cell markers are thought to have greater clinical relevance not only as independent prognostic indicators but also in identifying CD105 and Tie2 positive tumour vessels to enable specific targeting for cancer treatment. Therapy would entail blocking CD105 and Tie2 pathways of endothelial cell activation, therefore, specific patients positive to the markers could be selected for treatment. The use of these markers in colorectal cancers could further support the suggestion that tumour vessels of this cancer may bear prognostic relevance (Adam *et al*, 2003).

VEGF is a potent modulator of angiogenesis and the expression of VEGF is correlated with MVD in the colon, ovary, prostate and testis (Takahashi *et al*, 1995; Boocock *et al*, 1995; Viglietto *et al*, 1996; Ferrer *et al*, 1997; Kilic *et al*, 1997).

This protein binds to and activates the high affinity tyrosine kinase receptors flt-1 and flk-1/kdr which are almost exclusively expressed on the surface of endothelial cells (Adam *et al,* 2003). It would be useful to conduct further studies by highlighting VEGF in tumour vessels as VEGF, Tie and CD105 are weakly expressed or non-existent on normal endothelial cells (quiescent endothelial cells) and establish if angiogenesis in tumours is correlated to colorectal cancer prognosis (Dales *et al*, 2004).

Convincing evidence for tumour lymphangiogenesis has begun to accumulate. This study found that the two common endothelial markers CD34 and CD31 could be used to establish the presence of lymph vessels in tumours, although, a specific marker to highlight these vessels have been scarce. However, with the discovery of LYVE-1 (lymph vessel endothelial HA receptor) a lymphatic endothelial specific receptor, this could provide new impetus to study the biology and prognostic implications of tumour lymphangiogenesis (Jackson *et al,* 2001).

A better understanding of the angiogenic process and its role in disease states help to reveal specific targets for treatments. The variety of *in vivo* assays has enabled investigators to make rapid progress in elucidating the mechanism of a variety of angiogenic factors (Veronese and O'Dwyer, 2004).

Traditional methods such as Sandison window in the rabbit ear (Zawicki *et al,* 1981) or Algire window in the dorsal skin of a rodent (Leunig *et al*,1992) and subcutaneously grown tumours in mice are still used to assess anti-angiogenic treatments with the latter being more frequently used (Munn 2003). These models have also helped in assessing spatial distribution in tumours and how tumour vasculature may be implicated in metastasis (Folkman *et al*, 1995).

Newer methods are constantly being devised to assess tumour vessels. Until recently spatial distribution was assessed using tumour blood flow by using nuclear magnetic resonance, positron emission tomography and functional computerized tomography (Jain 1997). A recent method to quantify the complexity of tumour microvasculature by using multiphoton microscopy was presented. This approach may allow vessel quantification and assessing the model of tumour vessel network. The method may also aid measurement of tumour vasculature across a time series (Tyrrell *et al*, 2005).

This would help in investigating the response of the tumour to anti-angiogenic treatment but also allow the observation of tumour angiogenesis over time (Tyrrell *et al*, 2005). Nevertheless MVD is still the recommended method for angiogenesis quantification as assessed by international consensus panels (Vermeulen *et al*, 1996; Hansen 2000; Vermeulen 2002; West *et al*, 2005).

Other traditional methods such as monolayer cell cultures are still frequently used due to it being more practical and cost-effective. However, this method is far removed from the *in vivo* situation such as cell-cell interactions, cell-matrix interactions and gross morphology (Krueger *et al*, 2005).

These factors influence the very pathways that are crucial for tumour development. Therefore, a major advancement known as 3-dimensional cell culture has now been devised. The cells under particular growth conditions form spheroids and studies show that they resemble tumour of origin and exhibit similar growth dynamics of tumours. Hudson *et al*, (2006) used HUVECs culture cells coated cytodex micro-carrier beads (Sigma U.K.) to assess intercellular interactions which may modulate angiogenesis. These models may provide a breakthrough in the exact understanding of the effects of proteases between tumour cells and tumour endothelial cells as well as influencing factors of angiogenesis (Krueger *et al*, 2005).

Needless to say continued advances in methods assessing angiogenesis has provided extensive knowledge of cancer and it has also provided new methods of treatments therefore, it is crucial that research into the process of tumour angiogenesis, by possibly using techniques suggested in future studies, must be continued.

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