# TUMOUR INVASION AND METASTASIS IN COLORECTAL CANCER: AN IMMUNOHISTOCHEMICAL STUDY INTO THE ROLE OF MATRIX METALLOPROTEINASE ENZYMES, TIMP-1, AND CIRCULATING TUMOUR CELLS

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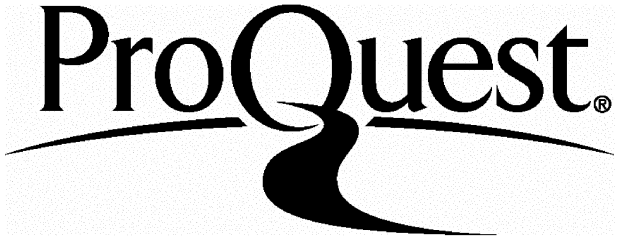

**Abstract**

The enzymatic degradation of surrounding connective tissue followed by the intravasation and circulation of tumour cells represent initial steps in metastasis. In a study of colorectal cancer the technique of immunohistochemistry was used to examine the role played by the matrix metalloproteinase enzymes interstitial collagenase, gelatinase and stromelysin and their inhibitor TIMP-1 in tumour invasion, and to detect and quantify blood borne tumour cells in mesenteric and peripheral vein blood samples.

A role for stromelysin and $M_r$ 72,000 gelatinase in tumour invasion was supported by immunolocalizing both enzymes to the connective tissue matrix, fibroblast-like cells or mononuclear cells within biopsies of tumour tissue and not to those of normal mucosa. There was no evidence that the tumour cells were responsible for the synthesis of these enzymes. By contrast interstitial collagenase was not identified. TIMP-1 predominantly immunolocalized to endothelial cells and was identified most frequently at the tumour/mucosal interface implying a possible role in modulating tumour invasion. Additionally evidence from immunostained Western blots prepared from the polyacrylamide gel electrophoresis of colonic mucus suggested that TIMP-1 was present in colonic mucus.

Circulating tumour cells were identified in 4 of 14 patients using the anti-cytokeratin antibody 8.13. Cells were present in concentrations ranging from 40 cells $ml^{-1}$ to 954 cells $ml^{-1}$.

These studies provide new information on the mechanism of tumour invasion and form the basis for further investigations aimed at correlating matrix metalloproteinase and TIMP-1 expression with the biological behaviour of colorectal tumours. The ability to detect and quantify circulating tumour cells with increased reliability also provides a foundation for a further study into the characteristics of these cells.

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**Statement of Originality**

To the best of the author's knowledge each of the studies detailed in the following Chapters represents an original investigation into the subject concerned.

In the case of the immunohistochemical studies into the matrix metalloproteinases no previous investigation has simultaneously examined the presence of the enzymes collagenase, stromelysin and gelatinase, and their specific inhibitor TIMP-1 in human colorectal cancer. Nor has there been any previous attempt to examine human colonic mucus for the presence of TIMP-1.

Although previous studies have been mounted to detect circulating tumour cells, no previous investigation has used the technique of immunocytochemistry to identify blood borne cancer cells in patients with colorectal cancer. There has also been no previous attempt to calculate the concentration of such cells if found.

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# CHAPTER 1
# INTRODUCTION

Colorectal cancer is second only to lung cancer in males and breast cancer in females as the commonest cause of death from malignancy in the United Kingdom. Nearly 24,000 new cases of colorectal cancer are registered each year in England and Wales (HMSO, 1990) and in 1989 over 17,000 people died from the disease (HMSO, 1991). Amongst other variables the mortality figures for colorectal cancer reflect the outcome of two opposing influences - the capacity of a malignant tumour to invade and metastasize, and the therapeutic strategies deployed to control these phenomena.

The mechanisms whereby a primary malignant tumour establishes a metastatic deposit have become conceptualized into a series of defined, sequential steps. This rationalization of the metastatic process has allowed investigators to study each step in turn, often with the aid of experimental models, in the hope that new approaches to treatment will emerge.

It is the aim of this thesis to examine two aspects of the invasive and metastatic process as they occur in colorectal cancer. The first part of the thesis is concerned with an investigation into the role played in tumour invasion by a group of connective tissue degrading enzymes, known as the matrix metalloproteinases, and their specific inhibitor, tissue inhibitor of metalloproteinases (TIMP). The second portion of the thesis reports on a study into the presence and enumeration of circulating tumour cells as detected in both local and peripheral venous blood samples. In investigating both of these themes emphasis has been placed on detailing events as they occur *in vivo*; by so doing it is hoped that a clearer perspective might be placed on the results of previous investigations, many of which have been conducted either *in vitro*, or using animal models. Through a greater understanding of some of the mechanisms

behind metastasis it may also be possible to augment current staging systems with information which more closely reflects the biological aggressiveness of the tumour.

The following introduction commences with an overview of the current methods available to treat primary colorectal cancer, and then continues with a review of matrix metalloproteinase enzymes, and circulating tumour cells.

## 1.1 THE TREATMENT OF COLORECTAL CANCER: AN OVERVIEW.

### 1.1.1 *Surgery*

Franks (1889) records the first published account of a colonic resection for cancer as taking place in France in 1843. The operation was performed for a carcinoma of the sigmoid colon; three inches of bowel were removed and the patient was reported to have recovered by the tenth day. Unfortunately, a recurrence developed six months later, and one year after the initial operation the patient died. Although further colonic resections were performed at intervals thereafter, the results were often similar; those patients who were fortunate enough to survive their surgery subsequently succumbed from recurrent disease.

These early operations disappointed as curative procedures because they failed to take account of the way in which colonic tumours spread, and it was not until the first decade of this century that the principles guiding curative resection were founded. The work of Jamieson and Dobson (1909) on the lymphatic drainage of the colon established the need to remove both the primary tumour and its associated "lymphatic field" if recurrent disease was to be avoided. Similar work by Ernest Miles, on the spread of rectal cancer, led him to develop the operation of abdominoperineal excision (Miles, 1908). However, because of the operative mortality associated with them, these operations had a disappointing influence on the death rate from colorectal cancer. At the London Hospital between 1901 and 1911 the mortality of radical operations for large bowel cancer was nearly 50% (Bland-Sutton, 1917).

Improvement in survival had to await the introduction of antibiotics, advances in anaesthesia and refinements of surgical technique. When these arrived, the mortality from colorectal cancer was cut dramatically. Between 1936 and 1965 the age standardised death rate per 100,000 of the population fell by over 35 per cent.

To further reduce the frequency of recurrent disease and enhance survival, myriad modifications of surgical technique have been devised with varying degrees of success.

Anxious to remove the most proximal lymph nodes likely to be affected by the spread of rectal cancer, Moynihan introduced ligation of the inferior mesenteric artery at a level above the origin of the left colic artery; the so called "high tie" (Moynihan, 1908). However, Pezim and Nicholls (1983) in a review of 1370 patients with cancer of the rectum or rectosigmoid, found that the level at which the inferior mesenteric artery was ligated made no difference to the 5-year survival of patients.

Fisher and Turnbull argued that manipulation of the tumour during surgery might release malignant cell emboli. To reduce this risk they advocated a "no-touch" isolation technique with early ligation of the mesenteric vessels. In their series of unrandomised patients with C stage tumours, the crude 5-year survival was 57.84% when this technique was used, compared to 28.06% when it was not (Turnbull et al, 1967). A subsequent prospective randomised trial of the no-touch isolation technique has failed to confirm this survival advantage. However, a trend towards a reduction in the number of liver metastases seen in the no-touch group, particularly in sigmoid carcinomas and those with evidence of vascular invasion, was noted (Wiggers et al, 1988).

More radical operations may also influence survival and the incidence of recurrent disease. In rectal cancer, wide anatomical resection coupled with radical lymphadenectomy to remove the aortic and internal iliac nodes may improve the outcome (Enker et al, 1979), although this is disputed by others (Glass et al, 1985); while removal of the mesorectum

reduces the incidence of at least local recurrence in rectal cancer (Heald and Ryall, 1986).

Whatever the operative technique, surgery remains the principal treatment for large bowel cancer with prognosis directly related to the extent of tumour spread at the time of operation (Dukes, 1958). Nevertheless, despite the good prognosis associated with early stage disease, the influence of surgery on the overall mortality from colorectal cancer has remained virtually unchanged for the last 25 years. At least two factors are responsible for this, i) the advanced stage of many tumours at presentation and ii) the high relapse rate amongst patients undergoing apparently curative operations. As many as 50% of patients present with tumours that are suitable for palliative treatment only (Clarke et al, 1980), and even when a "curative resection" is possible, up to 50% can still be expected to die from their disease within 5 years (McArdle et al, 1990).

The natural history of colorectal cancer within the population coupled with its biological behaviour has imposed a limit on the effectiveness of surgical treatment alone. Population screening and the use of adjuvant chemotherapy or radiotherapy have been seen as ways in which this impasse can be broken.

### *1.1.2 Screening for colorectal cancer*

Mass population screening seeks to exploit the excellent results of surgery in early stage disease (benign adenomas and Dukes' stage A tumours). The natural history of colonic carcinoma is such that most patients present only when their tumours are at an advanced stage and causing symptoms: less than 10% of patients undergoing surgery have Dukes' stage A tumours (Stower and Hardcastle, 1985; McArdle et al, 1990). By screening asymptomatic individuals an increased proportion of early lesions might be identified and treated.

In the Nottingham study faecal occult blood testing was used to screen individuals aged over 50 years for large

bowel neoplasia and the results compared to a control group. Of the 52,258 people offered screening 27,651 completed the tests. Investigation of the 2.3% who had a positive result led to the detection of 63 cancers, 52% of which were Dukes'A. In the control group only 11% of the 123 cancers detected were Dukes' stage A (Hardcastle et al, 1989). Although this result is promising it is not yet known whether screening will improve survival in those populations who receive it (The Seventh King's Fund Forum, 1990).

### *1.1.3 Adjuvant Chemotherapy*

Many patients develop recurrent disease despite an apparently curative resection, and metastases either present and undetected, or initiated at the time of operation are thought to be responsible (Finlay and McArdle, 1982). The administration of adjuvant chemotherapy offers a way in which occult disease might be destroyed.

5-Fluorouracil, either alone or in combination with other agents, has been the most extensively used drug. A meta-analysis of 20 trials involving systemic 5-fluorouracil suggested that the mortality rate might be reduced by up to 15 per cent, although the confidence interval for this result ranged from 0 to 20% (Buyse et al, 1988). More encouraging are the results of the intergroup trial from the United States in which 5-fluorouracil was combined with levamisole. Nearly 1300 patients with either stage B2 or C cancer of the colon were randomized to a control arm or to levamisole alone or levamisole with 5-fluorouracil. After a median follow up of 3 years those patients with stage C disease who had been treated with 5-fluorouracil and levamisole showed a 41% reduction in recurrence and a 33% reduction in mortality (Moertel et al, 1990).

Hepatic metastases are a frequent component of recurrent disease and this has prompted the use of intraportal adjuvant chemotherapy. A randomized trial in

which portal vein perfusion with 5-fluorouracil was used for 7 days reported a reduction in the number of metastases in the perfused group, and a survival advantage for patients with Dukes' stage B tumours (Taylor et al, 1985). Further evaluation of this approach, which carries the advantage of a much shorter treatment interval compared to systemic administration, is currently being conducted by the United Kingdom Coordinating Committee for Cancer Research through the AXIS trial (Taylor and Northover, 1990).

### *1.1.4 Adjuvant radiotherapy*

The objective of adjuvant radiotherapy is to prevent the growth of tumour cells which either cannot be removed, or are inadvertently left behind at the time of operation. In so doing it is hoped that local recurrence rates will be reduced and survival improved.

Adjuvant radiotherapy may be given either preoperatively or postoperatively, and has been used predominantly in rectal cancer. With the exception of the Veterans Administration Surgical Adjuvant Group (VASAG), none of the randomized studies involving preoperative or postoperative radiotherapy has demonstrated a significant increase in survival. In the VASAG study reported in 1975 patients were randomized to receive radiotherapy followed by abdominoperineal excision of their tumour or abdominoperineal resection alone. Of the irradiated patients 43% survived 5 years compared to 32% in the surgery only group (Higgins et al, 1975). However, a repeat study using a higher dose of radiation failed to confirm this result; both the irradiated and the control group had a 50% 5-year survival (Higgins et al, 1986). The favourable result observed in the first study was attributed to the inclusion of an excess number of patients with nodal metastases in the control group.

Although survival may not be improved, preoperative radiotherapy can reduce the incidence of local recurrence.

In the European Organization for the Research and Treatment of Cancer trial (EORTC) those patients undergoing curative resections who had received preoperative radiotherapy had a pelvic recurrence rate of 15% compared to 35% in the non-irradiated group (Gerard et al, 1985). However, accurate preoperative staging of a tumour is difficult and a policy of preoperative radiotherapy will inevitably result in some patients being irradiated unnecessarily.

Postoperative radiotherapy on the other hand can be given selectively to poor prognosis patients. Of the 3 randomized trials of postoperative radiotherapy only one, that of the National Surgical Adjuvant Bowel Project (NSAPB), has demonstrated a reduction in the incidence of local recurrence which approaches significance (Fisher et al, 1988).

Neither preoperative nor postoperative radiotherapy appear to influence the development of extrapelvic recurrence; their inadequacy in this respect underlines the importance of metastatic disease as a determinant of survival.

The success or failure of the above treatments depend ultimately on their effectiveness at removing or destroying tumour cells. Such cells may be solely confined to the primary tumour but in many instances they have spread to distant sites. The mechanisms which enable tumour cells to disseminate, and their susceptibility to therapeutic intervention are therefore of crucial importance in determining the outcome from colorectal cancer. As Isaiah Fidler has remarked, "You cannot fix it if you do not know how it works." (Fidler, 1991).

## 1.2 THE SPREAD OF COLORECTAL CANCER: AN OVERVIEW

The treatment of colorectal cancer is determined by the pattern and extent of its spread at the time of diagnosis.

Colorectal adenocarcinomas spread by four main routes: 1) Local spread, 2) lymphatic invasion, 3) venous invasion, and 4) transcoelomic spread.

### 1.2.1 *Local spread.*

The local spread of colorectal cancers occurs predominantly at right angles to the bowel wall (Black and Waugh, 1948). In contrast to other solid tumours, such as those of the breast, it is the extent of this spread, as measured by the depth of invasion of the bowel wall, rather than tumour size which appears to exert the greater influence on prognosis. As the depth of tumour invasion increases so the risk of lymph node metastases rises and the prognosis worsens. In a retrospective analysis of rectal cancer the incidence of lymph node metastasis in tumours confined to the bowel wall was 14.2% compared to 43.2% once the bowel wall had been breached (Dukes and Bussey, 1958). By comparison, in a study comparing the tumour volumes of Dukes' B and C lesions, i.e. those without lymph node metastases and those with, Dukes' B tumours were found on average to be larger than Dukes' C (Wolmark et al, 1983). The influence of local tumour invasion on prognosis has also been confirmed by a prospective study in which the survival of patients undergoing apparently curative surgery was significantly reduced when macroscopic extramural tumour invasion was present (Wood, 1981).

The extent of extramural spread also affects the incidence of recurrent disease. This is particularly so at those sites where clear lateral margins of resection are difficult to achieve, as in low rectal tumours. In a series of 39 patients undergoing a clinically curative resection for rectal cancer, 5 of the operative specimens were found to have involvement of the lateral resection margins, and four of these five patients subsequently developed local recurrence (Quirke et al, 1986).

### 1.2.2 *Lymphatic invasion*

The presence of lymph node metastases provides direct evidence of a tumour's capacity to disseminate and their detection provides important prognostic information for patients undergoing curative resection of rectal cancer (Jass et al, 1987). As the number of lymph nodes involved increases so the prognosis worsens; with metastasis in one node only, the 5-year survival rate is 63.3%, falling to 2.1% when more than ten nodes are affected (Dukes, 1958).

Anatomical evidence of a lymphatic network in solid tumours is lacking, and it seems likely that the tumour periphery is the principal site of lymphatic vessel invasion (Butler et al, 1975). Once access to the lymphatic channels has been gained, involvement of the draining lymph nodes occurs in a progressive manner, with successive lymph nodes affected according to their anatomical position (Dukes, 1940). Classically, this orderly process was considered to culminate in secondary haematogenous spread via the thoracic duct. However, lymphaticovenous and interlymphatic communications (Edwards and Kinmouth, 1969) may allow intervening lymph nodes to be bypassed, and make it likely that lymph node metastases can themselves disseminate at an early stage (Weiss, 1985a).

### 1.2.3 *Venous invasion*

Once colorectal cancer cells have invaded radicals of the portal venous system they have the opportunity to form secondary deposits in the liver (Willis, 1932).
Although hepatic metastases are present in 40% - 70% of patients dying of colorectal cancer (Ridge and Daly, 1985), they rarely occur in isolation and their presence is frequently associated with a wider pattern of spread. In a series of necropsies performed on 45 patients who had undergone "curative" colorectal surgery, 21 patients were found to have developed hepatic secondaries, but in only

three of these was the liver the sole site of recurrence (Gilbert, 1984). Similarly Welch and Donaldson (1979) observed that hepatic metastases were present as isolated lesions in only 9% of patients. The incidence of venous invasion in colorectal cancer and its effect on prognosis is discussed under the section dealing with circulating tumour cells.

### 1.2.4 *Transcoelomic spread and implantation*

Between 10% and 20% of patients undergoing curative resection for large bowel cancer can be expected to develop local recurrence of their disease (McDermott et al, 1985; Philips et al, 1984). Inadequate excision of the primary tumour may account for some these failures (Quirke et al, 1986), while tumour cell dissemination at the time of surgery may be responsible for others.

In the "tumour cell entrapment hypothesis" Sugarbaker (1989) has suggested that malignant cells, released from the primary tumour or from severed blood or lymphatic vessels, may be trapped by the layer of fibrin which forms on traumatised peritoneal surfaces. The trapped cells implant and grow, eventually forming secondary deposits. The demonstration that exfoliated cells, washed from the lumen of freshly resected bowel, are viable and capable of forming metastases (at least in nude mice; Fermor et al, 1986), provides some experimental support for this hypothesis. There is also evidence to suggest that peritoneal deposits can develop in the absence of prior peritoneal injury; Ehrlich ascites tumour cells have been observed to induce retraction of peritoneal mesothelial cells as part of the invasive process (Birbeck et al, 1964).

## 1.3 METASTASIS: AN OVERVIEW

Descriptions detailing the spread of malignant tumours exist from the time of Herodotus, around 520 B.C. (Herodotus, cited by Willis, 1948). However, even as late as the eighteenth century, anatomists such as Morgagni (1682 - 1771) failed to appreciate that distant foci of tumour observed, for example, in the liver and lymph nodes, originated from the primary cancer.

The term metastases does not appear until 1829, when Joseph Claude Recamier used it to describe the spontaneous eruption of a carcinoma succeeded by an identical eruption at another site (Recamier, 1829). In 1840 Langenbeck produced growths in the lungs of dogs by the intravenous injection of tumour material obtained from a carcinoma of the uterus (Langenbeck, 1841). The significance of this experiment was, however, either ignored or ambiguously interpreted and investigators as illustrious as Rudolf Virchow (1821 -1902) and Sir James Paget (1814 -1902) believed that metastasis was the result of a non-cellular infection or a "rudimentary fluid", which was transported in the circulation from the primary tumour (Virchow, 1863; Paget, 1863). This view held sway until the views of the surgeon Karl Thiersch (1822 - 1895) were published. Having observed venous invasion by cancerous growths and demonstrated tumour cells in the lymph of the thoracic duct, Thiersch advocated tumour cell embolization as the cause of metastasis (Thiersch, 1865). His views were subsequently supported by the anatomist Heinrich Waldeyer (1836 - 1921), and together their writings firmly established metastasis as an embolic phenomenon (Waldeyer, 1867).

A metastasis may be defined as a "neoplastic lesion that arises from another cancer with which it is no longer in continuity" (Poste and Fidler, 1980). The metastatic process is now recognised to be a multi-step one involving a sequence of complex interactions between tumour cells and the host tissues; the essential events are set out below.

**Figure 1.1**

Prominent personalities from the past whose observations have helped to shape current views on the process of metastasis. From top left, clockwise: Morgagni who observed distant foci of tumour in lymph nodes and liver; Recamier, the first worker to use the term mètastases; Virchow, who considered metastases to arise as a result of the circulation of a "rudimentary fluid"; Waldeyer, whose observations coupled with those of Thiersch established the embolic nature of metastasis.

**Figure 1.2**

Page 110 from Joseph Claude Recamier's book, Recherches sur le traitment du cancer, in which the term mètastases is used for the first time.

# RECHERCHES

SUR

LE TRAITEMENT

# DU CANCER,

PAR LA COMPRESSION MÉTHODIQUE SIMPLE OU COMBINÉE,

ET

SUR L'HISTOIRE GÉNÉRALE DE LA MÊME MALADIE;

SUIVIES DE NOTES

1°. SUR LES FORCES ET LA DYNAMÉTRIE VITALES;
2°. SUR L'INFLAMMATION ET L'ÉTAT FÉBRILE.

**PAR J. C. A. RÉCAMIER,**

MÉDECIN DE L'HOTEL-DIEU DE PARIS,

Chevalier de l'Ordre royal de la Légion-d'Honneur, Professeur de Médecine au Collége royal de France, Professeur de Clinique médicale à la Faculté de Médecine, Membre de l'Académie royale de Médecine, etc., etc.

(Carcinoma.) Quidam ferro admoverunt, quidam scalpelo exciderunt, neque illi unquàm medicina profecit. Adusta protinùs concitata sunt et increverunt donec occiderent. Exsecta, etiam post inductam cicatricem, tumor reverterunt et causam mortis attulerunt. (Celse, edit. Lugd. Bat., 1599, p. 806.)

Tota Chirurgia et Medicina desiderant medicamentum ad Cancrum, ut suppuretur, vel ut sanetur, ut aliquandò in anthracibus fit, vel ut substantia ejus vertatur in pus, et tamen continuum non salvatur: sed, prob dolor! Nullum datur.... (Boerhaave, *Prax. Med.*, t. II, p. 256.)

TOME SECOND.

**PARIS,**

CHEZ GABON, LIBRAIRE-ÉDITEUR,

Rue de l'École-de-Médecine, n° 10;

MONTPELLIER, MÊME MAISON;

BRUXELLES, au Dépôt de Librairie médicale française;

1829.



recouvré en grande partie dans cet endroit son organisation primitive. »

Le fait de M. Parent, en montrant la résolution spontanée d'un engorgement carcinomateux, suivie d'un autre engorgement de même nature, peut conduire à admettre *des métastases cancéreuses*.

Le fait suivant conduit à penser que dans certains cas la diathèse cancéreuse peut s'user sans métastase : madame G...., alors âgée de quarante-quatre ans, grêle, mal réglée et très-sanguine, portait, il y a douze ans, dans la région du colon droit, une tumeur fixe, qui était le siége d'élancemens spontanés très-vifs, avec des irradiations très-douloureuses, dont les exacerbations, malgré le régime le plus sévère, ramenaient souvent la nécessité de la saignée, seul moyen de soulagement véritable pour elle. Je crus à l'existence d'une tumeur de nature cancéreuse dans cette région du canal intestinal, et MM. Fouquier et Marjolin, qui ont vu la malade avec moi, ont partagé la même opinion. On a continué à saigner cette personne, très-affaiblie et très-maigre, lorsque l'intensité des accidens le demandait. L'époque de la cessation des règles est arrivée; les douleurs de la région cœcale ou colique ont diminué. Madame G.... a cessé d'être sai-

1. Growth of the primary tumour and neovascularization
2. Tissue invasion
3. Cell detachment
4. Intravasation of cells into the lymphatic or blood circulations
5. Entrapment of cells in the target organ
6. Invasion into the target organ
7. Growth of the metastasis

It is not possible within the confines of this introduction to discuss each of these steps in detail. The first two steps as well as the sixth are, however, relevant to the role played by enzymes in tumour invasion, while an outline of the remaining steps will be given in the section dealing with the fate of circulating tumour cells.

### 1.3.1 *Growth of the primary tumour and tissue invasion*

For tumour cells to reach and invade the vascular and lymphatic channels, which form the main conduits for their dissemination, the intervening connective tissues must be traversed (Liotta, 1984).

When an epithelial tumour starts to grow invasively the first structure to be breached is the basement membrane, which separates it from the underlying stroma. The structure of basement membranes is complex, but essentially they are composed of two distinct layers, i) a basal lamina which is derived from, and closely associated with, the parenchymal cells, and ii) a reticular lamina which is produced by the adjacent connective tissue cells (Bernfield, 1984). The principal constituents of basement membranes are: types IV and V collagen, heparan sulphate proteoglycans and the glycoproteins laminin and fibronectin (Hahn, 1990). Basement membranes are insoluble and relatively stable structures within the adult organism a property conferred on them by the hexagonal arrangement of type IV collagen molecules

within the basal lamina (Timpl et al, 1978). Based on the rate of loss of a silver stain, turnover rates for basement membranes have been estimated as ranging from 40 days in the colon to more than two years for skin (Walker, 1972). Once through the basement membrane malignant cells must negotiate the interstitial stroma, composed of a latticework of collagen and elastin fibres embedded in a ground substance of proteoglycans and glycoproteins (Hukins and Aspden, 1985). The collagen molecule is therefore common both to basement membranes and to the interstitial stroma, and its structure and the means whereby it can be degraded are important factors in tumour invasion.

The collagens form a family of related proteins of which there are at least 13 members. The basic unit of collagen is referred to as tropocollagen and consists of a triple helix of peptide chains, with each chain containing approximately 1000 amino acids. Within the chains every third residue is glycine and glycine, proline and hydroxyproline represent the commonest amino acids in the molecule. Peptide chains of different composition combine to form different collagen molecules. Type I collagen, for example, is composed of two different peptide chains, the helix being generated from two chains of one type ($\alpha_1$(I) 2) and one chain of the other ($\alpha_2$(I); Walters and Israel, 1979. At the ends of each triple helix are non-helical, or telopeptide regions, where cross-linking between the collagen molecules can take place to form collagen fibrils. It is the fibrillar structure of collagen which makes it resistant to the action of most proteinases including vertebrate collagenase, a feature possibly enhanced by its association in the tissue stroma with other macromolecules which may mask proteolytically sensitive sites in the molecule (Murphy and Reynolds, 1985; Pauli and Knudson, 1988).

Collagen types I, II and III are known as the interstitial collagens. Type I is the most abundant and is the principal structural protein of skin, tendons, bone and

the stroma of most organs (van den Hooff, 1988). Type II is found mainly in cartilage, and type III is usually found in association with type I collagen. The remaining collagens, often referred to as cell-associated collagens, occur in much smaller amounts and of these, types IV and V are of most interest in the study of tumour invasion as they are components of basement membranes. The structure of type IV collagen differs from that of the interstitial collagens in so far as it forms an open non-fibrillar network to which other proteins such as laminin and heparan sulphate proteoglycan can bind (Martin and Timpl, 1987).

Three theories have been advanced to explain how tumour cells invade the surrounding connective tissue stroma: i) the mechanical theory, ii) the migratory theory, and iii) the enzymatic theory (Pauli and Knudson, 1988).

The first of these proposes that tumour invasion and the degenerative stromal changes which accompany it are the result of an expanding tumour mass forcing its way between planes of natural cleavage and exerting pressure on the surrounding tissue. The process is seen as analogous to the downward thrust of a plant's roots through the soil (Eaves, 1973). However, the importance of tumour cell growth and pressure atrophy has been called into question by Thorgeirsson et al (1984). These workers were able to demonstrate in an experimental model that the ability of murine sarcoma cells to cross the basement membrane of human amnion was independent of DNA synthesis and therefore of cell proliferation, but dependent on the synthesis of enzymes or other proteins.

Evidence for the second theory, that of cell migration, has been provided by electron microscopy of the invasive edge of human colorectal cancers; cell locomotion has been inferred from ultrastructural features such as the presence of tumour cell cytoplasmic processes protruding through basement membranes (Carr et al, 1986). Studies of the V2 rabbit carcinoma have also implicated cell locomotion as an

important contributory factor in the spread of this tumour (Haemmerli et al, 1982).

However, although both mechanical and migratory properties undoubtedly have a part to play in tumour invasion, increasing evidence has accumulated relating the invasiveness of tumours to the production of matrix-degrading enzymes (Woolley, 1984).

### 1.3.2 *Enzymes and tumour invasion: historical perspective*

The idea that the production of a "cancerous juice" was essential to the development and spread of cancers was expounded as long ago as 1816 by Jean Cruveilhier (cited by Triolo, 1965). However, it was not until the discovery of the first enzymes, extracted from yeast by Büchner in 1897, that the concept acquired a firm basis. By the end of the nineteenth century several workers, principally in Germany, had formulated the idea that tissue invasion was mediated by enzymes secreted by the tumour (Petry, 1899). Although Boyland and McClean (1935) subsequently demonstrated that extracts from tumours could increase the spread of cancers transplanted into rabbits, the identity of the enzymes responsible remained elusive and they were instead referred to collectively as "spreading factors" (Gersh and Catchpole, 1949). Initial suspicion focused on the enzyme hyaluronidase; disappointingly, though, studies in which repeated injections of this enzyme were given to tumour bearing animals, failed to show that the invasiveness of their cancers was increased (Coman et al, 1947). Furthermore, assays of hyaluronidase revealed increased activity in only some of the human tumours examined (McCutcheon and Coman, 1947). Frustrated, investigators shifted their attention to another enzyme, collagenase, which was known to be produced by certain bacteria such as *Clostridium histolyticum*, and capable of degrading collagen fibrils at neutral pH (Birkedal-Hansen, 1988). Initial progress, however, was slow and great difficulty was

experienced in confirming that tumour extracts were capable of splitting the collagen molecule under anything other than the most unphysiological of conditions. It was not until Gross and Lapière (1962) demonstrated that living cells, cultured on collagen gels under physiological conditions, could produce collagenolysis. The advantage of this technique was that it allowed small amounts of collagenase, which would normally be undetectable, to accumulate to a level where its presence was apparent. Using this method the investigation of collagenase as a promotor of tumour invasion gained momentum. Riley and Peacock (1967) found that explants from a variety of tumours, including skin, breast and cervical cancers, could produce lysis of collagen gels. Not all tumours were positive, however, and these workers postulated the existence of other, as yet unidentified, factors which were required for the expression collagenase activity. The degree of collagen lysis was also noted to be greatest when both the epithelial and mesenchymal elements of a tissue were cultured together. Later Robertson and Williams (1969) were able to show that tumour extracts incubated with collagen solutions could produce the characteristic degradation products of tropocollagen as seen by electrophoresis. In 1972 there followed the first major study of collagenolytic enzymes in human neoplasms by Dresden (1972); one hundred and thirty five tumours were examined of which 53 had demonstrable collagenolytic activity.

Although enzymes from all the major classes of proteinases have now been implicated in tumour invasion, e.g. the serine proteinases (Zucker et al, 1985; Reich et al, 1988) and the cysteine and aspartic proteinases cathepsin B and cathepsin D (Poole et al, 1980), increasing interest has been directed on the role played by a family of enzymes known as the matrix metalloproteinases of which collagenase is a member.

## 1.4 MATRIX METALLOPROTEINASES

The matrix metalloproteinases are a family of enzymes characterized by the following features (Matrisian, 1990: Docherty and Murphy, 1990):

1) They are proteinases capable of degrading at least one component of the extracellular matrix.

2) They are dependent on zinc and calcium ions.

3) They can be activated by organomercurials or by treatment with trypsin or pepsin.

4) They are secreted in a latent form and require subsequent activation in order to realize their proteolytic activity.

5) They are inhibited by specific metalloproteinase inhibitors known as tissue inhibitors of metalloproteinases (TIMP).

6) Individual members share considerable amino acid sequence homology with one another.

7) Act at neutral pH.

The principal members of the matrix metalloproteinases are outlined, together with their substrates, in Table 1.1.

Table 1.1.

**Principal members of the Matrix Metalloproteinase family**

| Name | Substrate |
|---|---|
| Interstitial collagenase | Types I, II and III collagen |
| $M_r$ 72,000 gelatinase | Types IV, V and VII collagen, fibronectin and gelatins. |
| $M_r$ 95,000 gelatinase | Types IV and V collagen and gelatin |
| Stromelysin | Proteoglycans, the glycoproteins laminin and fibronectin, types III, IV and V collagen, gelatin and elastin. |
| PUMP-1 | Gelatins and fibronectin |

(Matrisian, 1990; Woessner, 1991)

Using the cDNAs for the human matrix metalloproteinases the primary amino acid sequences of both the latent and active forms of the enzymes have been determined. Not only is there a considerable degree of sequence homology between different members of the matrix metalloproteinases, but each may be considered to consist of three functional domains (Docherty and Murphy, 1990).

Secreted as latent enzymes the MMPs require subsequent activation if they are to realize their proteolytic potential. The process of activation is accompanied by the cleavage of domain 1, a sequence of about 80 amino-terminal amino acids, which act as a propeptide (Docherty and Murphy, 1990).

By virtue of its content of histidine residues, which can coordinate the zinc ion on which MMPs activity is dependent, domain 2 is thought to harbour the active site of the enzymes (Woessner, 1991). For $M_r$ 72,000 gelatinase and $M_r$ 95,000 gelatinase only, domain 2 also contains sequences of amino acids which may facilitate their binding to gelatin (Collier et al, 1988).

The third, or carboxy-terminal domain, may be responsible for binding the enzymes to the connective tissue matrix (Matrisian, 1990; Allan et al, 1991).

Between them the members of the matrix metalloproteinase family can degrade virtually all the components of connective tissue. This has led to their being implicated in a number of physiological as well as pathological processes in which connective tissue remodelling plays a part. The involvement of metalloproteinases (MMPs) has been shown in situations as diverse as embryogenesis (Brenner et al, 1989), angiogenesis (Liotta et al, 1991), rheumatoid arthritis (Woolley et al, 1980), atherosclerosis (Henney et al, 1991), aortic aneurysm (Brophy et al, 1991) and tumour invasion (Stetler-Stevenson, 1990).

The extent to which MMPs can influence the course of these conditions is dependent on factors which modulate the expression of their proteolytic activity. Under normal physiological conditions MMPs activity is strictly controlled (Murphy and Reynolds, 1985). In pathological situations, however, these control mechanisms fail, and matrix degradation proceeds relatively unchecked both spatially and temporally (Murphy et al, 1990; Ponton et al, 1991).

The principal points at which the activity of the MMPs can be controlled are during: i) their synthesis and secretion, ii) the activation of the proenzyme form to the active moiety, and iii) their inhibition by both specific and non-specific enzyme inhibitors (Murphy et al, 1990; Woessner, 1991). Other possible control mechanisms include the immobilization of enzymes on the extracellular matrix, a short half life, achieved by arranging for their rapid proteolytic degradation, and the existence of feedback controls to limit further production (Alexander and Werb, 1989).

### 1.4.1 *The synthesis of matrix metalloproteinases.*

Matrix metalloproteinases are produced by a number of cell types including polymorphonuclear leucocytes (Murphy et al, 1989a), chondrocytes and endothelial cells (Murphy et al, 1989a), fibroblasts (Hipps et al, 1991) and macrophages (Hibbs et al, 1987), as well as tumour cells (Gavrilović et al 1985). Whether or not these cells actually synthesize MMPs may depend on their ability to respond to specific stimuli within their microenvironment. Interleukin-1, epidermal growth factor, tumour necrosis factor-α and fibroblast growth factor are just some of the factors which have been shown to induce collagenase and/or stromelysin gene expression in a variety of connective tissue cells (reviewed by Frisch and Werb, 1989; Overall et al, 1989). Some of these growth factors and cytokines may be produced

by the connective tissue cells themselves or by macrophages and monocytes, and so provide a means for the autoregulation of connective tissue turnover (Murphy et al, 1990). Other factors, such as epidermal growth factor, may be produced by tumour cells, indicating a way in which these cells may induce MMPs synthesis in surrounding host cells to facilitate the process of tumour invasion. Dabbous et al (1988) have demonstrated that the medium in which tumour cells have been cultured can enhance the collagenolytic activity of fibroblasts, while Bauer et al (1979) found that fibroblasts isolated from human basal cell carcinomas had an increased capacity to produce collagenase. There is also evidence to suggest that host cells can stimulate collagenolytic activity in tumour cells. Macrophages, for example, were able to stimulate Lewis lung carcinoma cells to degrade the collagen gels on which they were cultured (Henry et al, 1983). A further possibility is that different subpopulations of tumour cells within the same tumour may interact with one another to produce a more aggressive phenotype. Studies performed on cultures of the BC1 rat mammary carcinoma cell line indicate that two phenotypically different subpopulations may exist; one secreting a growth factor required for the survival of the second, which in its turn secretes a factor able to stimulate collagenase production by the other (Lyon et al, 1989).

As well as stimulating the synthesis of collagenolytic enzymes, growth factors can also diminish their production. Transforming growth factor B can reduce the expression of procollagenase by human fibroblasts and additionally stimulate these cells to increase their output of tissue inhibitor of metalloproteinases (TIMP) (Overall et al, 1989).

Oncogenes can regulate the transcription of MMPs genes. In a mouse carcinogenesis model McDonnell and Matrisian (1990) have proposed that the increase in stromelysin mRNA that accompanies the conversion of a papilloma to a carcinoma requires both the activation of an oncogene that

induces stromelysin and the loss of a factor that inhibits stromelysin transcription. A possible candidate for such an oncogene is the Ha-*ras* oncogene which has been shown to induce stromelysin and gelatinase expression (Matrisian et al, 1985; Collier et al, 1988).

### 1.4.2 *Secretion of matrix metalloproteinases*

The synthesis of matrix metalloproteinase enzymes is almost invariably followed by their secretion. Studies of collagenase synthesis in human fibroblasts demonstrated that intracellular enzyme appeared after 15 minutes and that after a further 15 minutes it was present extracellularly (Valle and Bauer, 1979). The interval between synthesis and secretion suggests that little intracellular storage of the enzyme takes place, a feature which contributes to the difficulty in identifying the cells responsible for metalloproteinase synthesis. Polymorphonuclear leucocytes are an exception to this, and appear to store the enzyme in secretory granules.

Secretion of MMPs may be accompanied by the cleavage of a signal sequence of twenty or so amino acids from the N-terminal of the enzyme (Docherty and Murphy, 1990).

### 1.4.3 *Activation of latent matrix metalloproteinases.*

The mechanism(s) whereby the secreted latent form of the metalloproteinases are activated are still unclear. There is, however, evidence to suggest that the proteinase plasmin may be important. For collagenase and stromelysin a sequence of reactions involving the initial generation of plasmin from plasminogen by plasminogen activator, and culminating in the activation of the latent form of the enzyme has been envisaged (Frisch and Werb, 1989). Other enzymes such as cathepsin B and L, and trypsin have also been implicated in the activation of collagenase and stromelysin (Frisch and Werb, 1989).

Once activated stromelysin may itself activate procollagenase and thereby provide the opportunity for a cascade of activation reactions to occur (Murphy et al, 1987). However, although plasmin can convert both procollagenase and prostromelysin to their active forms it is relatively ineffective against progelatinase as indeed is stromelysin (Collier et al, 1988). Quite how progelatinase is activated remains unclear, but the process may be autocatalytic involving self-degradation to the active species (Hipps et al, 1991).

A requirement for additional proteinases, such as plasmin, allows for another level of control to be placed on the degradative activity of the metalloproteinases.

### 1.4.4 *Inhibition of matrix metalloproteinase activity*

The activity of matrix metalloproteinase enzymes may be further controlled by both non-specific and specific inhibitors. Of the non-specific inhibitors $\alpha_2$-macroglobulin is the most important and in human plasma accounts for over 95% of metalloproteinase inhibitory activity (Cawston and Mercer, 1986). Although $\alpha_2$-macroglobulin may have a role in limiting metalloproteinase activity its large size ($M_r$ 725,000) is likely to confine its action to in and around the vasculature (Cawston, 1986). There is therefore a requirement for smaller molecules to control metalloproteinase activity within the tissue stroma, and the existence of specific matrix metalloproteinase inhibitors, tissue inhibitors of metalloproteinases (TIMP) provide an appropriate system.

The TIMP family consists of at least two members, TIMP-1 and TIMP-2 (Ward et al, 1991).

TIMP-1 is a glycoprotein with a molecular weight of approximately 28 kDa. It was originally purified from rabbit bones in tissue culture (Cawston et al, 1981) and has since

been identified in a variety of human fluids including plasma, amniotic fluid and saliva (Clark et al, 1991). TIMPs act specifically against the matrix metalloproteinases by combining with them on a one to one basis to form irreversible complexes (Cawston, 1986).

TIMP-1 appears to be synthesized constitutively by most connective tissue cells, and its presence within the extracellular matrix may provide a final fail safe mechanism for the control of matrix metalloproteinase activity (Murphy and Reynolds, 1985). Net matrix proteolysis is only likely to occur when the level of activated enzyme exceeds that of the inhibitor. Thus chondrocytes stimulated to produce collagenase fail to degrade collagen films unless the action of TIMP is blocked by the addition of an anti-TIMP antibody (Gavrilovic et al, 1987). Endothelial cells are known to synthesize high levels of TIMP-1 and this may help to explain why these cells, despite also producing high concentrations of collagenase and stromelysin, show little evidence of their proteinase activity (Scott Herron et al, 1986). As well as combining with the active enzyme TIMP-1 has also been shown to form a complex with the latent pro form of $M_r$ 95,000 gelatinase (Wilhelm et al, 1989). The in vivo function of this complex is uncertain, but it may provide a way in which the inhibitor can be sequestered in the tissues. When required TIMP-1 may dissociate from the relatively weaker TIMP-1 progelatinase complex and combine with the higher affinity binding sites of the active enzymes (Ward et al, 1991).

TIMP-2 has been isolated from, among others, human melanoma cells (Stetler-Stevenson et al, 1989) and mouse colon 26 tumour cells (Kishi et al, 1991). Unlike TIMP-1 it is not glycosylated, a fact reflected in its lower molecular weight of 21 kDa. However, it does share considerable similarity to TIMP-1 at the amino acid sequence level (Stetler-Stevenson et al, 1990). TIMP-2 is able to form a complex with both the latent pro form of $M_r$ 72,000 gelatinase and the active enzyme. There is evidence to

suggest that the relative levels of TIMP-2 to $M_r$ 72,000 gelatinase are important in determining the level of $M_r$ 72,000 gelatinase proteolytic activity (Albini et al, 1991).

Recently a further metalloproteinase inhibitor has been identified with an apparent molecular weight of 76 kDa. In view of its larger size compared to TIMP it is referred to as large inhibitor of metalloproteinases, or LIMP, and has been shown to inhibit collagenase, stromelysin and gelatinase (Cawston et al, 1990).

### 1.4.5 *Connective tissue degradation by matrix metalloproteinases*

The action of the matrix metalloproteinases on collagen is perhaps best illustrated by the degradation of type I collagen by interstitial collagenase. The triple helix structure of tropocollagen is cleaved by interstitial collagenase at a locus three quarters of the way between the amino and carboxy termini of the molecule. This occurs at a Gly-Ile bond in the $\alpha_1$(I) chains and at a Gly-Leu bond in the $\alpha_2$(I) chain. The resultant degradation products are designated ¾($TC_A$) and ¼($TC_B$) (Harris and Krane, 1974). These fragments are unstable and spontaneously unravel to allow further degradation by gelatinase and other proteases. However, although interstitial collagenase can degrade individual tropocollagen molecules it is less able to do so when these are combined to form intact collagen fibrils. This has led to the theory that for interstitial collagenase to act *in vivo* other non-specific proteases, such as cysteine and serine proteases, may be required (Harris and Krane, 1974). These proteases would act to break the cross-links which exist between the telopeptide, or non-helical regions, of the tropocollagen molecules. Once released the collagen molecules would then be open to attack by interstitial collagenase (Gross, 1981).

As well as extracellular degradation, collagen fibrils may also be phagocytosed and digested by lysosomal enzymes such as the cathepsins (Pauli and Knudson, 1988).

Types IV and V collagen, which are present in basement membranes, can also be denatured by two mechanisms, one specific and involving gelatinase (type IV collagenase) (Murphy et al, 1985), and the other less specific and due to the action of proteases with a broader substrate specificity such as plasmin and the cathepsins (Crouch et al, 1980). *In vivo* there may be a requirement for both if the insoluble basement membrane is to be successfully degraded. Gelatinase lyses type IV collagen to produce fragments resembling the 3/4 - 1/4 cleavage products produced by interstitial collagenase (Murphy et al, 1989b), while plasmin is likely to attack the non-helical regions of the molecule. The cross-linked regions of type IV collagen are also susceptible to stromelysin, although this enzyme appears generally less potent against this substrate than gelatinase (Murphy et al, 1991).

In addition to its action against type IV collagen stromelysin can also degrade another component of basement membranes, proteoglycans, cleaving the central protein core of these molecules at several sites along their length (Murphy et al, 1990).

$M_r$ 72,000 gelatinase, $M_r$ 95,000 gelatinase and stromelysin all show some activity against elastin although in the case of stromelysin this is slight (Murphy et al, 1991).

The ability of the matrix metalloproteinases to degrade most of the components of connective tissue make them ideal enzymes to mediate the process of tumour invasion. The evidence to suggest that they do so has been derived indirectly from histological and ultrastructural studies of tumour invasion, in vitro studies of tumour cell invasion across artificial basement membranes, the biochemical assay of enzyme activity in tumour explants, and tissue

immunolocalization and *in situ* hybridization studies of the enzymes concerned. The evidence from each of these sources will be considered in turn with particular reference, where possible, to colorectal cancer.

### 1.4.6 *Histological studies of tumour invasion*

Although histological studies cannot provide direct evidence for the involvement of enzymes in tumour invasion they nevertheless demonstrate changes which might be attributable to their action. Perhaps because the basement membranes represent the first of the connective tissue barriers to be breached, considerable interest has been paid to the alterations which occur in them during tissue invasion. Studies based on the immunostaining of basement membrane have consistently revealed changes in its structure in invasive tumours. In breast cancer and cervical cancer true carcinomas in situ can be distinguished from their microinvasive counterparts by failing to demonstrate the irregularities in, and fragmentation of the basement membrane that accompany the latter condition (Siegal et al, 1981; Yavner et al, 1990).

Using antisera to type IV collagen, laminin and heparan sulphate proteoglycan, Burtin et al (1982) demonstrated that the basement membrane antigens of colonic adenocarcinomas were irregular and fragmented and the staining often weaker than that seen in normal colonic mucosa. These changes also appeared to be enhanced as the degree of tumour differentiation diminished. A further study of tumour deposits in the lymph nodes draining colorectal cancers also revealed defects in their associated basement membranes (Burtin et al, 1983). These results were extended by Forster et al (1984) who investigated the relationship between basement membrane staining and tumour grade, stage and metastasis formation for rectal adenocarcinomas. An antiserum to laminin was used and linear staining, presumed to reside in the basement membrane, was observed in the

majority of well and moderately differentiated carcinomas (24/35). By contrast only 3 of the 15 poorly differentiated tumours exhibited a similar pattern of staining. There was also a highly significant correlation between basement membrane staining and the likelihood of developing distant metastases; when laminin was present in relation to tumour cells only 30% of patients developed metastases compared to 80% when it was absent. Additionally, an increased five year survival was noted amongst patients whose tumours had laminin staining. In a similar study by Offerhaus et al (1990) the degree of expression of type IV collagen in colorectal adenocarcinomas, when analyzed as a single variable, was significantly related to a better prognosis. Although the significance of this observation was abolished on multi-variate analysis there was still a trend towards a better prognosis (P=0.12). Finally, Hewitt et al (1991) found that breaks in the epithelial basement membranes of colonic carcinomas were almost invariably more prominent at the periphery, or invading edge, of the tumour.

Defects in basement membranes have also been demonstrated at the electron microscope level. In basal cell carcinomas the basement membranes delimiting islands of tumour cells have been shown to be either absent, or degenerate and ill defined (Hashimoto et al, 1972). In the same study normal collagen fibrils were also noted to be absent in areas adjacent to the tumour cells, a finding considered to support the role of collagenase in the invasive process.

It is possible that the defects observed in the basement membranes of invasive tumours are the result of alterations in the synthesis and secretion of their component parts (Daneker et al, 1987). However, in many of the studies the tumours appeared to retain the ability to synthesize essential molecules such as laminin and type IV collagen (Siegal et al, 1981; Burtin et al, 1982). The changes in basement membrane integrity must therefore

represent either an inability to assemble the component molecules, or degradation by enzymes.

### *1.4.7 In vitro and in vivo studies of the involvement of matrix metalloproteinases in tumour cell invasion.*

Liotta (1986) has proposed a three-step theory to explain how the biochemical events leading to basement membrane degradation might take place. The first step requires that the tumour cell anchors itself to components of the matrix. This would be mediated by cell surface receptors able to recognize the laminin component of basement membranes or perhaps the fibronectin content of the interstitial stroma. Once anchored, the tumour cell either secretes proteolytic enzymes, or induces host cells to do so, which lyse the extracellular matrix in the immediate vicinity of the cell. With the matrix degraded the tumour cell then detaches itself and migrates forward (Liotta, 1986).

Support for this theory has been derived from studies on the role of laminin as an attachment factor and the part played by matrix metalloproteinase enzymes in the degradation of basement membranes and the connective tissues.

Laminin binding receptors have been identified in the cell membranes of a human colon adenocarcinoma cell line (Stallmach et al, 1990) and the levels of mRNA for these receptors as well as the protein product are found to be elevated in colonic adenocarcinomas compared to those of the adjacent normal mucosa (Mafune et al, 1990). Furthermore, cells selected for their ability to attach to type IV collagen in the presence of laminin produce more metastases than unattached cells, an effect which can be attenuated by prior incubation of the cells with an antibody to laminin (Terranova et al, 1982). I*n vitro* studies have also shown that the addition of laminin to the culture supernatants of a human melanoma cell line can produce a significant

increase in the release of type IV collagenase activity; a similar increase was not observed with fibronectin and again the effect of laminin could be blocked with an antibody directed against the human laminin receptor (Turpeenniemi-Hujanen et al, 1986). The Ha-ras oncogene, which has been implicated in the induction of stromelysin and gelatinase expression (Matrisian, 1985), has also been postulated to increase the invasiveness of cells by boosting the number of laminin receptors (Albini et al, 1986). However, although the above evidence suggests that an important component of the invasive phenotype involves the increased expression of the laminin receptor, a study of human papillomavirus associated cervical neoplasms indicates that expression of this receptor may be related to the proliferative rather than the invasive properties of tumour cells (Demeter et al, 1992).

As well as studies on the role of laminin in tumour invasion many investigators have developed models to examine the influence of matrix metalloproteinase expression and their inhibitor TIMP on tumour cell invasion and metastasis formation.

Liotta et al (1980) have shown that the metastatic capacity of various tumour cell lines *in vivo*, as measured by their ability to produce lung colonies following tail vein injection into mice, can be correlated with their ability to secrete an enzyme *in vitro* which specifically degrades type IV collagen. Subsequent studies, using artificial basement membranes, confirmed that tumour cells capable of penetrating such barriers showed not only an increase in their laminin binding capacities but also an enhanced ability to degrade type IV collagen compared to their non-invasive counterparts (Terranova et al, 1986).

Similar systems have been used to investigate the effect of specific inhibitors on the invasiveness of cells. A type IV collagenase inhibitor (SC44463), designed to mimic the cleavage site on the collagen molecule, can reduce the ability of malignant cells to invade across reconstituted

basement membranes (Reich et al, 1988). When given intraperitoneally SC44463 also reduces the number of lung nodules forming when melanoma cells are injected intravenously into mice (Reich et al, 1988). Mignatti et al (1986) used the invasion of cells across human amniotic membrane to confirm a requirement for the plasminogen activator urokinase in the invasive process. While tissue invasion was blocked by anti-urokinase antibodies the effect could be reversed by using a mercurial compound (Mersalyl) to activate type IV procollagenase. This implied that the plasminogen activator system was important in activating type IV collagenase. Monoclonal antibodies raised against type IV collagenase have also been used to decrease the penetration of melanoma cells across reconstituted basement membranes (Hoyhtya et al, 1990).

The participation of matrix metalloproteinases in tumour cell invasion can also be demonstrated through the use of specific inhibitors of these enzymes, principally tissue inhibitors of metalloproteinases (TIMP). By altering the balance between inhibitor and enzyme both TIMP-1 and TIMP-2 have been shown to block the invasion of tumour cells across either human amnion or reconstituted basement membranes (Schultz et al, 1988; Albini et al, 1991). TIMP can also modify the invasive behaviour of malignant cells in vivo. When administered intraperitoneally recombinant TIMP (rTIMP) reduced lung colonization by murine B16-F10 melanoma cells by 50% and by c-Ha-ras1 transfected cells (4R) by 83% (Schultz et al, 1988; Alvarez et al, 1990). Interestingly, rTIMP had no effect on the size of the B16-F10 lung tumours which did form, nor on the growth of 4R cells in culture, suggesting that the effect was one of inhibiting tumour invasion rather than tumour growth. Also notable was the necessity to administer rTIMP after the tumour cells had been injected. Neither the treatment of tumour cells with rTIMP before their injection nor the prior administration of rTIMP reduced the number of lung colonies forming. Recombinant TIMP is therefore unlikely to interfere with

earlier steps in the metastatic cascade such as those involving cell adhesion.

In addition to the use of exogenously applied TIMP the invasiveness of tumour cell lines can be altered through the techniques of molecular biology. Noninvasive Swiss 3T3 cells have been altered genetically to become metastatic in athymic mice. This was achieved by transfecting them with a plasmid which caused them to form an RNA complementary to the mRNA for TIMP, effectively down-modulating them for TIMP expression (Khokha et al, 1989). Examination of the mRNA levels for TIMP also reveals a correlation between the pattern of TIMP expression and the different metastatic phenotypes derived from a mouse mammary adenocarcinoma cell line; levels of TIMP mRNA were found to be lower in metastatic cell lines compared to nonmetastatic ones (Ponton et al, 1991).

### 1.4.8 *Assays of matrix metalloproteinase activity in colorectal tumours*

The studies described in the last two sections have shown that histological lesions compatible with the action of metalloproteinase enzymes exist within tumours and that such enzymes appear at least in vitro to be important mediators of the invasive process. Alongside these experiments investigators have attempted to assay matrix metalloproteinase activity, particularly that of interstitial collagenase, in a variety of malignant tumours. This has been done with the intention of not only confirming that these enzymes are synthesized by human cancers but also with the hope of relating the findings to the biological behaviour of the tumour.

There have to the author's knowledge been six studies which have attempted to assay the collagenolytic activity of colorectal tumours. The first by Dresden et al (1972) studied biopsies from 140 human tumours of which 18 were colonic adenocarcinomas. Two methods were used to determine

collagenolytic activity. In the first, based on the method of Gross and Lapiere (1962), tissue fragments were cultured on reconstituted fibrillar collagen gels; at the end of four days a specimen was scored as positive if at least one half of the explant had produced a clear zone of collagenolysis. For the second method collagenolytic activity was measured according to the reduction in viscosity produced when a solution of acid extracted collagen was treated with the medium in which the tumour biopsies had been cultured. Twelve of the 18 colonic tumours examined were regarded as positive for collagenase production and six negative. However, no control group was used for comparison and no quantitative data given. There is also no indication in the paper as to whether or not similar results were obtained with the two methods used.

This study was followed by that of Sturzaker and Hawley in 1975. Again collagen gels were employed, only this time quantitative data was obtained by measuring the area of lysis. Gross and Lapiere (1962) had previously shown that the amount of collagenase produced was proportional to the area of collagen degradation. Thirty seven colorectal tumours were examined along with 10 biopsies from cases of familial polyposis coli (without malignancy). Eight control tissues were also available, obtained from patients undergoing colectomy for non-malignant and non-inflammatory conditions. Of the tumours examined 18 were positive for collagenolytic activity and 19 negative. In the positive cases, though, the mean collagenolytic activity was approximately half that observed in the control biopsies. Only three of the familial polyposis coli biopsies were positive but again the mean level of activity was less than the controls. Interestingly, in the same study tissue from patients with inflammatory bowel disease was found to have a greatly increased level of collagenase activity. The conclusion from these results was that far from being increased, collagenase activity was in fact reduced in colorectal cancer.

Tighe et al (1981), also using an assay based on the lysis of collagen gels, studied the differences in collagenase activity between biopsies taken from neoplastic and normal colonic tissue. Sixteen tumours with samples of adjacent normal mucosa were examined. By comparison with normal mucosa, in eleven of the tumours the level of collagenase activity was elevated, in four it was equal and in one instance it was less. The overall activity of collagenase in colorectal tumours was found to be significantly greater than normal colonic tissue.

The study by Durdy et al in 1985 was the first to relate the collagenase activity in tumours to pathological variables such as Dukes' staging, degree of local spread and venous invasion. Fifty rectosigmoid cancers, along with biopsies of normal mucosa taken as far distant from the tumour as possible, were examined. The assay used measured the ability of the homogenized biopsies to degrade synthetic collagen substrates. Median values for collagenase activity were found to be significantly elevated in malignant as compared to normal tissue. There was, however, no clear relationship between the level of activity and Dukes' stage, the differentiation of the tumour, the degree of local spread, or the presence or absence of venous invasion.

Irimura et al (1987) as well as examining colorectal tumours for their ability to lyse type I collagen also looked at their capacity to degrade type IV collagen. The methods of assay were, however, different from those used by Durdy et al (1985), and involved analysis of both detergent extracts from the tissues and an examination of the media in which the biopsies had been cultured. The collagenolytic activity present in samples from both these sources was assessed according to their ability to release $^{3}H$-labelled collagen from type I and type IV collagen gels. The results obtained from the detergent extracts revealed no significant difference between tumour and normal mucosa, in either their type I or their type IV collagenolytic activities. It is, however, of interest to note that whereas some type IV

collagenolytic activity was apparent in all of the 24 tumours examined it was undetectable in 6 of the corresponding normal mucosa specimens. When the collagenolytic activity of tumour conditioned media was examined significantly higher levels of collagenolysis were present compared to those in media obtained from the culture of normal mucosa. No correlation was found between collagenolytic activity and the stage of the tumours. There was also no difference in the collagenolytic activity of biopsies taken from the deep or superficial portions of the tumours nor between primary tumours and their metastases.

van der Stappen et al (1990) examined the relationship between the grade of histological differentiation and collagenolytic activity against soluble and fibrillar type I collagen and fibrillar type III collagen. Collagenolytic activity was determined by the release of radioactivity from $^{14}C$-labelled collagens. The results obtained were expressed as the ratio of the collagenolytic activity in the tumour biopsy to that in its complementary control of normal colonic mucosa. This ratio was expressed in terms of the wet weight of the tissue examined, or per μg of DNA or protein present. Biopsies from 55 malignant colorectal tumours were taken along with control samples of normal mucosa from the resection edge of the colectomy specimens. Collagenolytic activity was found to be significantly higher in the tumour biopsies compared to the control samples when the results were expressed according to the wet weight of the tissue examined. However, this significance disappeared when values were recalculated according to the amount of protein or DNA present. With the exception of the results obtained from the fibrillar type I assay and recorded on a wet weight basis, significant correlations were present between the histological differentiation of the tumour and the tumour to control ratio for collagenolytic activity. In other words tumour collagenolytic activity appeared to rise with respect to normal mucosa as the tumours became less differentiated. When collagenolytic activity and stage were compared a

significant correlation was present only when the results were expressed on a DNA basis and fibrillar substrates had been used; with these criteria the more advanced the Dukes' stage of the tumour the greater the collagenolytic activity.

The above studies provide a contrasting picture of collagenolytic enzymes in colorectal cancer with some reporting an increase and others a decrease in their activity. These conflicting results reflect the biology of the matrix metalloproteinase enzymes and the difficulties present in assessing their action under *in vitro* conditions. Different results are likely depending on whether the collagen substrates used are soluble, or have been reconstituted into fibrils. The action of collagenase on both forms may be relevant *in vivo* since, as mentioned previously, fibrillar collagen may undergo initial degradation by non-specific proteases such as plasmin. Indeed in the study by van der Stappen (1990) intact collagen molecules, likely to have been released by the action of peptidases with a broad substrate specificity, were detected by SDS-PAGE in both extracts of tumour and normal mucosa. Whether or not collagenolytic activity is detected may also be influenced by the relative amounts of latent and active enzyme present in the biopsy as well as the prevailing levels of inhibitors such as TIMP and $\alpha_2$-macroglobulin. The requirement for several days of culture before collagenase was detectable may represent both the time taken for sufficient activation of the latent enzyme to take place and for any inhibitors in the tissue, or the culture medium if serum was used, to be saturated (Harris and Vater, 1980). It is also possible that when collagenase is detected in culture medium it simply represents overspill of enzyme which is unbound either to collagen or TIMP (Fell et al, 1986).

In contrast to the metalloproteinase enzymes there has to the authors knowledge been only one attempt to measure

the level of TIMP in colorectal tumours, that of Lu et al (1991), who found levels to be elevated over those prevailing in the surrounding normal mucosa.

Although the above studies have provided data to support the first two stages of the three stage theory of tumour invasion and for the involvement of the matrix metalloproteinases, it must be remembered that much of the evidence has been derived *in vitro*. Experiments involving reconstituted basement membranes, malignant cell lines in culture or athymic mice need not necessarily reflect the *in vivo* position. Similarly the assay of metalloproteinase activity through the culture of tumour explants can only be expected to show that the cells involved have the potential to produce such enzymes.

To obtain more information about their *in vivo* expression investigators have used the techniques of immunolocalization or in situ hybridization. Both of these methods have been applied to the study of matrix metalloproteinase enzymes in colorectal cancer (Hewitt et al, 1991; Levy et al, 1991; Poulsom et al, 1992) and a detailed assessment of the results is presented in Chapter 2.

## 1.5 CIRCULATING TUMOUR CELLS

The recognition by Thiersch in 1865 that metastasis was an embolic phenomenon secondary to venous invasion opened a new chapter in the investigation of tumour behaviour. However, despite the undoubted importance of the vasculature as a conduit for the dissemination of malignant disease relatively little attention has been focused on the circulating tumour cells themselves. Nearly one hundred and thirty years later many outstanding questions remain. By what mechanism(s) do tumour cells gain access to the blood stream and what factors influence the process? Does vascular invasion automatically imply the presence of circulating cells? In what numbers do tumour cells enter the circulation and do they bear a relation to the likelihood of metastasis? Finally, what factors determine the subsequent fate of circulating tumour cells?

### 1.5.1 *Angiogenesis and tumour vascularity*

In order that neoplastic cells might disseminate they must gain access to the circulation, and for this to happen two inextricably linked events must occur: the tumour must grow and a functional microcirculation must be induced from the surrounding host tissues (Folkman, 1985). Provided a solid tumour does not grow beyond a millimetre or so in diameter its supply of oxygen and nutrients can be maintained by simple diffusion alone. This avascular phase is typified by *in situ* carcinomas, which are associated with limited tumour growth and an absence of metastases. With the induction of neovascularization *in situ* carcinomas become invasive. They acquire the ability to breach the basement membrane and with this the potential to metastasize (Folkman,1976). A paradigm for this sequence of events is seen in the behaviour of malignant melanoma. Provided the vertical growth of a malignant melanoma does not exceed 0.76mm surgical excision carries an excellent prognosis (Breslow, 1970); with thicker

lesions the prognosis worsens. It has been demonstrated that up to a level of about 0.75mm the basement membrane remains intact and the melanoma avascular (Kirkham, 1987; Srivastava et al, 1986). At greater levels of penetration the basement membrane is breached, either by the tumour itself or by new capillary vessels and thereafter, the melanoma acquires the potential for rapid growth and dissemination (Folkman, 1987).

The mechanisms regulating tumour angiogenesis are not precisely understood. It seems likely that neovascularization is the result of a favourable balance between angiogenic polypeptides, angiogenesis inhibitors, proteolytic enzymes and the matrix proteins of the tumour stroma rather than a single angiogenesis factor (Mahadevan and Hart, 1991). This multiplicity of requirements may explain the heterogeneity observed in the vascular composition of individual tumours which in turn might influence their metastatic potential (Weidner et al, 1991).

Changes in the vasculature of a tumour particularly at its periphery have been noted by a number of investigators. Goldmann (1907), based on an X-ray study of vessels rendered radio-opaque by injections of bismuth and oil, observed the vascular architecture of tumours. He drew three principal conclusions; i) that the normal distribution of blood vessels was disturbed by the invading growth, ii) that the formation of new blood vessels was most apparent at the periphery of infiltrating tumours and iii) that as the volume of a tumour increased its centre became necrotic and newly formed vessels merely occupied the periphery. The increased density of vessels at the edge of malignant tumours compared to their benign counterparts was also observed by Thiessen (1936) in his study of gastric carcinomas. Another striking example of the differences between the microvasculature of metastatic and non-metastatic tumours was provided by Urbach and Graham's (1962) study of the capillaries supplying a variety of skin tumours. Dilated, elongated and tortuous vessels were common

to basal cell carcinomas, squamous cell carcinomas and malignant melanomas. However, whereas for the non-metastatic basal cell tumours the capillaries only surrounded clusters of tumour cells, in squamous cell carcinomas and melanomas they were interweaved with the cancer cells themselves. Lindgren (1945) in a series of observations on the vasculature of human tumours noted, although not invariably, that the extent of the capillary network within a tumour was also dependent on the nature of the surrounding stroma, being greater when this was loose and rich in cells rather than fibrous and relatively acellular. Additionally, the degree to which the capillary angioarchitecture of tumours deviated from that of the parent normal tissue was related to the cytological differentiation of the tumour; differences were most marked in poorly differentiated tumours.

The predominance of vessels at the tumour margin is in keeping with observations which suggest that neovascularization must precede tumour growth and invasion (Folkman, 1990). However, whether or not vascular invasion takes place will also depend on the penetrability of the vessels encountered. The resistance of arteries to tumour invasion is well known, and was recognized by Virchow who considered them to act as "isolators of pathological processes" (cited by Goldmann, 1906). Were tumours to invade arteries then malignant cells would be carried into the circulation peripheral to the point of penetration. Pelvic tumours, for example, invading the iliac arteries would be expected to produce metastases throughout the lower limb. The fact that this and similar situations are not observed clinically testifies to the immunity of arteries, and indeed arterioles, to neoplastic invasion (Willis, 1952); why this should be so is not clear. One possible explanation is that arteries are protected by their higher intraluminal pressure (Shivas and Finlayson, 1965). Warren (1979) provides a comprehensive listing of the other types of blood vessel which may be found in tumours. The varieties include

capillaries with basement membranes in which there are well formed cell-junctions between the endothelial cells; giant capillaries, found at the edge of tumours and referred to as forming a "venous capsule", with walls composed only of endothelial cells; venules and veins often tortuous and ectatic; and also arterio-venous anastomoses. Warren (1979) suggests that those vessels, such as the "giant capillaries", lacking a basement membrane may be the principle location for the migration of tumour cells into the circulation. Post-capillary venules may also be at risk of invasion since the junctions between endothelial cells are less well organized compared to their capillary counterparts (Simionescu et al, 1975).

The mechanisms by which tumour cells invade blood vessels are not clear. The deployment of proteolytic enzymes to lyse the basement membrane scaffold on which endothelial cells rest has already been discussed. However, their action may not always be necessary, particularly if the vessels most likely to be invaded have no basement membrane. Active cell movement could be of more importance. DeBruyn and Cho (1982) in a scanning electron microscope study of vascular invasion in murine tumours, found that cancer cells could intravasate through intact endothelial cells, particularly those lining tumour rather than host blood vessels. The suggested mechanism was the penetration of endothelial cells by way of migration pores, which sealed once the tumour cell had reached the lumen of the vessel. Should this observation also hold for human tumours, then the invasion of blood vessels could occur without subsequent visible evidence of it. A role for active migration in the invasion of colonic tumours is also supported by ultrastructural studies which show evidence of carcinoma cells extending pseudopodia-like cytoplasmic processes through the underlying basement membrane (Carr et al, 1986). Additionally, tumour cells may be seen insinuating themselves into narrow host tissue spaces including gaps in vessel walls, in a process analogous to the diapedesis of leucocytes (Gabbert, 1985).

In summary the evidence suggests that intravasation is most likely to occur at the tumour periphery, where new vessel formation is most pronounced and the vessels more susceptible to penetration.

### 1.5.2 *Vascular invasion in colorectal cancer*

The foregoing suggests that for haematogenous spread to occur a tumour must first develop an organized vascular network. Observation of the T241 fibrosarcoma indicates that the day after such a network is established tumour cells are detectable in the venous effluent (Liotta et al, 1974). It would appear that from an early stage in their natural history colorectal cancers can also shed cells into the circulation. Engell (1955) found 41% (11/27) of Dukes' stage A tumours to be associated with circulating tumour cells (including "atypical tumour-like cells"); and in an immunocytochemical study of bone marrow aspirates, tumour cells were detected in 16% of Dukes' A cases (Lindemann et al, 1991). These observations indicate that the invasion of extramural veins may not be necessary for the dissemination of potentially significant numbers of tumour cells.

If it is assumed that hepatic metastases from colorectal carcinomas arise as a result of the embolization of tumour cells into the portal circulation, then clearly the tumour must invade tributaries of this system. The importance of this was recognized by Willis (1932) and since then several studies have dealt with the incidence of venous invasion occurring in colorectal cancer. The reported figures vary widely, being influenced by both the histological techniques, for example the use of elastic tissue stains to distinguish blood from lymphatic vessels (Minsky et al 1988), and whether or not intramural or extramural venous invasion is being recorded (Talbot et al, 1980). Various studies have also used different definitions of blood vessel invasion. Thus, Minsky et al (1988) defined it as tumour present within a round or ovoid endothelial-

lined channel with a smooth muscle wall; while Krasna (1988) defined blood vessel invasion as having one or more of the following criteria: tumour cells lining the venous endothelial surface, tumour cell thrombi inside the lumen of the vein, or destruction of the vein wall by tumour cells. However, as noted above, those vessels most vulnerable to invasion need not have smooth muscle or elastic tissue components to their walls, and mechanisms exist whereby tumour cells can intravasate without leaving an imprint of structural damage on the vessel.

The highest incidence of venous invasion is that recorded by Brown and Warren (1938). Based on a study of the autopsy records of patients dying from rectal carcinoma, they reported the frequency of venous invasion to be 61%. However, Dukes and Bussey (1958) on examining operative specimens from 1800 cases of rectal cancer found the incidence of venous spread to be much lower at 11%. Subsequent studies have indicated intermediate figures; Talbot et al (1980) on an assessment of 703 surgical specimens record invasion of the veins by the primary growth in approximately 52%, while an examination of 709 colorectal tumours by Chapuis et al (1985) showed venous invasion to be present in only 19%.

There is general agreement that the incidence of blood vessel invasion rises as tumour differentiation deteriorates and the depth of infiltration of the bowel wall increases (Minsky and Mies, 1989). However, the frequency with which intramural, extramural or both types of vessel are invaded, appears to vary with the depth of tumour invasion. Minsky et al (1988) found the incidence of intramural vessel invasion to diminish with increasing tumour penetration, while that of extramural vessel invasion increased; the overall incidence of intramural vessel invasion was 61% compared to 23% for extramural. These figures are almost the reverse of those reported by Talbot et al (1980); when vascular invasion was present intramural vessel invasion accounted for 31% of cases and the extramural type for 69%. The

invasion of extramural blood vessels is particularly associated with the formation of hepatic metastases (Talbot et al, 1980; Minsky et al, 1988; Shirouzu et al, 1991).

A recent prospective study of venous invasion in colorectal cancer has indicated that the degree of venous invasion may also influence tumour spread (Shirouzu et al, 1991). The degree of venous invasion was expressed as the number of confirmed sites of venous invasion divided by the number of sections examined, i.e. the average number of sites of venous invasion per section. Using this method these authors were also able to show that as the frequency of venous invasion rose so too did the incidence of metachronous liver metastasis. When the average number of confirmed sites of venous invasion per section of the tumour examined (and the whole tumour was sectioned) was 3 or less, only 4.2% (15 of 354) patients developed hepatic metastases; with an average of greater than three sites per section the incidence of hepatic metastases rose to 29% (21 of 72 patients). For both Dukes' stage B and C tumours, survival within a particular stage was also related to the degree of venous invasion. Six-year survival decreased from 76.9% to 44.2% for Dukes' stage C tumours and from 88.2% to 74.1% for Dukes' B tumours, as the degree of venous invasion increased from slight to marked. These figures also provide some support for an observation made earlier by Krasna et al (1988) that the survival of patients with Dukes' B tumours showing vascular invasion is less than that of Dukes' C tumours without vascular invasion.

The relationship between venous invasion, circulating tumour cells and the development of hepatic metastases is not a straightforward one; hepatic metastases may occur without demonstrable venous invasion, while penetration of the vasculature need not necessarily imply distant spread. Although Brown and Warren (1938) found that hepatic secondaries were invariably associated with intravascular invasion, Talbot et al (1980) established that 14.2% of tumours without evidence of venous invasion also developed

liver metastases; and Minsky et al (1988) showed that for both $B_2$ and $C_2$ tumours (modified Astler-Coller staging system), liver metastases as well as dissemination to other distant sites could arise in the absence of detectable vascular invasion. Similarly, circulating cancer cells have been detected in patients with colorectal cancer whose tumours have no associated histological evidence of vascular permeation. In the study by Fisher and Turnbull (1955) two of eight tumours showed no evidence of venous invasion despite the presence of circulating cells in the mesenteric blood. Griffiths et al (1973) also found circulating tumour cells in 13 of 16 tumours without venous invasion. Moore and Sako (1959), however, record all 7 of their tumours, associated with blood borne tumour cells, as showing venous invasion, (although in 2 of the 7 instances venous invasion was described as "questionable"); no tumour cells were found in 5 carcinomas without evidence of venous invasion. Even the infiltration of extramural veins, regarded as particularly likely to result in haematogenous dissemination (Talbot et al, 1980) only gave rise to hepatic metastases in between 20% and 40.2% of cases (Shirouzu et al, 1991; Talbot et al, 1980).

Some of the above "inconsistencies" relating vascular invasion to the incidence of circulating tumour cells and hepatic metastases are doubtless due to difficulties in sampling tumour specimens adequately, and in reliably identifying invasion particularly when capillaries and venules are involved. It is also possible that patients recorded as having no overt evidence of liver metastases actually harbour microscopic foci of tumour (Finlay and McArdle, 1986). The remaining inconsistencies, however, are likely to reflect the biology of the intravasation process itself and the subsequent fate of tumour cells entering the circulation. In this respect the inefficiency of the metastatic process may help to explain why divergent views exist as to whether or not venous invasion can be held as an independent prognostic variable in colorectal cancer;

Chapuis et al (1985) consider that it is, while Jass et al (1987), Shepherd et al (1989) and Minsky et al (1988), do not.

It is also possible that other histological features may need to be examined when considering vascular invasion in colorectal tumours.

Amongst other variables, the number of cells entering the circulation may depend on two anatomical features: i) the density of vessels within the tumour, including those formed by angiogenesis and those enveloped by tumour invasion, and ii) the diameter of the vessels. Liotta et al (1976) found that the proportion of tumour cell clumps, of a certain size, entering the circulation was linearly related to the number of vessels within the tumour larger than the size of the clump. The efficiency with which tumour cell clumps could produce metastases was also shown to be related to the number of cells within them. It follows that some cancers, with a vascular architecture containing a larger proportion of vessels whose diameter is appropriate to the passage of tumour cells or tumour cell clumps, might shed cells into the circulation more readily than those that do not. By allowing more cells into the circulation, these tumours could overcome the inherent inefficiency of the metastatic process and form metastases more easily. The observation that the incidence of distant metastases in rectal cancer is particularly high when thick walled extramural veins have been invaded (Talbot et al, 1981) may represent one extreme of this relationship.

Some support for the importance of vascular architecture in the metastasis of human tumours is provided by studies on malignant melanoma and breast cancer. The maximum diameter of vessels in metastatic malignant melanomas (49.5 μm) was found to be greater than in non-metastatic tumours (37.2 μm) (although the difference just failed to reach statistical significance, $p = 0.07$) (Srivastava et al, 1988); and in a study of invasive breast carcinomas vessel density, defined as the number of vessels

per 200x microscopic field, was found to be an independent predictor of metastatic disease (Weidner et al, 1991).

Thus, the importance of the tumour vasculature to haematogenous metastasis may not lie solely with the presence or absence of venous invasion but also with the density of the vessels available. With more vessels present the greater is the opportunity for venous invasion to occur. This theme is pursued in Chapter 5.

### 1.5.3 *Tumour manipulation and metastasis*

Although clinical observations had suggested that tumour massage may be an important factor in promoting the metastatic spread of tumours (Knox, 1922) it was not until the studies of Tyzzer (1913) that the experimental basis for this concept was established. Tyzzer was able to show that massage of a transplanted carcinoma in the mouse was associated with a greatly increased incidence of pulmonary metastases. Similar experiments were performed by Knox (1922) who confirmed that tumour massage led to a greater incidence of tumour emboli and metastases in the lungs compared to control cases. More recent studies have indicated that massage only produces an increase in pulmonary metastases in tumours derived from cell lines which are already highly metastatic (Weiss, 1985b).

The influence which operative manipulation exerts on the dissemination of cancer cells from human tumours is uncertain. Most of the information available relates to the detection of circulating tumour cells following handling of the tumour and therefore need not necessarily reflect the subsequent formation of metastases. Jonasson et al (1961) studied the effect of rectal massage on the release of cells from prostatic cancers and observed showers of cancer cells in blood samples from the inferior vena cava in 4 of 11 patients. West et al (1964) in a study of tumour cell embolization occurring before, during and after operation found that blood samples taken during the induction of

anaesthesia were more likely to be positive for cells than those removed at operation. Engell (1955) in his study of tumour cells in patients with colorectal cancer concluded that there was no definite increase in the escape of tumour cells into the circulation following operative manipulation. Turnbull regarded the finding of cancer cells in the mesenteric venous blood draining colorectal tumours as potentially significant for the development of liver metastases (Fisher and Turnbull, 1955). Although unable to prove that the circulating tumour cells arose as a result of operative manipulation he used the observation for his subsequent advocacy of the no-touch isolation technique in colonic resection (Turnbull et al, 1967). The aim of this procedure was to minimise the risk of tumour cell embolization during mobilization of the tumour by first securing the vascular pedicle. Wiggers et al (1988) have since shown that application of this technique has a tendency to reduce the number of metastases subsequently forming after resection. There was also a tendency for patients treated by the no-touch technique to survive longer, although there was no significant difference between them and control cases.

### 1.5.4 *The fate of circulating tumour cells*

Once vascular invasion has taken place a variety of host responses can occur. At the site of penetration inflammatory damage to the wall of the invaded vessel, a thrombus cap covering the tip of the intravenous tumour and an endothelial mantle covering extramural intravenous tumour cells all appear to exert a protective influence on patient survival (Talbot et al, 1981). Changes in the expression of cell adhesion molecules may mediate the actual release of tumour cells into the blood stream (Ruoslahtii et al Giancotti, 1989). There, their fate will depend on the avoidance of random destruction and the ability to complete further steps in the metastatic process.

Undoubtedly the first insult to circulating tumour cells is that of mechanical trauma. Weiss (1990a) has hypothesised that the death of cancer cells occurs in two phases, a rapid one in which cells are killed within 5 minutes or less of their entry into the blood stream, and a subsequent slower phase, taking place over a period of days. Rapid destruction is thought to be the result of shear forces set up by a failure of tumour cells to adapt their shape sufficiently to negotiate the microvasculature of the organ which they first encounter (Weiss, 1990b). Those cells which survive this process may subsequently fall prey to host defence mechanisms, amongst which are cytotoxic natural killer cells. These cells, which are derived from their non-toxic precursors when these are subjected to the influence of growth factors such as interferon and interleukin 2, may be particularly active against metastatic tumour cells (Guillou, 1987; Hanna and Fidler, 1980). However, the cytocidal action of natural killer cells may be thwarted by the ability of tumour cells to form multi-cell emboli, a process likely to protect cells at the centre of such aggregations (Liotta et al, 1974).

The eventual sites of metastasis formation are determined by both haemodynamic factors and site selectivity (Murphy et al, 1988). Following their release into the portal circulation colorectal cancer cells are trapped in the microvasculature of the liver - the organ of first encounter. Animal experiments involving the injection into the portal vein of radiolabelled B16 melanoma cells indicate that the majority of these arrested cells will subsequently be released although few (<1%) will be viable (Weiss et al, 1983). Those that remain may have been influenced by carcinoembryonic antigen (CEA), a cell surface adhesion molecule. CEA may facilitate the arrest of tumour cells in two ways, i) by coating the surfaces of Kupffer cells and hepatocytes during its clearance by the liver (Johnson JP, 1991) and ii) by controlling the functional activity of a collagen receptor expressed by colonic epithelial cells

(Pignatelli et al, 1990). Tabuchi et al (1991) have shown that elevated levels of CEA (> 5 ng/ml) in the venous blood draining the primary tumour, rather than CEA levels in the peripheral blood, provide a better indicator of the likelihood of developing liver metastases. The organ specificity and entrapment of tumour cells may also be dependent on their ability to recognize specific endothelial cell surface adhesion molecules (Streeter et al, 1988). Tumour cell lines selected for their ability to metastasize to a particular organ *in vivo*, can be shown *in vitro* to adhere preferentially to endothelial cells grown on extracellular matrix components from that organ (Pauli and Lee, 1988).

To complete the intravascular phase of the metastatic cascade tumour cells must next breach the endothelium of the vessel and penetrate the underlying basement membrane. It is uncertain how this process is achieved but in the liver the fenestrated nature of the sinusoids may aid extravasation (Carter, 1975). In an animal model involving the Rd/3 anaplastic rat neoplasm Carr et al (1976) have observed tumour cells insinuating themselves between the endothelial cells of liver sinusoids. Also, in vitro experiments indicate that tumour cells have the ability to cause retraction of endothelial cells (Nicolson, 1978); this would expose the basement membrane beneath, to which the tumour cells could then adhere. The capacity of colorectal carcinoma cells to adhere to and invade through basement membranes appears inversely related to their degree of differentiation (Daneker et al, 1989). A particular matrix macromolecule which may be important in mediating this adherence is fibronectin. The synthetic peptide sequence, Gly-Arg-Gly-Asp-Ser, from the cell-binding domain of fibronectin can be used to block tumour cell adhesion and reduce the incidence of metastasis. Mice injected with melanoma cells and the peptide were still alive after 15 months whereas mice injected with melanoma cells alone died within 6 weeks (Humphries, 1988). The glycoprotein laminin

also mediates cell attachment and again a peptide sequence derived from its cell attachment site, tyrosine-isoleucine-glycine-serine-arginine (YIGSR), has been found to inhibit the invasion of tumour cells in vitro (Iwamoto et al, 1987). Proteolytic enzymes such as $M_r$ 72,000 gelatinase would enable the tumour cells to complete the extravasation process by degrading the basement membrane. This would be followed by migration of the tumour cells into the host organ's stroma, a process which may be facilitated by the generation of chemotactic factors derived from the lysis of matrix macromolecules such as collagen and elastin (Zetter, 1990). Within the stroma the tumour cells proliferate to form a micrometastatic colony. Provided these colonies are able to induce new vessel formation and respond to organ specific growth factors they will develop into macroscopic lesions (Price et al, 1988). Once established, metastases in the liver, for example, can then act as generalizing sites disseminating cells into the lung.

The above account can only provide an outline of the many steps involved in establishing a secondary deposit in a distant organ. Failure of blood borne tumour cells to complete any one of these steps will frustrate the process and this has lead to the concept of metastatic inefficiency; the majority of tumour cells released into the circulation do not form metastases (Weiss, 1990a). Studies using animal tumour models have provided quantitative data on the inefficiency of the metastatic process. However, there is little information relating to human tumours where investigators have principally concerned themselves with the detection rather than the quantification of circulating tumour cells.

### 1.5.5 *Circulating cancer cells in human tumours with special reference to colorectal cancer.*

Initial observations on circulating tumour cells were confined to post-mortem material. Ashworth writing in the Australian Medical Journal in 1869 reported on a case in which a man had died with multiple subcutaneous tumours over his thorax, abdomen and left leg. Blood taken from the saphenous vein of the good leg was found to contain cells, ... exactly in shape, size, and appearance like to those of the tumour." This was an important observation and the first to suggest that tumour cells had the capacity to traverse the entire circulation. Subsequent reports (reviewed by Engell, 1955) appeared of tumour cells in the post-mortem blood of patients with myeloma, gastric cancer and bronchogenic carcinoma. It was not until 1934, however, that putative cancer cells were identified in the blood of living subjects. In that year Poole and Dunlop identified large spherical cells with hyperchromatic nuclei in 17 of 40 patients with advanced carcinomas. Although at least some of these cells were likely to have been malignant, Poole and Dunlop refrained from this diagnosis since a similar cell was subsequently discovered in a patient with a benign gastric ulcer. As a result, this investigation was the first to highlight a problem which was to hamper studies of circulating tumour cells until the 1980s, namely the ability reliably to distinguish tumour cells from those of the normal circulating cell population.

The first reported demonstration of free tumour cells in the portal circulation was probably that of Cole et al (1954) who identified them in saline perfusates used to irrigate the vessels of resected colonic tumours. This observation was followed by a number of studies which sought to identify tumour cells not only in the mesenteric blood but also in peripheral blood samples of patients with colorectal cancer.

Fisher and Turnbull (1955) studied 25 cases of colorectal cancer. Rather than withdrawing blood from the relevant vessels these investigators isolated the draining mesenteric vein within the mesentery without prior mobilization of the tumour. After excision of the tumour, the blood trapped in the vein was released and the vein irrigated with saline. Tumour cells were detected in the combined blood and perfusate samples in 8 of the 25 (32%) cases examined. Five of these positive findings came from rectal tumours of which there were 14. Cancer cells were also found in one of the five Dukes' stage A tumours. A discrepancy was also noted between the presence of vascular invasion and the detection of tumour cells in the blood; in 2 cases cells were found in the absence of demonstrable venous invasion and, conversely, the blood was negative in 3 of the 7 cases with venous invasion. There is no mention of the incidence of hepatic metastases in this study and no follow-up details are provided.

The most comprehensive investigation into circulating tumour cells was published by Engell in 1955. One hundred and eight patients with colorectal cancer were studied (76 rectal and 32 colonic) with blood samples being taken from the vein draining the tumour both before and after mobilization; samples were also taken from a peripheral vein during the course of the operation. The analysis of Engell's results are complicated by two factors. Firstly, the reported positive cytological findings include cells labelled either as "definite tumour cells" or as "atypical tumour-like cells". Secondly, only 47 of the 108 patients (43%) had a complete set of blood samples taken before and after manipulation of the tumour.

Excluding the "atypical tumour-like cells", 32 of the 76 patients (42%) with rectal carcinomas and 20 of the 31 patients (65%) with colonic cancers, had tumour cells detected in the mesenteric vein samples. Eight patients had liver metastases at the time of surgery and all but two of these had circulating tumour cells. Of the 72 patients who

had a peripheral blood sample taken only 3 (4%) were positive.

Three years later Roberts et al (1958) conducted the first study of circulating tumour cells in which the technique of density gradient centrifugation was used. The study comprised 100 cancer patients including 26 with colorectal tumours, four of whom were considered incurable. No histopathological details of the tumours are provided. Despite sampling blood from the mesenteric vein only one of these 26 patients (3.8%) is recorded as having tumour cells in the blood.

In 1959 Moore and Sako published their study into the spread of carcinoma of the colon and rectum. None of 40 curable cases of colorectal cancer had positive cells in peripheral blood samples but when blood from the vein draining the tumour was examined 7 of 44 resectable cases had tumour cells detected.

The next significant study relating to the detection of circulating cells during excision of colorectal carcinomas was published by Salsbury et al in 1965. These investigators were interested in the effect which high ligation of the inferior mesenteric vein would have on the presence of malignant cells in common iliac venous blood during resection of sigmoid and rectal carcinomas. As well as sampling blood in the iliac veins they also looked for tumour cells in samples of mesenteric venous blood taken from the specimen after resection had been carried out. Fifty tumours were studied, from which 24 post-resection mesenteric samples were obtained. Of these 24 samples 16 (66%) contained tumour cells. It was demonstrated that early ligation of the inferior mesenteric vein produced an approximately fivefold increase in the incidence of malignant cells detected in the common iliac vein blood samples. The suggestion was that ligation of the inferior mesenteric vessels resulted in the retrograde flow of blood along the marginal veins and thence into the systemic circulation via the middle rectal and common iliac veins.

In an extension of Salsbury's series (1965) Griffiths et al (1973) reported on the five-year follow-up of patients in whom cells had, or had not, been detected in the iliac veins. Patients without tumour cells in the blood showed a tendency towards developing liver metastases, while those with circulating cells in the pelvic veins, appearing only after ligation of the mesenteric vessels, had a tendency to produce bony metastases.

### 1.5.6 *Methods to isolate and detect circulating tumour cells.*

To demonstrate the presence of circulating tumour cells the above studies had to contend with two problems, i) that cancer cells, if present, were likely to form only a small proportion of the total circulating cells, and ii) the cancer cells had to be distinguished form those cells present normally in the circulation.

To combat the first of these problems investigators employed a variety of methods to increase their chances of detecting tumour cells in collected blood samples. Initial studies achieved this by haemolyzing the red cells present. A variety of agents were used, including distilled water (Quensel 1921, quoted by Engell, 1955), acetic acid (Poole and Dorset, 1934) and saponin (Engell, 1955). Although effective in their removal of erythrocytes these agents tended to damage both leucocytes and tumour cells (Engell, 1955), thereby confusing the distinction of the two on cytological grounds. Problems of identification would also have been compounded by degeneration of the cells caused by storage of the samples prior to examination (Engell, 1955). A less traumatic method of removing red blood cells was by the addition of fibrinogen (Sandberg and Moore, 1957). This resulted in rouleaux formation and accelerated their sedimentation under gravity. This technique was subsequently considered to result in a considerable loss of tumour cells and abandoned as a result (Roberts et al, 1961). Other

procedures followed, including the use of streptolysin-O to lyse red blood cells and polymorphonuclear leucocytes (Malmgren et al, 1958; Long et al, 1960); this left a suspension containing lymphocytes and any tumour cells, both of which were considered comparatively resistant to such treatment; however, the dose of streptolysin-O was critical if these cells were not to suffer damage. An ingenious method for the isolation of tumour cells was devised by Kuper et al (1961). Following the lysis of erythrocytes by saponin, carbonyl iron was added to the suspension of cells. The iron was ingested by the polymorphonuclear leucocytes, which were then removed by means of a magnet placed in the container.

The above methods have now been superseded by the use of differential centrifugation in which cells are separated on the basis of their specific densities. The technique was first used to isolate circulating cancer cells by Roberts et al (1958) and is discussed more fully in Chapter 4. In contrast to previous methods, it minimised trauma to the cells.

Running alongside efforts to isolate tumour cells from whole blood were attempts to improve the reliability of their identification. Until comparatively recently all such attempts at identification were based on the appearance of the cell as seen using standard cytological techniques. To aid in their diagnosis, isolated tumour cells were often compared with cells obtained from a direct smear of the tumour (Roberts et al, 1958). Amongst the most commonly reported features used to recognize tumour cells were the variable size of their nuclei, the presence of deep staining nuclei with coarse irregular chromatin patterns, a reduced nuclear/cytoplasmic ratio, and prominent frequently multiple nucleoli (Engell, 1955; Fisher and Turnbull, 1955; Salsbury et al, 1965). Nevertheless, "atypical cells" were often reported and frequently frustrated attempts at an accurate diagnosis. Griffiths et al (1973) reported on the presence of a variety of cells in blood samples which could be

confused with tumour cells including myeloblasts, promyelocytes, atypical mononuclear cells, endothelial cells and occasional epithelial cells introduced at the time of venepuncture. They even identified hepatocytes in one sample obtained by the retrograde cannulation of the inferior mesenteric vein. Megakaryocytes were considered particularly liable to be confused with tumour cells, a problem exacerbated by their increased incidence in patients with malignant disease (Scheinin and Koivuniemi, 1962)

To overcome the disadvantages of conventional cytology a variety of other techniques have been developed to improve the detection of circulating tumour cells. Giraldi et al (1984) used flow cytometry to study circulating Lewis lung carcinoma cells in an animal model. This technique does not require the tumour cell population to be enriched but is dependent on the existence of clear differences in the ploidy/DNA status of tumour cells compared to normal cells and errors may occur when cell clumping is present (Glaves, 1986).

Schirrmacher and Waller (1982) investigating tumour cells disseminated into the blood of tumour-bearing mice, devised a microradioassay for their quantitative detection. Tumour cells were distinguished from normal cells by their higher proliferative activity, which was measured by the uptake of tritiated thymidine. An approximately linear relationship was established between the number of tumour cells present in blood and the uptake of tritiated thymidine. Twenty five mice were studied and in 17 a significant increase in the radioactivity of blood samples was found just prior to their death. Six mice died without increased levels being detected. In these experiments no attempt was made to translate the radioactivity of a given sample from the mice into tumour cells per ml. The Lewis lung carcinoma is associated with the shedding of large numbers of cells into the circulation (Butler and Gullino, 1975), a factor which may have contributed to the relative success of the technique. Its applicability to the study of

human cancers, where the concentration of blood borne tumour cells is likely to be less, is debatable. As well as the radioassay Schirrmacher and Waller (1982) also used the growth of tumour cells in agar to quantify their presence in blood. In prior titration experiments the number of colonies formed bore a linear relationship to the number of cells plated. The success of this technique is, however, dependent on the plating efficiency of the cancer cells, which in the experiments of Schirrmacher and Waller varied from 30% to 100%. The technique, nevertheless, gives an indication of the clonogenic potential of the tumour cells. Although, Pretlow et al (1986) were able to grow colonies of pigmented cells in soft agar using cells originally isolated from the blood of patients with malignant melanoma, no similar success has been reported for tumour cells isolated from the blood of patients with colorectal cancer.

Bioassays provide another means of estimating numbers of circulating tumour cells. Mayhew and Glaves (1984), in a series of experiments in which known numbers of cells were injected intraperitoneally into normal mice, established a relationship between the size of the inoculum and the subsequent survival of the animal. Thus, for B16F10 melanoma cells the mean survival time was 39.3 days when $10^3$ cells were injected, 17.3 days when $10^6$ cells were used. The number of tumour takes could be enhanced when low numbers of cells were present by mixing them with carrier tumour cells previously exposed to radiation. The threshold of sensitivity for this assay was in the range of 10-100 cells. As well as providing the means for their quantification, bioassays also testify to the tumourgenicity of the inoculated cells. The author is not aware of similar methods being used to quantify circulating tumour cells in humans with colorectal or indeed any other cancer. However, cells exfoliated from human colorectal cancers and injected intravenously have been shown to proliferate in immune deprived mice; the numbers used, though, were 190,000 cells or more (Symes et al, 1984), and such quantities may be

difficult to isolate from the blood of patients with circulating tumour cells. Gazet (1966) attempted to establish the growth of tumour cells, derived from the blood of patients with lung carcinomas, in recipient mice but without success.

Recent attempts to detect circulating tumour cells have focused on the use of immunocytochemical techniques. Tumour specific or epithelial cell specific antibodies can be used to detect small numbers of neoplastic cells in body fluids or tissue sections. Glaves et al (1988) chose to use an indirect immunofluorescence technique with an antibody directed against keratins for the detection of blood borne cancer cells shed from renal adenocarcinomas. Keratins are intermediate filaments, which are constitutively expressed by epithelial cells, and not by endothelial cells or cells of haemopoietic origin (Lazarides, 1980). The use of an anti-keratin antibody therefore provides a more certain way of identifying epithelial derived tumour cells and avoids some of the morphological ambiguities encountered when conventional cytology is used. Anticytokeratin antibodies have also been used to detect micrometastatic deposits in the bone marrow of patients with colorectal cancer (Schlimok et al, 1990) and breast cancer (Cote et al, 1991).
The use of anticytokeratin antibodies to detect tumour cells in the blood of patients with colorectal cancer forms the basis of the studies described in chapters 4 and 5 of this thesis.

### 1.5.7 *Quantitative studies of circulating tumour cells.*

One of the first attempts to quantify the release of tumour cells from a primary cancer into the blood stream was made by Butler and Gullino (1975). MTW9 mammary carcinomas were transplanted on to the end of the ovarian pedicle in rats in such a way that all the draining efferent blood could be collected and examined. Samples of aortic blood were also taken. As blood was withdrawn so was it replaced

from a reservoir containing heparinised blood. By removing the erythrocytes with a combination of dextran sedimentation and hypotonic lysis, concentrated preparations of leucocytes and tumour cells were prepared. The tumour cells were identified with an indirect immunofluorescent technique using antibodies raised to antigens on the ascites form of the MTW9 carcinoma. Initial experiments to determine the rate of tumour cell recovery for the whole process showed that when $10^6$ cells were added to whole blood containing $10^8$ leucocytes, approximately 60% of both types of cell could be recovered; furthermore, the ratio of tumour cells to normal leucocytes was not altered. By examining aliquots of the concentrated sample containing a known number of cells, the number of tumour cells could therefore be expressed as a function of the number of leucocytes present. The number of tumour cells per ml of blood was calculated according to Equation 1.

Equation 1
Tumour cells/ml =
(No. of tumour cells detected / 100,000 cells) X
(No. of leucocytes / ml).

The mean number of cancer cells in the blood draining from the tumours was 16,830 per ml.

From their results Butler and Guillino drew two principle conclusions: i) MTW9 carcinomas lost between 3 and 4 million cells per day for each gram of tumour tissue, ii) tumour cells were rapidly removed from the circulation.

Liotta et al (1974) studied the relationship between tumour growth, the density and size distribution of the tumour vasculature and the rate of entry of tumour cells into the circulation. The tumour chosen was the highly metastatic T241 fibrosarcoma which was transplanted into the femoral region of a mouse. Blood samples were collected from

the external iliac vein and passed over a nucleopore filter to isolate the cells within them. The cells were then stained using a Papanicolaou technique and cancer cells identified on the basis of their cell area; computerised image analysis had predetermined that the surface area of fibrosarcoma cells was greater than that of blood cells normally found in the circulation. The number and size of blood vessels within the tumours was also determined; vessels being identified by their content of crystal violet, a solution of which was used to perfuse the tumour prior to its excision.

Tumour cells in the blood draining these tumours occurred either singly or in clumps and their concentration increased with tumour growth. By day 15 after transplantation of the fibrosarcoma the concentration of tumour cells released was approximately 100 tumour cells per ml. A strong linear relationship ($r=0.94$) was also found between the density of vessels $\geq$ 30 μm in diameter and the concentration of tumour cells. The relationship between the density of vessels of all sizes per square mm and total cell concentration was less strong ($r=0.79$).

In similar experiments, only this time using the Lewis lung carcinoma and B16 melanoma, Glaves (1983a) explored the relation between circulating tumour cells and the incidence of metastases. Tumours were established in mice by the intramuscular injection of suspensions of the tumour cells. Tumour cells were then isolated from peripheral blood samples using density gradient centrifugation, and suspended in fetal calf serum. Known volumes (50 μl) of the suspension were then pipetted onto polycarbonate filters, which were then fixed and stained by the Papanicolou technique. Tumour cells were identified according to morphological criteria, e.g. size, hyperchromicity, nucleolar number and prominence. The tumour cells on the filters were counted and the number of tumour cells per ml of original blood calculated according to the following formula:

(Mean no. cells / filter ÷ vol. cell suspension filtered) X (vol. cell suspension ÷ vol. blood sample) X efficiency = cancer cells per ml

The efficiency of the procedure was estimated by seeding known numbers of tumour cells into whole blood, which was then subjected to the full isolation procedure; for the Lewis carcinoma cells the efficiency for $10^2$ and $10^4$ cells was approximately 16%.

Circulating tumour cells were isolated singly, or as formed clumps involving both tumour cells and leucocytes. By using the trypan blue exclusion technique, 88% of the tumour cells were shown to be viable. The number of circulating Lewis cancer cells was found to rise as tumour growth progressed, with a peak of $5.2 \times 10^4$ cells per ml being reached 19 to 21 days after transplantation. After this time the number of cells fell, presumably as a result of central tumour necrosis. Pulmonary metastases were not detected with any regularity until day 16, after which almost all the transplanted mice had them. However, a gross disparity was observed between the numbers of circulating tumour cells and the numbers of pulmonary metastases, illustrating the inefficiency of the metastatic process. Glaves reached the conclusion that the release of cancer cell clumps was the variable best quantitatively related to overall rates of metastasis formation. Also of interest was the observation that the median number of cells shed into the circulation throughout tumour growth from the B16 melanoma tumours was less than for the Lewis carcinomas. This may be a reflection of the fact that B16 melanoma cells tend to produce relatively avascular tumours compared to the Lewis carcinoma.

These animal experiments indicate both the variety of ways in which investigators have attempted to isolate and quantify circulating tumour cells, and some of the factors on which tumour cell shedding into the circulation depend. From clinical experience it is likely that the inefficiency

of the metastatic process observed in animal models is also true of human cancers, although there are surprisingly few quantitative data to support such a concept. To the author's knowledge there has been only one attempt to detect and quantify tumour cell release in a human cancer, that of Glaves et al (1988) who studied renal adenocarcinomas. Blood samples were taken from the renal vein during surgery just prior to nephrectomy. As before, the tumour cells were separated by density gradient centrifugation, collected onto polycarbonate filters and identified using monoclonal antibody directed against their content of keratin proteins. Ten patients were studied and in eight of these circulating tumour cells were detected. The number of tumour cell emboli per ml of blood varied between 0 and 7309. As with the animal experiments, both single cells and clumps of cells were observed, although the latter were not ubiquitous. The smallest tumour studied was 5 cm in diameter and the largest 10 cm. Glaves et al estimated that, with a doubling time of 60 days, it would take 180 days for a tumour of 5 cm diameter to grow to one of 10 cm and that during this period approximately $7 \times 10^9$ tumour cells would be released into the renal vein. Despite this huge input of cells only 3 of the patients had evidence of extraperitoneal metastases prior to surgery and only one patient previously disease free developed metastases subsequent to surgery.

The inefficiency of the metastatic process has also been illustrated by Tarin et al (1984), who studied the incidence of metastasis formation in patients receiving peritoneovenous shunts to relieve the pain and distension of malignant ascites. The insertion of such shunts means that large numbers of malignant cells are transfused from the peritoneal cavity into the circulation. Twenty nine patients were studied with post mortem data subsequently available on 15. Despite estimated infusions of malignant cells ranging from $1.6 \times 10^8$ to $4 \times 10^9$ cells per week, 8 of the 15 patients who had autopsies showed no evidence of haematogenous metastases. This study also highlighted

potential differences in the metastatic capacity of malignant cells even when these were derived from histologically similar tumours. Thus, while tumour cells from some patients with ovarian carcinomas failed to colonize the lungs, others had easily recognizable metastatic deposits. The apparent influence of tissue type on the success or failure of metastasis was also apparent. According to Paget's seed and soil hypothesis metastatic deposits are only likely to form when tumour cells encounter a tissue capable of supporting their growth requirements (Paget, 1889). In one patient with pre-existing hepatic and spinal secondaries, which testified to the capacity of her tumour to metastasize via the blood stream, no metastasis was found in the lungs or other organs 5 months after the shunt was established.

Although the above experiments support the role of tumour cells in the blood stream in establishing metastatic deposits, there are surprisingly little quantitative data relating to circulating tumour cells in colorectal cancer. In chapters 4 and 5 of this thesis experiments are presented in which immunocytochemical methods have been used to increase the reliability of circulating tumour cell identification so enabling quantitative estimates of their numbers to be made.

The above introduction has focused on two aspects of metastasis, namely matrix metalloproteinase enzymes and circulating tumour cells. Although much information is available on each, relatively little is concerned with colorectal cancer specifically. It is the aim of this thesis to redress this imbalance. Increased knowledge of the mechanisms which permit tumour cell dissemination may allow the biological behaviour of individual tumours to be predicted with greater certainty, and as a consequence therapeutic regimens to be tailored more exactly to the patient's requirements.

# CHAPTER 2

## PART 1: THE IMMUNOHISTOCHEMICAL LOCALIZATION OF MATRIX METALLOPROTEINASES (MMPs) AND TIMP-1 IN FRESH FROZEN BIOPSIES OF COLORECTAL CANCER.

### 2.1 AIM

To investigate the presence and distribution of the MMPs, interstitial collagenase (CL), gelatinase (GL) and stromelysin (SL), and their inhibitor, tissue inhibitor of metalloproteinases 1 (TIMP-1), in colorectal adenocarcinomas using an indirect immunolocalization technique.

### 2.2 INTRODUCTION

Experiments, based mainly on biochemical techniques, have demonstrated the ability of malignant cells and tissues to produce matrix metalloproteinase enzymes *in vitro*. However, although such studies are valuable, they do no more than indicate the capacity of the cells and tissues examined to manufacture these enzymes under the experimental conditions used. Furthermore, *in vitro* studies provide only limited information about which metalloproteinases are functionally important *in vivo*. The cellular heterogeneity of tissues also makes it difficult to know which cells are responsible for MMPs synthesis, and also the distribution of MMPs within different parts of a tumour. Specific antibodies, raised against the MMPs and TIMP-1, and used to perform immunolocalization studies, have the potential to redress these deficiencies, allowing a more complete assessment to be made of the *in vivo* role of MMPs and TIMP in tumour invasion.

## 2.3 MATERIALS AND METHODS

### *Chemicals*

All chemicals were supplied by Merck Ltd (Poole, Dorset, UK) unless otherwise stated, and were of AnalaR grade.

### 2.3.1 *Antibodies to interstitial collagenase (CL), gelatinase (GL), stromelysin (SL) and TIMP-1.*

The antibodies used for the immunolocalization of the matrix metalloproteinases and TIMP-1 were all developed and generously supplied by Dr J.J. Reynolds and Dr R.M. Hembry of Strangeways Research Laboratory, Cambridge.

Each of the polyclonal antibodies was raised according to the method described by Hembry et al (1985). In brief, a purified sample of the whole antigen was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into an adult sheep. At varying intervals, depending on the antibody being produced, blood samples were taken, the serum removed and the immunoglobulin fraction purified. The specificities of the anti-sera obtained were verified by electrophoretic immunoblotting, inhibition studies and immunolocalization experiments; a summary of these studies is provided in Table 2.1. In addition, the antibodies used in this study have been further validated in a number of investigations. *In vitro*, the antibodies to TIMP-1 and CL have been shown to inhibit the degradation of type I collagen by rabbit chondrocytes and endothelial cells, which have been stimulated to produce collagenase (Gavrilovic et al, 1987); similarly these antibodies also inhibit the degradation of type I collagen films by rabbit VX2 tumour cells (Gavrilovic et al, 1985). The antibodies to CL, SL, GL and TIMP-1 have also been used to conduct immunolocalization studies of the MMPs in the rabbit growth plate, human hypertrophic scar tissue and gut from patients with Crohn's disease (Brown et al, 1989; Hembry and Ehrlich,

1986; Bailey et al, 1990). Experiments using antibodies against the rabbit MMPs and TIMP-1 have been mentioned here as these antibodies were raised and characterized in a similar way to their human counterparts.

As detailed previously, the MMPs and TIMP-1 may exist *in vivo* in different forms. To interpret the results of immunolocalization studies it is therefore necessary to know which forms are recognisable by the antibodies used; Table 2.2 summarises the available information and provides supporting references.

### 2.3.2 *Indirect immunohistochemistry*

Immunohistochemistry may be defined as the use of labelled antibodies as specific reagents for the localization of tissue constituents (antigens) *in situ* (Polak and Van Noorden, 1988).

Coons (1941) was the first to label an antibody with a fluorescent dye in order to identify an antigen in tissue sections. By exploiting the specificity of the antigen - antibody reaction the identification of tissue components can be achieved more reliably than with conventional histological techniques.

Coons' original work made use of a one step, or direct, technique in which the antibody was conjugated directly with fluorescein isothiocyanate (FITC) (Coons and Kaplan, 1950). Binding of the antibody, within the tissue section, was then revealed by illumination with light at a wavelength of 490 nm, which excites fluorescein compounds to emit a bright green fluorescence.

The direct method was later modified to become an indirect technique in which the primary antibody was no longer conjugated with the dye (Coons et al, 1955). Instead, detection of the primary antibody relied on incubation of the tissue with a second antibody, labelled with FITC, and raised against the gamma-globulin of the species from which the primary antibody was derived.

Two main advantages accrue from the indirect technique: i) two labelled anti-immunoglobulin molecules can bind to each primary antibody molecule, amplifying and thus increasing the sensitivity of the reaction, and ii) the binding strength, or avidity, of anti-IgG sera is usually high making them less likely to be washed off during the staining process.

Table 2.1

## Characteristics of antibodies raised to the matrix metalloproteinases and TIMP-1

| Antigen ▸ / Antibody ▾ | Latent proenzyme (free TIMP-1) | Active enzyme | Enzyme (or TIMP-1) bound to collagen fibrils | Enzyme : TIMP-1 complex |
|---|---|---|---|---|
| **Interstitial collagenase. (Whitham et al, 1986; Hembry et al, 1986)** | Yes | Yes | Yes: only the active form of collagenase is collagen bound | Uncertain *in vivo*, although it does recognise the complex *in vitro* |
| **Gelatinase $M_r$ 72,000 & (Hipps et al, 1991; Murphy et al, 1989a)** | Yes | Yes | Yes: both active and latent $M_r$ 72,000 gelatinase bind to collagen | Information not available |
| **Gelatinase $M_r$ 95,000. (Murphy et al, 1989a)** | Yes | Yes | Yes: both active & latent forms bind to collagen | Yes |
| **Stromelysin. (Allen et al, 1991)** | Yes: both high & low molecular mass forms | Yes | Yes: both active and latent forms bind to collagen | Yes |
| **TIMP-1. (Hembry et al, 1985; Allan et al, 1991)** | Yes | Not applicable | No: TIMP-1 is unable to bind to reconstituted collagen fibrils | Yes: for complexes with active collagenase, active $M_r$ 95,000 & $M_r$ 72,000 gelatinase & active high molecular mass stromelysin |

Table 2.2

## Tests of specificity for antibodies raised against the matrix metalloproteinases and TIMP-1

| Test ▸ / Antibody ▾ | Inhibition of MMP activity | Double immunodiffusion and immunoprecipitation | Electrophoretic immunoblotting | Immunocytochemistry |
|---|---|---|---|---|
| **Interstitial collagenase. (Whitham et al, 1986; Hembry et al, 1986)** | Ab inhibits human fibroblast collagenase activity. Only slight inhibition of human PMNL collagenase | Single precipitin line formed against human collagenase | Collagenase appears as two doublets indicating latent and active forms can be identified | Immunoprecipitation of secreted collagenase from chondrocytes & intracellular localization in chondrocytes. |
| **Gelatinase $M_r$ 72,000 & $M_r$ 95,000. (Hipps et al, 1991; Murphy et al, 1989a)** | Inhibits gelatinolysis by purified HGF gelatinase, but not collagenase or stromelysin activity | Immunoprecipitates $M_r$ 72,000 gelatinase from cell culture media | $M_r$ 72,000 gelatinase from human fibroblasts identifiable | Juxtanuclear fluorescent granules observed in human gingival fibroblasts. |
| **Gelatinase $M_r$ 95,000. (Murphy et al, 1989a)** | Inhibition of human polymorph & monocyte gelatinase | Recognises human polymorph but not human gingival fibroblast gelatinase | | Juxtanuclear fluorescent granules observed in human polymorphs. |
| **Stromelysin. (Allan et al, 1991)** | Detection of latent and active, high & low molecular mass forms of stromelysin | | Both active and latent forms of stromelysin from human gingival fibroblasts | |
| **TIMP-1. (Hembry et al, 1985)** | Inhibits TIMP action amniotic fluid | Single line of identity formed when run against pure inhibitor from amniotic fluid | Single band detected when pure TIMP was electroblotted onto nitrocellulose paper | Intracellular localization of TIMP-1 in HFL-1 |

### 2.3.3 *Details of patients and their tumours.*

Forty colorectal adenocarcinomas were studied by the technique described below. The pathological details of these tumours together with details of the patients from which they were obtained are summarized in Table 2.3. As well as the primary adenocarcinomas, three benign polyps (tubulo-villous adenomas) and two liver metastases were also examined; the patients who provided this additional material are identified in Table 2.3.

### 2.3.4 *Tissue collection*

Full thickness biopsies 1 cm in diameter were cut from fresh colectomy specimens, which had been removed from patients with colorectal adenocarcinomas. The biopsies, taken within one hour of resection, were cut from the following sites: the tumour centre, the tumour/mucosal junction, so that both normal and malignant tissue were represented equally on the same biopsy, and from macroscopically normal mucosa 10cm distant from the primary tumour. Areas of tumour which were obviously necrotic were avoided.

To eliminate bacteria, whose production of collagenase could confound the interpretation of any observed immunostaining, each biopsy was cleansed by washing in five changes of Hank's balanced salt solution containing antibiotics (see Appendix 2.1). The tissue was then orientated on a cellulose nitrate filter (Whatman, Maidstone, UK) so that the mucosal surface was uppermost, placed in a 7ml bijou (Bibby Sterilin, Stone, UK), covered with a solution of 7% gelatin in 0.9% sodium chloride containing 0.02% sodium azide as an antibacterial agent, and then snap frozen in liquid nitrogen for 90 seconds. The frozen tissue was stored at -20°C until required.

### 2.3.5 *Immunocytochemistry*

Frozen sections 5μm thick were cut from each tissue block on a cryostat (Bright, Huntingdon, UK). Each section was

allowed to dry onto the slide for 3 minutes before being fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), (Appendix 2.1) for 30 minutes at room temperature. The sections were then washed for 5 minutes in each of three changes of PBS before being placed for 10 minutes in a 0.1% solution of the detergent Triton X-100 in PBS to facilitate tissue penetration by the antibodies. The sections were again washed in PBS and then treated for 20 minutes with a 2.8 mM solution of 4-chloro-1-naphthol in methanol/PBS with 0.01% $H_2O_2$ (Appendix 2.1) to prevent the non-specific binding of fluorescein labelled antibody by inflammatory cells (Johnston and Bienenstock, 1974). To reduce background staining caused by the non-specific binding of the second antibody to the tissue, the sections were washed and then incubated with a 2% solution of rabbit albumin in PBS (Sigma, Poole, UK) for 10 minutes; this and all subsequent incubations were performed in a humidified chamber at room temperature. Excess rabbit albumin was then drawn off with a tissue and sequential sections incubated with the antibodies to TIMP-1, stromelysin, gelatinase and interstitial collagenase for 40 minutes. Each of these antibodies was used at a concentration of 50µg/ml in PBS. After thorough washing, the sections were incubated for 40 minutes with the second antibody, a fluorescein isothiocyanate (FITC) - labelled rabbit F(ab')2 anti-sheep IgG (Southern Biotechnology Associates Ltd, supplied by Bio-nuclear Services Ltd, Bude, UK) diluted to 1:100 with 10% human serum in PBS. The addition of human serum reduces the binding of the second antibody to immunoglobulins within the tissue section. Three further washes were then performed before counterstaining with a 1% solution of methyl green in water, which produces red fluorescent nuclei when the sections are viewed under a standard wide band FITC filter.

Control sections were prepared in an identical manner except that the primary antibody was replaced by normal (non-immune) sheep serum (Sigma, Poole, UK) also used at a concentration of 50µg/ml.

The sections were mounted in Citifluor AF1 (Citifluor Ltd, City University, London, U.K.) and the coverslips

sealed with glyceal. A Leitz Laborlux S microscope (Leica, Milton Keynes, U.K.) fitted with epifluorescence and standard FITC and rhodamine filters was used to view the slides, and photographs were taken using Agfa Agfachrome 1000 RS film.

In selected cases the co-ordinates of staining within the tissue section were noted using the Vernier scale on the microscope stage. On completion of the fluorescent studies the coverslips were removed and the sections stained with haematoxylin and eosin. Using transmitted light, previously fluorescent areas were relocated from their co-ordinates and photographed on Kodak Ektachrome 160T film. In one instance where a particular group of cells had been shown to stain for the presence of metalloproteinases an attempt was made to determine if they were of epithelial origin, and therefore likely to be tumour cells. As discussed in Chapter 4 this can be achieved by demonstrating the presence or absence of keratin proteins which are synthesized constitutively by epithelial cells and not by stromal cells or cells of a haemopoietic origin. An indirect immunofluorescent technique was used employing an anti-cytokeratin 8.13 antibody (Sigma, Poole, UK) at a dilution of 1:50 in Tris-buffered saline. Binding of this antibody was detected by incubating the sections with a 1:100 dilution of a goat anti-mouse antibody labelled with horseradish peroxidase (full details of the method used are given in Chapter 4 in the section entitled:- Selection of cell line and antibody).

### 2.3.6 *Method of assessing MMP and TIMP-1 immunolocalization*

Based on the results of the immunostained sections, each biopsy was assessed as follows. The presence or absence of fluorescent staining for stromelysin, gelatinase, interstitial collagenase and TIMP-1 was recorded and the location of the staining, whether it was intracellular or extracellular noted. If the staining was intracellular an attempt was made to identify the cell type. The resolution of cytological detail in fluorescent microscopy is poor, but

with the aid of the methyl green counterstain two populations of cells could be distinguished on the basis of their distribution and nuclear morphology, namely, tumour cells and stromal cells. In some cases further identification of the stromal cells was possible, aided by restaining positive sections with haematoxylin and eosin, or through the use of an immunocytochemistry, as described above.

Before deciding that a biopsy was negative for a particular MMP or TIMP-1 the staining process was repeated on at least one other occasion using sections cut from a deeper level.

### 2.3.7 *Statistics*

The respective frequencies with which the MMPs and TIMP-1 were observed in biopsies from normal mucosa, the tumour centre and the tumour mucosal junction, as well as in tumours of different stage and differentiation, were compared using the chi-squared test and Fisher's exact test.

## 2.4 RESULTS

### 2.4.1 *Details of patients and tumours*

Forty patients with colorectal adenocarcinomas were studied. The median age of the patients was 68.5 years (range 34 - 91 years) and the male to female ratio 22 to 18.

Of the forty tumours examined 14 were from the rectum, 13 from the descending colon, 7 from the ascending colon and 6 from the caecum. According to Dukes' classification the tumours were staged as follows; 4 were stage A tumours, 16 stage B, and 20 stage C. Three of the tumours were well differentiated, 29 moderately differentiated and 8 poorly differentiated (Table 2.3).

### 2.4.2 *Specificity of the staining*

Control sections were prepared by replacing the primary antibody with non-immune sheep serum. The only "staining" discernable on these control sections was a faint green autofluorescence associated with the connective tissue. In all cases the control sections enabled autofluorescence to be distinguished easily from specific immunostaining. The control sections also confirmed that normal (non-immune) sheep serum proteins do not bind to tumour cells, stromal cells, connective tissue or blood vessels.

The ideal negative control is made by applying to the tissue section antibody which has first been incubated with the antigen (Heyderman, 1979); negative control sections under these circumstances indicate that positive staining is due to the presence of that antigen (or perhaps a shared or cross-reactive epitope). This test was not performed here, however, each of the primary antibodies used has been absorbed with its antigen and the specificity of the immunoreactivity confirmed in other studies (see Table 2.1 for references).

The presence or absence of the MMPs and TIMP-1 in biopsies taken from normal colonic mucosa, the tumour mucosal junction and the tumour centre are recorded for each patient in the study in Tables 2.4 to 2.7. The frequencies with which the MMPs and TIMP-1 were encountered at each biopsy site are summarised in Table 2.8.

### 2.4.3 *Stromelysin immunolocalization*

Of the biopsies taken from each of the 40 tumours, stromelysin was observed in 18 of those taken from the tumour centre and in 14 from the tumour mucosal junction (Table 2.4). Stromelysin was not identified in biopsies of normal colonic mucosa, the benign polyps or the two liver metastases.

Stromelysin staining was confined to one or two small areas of those sections in which it was seen (Figs 2.1 & 2.2). The staining was always associated with the

extracellular matrix and was typically observed as streaks of green fluorescence running between or around small groups of malignant glands (Figs 2.3, 2.4 & 2.5). However, despite the invariably close relationship between stromelysin staining and tumour cells, no tumour cells were ever identified as positively staining for the enzyme. In biopsies from the tumour mucosal junction stromelysin staining was only ever seen on the tumour side of the junction.

In the one instance where intracellular stromelysin was clearly observed (TMJ, Patient ES), granules of the enzyme were identified in mononuclear cells lying within the tumour stroma (Fig 2.6). Although these mononuclear cells could be distinguished from neoplastic cells by their smaller nuclei, further identification was impossible. On other occasions intracellular stromelysin may well have been present but concomitant extracellular staining for the enzyme made it difficult to see conclusively (Fig 2.4).

When seen at both sites within the same tumour there was no suggestion that the expression of stromelysin was enhanced at the tumour mucosal junction compared to the tumour centre.

From the characteristics of the anti-stromelysin antibody detailed in Table 2.1 both the active and latent forms of stromelysin may be present, bound to the collagen matrix within the tumour.

### 2.4.4. *Gelatinase immunolocalization: Part A*

The following results refer to the immunolocalization studies performed using the antibody which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase.

Gelatinase staining, in the form of intracellular fluorescent granules, was most frequently seen in polymorphonuclear leucocytes, which were identified by their multi-lobed nuclei. Positively stained polymorphonuclear leucocytes were present in several locations: within the lumens of blood vessels (Fig 2.7), between smooth muscle cells at the deep margin of tumour invasion (Fig 2.15), in the stroma surrounding neoplastic glands (Fig 2.8), and occasionally in the lamina propria of normal colonic mucosa. Mononuclear cells, either in the stroma or within blood vessels (Fig 2.9, 2.10), also demonstrated intracellular fluorescent granules. Some of these stromal cells resembled fibroblasts, in that they were spindle shaped and orientated along the line of the connective tissue elements (Fig 2.11). These fibroblast-like cells were often closely aligned to the periphery of neoplastic cell clusters or present at the deep margin of tumour invasion (Fig 2.12 & 2.13). However, immunostaining was never seen within the tumour cells themselves.

Extracellular staining was also observed. As with stromelysin, the fluorescence was associated with the connective tissue matrix (Fig 2.14). Positive regions were confined to one or two small areas of a section, always in close proximity to clusters of neoplastic cells (2.14). Generally, the extent of extracellular staining for gelatinase was less than that observed for stromelysin. Whereas staining for stromelysin might completely surround a neoplastic gland (Fig 2.4), that for gelatinase would appear adjacent to only one portion of a gland's periphery, often surrounding positively stained fibroblast-like cells (Fig 2.14). Extracellular gelatinase was never observed in biopsies taken from normal colonic mucosa, benign polyps or the two liver metastases.

Table 2.5 records the presence or absence of extracellular gelatinase staining in biopsies from the tumour centre and the tumour mucosal junction. Extracellular gelatinase staining was observed in 16 of the biopsies from the tumour/mucosal junction and in 17 of the tumour centre biopsies.

### 2.4.5 *Gelatinase immunolocalization: Part B*

A comparison was made of the immunostaining results obtained using the antibody which recognises both the $M_r$ 95,000 and $M_r$ 72,000 forms of gelatinase and the antibody which recognises only the $M_r$ 95,000 form. The tumours of nine patients were studied using biopsies taken from the tumour centre. Serial sections were cut, and adjacent sections incubated with one or other of the two antibodies. The results obtained are tabulated in Table 2.6.

The most important difference between the two antibodies was the absence of extracellular staining when the $M_r$ 95,000 gelatinase antibody was used. Of the nine biopsies studied, five showed extracellular fluorescent staining when the antibody which recognises both forms of gelatinase was used. When adjacent sections from these five biopsies were stained with the antibody recognising only the $M_r$ 95,000 gelatinase, neither extracellular staining nor staining of fibroblast-like cells was observed (Fig 2.15 & 2.16). Extracellular staining was therefore concluded to be due to the presence of $M_r$ 72,000 gelatinase.

Intracellular fluorescence was observed with both antibodies. However, no cells other than polymorphonuclear leucocytes appeared to contain fluorescent granules when the anti - $M_r$ 95,000 gelatinase antibody was used. By contrast the antibody to both forms of gelatinase produced fluorescent granules, not only in polymorphonuclear leucocytes but also in various stromal cells, including fibroblast-like cells and mononuclear leucocytes; these latter cell types were therefore deduced to contain the $M_r$ 72,000 gelatinase. No fluorescent granules were observed in tumour cells with either antibody.

Reference to Table 2.1 indicates that the anti- $M_r$ 95,000 gelatinase antibody recognizes not only the active and latent forms of the enzyme but also these forms when they are bound to the collagen matrix. The absence of extracellular staining for $M_r$ 95,000 gelatinase indicates that matrix-bound enzyme, be it the latent or active form, was was not identified in the biopsies. The observed extracellular fluorescence, marking the presence of the $M_r$ 72,000 gelatinase, supports *in vitro* evidence which indicates that the antiserum against both forms of gelatinase is able to recognize both the latent and active forms of $M_r$ 72,000 gelatinase when they are bound to collagen.

The sequence of figures 2.17 to 2.21 show the relative expressions of $M_r$ 72,000 and $M_r$ 95,000 gelatinase (as detected using both of the antibodies to gelatinase available), stromelysin, collagenase and TIMP-1 as shown immunohistochemically in one particular portion of a biopsy taken from the tumour/mucosal junction of a Dukes' B, moderately differentiated adenocarcinoma of the rectum.

### 2.4.6 *Interstitial collagenase immunolocalization*

Interstitial collagenase was not detected in biopsies of either normal or malignant colorectal tissue.

### 2.4.7. *TIMP-1 immunolocalization*

Immunofluorescent staining for TIMP-1 was generally associated with the endothelial cells of blood vessels. It was seen in all types of vessel from capillaries to extramural arteries and veins (Figs 2.22, 2.23, 2.24).

In only 3 tumours (SE, SP, ES) was extracellular staining for TIMP-1 observed, and in each of these it occurred in an area where MMPs had been identified. In each case it was associated with small areas of the connective tissue matrix. An example is presented in Figures 2.25 to 2.31 which show the expression of the MMPs and TIMP-1 as viewed on adjacent sections through the same malignant

gland. Extracellular staining for TIMP-1 was absent from biopsies of normal colonic mucosa.

No immunofluorescent staining for TIMP-1 was recognizable in carcinoma cells.

TIMP-1 was detected in the vascular endothelium of 27 of the biopsies from the tumour mucosal junction, in 12 of the biopsies from the tumour centre and in 8 biopsies from normal colonic mucosa (Table 2.7).

The anti-TIMP-1 antibody is able to recognize complexes between TIMP-1, interstitial collagenase, active $M_r$ 95,000 gelatinase, active $M_r$ 72,000 gelatinase and high molecular mass stromelysin. The failure to detect either interstitial collagenase or extracellular $M_r$ 95,000 gelatinase suggests that when TIMP-1 staining was present in the stroma (as opposed to within endothelial cells) it indicated the presence of either free TIMP-1 (unbound to collagen fibrils) or a complex between TIMP-1 and either stromelysin or $M_r$ 72,000 gelatinase.

### 2.4.8 *Comparison of staining patterns for TIMP-1, stromelysin and gelatinase.*

The distribution of immunofluorescent staining for stromelysin differed from that for gelatinase ($M_r$ 72,000 gelatinase). In biopsies in which both stromelysin and gelatinase were detected when adjacent sections were compared those portions which stained positively for extracellular stromelysin on one would be negative for extracellular gelatinase on the other and vice versa. In two of the patients, however, the simultaneous expression of stromelysin and gelatinase was observed, together with the presence of extracellular TIMP-1.

Although the presence of stromelysin was invariably confined to small areas of tumour it was usually represented more prominently than extracellular gelatinase ($M_r$ 72,000 gelatinase). Whereas streaks of stromelysin immunostaining were often observed surrounding several malignant glands (Fig 2.5), gelatinase was rarely seen adjacent to more than a few tumour cells at a time (Figs 2.11, 2.12).

The staining pattern for stromelysin and gelatinase did not differ when biopsies from the tumour centre and tumour mucosal junction were compared. There was also no indication that the presence of either of these enzymes was enhanced at the invading edge of the tumour compared to the tumour centre.

Although extracellular stromelysin and gelatinase were frequently observed, their presence was rarely accompanied by extracellular TIMP-1, which for the most part was confined to vascular endothelium. When extracellular TIMP-1 immunofluorescence was detected its distribution bore a variable relationship to that of the prevailing enzymes, being more prominent in one case (Figs 2.26 to 2.31) and less so in the other two.

### 2.4.9 *MMPs expression in relation to biopsy site*

Table 2.8 summarises the frequency with which each of the MMPs studied and TIMP-1 were encountered in the biopsies taken from the 40 colectomy specimens.

There was a significant difference in the frequency with which TIMP-1 was detected in biopsies from the tumour mucosal junction (27/40) compared to the tumour centre (12/40), ($X^2$ = 18.27, $p < 0.001$). In addition, for each of the 8 tumours in which TIMP-1 was detected in the normal colonic mucosa, TIMP-1 was invariably present in the blood vessels of the tumour/mucosal junction. However, of the 32 tumours in which the associated normal mucosa was negative for TIMP-1, only 19 had detectable TIMP-1 at the tumour/ mucosal junction. This difference in the proportion of tumours expressing TIMP-1 at the tumour mucosal junction, according to its presence in the normal mucosa, was statistically significant, (Fisher's exact test, p= 0.03) (Table 2.8).

The frequency with which extracellular stromelysin was detected in biopsies from the tumour centre and from the tumour mucosal junction did not differ significantly. There was also no significant difference in the frequency with

which extracellular gelatinase was detected between these two sites.

### 2.4.10 *MMPs expression and tumour differentiation*

The relationship between MMPs expression and tumour differentiation is set out in Table 2.9. There was no significant difference in the incidence with which either TIMP-1 or the MMPs was detected when well or moderately differentiated tumours were compared with poorly differentiated tumours.

### 2.4.11 *MMPs expression in relation to Dukes' stage*

The frequencies with which TIMP-1, stromelysin and extracellular gelatinase were detected in tumours of different Dukes' stage are recorded in Table 2.10 There was no significant difference in the frequency with which either of these enzymes or TIMP-1 was detected when Dukes' stage A and B tumours were compared with Dukes' C.

| PATIENT | AGE | SEX | SITE OF TUMOUR | DIFFERENTIATION | DUKES' STAGE |
|---|---|---|---|---|---|
| JB (LM) | 60 | M | DESC COLON | MODERATE | C |
| DW | 48 | M | SIGMOID | MODERATE | C |
| LS | 64 | M | RECTUM | MODERATE | B |
| CM | 81 | F | RECTUM | MODERATE | A |
| PD | 61 | F | RECTUM | MODERATE | B |
| AA | 61 | F | CAECUM | POOR | B |
| EH (P) | 72 | F | ASCEN COLON | MODERATE | C |
| SP | 57 | F | DESC COLON | MODERATE | C |
| ZE | 74 | F | RECTUM | POOR | B |
| MP | 67 | F | CAECUM | POOR | C |
| DM | 72 | F | ASCEN COLON | MODERATE | B |
| RS | 64 | M | DESC COLON | MODERATE | B |
| SE | 91 | M | DESC COLON | MODERATE | C |
| SP | 75 | F | ASCEN COLON | MODERATE | B |
| JT | 62 | M | CAECUM | MODERATE | B |
| LM | 57 | M | ASCEN COLON | POOR | C |
| AG | 60 | F | RECTUM | MODERATE | B |
| DG | 70 | M | CAECUM | MODERATE | C |
| RS | 43 | M | RECTUM | MODERATE | A |
| HF | 72 | M | DESC COLON | POOR | C |
| CR | 78 | F | ASCEN COLON | MODERATE | B |
| RM | 72 | F | DESC COLON | MODERATE | A |
| JF (P) | 69 | M | RECTUM | MODERATE | B |
| JF | 89 | M | DESC COLON | MODERATE | B |
| ES | 81 | M | CAECUM | MODERATE | C |
| WT | 68 | M | DESC COLON | WELL | A |
| KA | 61 | M | CAECUM | POOR | C |
| GK (P) | 71 | F | DESC COLON | MODERATE | C |
| WL | 68 | M | RECTUM | WELL | B |
| JL | 67 | M | DESC COLON | MODERATE | C |
| EF | 86 | F | RECTUM | POOR | C |
| TG | 48 | F | RECTUM | MODERATE | C |
| BM (LM) | 65 | F | DESC COLON | WELL | B |
| DB | 76 | F | DESC COLON | POOR | C |
| SS | 67 | M | RECTUM | MODERATE | C |
| NG | 77 | M | RECTUM | MODERATE | B |
| PL | 34 | M | ASCEN COLON | MODERATE | C |
| MS | 70 | F | RECTUM | MODERATE | C |
| MS | 74 | M | ASCEN COLON | MODERATE | C |
| ES | 91 | M | RECTUM | MODERATE | B |

**Table 2.3:** MMP and TIMP-1 immunolocalization study: patient and tumour details. Shaded patients were involved in the tissue culture study. Additional material; P, Polyp; LM, Liver metastasis.

| PATIENT | DIFFERENTIATION | DUKES' STAGE | TMJ | TC |
|---|---|---|---|---|
| JB | MODERATE | C | - | - |
| DW | MODERATE | C | - | + |
| LS | MODERATE | B | - | + |
| CM | MODERATE | A | - | - |
| PD | MODERATE | B | - | - |
| AA | POOR | B | + | - |
| EH | MODERATE | C | - | + |
| SP | MODERATE | C | - | - |
| ZE | POOR | B | + | + |
| MP | POOR | C | - | + |
| DM | MODERATE | B | - | + |
| RS | MODERATE | B | - | - |
| SE | MODERATE | C | + | + |
| SP | MODERATE | B | + | + |
| JT | MODERATE | B | + | + |
| LM | POOR | C | - | - |
| AG | MODERATE | B | + | - |
| DG | MODERATE | C | - | + |
| RS | MODERATE | A | - | - |
| HF | POOR | C | - | - |
| CR | MODERATE | B | - | + |
| RM | MODERATE | A | - | + |
| JF | MODERATE | B | - | - |
| JF | MODERATE | B | + | + |
| ES | MODERATE | C | + | - |
| WT | WELL | A | - | + |
| KA | POOR | C | + | + |
| GK | MODERATE | C | + | + |
| WL | WELL | B | + | + |
| JL | MODERATE | C | + | + |
| EF | POOR | C | - | - |
| TG | MODERATE | C | - | - |
| BM | WELL | B | - | - |
| DB | POOR | C | + | - |
| SS | MODERATE | C | - | - |
| NG | MODERATE | B | - | - |
| PL | MODERATE | C | - | - |
| MS | MODERATE | C | - | - |
| MS | MODERATE | C | - | + |
| ES | MODERATE | B | + | - |

Table 2.4: The presence of stromelysin staining in biopsies taken from the tumour mucosal junction (TMJ) and tumour centre (TC). +, staining present; -, staining absent.

| PATIENT | DIFFERENTIATION | DUKES' STAGE | TMJ | TC |
|---|---|---|---|---|
| JB | MODERATE | C | - | - |
| DW | MODERATE | C | - | + |
| LS | MODERATE | B | - | - |
| CM | MODERATE | A | + | - |
| PD | MODERATE | B | - | - |
| AA | POOR | B | + | + |
| EH | MODERATE | C | + | + |
| SP | MODERATE | C | - | - |
| ZE | POOR | B | + | + |
| MP | POOR | C | - | + |
| DM | MODERATE | B | - | - |
| RS | MODERATE | B | + | - |
| SE | MODERATE | C | + | + |
| SP | MODERATE | B | - | - |
| JT | MODERATE | B | + | - |
| LM | POOR | C | + | + |
| AG | MODERATE | B | - | - |
| DG | MODERATE | C | - | + |
| RS | MODERATE | A | - | - |
| HF | POOR | C | - | + |
| CR | MODERATE | B | + | + |
| RM | MODERATE | A | - | + |
| JF | MODERATE | B | - | - |
| JF | MODERATE | B | - | - |
| ES | MODERATE | C | + | - |
| WT | WELL | A | - | + |
| KA | POOR | C | + | - |
| GK | MODERATE | C | + | + |
| WL | WELL | B | - | + |
| JL | MODERATE | C | + | + |
| EF | POOR | C | - | - |
| TG | MODERATE | C | + | + |
| BM | WELL | B | - | - |
| DB | POOR | C | + | - |
| SS | MODERATE | C | - | - |
| NG | MODERATE | B | - | - |
| PL | MODERATE | C | - | - |
| MS | MODERATE | C | - | - |
| MS | MODERATE | C | - | - |
| ES | MODERATE | B | + | + |

**Table 2.5:** The presence of extracellular gelatinase staining as detected using an antibody which recognises both the Mr 72,000 and Mr 95,000 forms of the enzyme. +, staining present; -, staining absent.

| PATIENT | DIFFERENTIATION | DUKES' STAGE | ANTIBODY TO Mr 95,000 & Mr 72,000 GELATINASE | ANTIBODY TO Mr 95,000 GELATINASE |
|---|---|---|---|---|
| EH | MODERATE | C | EC | IC in PMNL |
| AG | MODERATE | B | No staining | IC in PMNL |
| DG | MODERATE | C | EC | No staining |
| RM | MODERATE | A | EC | No staining |
| JF | MODERATE | B | No staining | No staining |
| JL | MODERATE | C | EC and IC in PMNL & other stromal cells | IC in PMNL |
| EF | POOR | C | IC in PMNL & other stromal cells | IC in PMNL |
| MS | MODERATE | C | IC in PMNL & other stromal cells | IC in PMNL |
| ES | MODERATE | B | EC and IC in PMNL & other stromal cells | IC in PMNL |

**Table 2.6:** The staining patterns observed when biopsies from the tumour centre were stained with the two different antibodies to gelatinase. EC, Extracellular; IC, Intracellular; PMNL, Polymorphonuclear leucocyte.

| PATIENT | DIFFERENTIATION | DUKES' STAGE | NORMAL | TMJ | TC |
|---|---|---|---|---|---|
| JB | MODERATE | C | - | + | - |
| DW | MODERATE | C | - | + | + |
| LS | MODERATE | B | + | + | + |
| CM | MODERATE | A | - | + | - |
| PD | MODERATE | B | - | + | + |
| AA | POOR | B | - | + | - |
| EH | MODERATE | C | - | - | - |
| SP | MODERATE | C | - | + | - |
| ZE | POOR | B | - | + | - |
| MP | POOR | C | - | + | - |
| DM | MODERATE | B | - | - | - |
| RS | MODERATE | B | + | + | + |
| SE | MODERATE | C | + | + | + |
| SP | MODERATE | B | - | + | - |
| JT | MODERATE | B | - | + | + |
| LM | POOR | C | - | + | + |
| AG | MODERATE | B | - | - | - |
| DG | MODERATE | C | - | - | - |
| RS | MODERATE | A | - | - | - |
| HF | POOR | C | + | + | - |
| CR | MODERATE | B | - | - | - |
| RM | MODERATE | A | + | + | + |
| JF | MODERATE | B | - | - | - |
| JF | MODERATE | B | - | - | - |
| ES | MODERATE | C | - | - | - |
| WT | WELL | A | - | - | - |
| KA | POOR | C | + | + | + |
| GK | MODERATE | C | + | + | - |
| WL | WELL | B | - | + | + |
| JL | MODERATE | C | - | - | + |
| EF | POOR | C | - | + | - |
| TG | MODERATE | C | + | + | - |
| BM | WELL | B | - | + | - |
| DB | POOR | C | - | - | - |
| SS | MODERATE | C | - | + | - |
| NG | MODERATE | B | - | - | - |
| PL | MODERATE | C | - | + | - |
| MS | MODERATE | C | - | + | - |
| MS | MODERATE | C | - | + | - |
| ES | MODERATE | B | - | + | + |

**Table 2.7**: The presence of vascular TIMP-1. +, TIMP-1 present; -, TIMP-1 absent.

| | Tumour centre | Tumour/mucosal junction | Normal mucosa |
|---|---|---|---|
| Stromelysin$_{EC}$ | 19 | 14 | 0 |
| Gelatinase$_{EC}$ | 17 | 16 | 0 |
| TIMP-1 | 12 | 27 | 8 |
| Interstitial collagenase | 0 | 0 | 0 |

**Table 2.8:** The incidence of MMPs and vascular TIMP-1 in biopsies from 40 colorectal tumours and associated normal mucosa. EC, extracellular.

| | | Tumour Mucosal Junction | | | Tumour | |
|---|---|---|---|---|---|---|
| | | WELL \MOD | POOR | | WELL \MOD | POOR |
| TIMP | + | 20 | 7 | + | 10 | 2 |
| | - | 12 | 1 | - | 22 | 6 |
| | | $X^2$=0.86, P=0.64 | | | $X^2$=0.0074, P=0.93 | |
| | | WELL \MOD | POOR | | WELL \MOD | POOR |
| SL | + | 10 | 4 | + | 16 | 3 |
| | - | 22 | 4 | - | 16 | 5 |
| | | $X^2$=0.3365, P=0.57 | | | $X^2$=0.056, P=0.81 | |
| | | WELL \MOD | POOR | | WELL \MOD | POOR |
| $GL_{EC}$ | + | 11 | 5 | + | 12 | 5 |
| | - | 21 | 3 | - | 20 | 3 |
| | | $X^2$=1.1, P=0.29 | | | $X^2$=0.77, P=0.62 | |

Table 2.9: The distribution of TIMP-1, stromelysin (SL) and extracellular gelatinase ($GL_{EC}$) according to biopsy site and tumour differentiation. WELL\MOD, well or moderately differentiated tumours; POOR, poorly differentiated tumours; +, present; -, absent.

| | | Tumour mucosal junction | | | Tumour | |
|---|---|---|---|---|---|---|
| | | A/B | C | | A/B | C |
| TIMP | + | 12 | 15 | + | 7 | 5 |
| | - | 8 | 5 | - | 13 | 15 |
| | | $X^2$=0.45, P=0.51 | | | $X^2$=0.12, P=0.73 | |
| | | A/B | C | | A/B | C |
| SL | + | 8 | 6 | + | 10 | 9 |
| | - | 12 | 14 | - | 10 | 11 |
| | | $X^2$=0.11, P=0.74 | | | $X^2$=0, P=1.0 | |
| | | A/B | C | | A/B | C |
| $GL_{EC}$ | + | 7 | 9 | + | 7 | 10 |
| | - | 13 | 11 | - | 13 | 10 |
| | | $X^2$=0.104, P=0.75 | | | $X^2$=0.41, P=0.51 | |

Table 2.10: The distribution of TIMP, stromelysin (SL) and extracellular gelatinase (GLEC) according to biopsy site and Dukes' stage. +, present; -, absent.

## PART 2

## THE IMMUNOLOCALIZATION OF THE MMPs AND TIMP-1 IN CULTURED BIOPSIES OF COLORECTAL ADENOCARCINOMA.

### 2.5 AIM

To immunolocalize the MMPs stromelysin, gelatinase and interstitial collagenase and their inhibitor TIMP-1 in biopsies of colorectal cancer which had previously been subjected to a period of tissue culture.

### 2.6 INTRODUCTION

In Part 1 of this study the MMPs stromelysin and Mr 72,000 gelatinase were identified in biopsies of colorectal adenocarcinoma. The majority of the immunostaining was, however, extracellular and therefore the cells responsible for the synthesis of the MMPs could not be identified. In a few tumours intracellular staining was observed and the cells variously identified as fibroblast-like cells and mononuclear leucocytes. Surprisingly, in view of the *in vitro* evidence to support the production of MMPs by tumour cells, none of the immunostaining detected was localized to carcinoma cells. One possible explanation for this is that the level of intracellular enzyme was insufficient for detection, as may arise if the enzyme were synthesised and rapidly secreted rather than stored within the cell. For example the synthesis and secretion of collagenase by human fibroblasts takes 30 minutes (Valle and Bauer, 1979). The comparative ease with which neutrophils containing $M_r$ 95,000 gelatinase can be identified is due to the fact that they store the latent form of the enzyme in secretory granules. To enhance the identification of those cells synthesizing MMPs enzymes, tissue biopsies can be subjected to a period of tissue culture with monensin, a monovalent ionophore, which prevents the translocation of secretory proteins. Its use in tissue culture results in a characteristic vesiculation of the Golgi apparatus and an accumulation of secretory granules (Ledger et al, 1980; Brown et al, 1989). The build up of secretory granules in cells synthesizing MMPs should make them easier to identify microscopically.

## 2.7 MATERIALS and METHODS

**Tissue culture of colonic biopsies for immunohistochemistry.**

All reagents were obtained from Merck, Poole, Dorset, U.K. unless otherwise stated and were of AnalaR grade.

### 2.7.1 *Details of patients*

Tissue from nine of the patients in Part 1 of this study was used. These nine patients are identified by shading in Table 2.3.

### 2.7.2 *Tissue collection*

The method of tissue collection and the biopsy sites were as described in Part 1. The biopsies were bisected and one half mounted in gelatin as previously described while the other prepared for tissue culture.

### 2.7.3 *Tissue culture*

All work involving tissue for culture was carried out in a Bassaire dust-free assembly cabinet (John Bass Ltd, Southampton, UK) using sterile instruments.

The biopsies were initially "sterilized" by washing thoroughly in five changes of Hanks balanced salt solution containing HEPES buffer and antibiotics; the first three of these washes used a solution, that carried ten times the antibiotic concentration of the last two (Appendix 2.1).

The colonic biopsies were set on sterile stainless steel grids and placed in the wells of a six-well tissue culture plate (Nunclon, Denmark) to each of which 4 ml of culture medium had been added. The medium chosen was Dulbecco's modification of Eagle's medium (ICN Flow, High Wycombe, UK) with 1% acid-treated fetal calf serum (ICN Flow, High Wycombe, UK): additions made to the medium are

given in Appendix 2.2. The fetal calf serum was acid-treated to destroy serum inhibitors of MMPs such as $\alpha_2$-macroglobulin. To each well 5μm of a fresh solution of monensin (Sigma, Poole, Dorset) in 100% ethanol was also added. The plates were then incubated in air at 37°C.

In a pilot study to examine the effect of culture on the integrity of the biopsy tissue three periods of incubation were chosen: 6, 12 and 24 hours. The degree of tissue decomposition seen at 12 and 24 hours precluded meaningful histological interpretation and therefore the definitive study used an incubation period of 6 hours.

At the end of six hours the cultured samples were snap-frozen in 7% gelatin with liquid nitrogen for 90 seconds as described in Part 1.

### 2.7.4 *Immunocytochemistry*

The immunohistochemical technique used as well as the method of assessing each tissue section were as described in Part 1.

## 2.8 RESULTS

Table 2.11 compares the immunolocalization results obtained with tissue culture to those of the uncultured portion of the biopsy.

### 2.8.1 *Stromelysin immunolocalization*

The principal difference between cultured and uncultured tissue was the presence of intracellular fluorescence, after incubation with the anti-stromelysin antibody. Intracellular stromelysin was observed in 4 of the cultured tumours (DG, HF, WL, JF), appearing at the tumour/mucosal junctions of each and within the tumour centres of two. As with the distribution of extracellular stromelysin these positive cells were confined to a small portion of the section

examined. The identity of the cells containing the fluorescence was difficult to determine. However, they appeared separate from clusters of tumour cells and were most likely part of the stromal cell population. In one instance it was possible to restain cells which contained intracellular stromelysin with the anti-cytokeratin antibody 8.13 (Sigma, Poole, UK). Cytokeratins are expressed constitutively by epithelial cells and may be used as markers for their presence. The cells with intracellular stromelysin did not stain with the 8.13 antibody strengthening the view that they were stromal in origin (Figs 2.32 & 2.33). No intracellular stromelysin was observed in the uncultured portions of these biopsies.

Despite the stimulus to MMPs synthesis, provided by tissue culture, no stromelysin was detectable in biopsies of normal colonic mucosa.

### 2.8.2 *Gelatinase immunolocalization*

Table 2.11 presents the results for gelatinase immunolocalization, which were obtained using both of the available antibodies to gelatinase. In both cultured and uncultured biopsy material intracellular and extracellular immunofluorescence was observed. Fluorescence, seen with the antibody recognizing only the $M_r$ 95,000 gelatinase, was always intracellular and confined to stromal cells resembling PMNL. By contrast intracellular staining,

| | CULTURED BIOPSIES | | | UNCULTURED BIOPSIES | | |
|---|---|---|---|---|---|---|
| Pt | Tumour | TMJ | NM | Tumour | TMJ | NM |
| DG | $GLB_{IC\&EC}$, $GL95_{IC}$ | $SL_{IC}$ | N | $SL_{EC}$, $GLB_{IC\&EC}$ $GL95_{IC}$ | N | N |
| HF | $GLB_{IC}$ $SL_{IC}$ | $SL_{IC}$ | N | $GLB_{EC}$ | TIMP | N |
| CR | N | N | N | $SL_{EC}$, $GLB_{EC}$ | $GLB_{IC\&EC}$ | N |
| RM | TIMP | TIMP | N | $SL_{EC}$, $GLB_{IC\&EC}$ $GL95_{IC}$, TIMP | TIMP - | N |
| JF | N | N | N | N | N | N |
| JF | N | $SL_{IC\&EC}$ $GLB_{IC\&EC}$ $GL95_{IC}$ | N | $SL_{EC}$ | $SL_{EC}$ | N |
| WL | $SL_{IC\&EC}$, $GLB_{IC}$, $GL95_{IC}$ | $SL_{IC\&EC}$, $GLB_{EC}$, $GL95_{IC}$ | N | $SL_{EC}$, $GL95_{IC}$, $GLB_{IC\&EC}$ | $SL_{EC}$, TIMP | N |
| JL | TIMP, $SL_{EC}$ | $SL_{EC}$, $GLB_{EC\&IC}$ $GL95_{IC}$, TIMP | N | $SL_{EC}$, $GLB_{IC\&EC}$ $GL95_{IC}$ TIMP | $SL_{EC}$, $GLB_{EC}$ | N |
| EF | $GLB_{IC}$ | N | N | N | TIMP | N |

**Table 2.11:** Comparison of the immunolocalization results between cultured and uncultured biopsies from the same tumour. Pt, patient; N, no immunostaining; SL, stromelysin; GLB, antibody recognizing both Mr 72,000 & Mr 95,000 forms of gelatinase; GL95, antibody recognizing only the Mr 95,000 form of gelatinase; IC, intracellular; EC, extracellular. TMJ, tumour/mucosal junction; NM, normal mucosa.

observed with the antibody to both $M_r$ 95,000 and $M_r$ 72,000 forms of gelatinase, appeared to recognize other stromal cells besides PMNL, either fibroblast-like cells or mononuclear leucocytes. That these cells were not of epithelial origin was shown by the use of the 8.13 anti-cytokeratin antibody (Figs 2.34 & 2.35).

Extracellular staining was also present in cultured biopsy material. As with uncultured tissue, the extracellular staining was present in small areas of the

sections and closely associated with clusters of carcinoma cells (2.36).

Apart from an occasional PMNL (presence not recorded in Table 2.11) no staining was observed in normal mucosa.

### 2.8.3 *Interstitial collagenase immunolocalization*

There was no evidence of either intracellular or extracellular immunofluorescent staining to indicate the presence of interstitial collagenase in the cultured tissue biopsies.

### 2.8.4 *TIMP-1 immunolocalization*

Tissue culture did not enhance the detection of TIMP-1. In no instance was TIMP-1 detected in cultured tissue when it was not already detectable in the uncultured portion of the biopsy. Furthermore, in three cultured biopsies from the tumour mucosal junction (HF, WL, EF), TIMP-1 went undetected despite being present in the corresponding uncultured tissue.

## 2.9 SUMMARY OF THE IMMUNOLOCALIZATION FINDINGS FOR TIMP-1 AND THE MMPs INTERSTITIAL COLLAGENASE, GELATINASE AND STROMELYSIN.

i) Apart from the occasional polymorphonuclear leucocyte containing Mr 95,000 gelatinase, MMPs were absent from normal colonic mucosa.

ii) Stromelysin and gelatinase were detected in tumour biopsies.

iii) Interstitial collagenase was not detected in malignant tissue.

iv) Stromelysin and Mr 72,000 gelatinase were predominantly associated with the extracellular matrix and only occasionally observed intracellularly.

v) When stromelysin and Mr 72,000 gelatinase were observed intracellularly the cells concerned were either mononuclear leucocytes or fibroblast like cells; there was no evidence of fluorescent granules in tumour cells for either enzyme even after culture with monensin.

vi) TIMP-1 was detected in biopsies of normal and malignant tissue although it occurred more frequently in the latter.

vii) TIMP-1 was usually observed as intracellular staining associated with endothelial cells but also appeared extracellularly.

vii) The frequency of detection of TIMP-1 was much greater in biopsies from the tumour edge than the tumour centre.

## 2.10 DISCUSSION

Although immunolocalization studies of the MMPs and TIMP-1 in tumours have been conducted previously, this investigation represents the first in which the metalloproteinases stromelysin, gelatinase and interstitial collagenase, and their inhibitor TIMP-1 have been examined together.

Four principal observations were made, i) stromelysin and Mr 72,000 gelatinase were detected in tumour biopsies while being absent from normal mucosa, ii) no interstitial collagenase was identified either in normal or malignant colonic tissue, iii) immunostaining for stromelysin and gelatinase was associated with the extracellular matrix or confined to stromal cells, there was no evidence of tumour epithelial cell staining and iv) TIMP-1 was not only identified in tumour tissue but found to occur more frequently at the tumour mucosal junction than at the tumour centre, or in normal mucosa.

These results must be compared with those of previous studies and also examined in the light of biochemical and molecular biology data relating to the expression of the MMPs in malignant disease.

### 2.10.1 *Interstitial collagenase immunolocalization: comparison with previous immunohistochemical studies.*

There have been only two previous immunohistochemical studies of interstitial collagenase in colorectal cancer namely those by Childers et al (1988) and Hewitt et al (1991).

The study by Childers et al (1988) is only reported in abstract form. Ten colorectal carcinomas were examined; staining for collagenase was confined to the connective tissue stroma, was observed in both normal and neoplastic tissue and appeared to be enhanced at the invading edge of the tumours.

Hewitt's study (1991) comprised twenty colorectal carcinomas and six specimens of normal colonic mucosa. Collagenase was seen as faint staining throughout the connective tissues of five of the normal mucosa specimens; there was no evidence of epithelial cell staining. Within the tumours collagenase staining was more intense, but again almost entirely restricted to the stroma where it was localized to fibroblasts and collagen fibres. As in Childer's study, staining for collagenase was enhanced at the invading edge of the tumour (7 of 10 cases in which this could be assessed). Apart from two carcinomas, in which small groups of tumour cells were positively stained, collagenase was generally absent from neoplastic epithelium. Seven tubular adenomas were also examined, collagenase was identified but the intensity of the staining was less than for the carcinomas. In two of the seven adenomas collagenase staining was also observed in association with the epithelial basement membrane.

The interpretation of MMPs immunolocalization data is critically dependent on the purity and specificity of the antibodies used. In comparing studies from different authors, therefore, a number of questions must be asked. What was the source and purity of the antigen used to raise the antibodies? Were the antibodies raised against the whole enzyme or a peptide sequence(s) from it? What tests of specificity were performed and what forms of the enzyme are recognized, e.g. pro- or activated? Does the antibody cross-react with other members of the MMPs family, and do the results of immunolocalization data agree with the expected biochemical findings?

Given the many points on which antibodies raised to collagenase and indeed other MMPs may differ, comparison between immunolocalization studies is difficult.

That collagenase may have a role in the invasive behaviour of tumours, as reported by both Hewitt and Childers for colorectal adenocarcinomas, is certainly supported by immunolocalization studies conducted on other

human cancers. Collagenase has been localized to the connective tissue elements of basal cell carcinomas (Bauer et al, 1977), cutaneous secondary melanomas (Woolley and Grafton, 1980a) as well as breast and gastric carcinomas (Woolley et al, 1980b). As with colorectal cancer virtually all the staining was confined to the connective tissue matrix and in the studies reported by Woolley the majority of the tumours examined were negative.

These observations contrast markedly with our own in which no interstitial collagenase was identified in either normal or malignant colorectal tissue, even after a period of tissue culture with monensin. It is also of note that in a similar immunolocalization study, using the same antibody as was employed here, no collagenase was identified in the intestines of patients with Crohns disease (Bailey et al, 1990).

The cause of these disparate results is likely to reside in the specificities of the respective antibodies. The anti-collagenase antibodies used in the Hewitt study (Clark and Cawston, 1989) and in the study by Childers (Stricklin et al, 1978) undoubtedly recognize collagenase in both its proenzyme and active forms. However, unlike the anti-collagenase antibody used in this study, neither has been tested for cross-reactivity with other MMPs. Such tests are important for two reasons. Firstly, the high degree of homology which exists between the amino acid sequences of the MMPs (Docherty and Murphy, 1990) predisposes to cross-reactivity, e.g. the degree of homology between human collagenase and human stromelysin is 55% (Whitham et al, 1986); and secondly, spurious antibodies might be produced to other MMPs if these were present as contaminants of the collagenase preparation used to raise the anti-collagenase serum. The significance of these problems is likely to increase as further MMPs are discovered, e.g. PUMP-1 and stromelysin-3 (Woessner and Taplin, 1988; Basset et al, 1990). The antibody used in this study has been shown by inhibition studies not to cross-react with either

stromelysin or gelatinase. This is not true of the antisera used in the other studies. It is therefore possible that the collagenase staining detected was in fact due to other MMPs.

As well as testing the specificity of a potential anti-MMP antibody using *in vitro* techniques, such as immunoblotting and immunoassay, it is also desirable that the antibody should be tested in a "biological system" which allows a correlation to be made between biochemically and immunochemically detectable enzyme. For collagenase, cultured chondrocytes provide such a system. Under normal circumstances these cells make negligible quantities of collagenase. Once stimulated, though, by the addition of conditioned medium from human blood mononuclear cells, large amounts of biochemically detectable collagenase are produced (Trechsel et al, 1982). When unstimulated and stimulated chondrocyte cultures were probed with the anti-collagenase antibody used in our study the amount of immunoprecipitable collagenase was found to correspond with the expected changes in biochemically detectable enzyme (Hembry et al, 1986).

Failure of immunolocalization observations to match biochemical data has been observed previously and has cast doubts on the specificity of the antibody used. In a biochemical study of the rat uterus Woessner (1979) observed that there was negligible collagenase activity in both the non-gravid uterus and in the uterus at the time of parturition. During post-partum involution, however, collagen degradation increased markedly and was accompanied by a rise in collagenase activity. A subsequent immunohistochemical study of collagenase in the rat uterus failed to confirm these findings (Montfort and Perez-Tamayo, 1975). In the reverse of what was expected, the non-gravid uterus demonstrated considerable staining for collagenase, which then diminished markedly during the first 24 hours after parturition. By comparison, when a similar experiment was conducted in the rabbit with the anti-collagenase antibody used in this study, (which cross-reacts with rabbit

collagenase), Woessner's findings were verified (Hembry et al, 1986), confirming the specificity of the anti-collagenase antibody. Without similar observations for the anti-collagenase antibodies used in the other studies, the significance of any immunostaining cannot be fully appreciated. This may be particularly relevant to the observation made by both Childers et al (1987) and Hewitt et al (1991) of collagenase immunostaining in the connective tissue of normal mucosa. Since only active collagenase binds to collagen (Stricklin et al, 1978; Hembry et al, 1986) these findings imply active resorption of the extracellular matrix of normal colonic mucosa. This is surprising as the turnover of formed collagen fibrils in quiescent tissues is thought to be exceedingly slow (Gross, 1981). Immunolocalization studies have failed to confirm the presence of collagenase in other normal tissues such as the rabbit colon (Chowcat et al, 1988) and human skin (Hembry and Ehrlich, 1986). Furthermore, Oyamada et al (1983), using a biochemical assay, failed to detect collagenase activity in normal rabbit colon **unless** it was cultured. Other studies, in which the lysis of collagen gels has been used as a measure of collagenase activity, have also indicated that collagenase is either absent from or present at very low levels in explants of normal colonic mucosa (Hawley et al, 1970; Horowitz et al, 1987). Horowitz et al (1987) for example detected collagenase activity in only 3 out of 34 biopsies of normal colonic mucosa, and then only after 72 hours incubation with the gel.

The progressive removal of amino acid residues results in the conversion of collagenase from its synthesized form as a preproenzyme, to the secreted proenzyme and thence to the active enzyme. Subsequent autolysis of the active enzyme then yields further fragments (Mr 22,000 and Mr 27,000) (Clark and Cawston, 1989). It is possible that the disparity between the observations of the present study and those of Hewitt et al (1991) reside in the ability of the latter's antibody to recognize these autolytic fragments of active

collagenase (Clark and Cawston, 1989); it is not known if our anti-collagenase antibody can also recognize them.

There is another possible explanation for the failure to detect collagenase in this study, and that is that tumour-produced collagenase might differ from that synthesized by normal mesenchymal cells. Under these circumstances antibodies raised against collagenase produced by fibroblasts, for example, might not identify the tumour derived enzyme. Although this possibility cannot be disproved, four pieces of evidence militate against it: i) antibodies raised to collagenase isolated from the medium of cultured rheumatoid synovium have been shown to recognize tumour-derived collagenases (Woolley et al, 1980b), ii) the collagenolytic activity of rabbit VX2 tumour cells in culture has been shown to be immunologically identical to that produced by rabbit articular chondrocytes (Gavrilović et al, 1985), iii) Whitelock et al (1989) failed to demonstrate collagenase in an immunolocalization study of an invasive rat mammary carcinoma despite using antibodies raised specifically to a tumour-derived collagenase and iv) with the exception of two base pairs human melanoma interstitial collagenase has been shown to be identical to human fibroblast collagenase (Templeton et al, 1990).

### 2.10.2 *Collagenase immunolocalization: comparison with biochemical studies.*

There have been six studies in which the collagenolytic activity of colorectal tumours has been assayed, namely Dresden et al, (1972); Sturzaker and Hawley, (1975); Irimura et al, (1987); Tighe et al, (1981); Durdy et al, (1985) and van der Stappen et al, 1990. The findings from these studies have already been discussed in Chapter 1. However, despite the different assays used for collagenase activity, it is worth repeating that each of these studies detected collagenolytic activity in some or all of the tumours examined. The absence of immunolocalizable

collagenase in the present study, therefore, contrasts with these biochemical data.

During the performance of biochemical assays tumour biopsies may variously be homogenized, cultured or incubated with necessary reagents (Birkedal-Hansen, 1987). Through the infliction of tissue damage or by removing the normal restraints on its *in vivo* activity, these processes may distort the natural expression of collagenase. The influence which an *in vitro* technique can have on collagenase expression has been clearly shown by Woolley et al (1980b), who found that a short period of tissue culture (2-4 days) can significantly increase the amount of immunolocalizable collagenase in tumour tissue. The importance of the prevailing microenvironment on collagenase activity has also been emphasised by Woessner (1991), who cites the quite disparate effects which estradiol and progesterone can have on collagenase expression in different circumstances. *In vivo* levels of collagenase were suppressed and collagen loss retarded from the uterus by estradiol whereas it had no effect *in vitro* on cultured uterine cells. Progesterone, by comparison, inhibited collagenase production by cultured uterine smooth muscle cells but had little effect *in vivo*.

The biochemical, or *in vitro*, studies would appear to indicate that colorectal tumours have the potential to produce collagenase. The result of the present immunolocalization study suggests that this potential is not necessarily realized *in vivo*. It is conceivable though that the sampling technique employed in this study is not sensitive enough to detect interstitial collagenase; the very specific action which this enzyme has in breaking the triple helix of the collagen molecule at one particular locus may mean that it has only a transient presence. By examining tumour at one instant in its natural history interstitial collagenase produced at an earlier time may be missed. Support for this theory comes from studies on a rabbit arthritis model in which collagenase is present on day one of the inflammatory process, but gelatinase the

predominant enzyme on days 7 to 28 (Hembry, personnel communication).

### 2.10.3 *Stromelysin immunolocalization*

Unlike collagenase there has been no previous attempt to immunolocalize stromelysin within colorectal tumours. There are, however, reports of members of the stromelysin family being identified in samples of human tumours through the use of molecular probes. PUMP-1 has been reported to be expressed by both stomach and colonic carcinomas while being absent from the adjacent normal tissue, and stromelysin expression has been observed in biopsies of malignant melanoma and glioblastoma (McDonnell and Matrisian, 1990). Basset et al (1990) have identified a gene within the stromal cells of breast carcinomas which encodes for a metalloproteinase enzyme designated stromelysin-3, and Miyazaki et al (1990) have shown that PUMP-1 is secreted by a human rectal carcinoma cell line. These observations suggest that stromelysin may have a role in mediating tumour invasion. By confirming the expression of stromelysin in colorectal carcinomas at the protein level as well as its absence from normal mucosa this study has reinforced that concept. However, it has not been possible to provide unequivocal evidence to support the synthesis of stromelysin by a particular cell type. The predominant observation of stromelysin localized to the extracellular matrix surrounding but not involving clusters of malignant cells, suggests that stromal rather than tumour cells may be responsible for its production. This view was strengthened by the tissue culture study with monensin, in which the intracellular fluorescence generated appeared to occupy cells which were not obviously neoplastic and which failed to stain with the anti-cytokeratin antibody 8.13, a marker for cells of epithelial origin. A stromal origin for stromelysin would agree with the distribution of "stromelysin-3" in breast carcinoma mentioned above but

contradict the findings of the only other immunolocalization study of stromelysin in malignant disease. Conducted on the rabbit VX2 tumour, this study also immunolocalized stromelysin only within the tumour tissue but the enzyme appeared to reside in a small population of tumour cells rather than on the extracellular matrix (Gavrilović et al, 1989).

Allan et al (1991) have shown that both the active and latent forms of stromelysin are capable of binding to the collagen component of the extracellular matrix. Additionally, TIMP only binds to collagen fibrils when the active rather than the latent from of stromelysin is present; by itself TIMP-1 cannot bind to collagen fibrils (Allan et al, 1991). Since the antiserum to stromelysin used in this study is able to recognize both the latent and active enzyme (Allan et al, 1991) it follows that the immunostaining observed on the extracellular matrix must represent one of these two forms. Also noted was that staining for TIMP was generally absent from those areas in which stromelysin was seen. This would imply that TIMP was either actively excluded from these areas, allowing the proteolytic activity of stromelysin to be freely expressed or that the stromelysin present was mainly of the latent form and therefore unable to bind TIMP. The first of these possibilities accords with the view that the promotion of tumour invasion is linked to a down-regulation of TIMP-1 expression (Khokha et al, 1989; Ponton et al, 1991). The alternative explanation, that much of the observed stromelysin is in fact in the latent form, is less easy to explain, and one can only hypothesize as to its significance. It could be that stromal cells synthesize and secrete the latent form of the enzyme as a prelude to tumour-induced matrix degradation. At the right time, given the appropriate microenvironmental conditions, the latent enzyme would then be activated either by the stromal cells themselves or by tumour cells (Reich, 1978).

In the present study there was no correlation between the incidence of stromelysin immunostaining and Dukes' stage or tumour differentiation, nor was there any obvious enhancement of its presence at the tumour/mucosal junction.

### *2.10.4 $M_r$ 72,000 gelatinase immunolocalization.*

This study has demonstrated the presence of $M_r$ 72,000 gelatinase in colorectal cancer. $M_r$ 72,000 gelatinase was seen associated with both the extracellular matrix and as fluorescent granules in stromal cells, particularly fibroblast-like cells. Even after a period of tissue culture there was no indication that the enzyme emanated from tumour epithelial cells. As with stromelysin the presence of $M_r$ 72,000 gelatinase in tumour tissue and its absence from normal mucosa provides support for its role in promoting tumour invasion.

$M_r$ 72,000 gelatinase was confined to small areas of the tissue sections in which it was observed and from which, in the majority of cases, staining for TIMP was absent. It is not known whether the extracellular gelatinase identified represents the active or latent form of the enzyme, or indeed both. $M_r$ 72,000 gelatinase was detected in only 23 of the 40 tumours examined and there was no relation between its presence and Dukes' stage or tumour differentiation.

The substantial body of evidence, much of it from *in vitro* studies, which supports a positive correlation between $M_r$ 72,000 gelatinase activity and tumour cell invasion has already been reviewed in Chapter 1. In addition there is also one study in which the active form of $M_r$ 72,000 gelatinase has been detected in the supernatant of cultured explants of colorectal carcinomas (Yamagata et al, 1991).

There are, however, relatively few studies which have investigated the expression of $M_r$ 72,000 gelatinase at the protein level in human tumours. Two studies have looked at the distribution of $M_r$ 72,000 gelatinase in malignant breast tissue (Barsky et al, 1983; Monteagudo et al, 1990) while

only one study has examined its presence in colorectal cancer (Levy et al, 1991).

In a study of 70 colorectal tumours Levy et al (1991) immunolocalized $M_r$ 72,000 gelatinase to the cytoplasm of tumour epithelial cells. There was a significant increase in the number of positive cells when adenomas were compared with carcinomas, and when Dukes' stage A/B tumours were compared with Dukes' C tumours. Monteagudo et al (1990) and Grigioni et al (1991) in studies of breast cancer and hepatocellular carcinoma respectively , and using the same antibody as was employed by Levy et al (1991), have also localized the source of $M_r$ 72,000 gelatinase to the malignant cells rather than to those of the stroma. These results clearly conflict with those presented here. As with the immunolocalization of collagenase the explanation is likely to reside in the specificity of the antibodies used.

The polyclonal antibodies employed by Levy et al (1991) were raised against two synthetic peptide sequences from $M_r$ 72,000 gelatinase: the 17 N-terminal amino acids of the pro-enzyme (Peptide $H_1$) and a sequence of 16 amino acids corresponding to the putative metal ion-binding domain (Peptide MBR) (Monteagudo et al, 1990).[1] The antibodies were characterized by Western blot analysis and found to react with a single species in human melanoma cell conditioned media that corresponded to purified $M_r$ 72,000 gelatinase. Employing similar methods, the effectiveness of using synthetic peptides to generate antibodies to $M_r$ 72,000 gelatinase has also been confirmed by Coetzer et al (1991). However, while these anti - $M_r$ 72,000 gelatinase antibodies undoubtedly recognize the enzyme *in vitro*, their specificity for the enzyme *in vivo* remains in question. In contrast to the antibodies used in the present study (Murphy et al,

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**[1]Monteagudo refers to the 17 N-terminal amino acids of type IV precollagenase ($M_r$ 72,000 gelatinase). These residues are in fact the N-terminal amino acids of the pro enzyme (Docherty and Murphy, 1990) and are not part of the 20 or so amino acids which precede the N-terminal amino acids and are thought to comprise a signal sequence which is cleaved off during secretion.**

1989a; Hipps et al, 1991) neither of the anti-peptide antibodies has been validated by the immunolocalization of $M_r$ 72,000 gelatinase in tissues or cells known to be synthesizing the enzyme. There is therefore the possibility that the antibodies used by Levy et al are inadvertently recognizing peptide sequences from other proteins, producing a false positive result. It is also not apparent why Levy et al (1991) failed to detect immunostaining for $M_r$ 72,000 gelatinase extracellularly. While the antibody to peptide H1 (which recognizes a sequence from the pro-domain of latent $M_r$ 72,000 gelatinase) would be expected to immunolocalize the enzyme intracellularly, it might also be anticipated that the antibody to the metal binding site would identify the enzyme extracellularly, which it clearly does not. These doubts have been given added weight by the findings of a recent study which sought to localize the mRNA for $M_r$ 72,000 gelatinase in colorectal cancers by the technique of *in-situ* hybridization (Poulsom et al, 1992). Contrary to what was anticipated, labelling for $M_r$ 72,000 mRNA was much stronger within the stroma of the tumours compared to tumour epithelial cells. The distribution of the labelling corresponded to that of fibroblastic cells and was heaviest at the lateral aspects of penetrating glands. This observation complements that of the present study where $M_r$ 72,000 gelatinase was immunolocalized to fibroblast-like cells often closely aligned to the periphery of neoplastic cell clusters.

It is possible to propose explanations for the disparity in the distribution of $M_r$ 72,000 gelatinase as defined by the *in-situ* hybridization study of Poulsom et al (1992) and the immunolocalization study of Levy et al (1991).[2] Tumour cells could, for example, contain only a small amount of mRNA the majority of which is translated

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**[2]In the same study Poulsom et al also confirmed the cytoplasmic immunolocalization of $M_r$ 72,000 gelatinase reported by Levy et al (1991) by using a monoclonal antibody, raised to a peptide fragment in the pro-domain of the enzyme.**

into gelatinase, while fibroblasts might contain relatively large quantities of mRNA with little being converted into the protein message. Failure to detect mRNA in tumour epithelial cells might also reflect differences in the stability of fibroblast mRNA compared to tumour cell mRNA in formalin-fixed paraffin tissue. Whether or not such alternative explanations exist remains to be seen. For the present, the correlation between the *in-situ* hybridization results of Poulsom et al (1992) and the immunolocalization observations reported here challenge the previously held concept that tumour cells are the principal source of matrix-degrading metalloproteinase enzymes.

### *2.10.5 $M_r$ 95,000 gelatinase immunolocalization*

$M_r$ 95,000 gelatinase was confined to neutrophils and macrophages in which were seen secretory granules containing the enzyme. No $M_r$ 95,000 gelatinase was observed in fibroblast-like cells and no extracellular $M_r$ 95,000 gelatinase was observed. These observations confirm that $M_r$ 95,000 gelatinase is not constitutively expressed by fibroblasts (Hipps et al, 1991). Unlike $M_r$ 72,000 gelatinase there are no other studies with which these results might be compared.

In common with tumour cells neutrophils have a requirement to cross connective tissue barriers, e.g. the basement membranes of capillary endothelium. It is perhaps not surprising, therefore, that they should contain $M_r$ 95,000 gelatinase which would enable them to degrade the type IV collagen content of basement membranes, thereby facilitating their migration.

### 2.10.6 *Immunolocalization of TIMP-1*

When seen, TIMP-1 was predominantly localized to the endothelial cells of blood vessels. In the three instances where TIMP-1 was localized to the extracellular matrix it was always to an area of the tumour in which MMPs had been identified on adjacent sections. Where TIMP-1 appeared to be bound to the matrix it was assumed that the anti-TIMP antibody was identifying TIMP-1 complexed to either $M_r$ 72,000 gelatinase or stromelysin since free TIMP-1 is unable to bind to collagen fibrils (Allan et al, 1991). There was a significant difference in the distribution of TIMP-1 not only between normal and neoplastic tissue but also within the tumours themselves. The incidence of TIMP-1 in biopsies from normal colonic mucosa compared to those taken from the tumour (tumour or tumour mucosal junction biopsies) was significantly greater (8/40 vs. 28/40, $X^2$ = 18.23, $p < 0.001$), as was the incidence of TIMP-1 at the tumour mucosal junction compared to the tumour centre (27/40 vs. 12/40, $X^2 = 18.27$, $p < 0.001$). These observations support the finding that levels of TIMP-1 mRNA in colonic adenocarcinomas were elevated compared to those of adjacent normal tissue (Stetler-Stevenson et al, 1990b). Measurements of TIMP-1 at the protein level have also been made using an enzyme-linked immunoabsorbent assay (ELISA), and these confirm that the expression of TIMP-1 in colonic tumours is elevated over that of the normal mucosa (Lu et al, 1991). Variations in the detection of TIMP-1 according to the biopsy site could also account for the wide range of the measurements (98 - 936 ng/mg) in the latter study. There was no correlation between the detection of TIMP-1 and either Dukes' stage or tumour differentiation in the present study. This is in agreement with the ELISA study (Lu et al, 1991) where in fact Dukes' B tumours showed the greatest abundance of TIMP-1. An unexpected observation was the significant relationship between the presence of TIMP-1 in normal mucosa vessels and its appearance at the tumour/mucosal junction.

All eight patients in whom TIMP-1 was detected in biopsies of their normal mucosa also expressed TIMP-1 at the invading edge of the associated tumours. This may indicate that patients who express TIMP-1 constitutively in their normal mucosa are better able to mount a TIMP response in areas where connective tissue remodelling is taking place, e.g. at the invading edge of the tumour.

As with the matrix metalloproteinases few studies have examined the expression of TIMP-1 in human colorectal cancers, or indeed other tumours, at either the protein level by immunolocalization or the molecular level by *in situ* hybridization. Hewitt et al (1991) in an immunolocalization study of TIMP found weak staining to mark its presence throughout the connective tissues of normal colonic mucosa, but no vascular staining. Unlike the present study some degree of staining for TIMP was present in all of the 20 colorectal tumours examined and was confined almost exclusively to stromal components, particularly collagen fibres, epithelial basement membranes and fibroblasts. Apart from a few small groups of cells in 3 of the carcinomas there was little evidence to point to TIMP synthesis by neoplastic cells. Vascular staining for TIMP was seen in 15 of the tumours. The inability of free TIMP to bind to collagen fibrils (Allan et al, 1991) suggests that the staining observed represents TIMP complexed to MMPs. This would imply that the majority of the collagenase staining, also reported (and discussed above) represents enzyme inhibited by TIMP since their staining patterns were similar. Interestingly, however, TIMP was relatively absent at the invasive edge of the tumours suggesting that collagenase activity here was unhindered. A similar distribution of TIMP has also been reported by Childers et al for colorectal cancers (1988) and for basal cell carcinomas (1987). In a study of malignant non-Hodgkin's lymphomas, TIMP-1 was localized to the tumour stroma by the technique of in-situ hybridization; endothelial cells were found to label particularly strongly, no labelling was

present in tumour cells (Kossakowska et al, 1991). In a similar study, but of colorectal tumours, TIMP-2 was found predominantly in the stroma with weak labelling of endothelial cells (Poulsom et al, 1992).

In so far as these studies have immunolocalized TIMP to the tumour stroma there is some agreement between them and the present investigation. The points of difference appear to be in the frequency with which TIMP is detected and the extent of its distribution particularly within the extracellular matrix. As before the individual characteristics of the antibodies used may be responsible for these dissimilarities. However, in this instance the *in vitro* specificity of the polyclonal antiserum used by Hewitt et al (1991) has been tested in the same way, and found to give similar results (Clark et al, 1991) to the anti-TIMP antibody used in this study (Hembry et al, 1985). The only difference between the two is that the specificity of the antibody used in the present study has been further validated *in vivo* by its successful detection of TIMP secreted by human foetal lung fibroblasts (Hembry et al, 1985). Neither of the two antibodies is able to recognize TIMP-2 (Clark et al,1991; Ward et al, 1991).

On the available evidence it is not possible to determine whether the study by Hewitt et al or the present work gives the truer picture of TIMP expression in colorectal carcinomas. Help in resolving the differences could come from an investigation in which the two antibodies were used under identical conditions on similar tissue sections.

### 2.10.7 *Overview of matrix metalloproteinase and TIMP-1 expression in colorectal cancer.*

This study has examined the presence of matrix metalloproteinases and TIMP-1 in colorectal adenocarcinomas. Notwithstanding the results of other investigations which have been discussed above, the view is presented that the

expression of these enzymes at the protein level need not necessarily correspond with that which might be inferred from either biochemical or molecular probe methods.

The potential of colorectal tumours to produce collagenase under conditions of culture in vitro (Dresden et al, 1972; van der Stappen et al, 1990) has not been confirmed in vivo. This would support the contention that there is no absolute requirement for the deployment of interstitial collagenase in the promotion of tumour invasion (Pauli et al, 1986; Zucker et al, 1985). Indeed interstitial collagenase is incapable of breaking the cross links which exist between collagen molecules and which are therefore essential to the integrity of the collagen fibril (Murphy and Reynolds, 1985). An alternative mechanism for the degradation of interstitial collagens must therefore exist. Enzymes with a broader substrate specificity than interstitial collagenase such as trypsin and plasmin, which have the capacity to degrade the non-helical regions of tropocollagen molecules where the intermolecular cross links are sited, might well have a role (Pauli and Knudson, 1988). Their action would destabilize collagen fibrils leaving them prey to further denaturation. The "loosening" of the matrix which would ensue might then be sufficient to allow tumour cells to migrate along a path of least resistance. This process of invasion could be facilitated by the development of interstitial oedema produced by the hydration of glycosaminoglycans, which had formerly been contained by an intact collagen network. Tissue oedema has been shown to be of importance not only in tumour invasion (Gabbert, 1985) but also in the cell movements which take place during embryogenesis (Toole BP, 1981).

While interstitial collagenase may have been absent from colorectal tumours the presence of two other MMPs enzymes was confirmed, namely stromelysin and gelatinase. I*n vitro* studies have correlated the metastatic potential of epithelial-derived tumour cells with their ability to secrete matrix-degrading enzymes, particularly gelatinase

(type IV collagenase) (Liotta et al, 1980; Turpeenniemi-Hujanen et al, 1985; Collier et al, 1988). It was therefore unexpected when both stromelysin and $M_r$ 72,000 gelatinase were immunolocalized either to the extracellular matrix or to cells of stromal origin, principally fibroblast-like cells and tissue macrophages. The findings for $M_r$ 72,000 gelatinase have been supported by an *in situ* hybridisation study in which the mRNA for this enzyme was found predominantly in the stroma of colonic tumours (Poulsom et al, 1992). The stromal localization of MMPs is in line with other studies which have immunolocalized proteolytic enzymes, thought to be important in tumour invasion, to the extracellular matrix. Urokinase-type plasminogen activator has been shown confined to fibroblast-like cells in colonic tumours (Grøndahl-Hansen et al, 1991) and cathepsin B has been localized to the fibroblasts and matrix surrounding tumour cell clusters in the rabbit V2 carcinoma (Graf et al, 1981). Collagenase also appears associated with the surrounding collagen matrix of tumours when its production is observed following tissue culture (Woolley et al, 1980b). These observations suggest that the remodelling of the connective tissue matrix which occurs as a part of tumour invasion may be the result of cellular interactions between tumour and stromal cells (Woolley, 1984). In the present study the immunolocalization of stromelysin and $M_r$ 72,000 gelatinase to the peritumoural stroma and fibroblasts may be evidence of the necessary close relationship which must exist between the two. A similar relationship has been observed in basal cell carcinomas, the fibroblasts of which show an increased capacity to synthesize and secrete collagenase which can be immunolocalized around islands of tumour cells (Bauer et al, 1977; Bauer et al, 1979). The ability of tumour cell lines to release collagenase from cultures of stromal fibroblasts has also been related to their metastatic potential (Dabbous et al, 1987).

If the mechanism of tumour invasion does involve

co-operation between tumour cells and fibroblasts, or indeed other stromal cells, then the nature of the cellular messengers which link them requires elucidation. A variety of stimuli have been identified which can induce the transcription of genes for stromelysin and collagenase, amongst them epidermal growth factor, platelet derived growth factor and the cytokine interleukin-1 (Alexander and Werb, 1989). Most of these agents appear to alter the actin cytoskeleton of fibroblasts (Werb et al, 1986), a change which has been correlated with the induction of metalloproteinase expression (Frisch and Werb, 1989). In this respect it is of interest to note that skin fibroblasts obtained from patients with familial polyposis coli show abnormal properties including disorganization of their actin content. There is also evidence to suggest that these skin fibroblasts exist in an "initiated state" due to a dominant mutation, and that the expression of malignancy requires only the addition of a tumour promoting agent such as a phorbol ester (Kopelovich et al, 1979). If similar changes were also present in fibroblasts from the colons of these patients it is tempting to speculate that they might be linked to the process of malignant transformation which characterizes this disease. The potential importance of the stroma to carcinogenesis was shown by Hodges et al (1977) in a series of transplantaion experiments involving carcinogen-treated rat urinary bladders. When untreated epithelium was placed on the stroma from such bladders its behaviour changed to resemble that of neoplastic epithelium. Schor et al (1985) have hypothesized that the presence of abnormal fibroblasts may disturb the normal relationship between stroma and epithelium in a way which makes malignant change more likely.

A separate signalling system, not linked to changes in cell shape or cytoskeletal architecture, also exists for the production of MMPs by fibroblasts. The binding of degradation products, arising from fibronectin, to the fibronectin receptor on fibroblasts induces the expression

of stromelysin and collagenase (Werb et al, 1989b). This shows the potential importance of a family of membrane receptors known as the integrins, of which the fibronectin receptor is a member, to the control of extracellular matrix remodelling.

The substrate specificities of stromelysin and $M_r$ 72,000 gelatinase, encompassing proteoglycans, laminin and type IV collagen, make them ideally suited to the degradation of basement membranes, seen as a key step in the transition from a benign to a malignant tumour (Liotta et al, 1983). The immunolocalization of stromelysin and gelatinase within colonic carcinomas as opposed to benign papillomas and normal mucosa lends support to the hypothesis that these enzymes are positively correlated with malignant transformation and the metastatic phenotype (Matrisian et al, 1991; Stetler-Stevenson, 1990). In a chemical carcinogenesis model that produced malignant tumours with a high probability of metastasis, the tumours were shown to contain exceptionally high levels of stromelysin mRNA transcripts Ostrowski et al (1988). Conversely down-regulation of $M_r$ 72,000 gelatinase activity by retinoic acid resulted in loss of the invasive phenotype by human melanoma cells (Nakajima et al, 1989).

When adjacent sections respectively stained for stromelysin and $M_r$ 72,000 gelatinase were compared, the distributions of the enzymes were found to differ. This suggests that their synthesis and secretion are not co-ordinately regulated at least spatially.

Both stromelysin and $M_r$ 72,000 gelatinase were confined to small areas of the tissue sections examined, often surrounding or adjacent to a single clump of neoplastic cells. This limited expression was also reflected in the infrequency (< 50%) with which either enzyme was detected in the various biopsies. Poste and Fidler (1980) have advocated that within a tumour there exist specialized subpopulations of cells with an enhanced capacity to metastasize. The circumscribed presence of stromelysin and $M_r$ 72,000

gelatinase may point to the existence of such cells, with the ability either to synthesize MMPs or else to induce MMPs synthesis in other cell types. As an alternative, the majority of cells in a tumour may be able to secrete stromelysin or gelatinase, but do so only on receipt of the appropriate signal; by viewing events at one instant only, i.e. the time of biopsy, only a small proportion of these cells are revealed.

The restricted expression of stromelysin and $M_r$ 72,000 gelatinase may also reflect the existence of *in vivo* controls to regulate their activity. The distribution of the mRNA for $M_r$ 72,000 gelatinase (Poulsom et al, 1992) was found to be more extensive than that of the protein product as demonstrated by the present study. This may indicate control of MMPs expression in colorectal tumours at the level of transcription. MMPs activity can also be regulated through the conversion of the latent proenzyme to the active form (Murphy et al, 1990). Although the antibodies to stromelysin and $M_r$ 72,000 gelatinase are able to identify both the active and latent forms of these enzymes, it is not certain which are being detected *in vivo* or in what proportion. Stromelysin, for example, could be present in both forms, with the relative amount of the active enzyme determined by the presence of appropriate activators. With the exception of gelatinase, plasmin has been cited as a possible *in vivo* activator of matrix metalloproteinases (Saksela and Rifkin, 1988; Gavrilović and Murphy, 1989) and a cascade of reactions, involving the generation of plasmin from plasminogen by the plasminogen activator urokinase, may be important in regulating local matrix metalloproteinase activity. *In vitro*, the enhanced secretion of urokinase has been shown to facilitate the metastatic process (Yu and Schultz, 1990), and like the MMPs, urokinase has also been immunolocalized to fibroblast-like cells in colonic cancers (Grondahl-Hansen et al, 1991). The mechanism whereby progelatinase is activated in vivo remains to be elucidated (Murphy et al, 1990). Recent in vitro work, however,

suggests that the activation of $M_r$ 72,000 gelatinase may be initiated by a fibroblast membrane-bound molecule which induces a conformational change in the enzyme followed by self-cleavage (Ward et al, 1991).

The MMPs may also be constrained by the nature of the physiochemical environment in which they operate. Classically the MMPs function at neutral pH, a feature which confines their action to the extracellular matrix. However, unlike the other members, stromelysin has the ability to degrade substrates over a wider range of pH values (Galloway et al, 1983). This property would enable it to act not just extracellularly but also within the pericellular environment where the pH is thought to be more acidic (Eden et al, 1955; Sutherland, 1988). The immunolocalization of stromelysin close to clusters of tumour cells may be evidence of such pericellular activity.

The infrequency with which stromelysin and $M_r$ 72,000 gelatinase were detected may also indicate the rate at which the extracellular matrix is degraded. Unlike the rapid post-partum resorption which takes place in the uterus (Woessner, 1979) matrix turnover in tumours is likely to be a much slower process. Teleologically, unrestricted MMPs activity, even in the context of a pathological process, may not be desirable. In his "three-step" theory of tumour invasion Liotta (1986) has emphasized the importance of tumour cell movement into regions of the matrix modified by proteolysis. Cellular locomotion requires the sequential attachment and detachment of the cell to a suitable matrix substrate, e.g. fibronectin (Rollins and Culp, 1979). It follows that the wholesale destruction of the extracellular matrix would deny tumour cells the essential scaffold they require for their motility.

The lack of correlation between detectable stromelysin and $M_r$ 72,000 gelatinase, and either the stage or the differentiation of the tumours examined is perhaps not surprising. The expression of both these enzymes is certain to be heterogeneous, varying both spatially and

temporally throughout the natural history of the tumour. The technique of immunohistochemistry provides only a "snap-shot" of the contribution of these enzymes to the proteolytic activity of the tumour at one instant in time. More meaningful comparisons between MMPs activity, tumour invasiveness and metastatic capability might be made if the potential of a tumour to synthesize the enzyme could be estimated. An analysis of the level of mRNA transcripts for stromelysin and $M_r$ 72,000 gelatinase might provide a base from which tumour behaviour could be predicted.

This study has gone some way to identifying the relative contributions made to matrix remodelling in tumours by the two types of gelatinase, $M_r$ 72,000 gelatinase and $M_r$ 95,000 gelatinase. Although some investigators have hinted at a relationship between $M_r$ 95,000 gelatinase and the metastatic phenotype (Moll et al, 1990; Bernhard et al, 1990), this study found that the enzyme was confined exclusively to polymorphonuclear leucocytes. No extracellular $M_r$ 95,000 gelatinase was observed. This may be an indication that the action of $M_r$ 95,000 is confined to mediating the dissolution of matrix necessary for the migratory behaviour of polymorphonuclear leucocytes.

The activity of the MMPs may be further controlled by the action of the specific matrix metalloproteinase inhibitor TIMP-1. By demonstrating a difference in the expression of TIMP-1 between normal and malignant tissue this immunohistochemical study has strengthened the case for TIMP-1 as a regulator of matrix degradation during tumour invasion. In the majority of cases examined TIMP-1 was absent from those areas of the tumour which had stained positively for MMPs. This would appear to support the hypothesis that the focal degradation of the extracellular matrix in tumour invasion takes place as a result of a down-regulation in TIMP-1 expression (Hicks et al, 1984; Khokha et al, 1991; Ponton et al, 1991).

Endothelial cells were by far the most frequent site for the immunolocalization of TIMP-1. Although the precise

role of TIMP-1 at this location is not known, the process of angiogenesis is not dissimilar to that of tumour invasion. To establish new vessels endothelial cells must invade adjacent tissues. In vitro studies have implicated MMPs in angiogenesis, and inhibitors of these enzymes can block the invasion of endothelial cells through artificial basement membranes (Mignatti et al, 1989). TIMP-1 is an inhibitor of angiogenesis *in vivo* (Bouck, 1990) and it may be in the discharge of this role that its expression is determined in colorectal cancer. For tumours to progress they must first initiate neovascularization of the host tissues, a process which will be most pronounced at the invading edge of the tumour (Folkman, 1985). It is perhaps significant then, that TIMP-1 was detected most frequently at the tumour/mucosal junction where it could potentially regulate the angiogenic process. This finding is in agreement with an earlier observation made by Ponton et al (1991) who showed that TIMP mRNA was concentrated near the edge of tumours derived from a murine mammary carcinoma cell line. *In vitro* studies indicate that endothelial cells produce both metalloproteinases and TIMP, suggesting that proteolytic activity is restricted by a balance between the two (Herron et al, 1986). The existence of a low molecular weight angiogenic factor, known as endothelial cell stimulating angiogenesis factor, which can reactivate TIMP/collagenase and TIMP/gelatinase complexes (McLaughlin et al, 1991) provides a further link between TIMP and new vessel growth. To what extent the presence or absence of TIMP-1 at the tumour periphery influences colorectal tumour behaviour is unknown. Consideration of the 40 tumours examined revealed no relationship between the likelihood of detecting TIMP-1 at the tumour edge and either Dukes' stage or tumour differentiation. Whether or not TIMP-1 expression affects survival remains to be seen. In a study based on Northern blot analysis of the mRNAs obtained from non-Hodgkin's lymphomas there was the suggestion that elevated TIMP-1 expression was associated with a more aggressive tumour

phenotype (Kossakowska et al, 1991). Although the presence of the mRNA need not necessarily reflect the level of the protein product this observation nevertheless contrasts with the proposal that TIMP-1 actually protects against invasive tumour behaviour. In a study of intracranial tumours there was a tendency for higher levels of TIMP to be produced by noninvasive meningiomas compared to their more invasive counterparts (Halaka et al, 1983); and Barsky et al (1988) have correlated the unusual resistance of cirrhotic livers to metastatic disease with their high endogenous concentration of a metalloproteinase inhibitor. With respect to this it is worth noting that no TIMP-1 was identified in the two cases of hepatic metastasis which were examined in the present study. Experimental evidence also indicates that the ability of highly metastatic cells to colonize the lungs, after their intravenous injection into nude mice, is greatly reduced by the repeated intraperitoneal administration of recombinant TIMP (Alvarez et al, 1990). It is therefore possible that the presence of TIMP-1 in endothelial cells offers a source of inhibitor which can attenuate the action of MMPs on the extracellular matrix or protect against vascular invasion by tumour cells. Jones et al (1981) have shown that the invasion of artificial blood vessels by fibrosarcoma cells can be markedly reduced by the addition of an endothelial cell layer. Endothelial cells also inhibited the collagenolytic behaviour of melanoma cells when the two were co-cultured in the presence of a collagen substrate (DeClerk and Lang, 1986).

Hall and Lemoine (1991) have developed four criteria which must be satisfied before an hypothesis based on the observations of molecular biology can be accepted: i) the cellular and molecular events common to a given pathological process must be identified; ii) the sequence of these events must be characterized spatially and temporally; iii) in a model system introduction of the observed changes leads to the altered phenotype; and iv) in a suitable model system

correction of the defect rectifies the abnormalities of the disease process.

This study by identifying the confinement of the MMPs (gelatinase and stromelysin) to malignant tissue may be said to provide evidence to satisfy the first of these criteria. Evidence, much of it from *in vitro* experiments, has also accumulated to support the theory that the acquisition of the malignant phenotype requires tumour cells to express matrix-degrading enzymes. This evidence may be said in its way to satisfy the other three criteria. However, this immunolocalization study, by failing to confirm the synthesis and secretion of MMPs and TIMP-1 by malignant epithelial cells in colorectal cancer has opened the door on a second hypothesis: the dissolution of connective tissue required for tumour invasion may be mediated by changes in MMPs production by the stromal cell population.

## ILLUSTRATIONS FOR CHAPTER 2

The following pages contain the figures for Parts A and B of Chapter 2. The illustrations have been prepared from the original photographs using a colour laser printer.

Apart from those figures which show sections stained with haematoxylin and eosin, or an immunoperoxidase technique, all the illustrations represent the view obtained when immunostained tissue was viewed with standard narrow or wide band FITC filters, or a rhodamine filter.

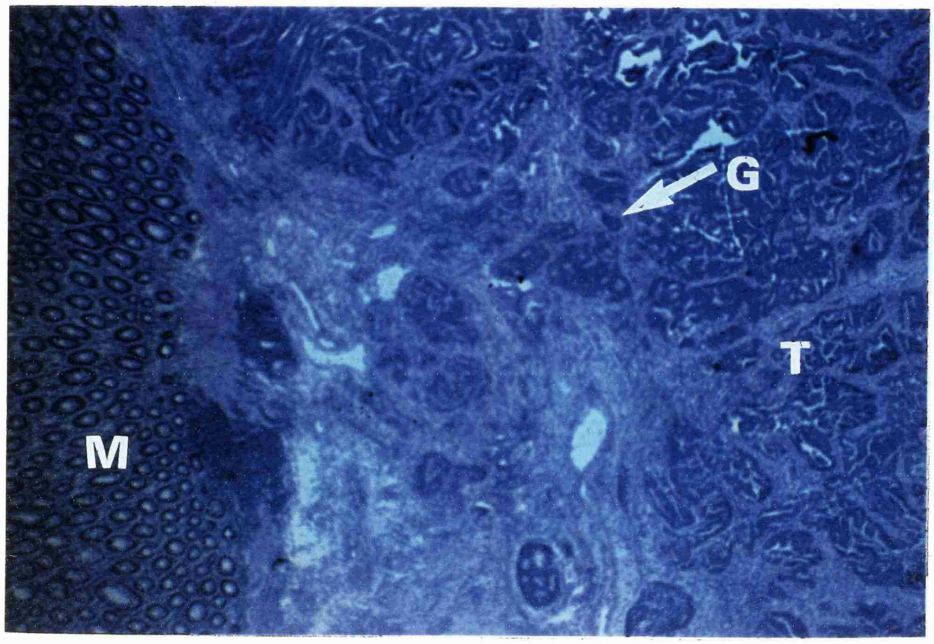
Sections stained using an immunofluorescent technique were counterstained with methyl green to aid visualization of the cellular architecture. When this technique is used and the section viewed under either a wide band FITC filter or a rhodamine filter the **nuclei of cells fluoresce red** and this is how they appear in the illustrations. Fluorescence marking **the presence of the MMPs or TIMP appears green** in the photographs.

**Figure 2.1**

Section taken through the tumour/mucosal junction of a moderately differentiated Dukes' C carcinoma of the descending colon; normal mucosa (M) is present on the left and tumour (T) on the right. Figures 2.2 to 2.4 refer to a malignant gland whose position is indicated by the arrow G (Haematoxylin and eosin; original magnification x20).

**Figure 2.2**

The malignant gland (G) identified by the arrow in Figure 2.1 (Haematoxylin and eosin; original magnification x250).


G
T
M

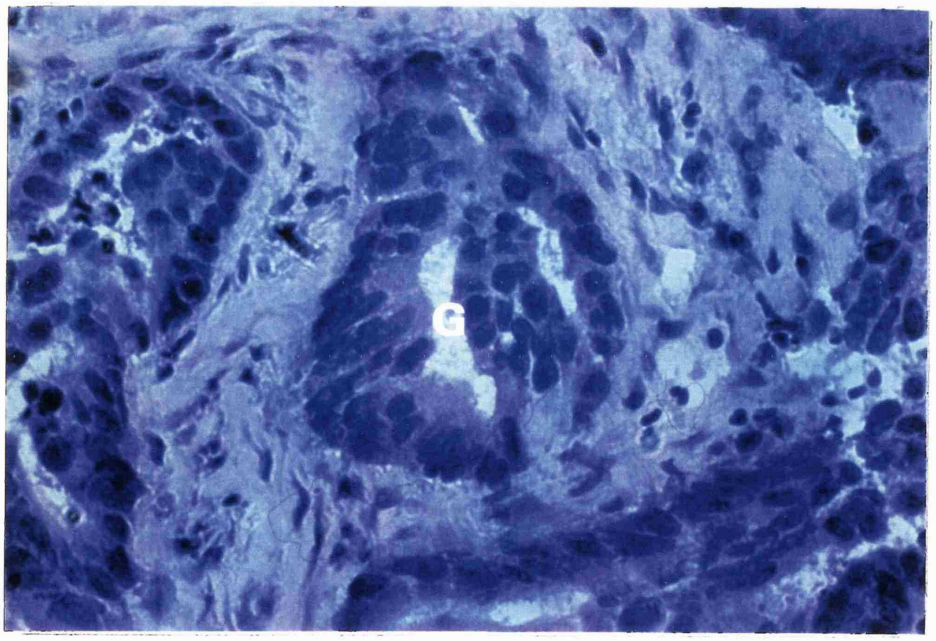
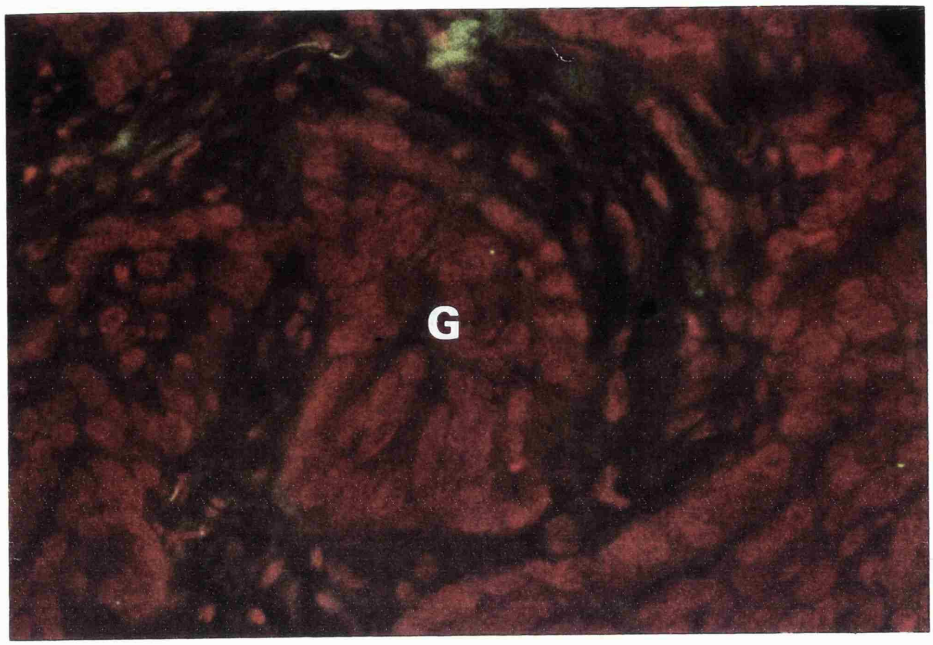

G

**Figure 2.3**

A control section, incubated with non-immune sheep serum, showing the malignant gland (G) identified in Figure 2.2. Apart from a tiny amount of non-specific staining present at the top of the photograph this control section was essentially negative in contrast to the appearance seen in Figure 2.4 (original magnification x250).

**Figure 2.4**

Section, incubated with the antiserum to stromelysin, showing the same malignant gland (G) as was identified in Figure 2.3. Stromelysin is identified as streaks of green fluorescence associated with the extracellular matrix surrounding the malignant gland. Some of the staining may reside within connective tissue cells although the density of the staining makes this difficult to see. No staining is seen within the tumour cells forming the gland G (original magnification x250).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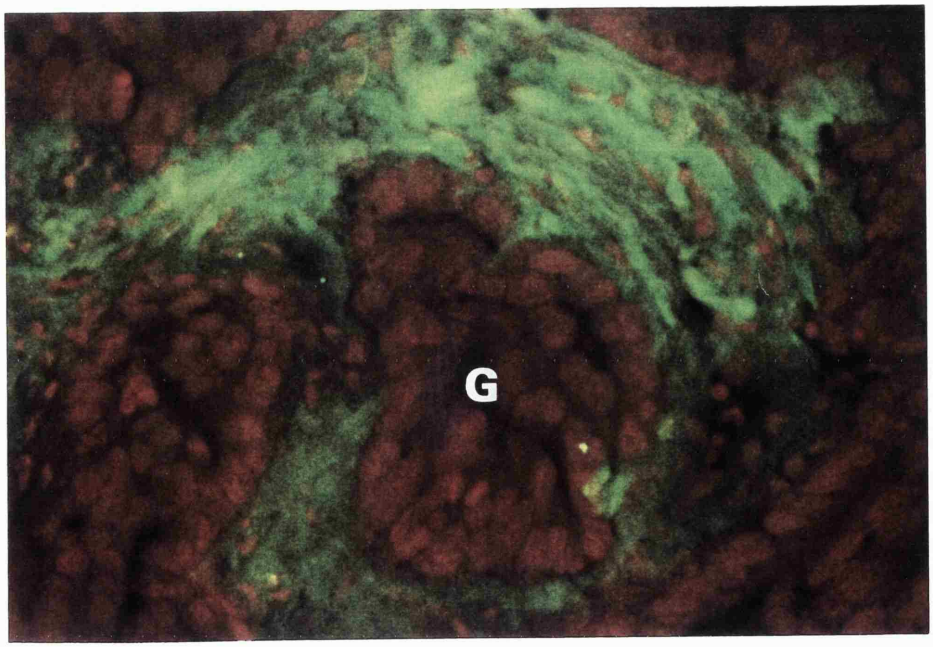

G

**Figure 2.5**

Section taken from a moderately differentiated adenocarcinoma of the colon. Stromelysin is identified as streaks of green immunofluorescence (arrows) which surround a neoplastic gland (NG). Once again the staining appears confined to the connective tissue stroma between adjacent glands (original magnification x250).

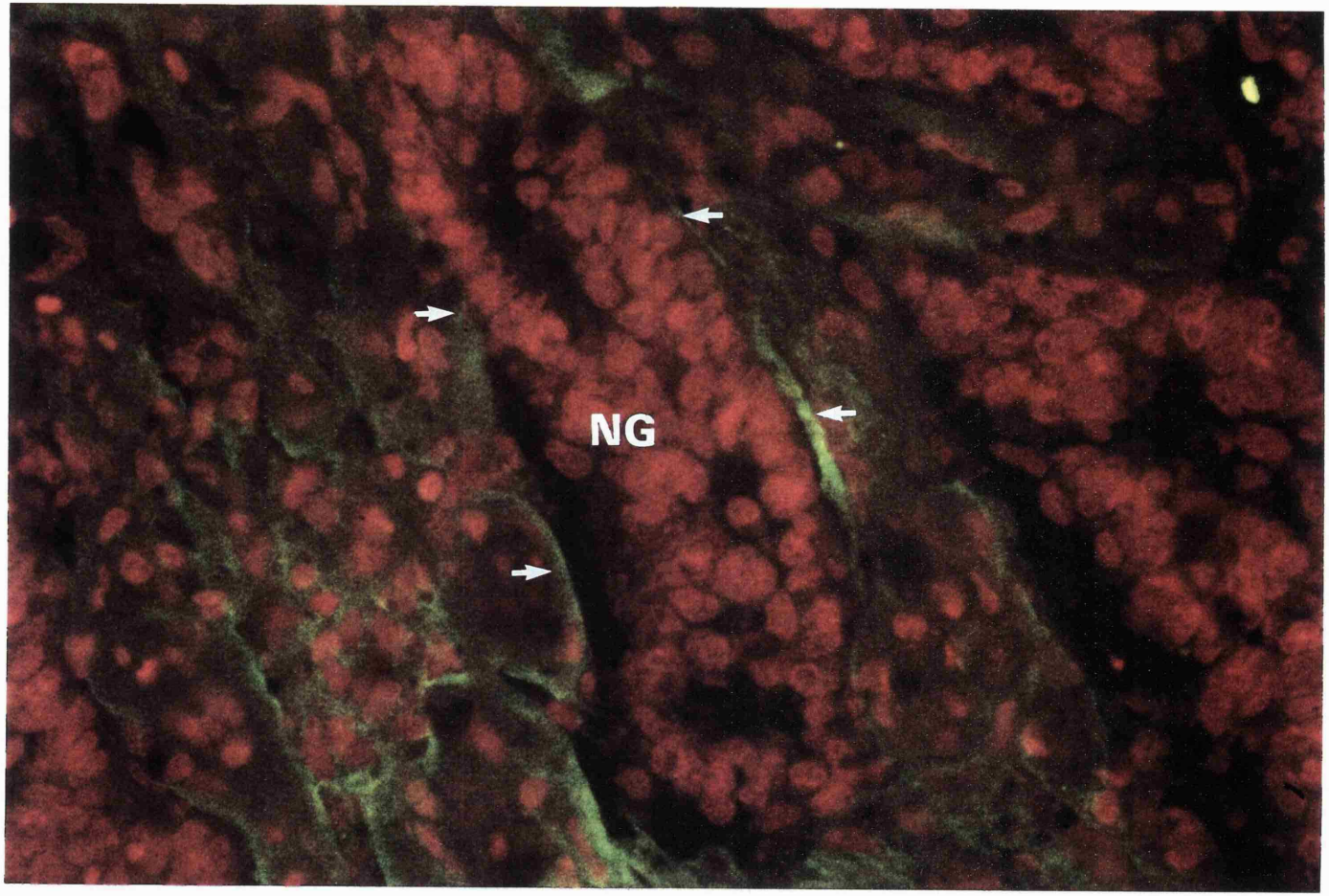
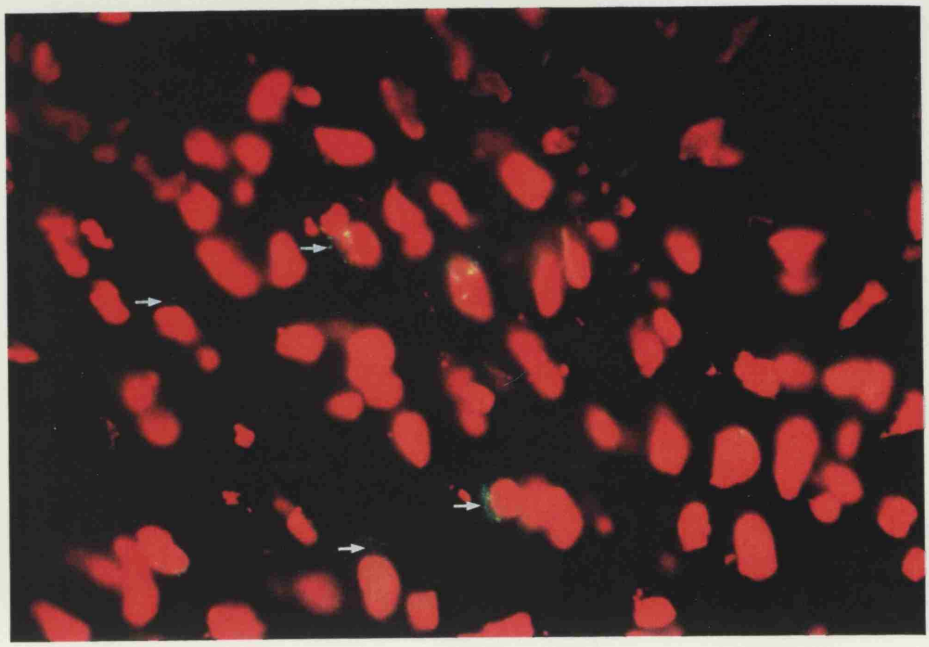

NG

**Figure 2.6**

Section from the tumour/mucosal junction of a moderately differentiated carcinoma of the rectum. The arrows indicate fluorescent granules indicating the presence of stromelysin associated with mononuclear cells lying in the stroma between malignant glands. The area of the tumour in which these cells were found is shown in Figure 2.17 (original magnification x500).

**Figure 2.7**

Section showing a blood vessel (bv) within a moderately differentiated Dukes' B carcinoma of the rectum. The section has been incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase, and viewed under a narrow band FITC filter. Within the lumen of the blood vessel cells containing brightly fluorescent granules are seen (arrows). The staining pattern suggests that these cells have multi-lobed nuclei and are therefore likely to be polymorphonuclear leucocytes (original magnification x500).



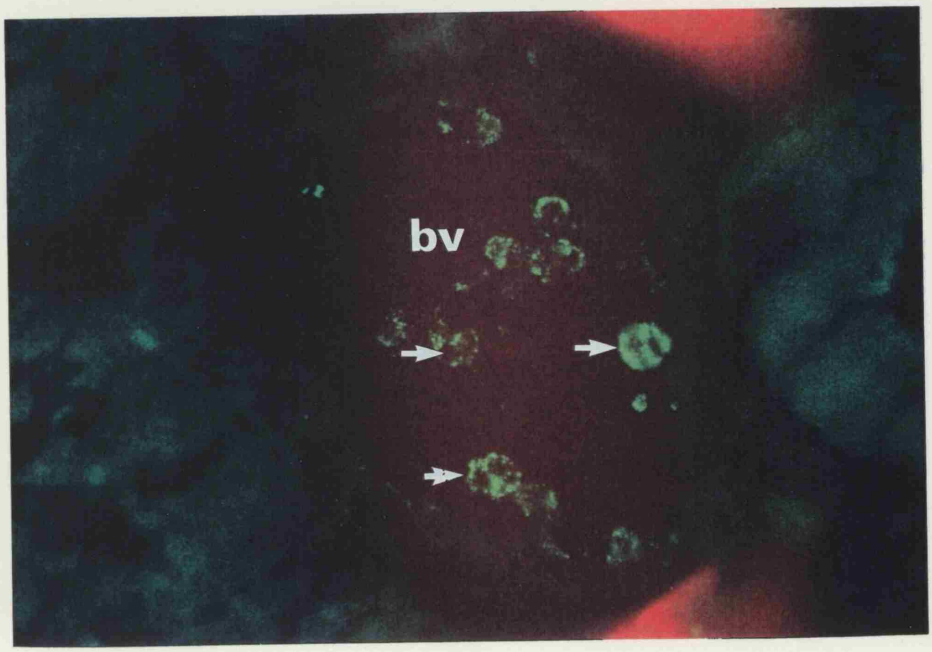
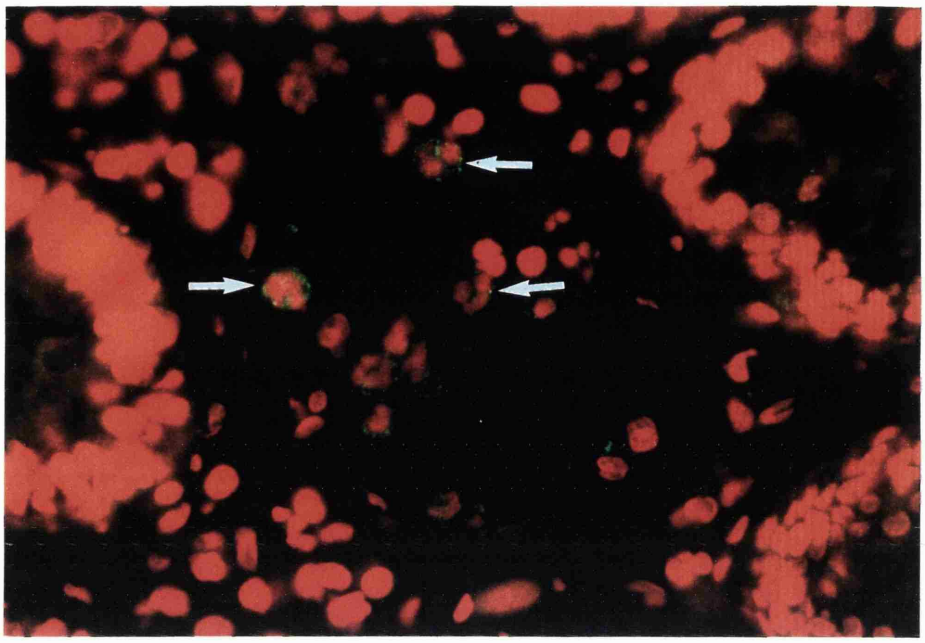

bv

**Figure 2.8**

Section from a well differentiated tumour incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase, showing polymorphonuclear leucocytes (arrows) containing fluorescent granules marking the presence of the enzyme (original magnification x500).

**Figure 2.9**

Section of normal colonic mucosa incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase, showing mononuclear cells (arrows) in the lamina propria containing fluorescent granules marking the presence of the enzyme (original magnification x500).



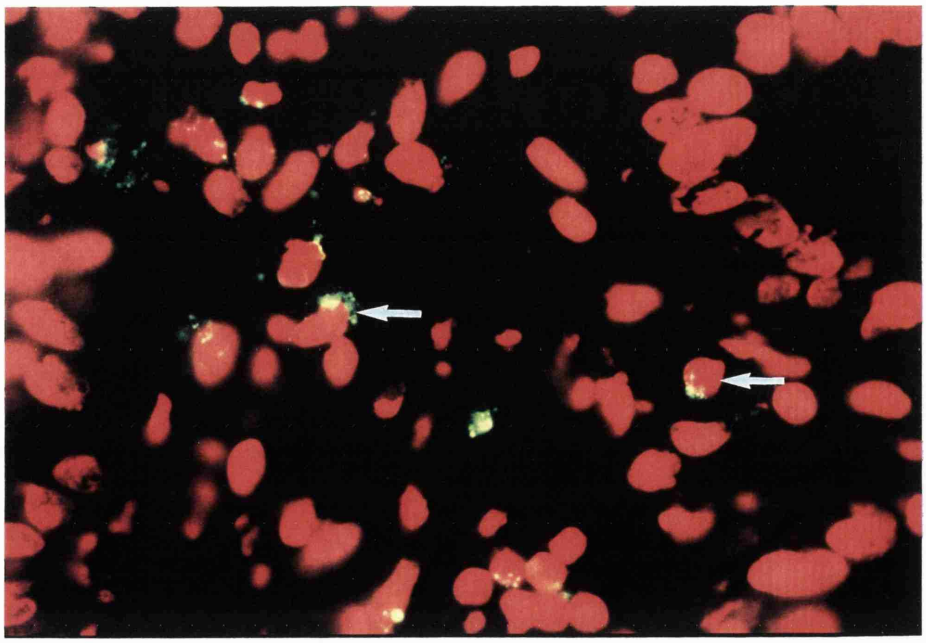
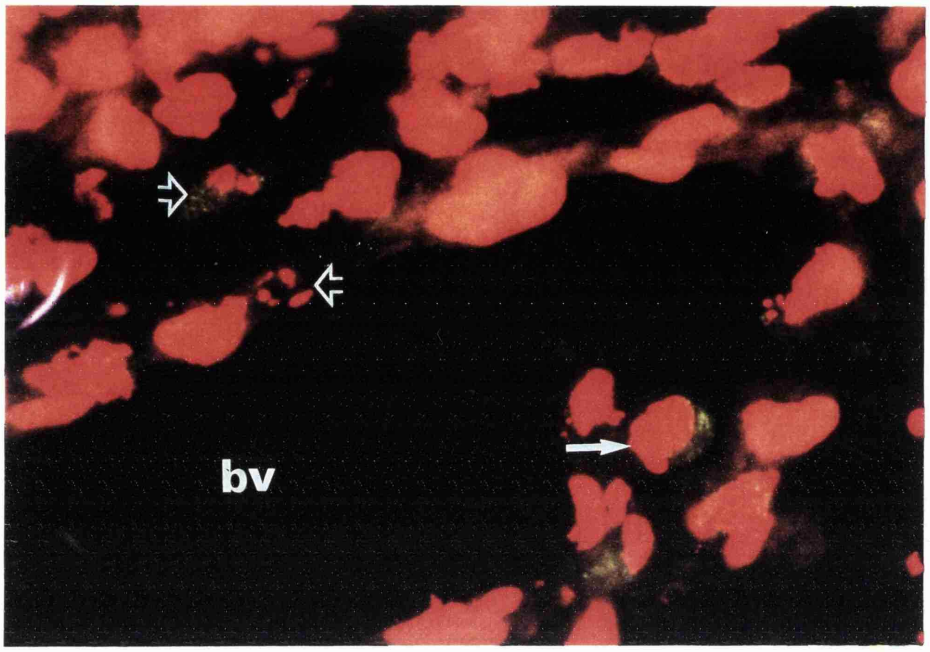


**Figure 2.10**

Section from a moderately differentiated adenocarcinoma of the colon incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase. A capillary (bv) in the submucosa is seen to be associated with cells containing fluorescent granules marking the presence of the enzyme. Two polymorphonuclear leucocytes (open arrows) are present, one lying just outside the capillary and containing fluorescent granules, the other apparently in the process of traversing the capillary wall. A mononuclear cell (closed arrow) containing fluorescent granules is also seen (original magnification x500).

**Figure 2.11**

Section from a moderately differentiated carcinoma of the rectum incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase. Spindle shaped cells (arrows) consistent with fibroblasts, and containing fluorescent granules marking the presence of the enzyme are seen aligned in the connective tissue stroma adjacent to a neoplastic gland (NG) (original magnification x500).


bv

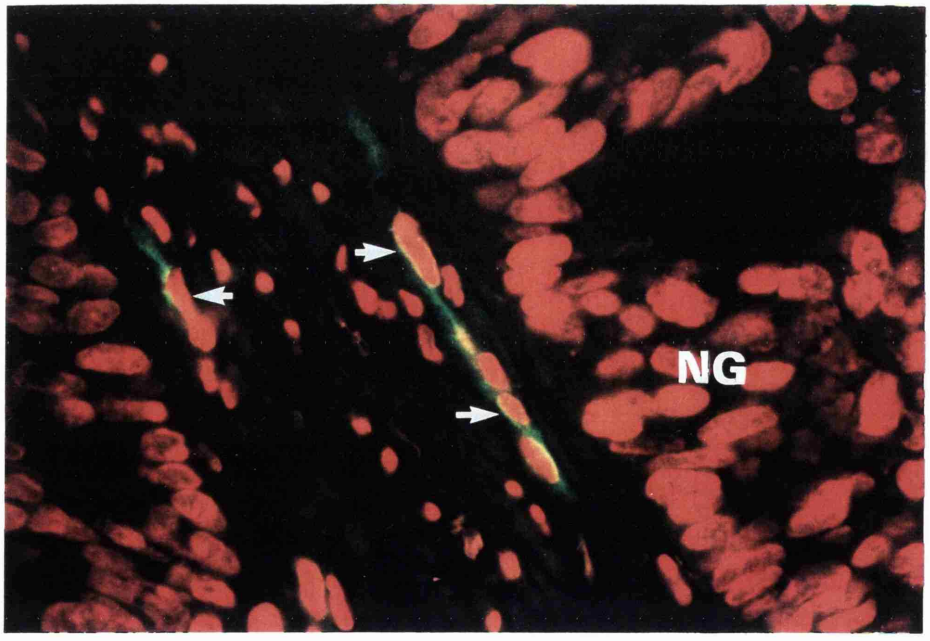
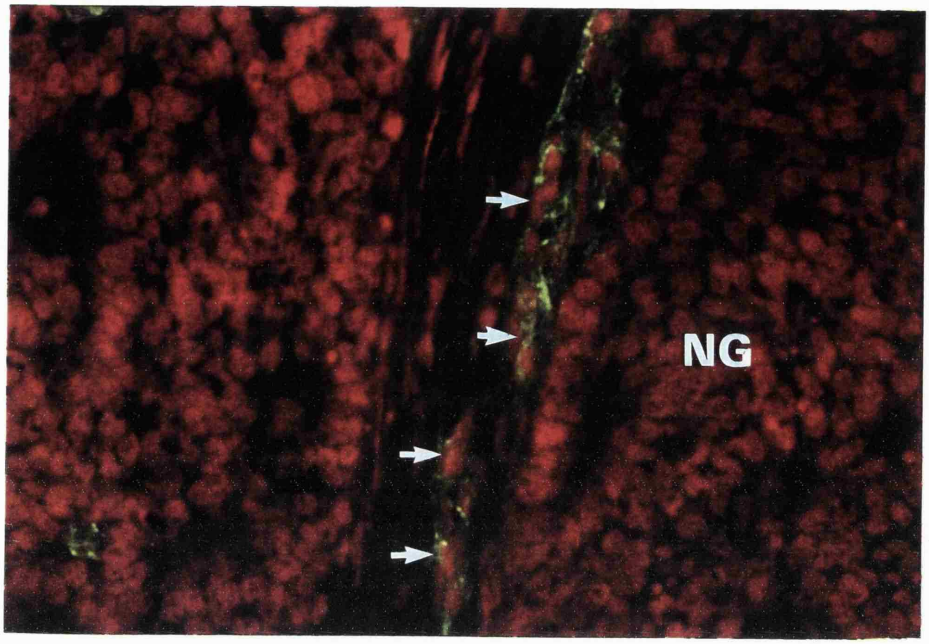

NG

**Figure 2.12**

Section from a moderately differentiated adenocarcinoma of the colon incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase. As in Figure 2.11 spindle shaped cells (arrows) probably fibroblasts and containing fluorescent granules, are seen to lie in the connective tissue stroma alongside a neoplastic gland (NG). There is no indication that the tumour cells are synthesizing gelatinase (original magnification x400).

**Figure 2.13**

Section from the tumour/mucosal junction of a moderately differentiated adenocarcinoma incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase. The view is from the deep margin of the tumour and shows a solitary fibroblast-like cell (arrowed) containing fluorescent granules marking the presence of the enzyme (original magnification x500).


NG

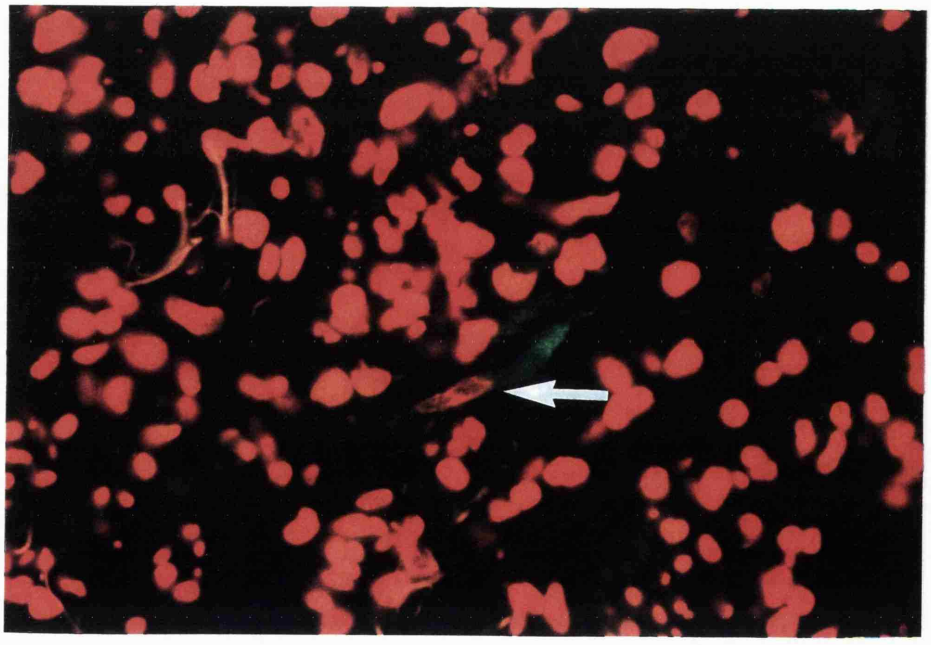


**Figure 2.14**

Section from a well differentiated adenocarcinoma of the rectum incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase. Fluorescence (arrowed) marking the presence of extracellular gelatinase is seen to lie between three neoplastic glands (ng) (original magnification x400).

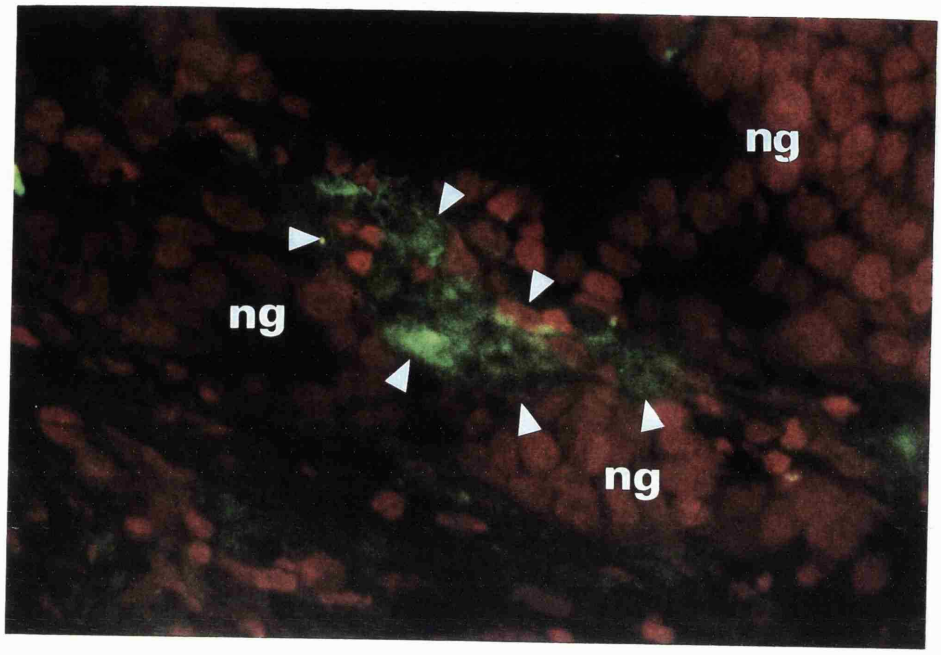
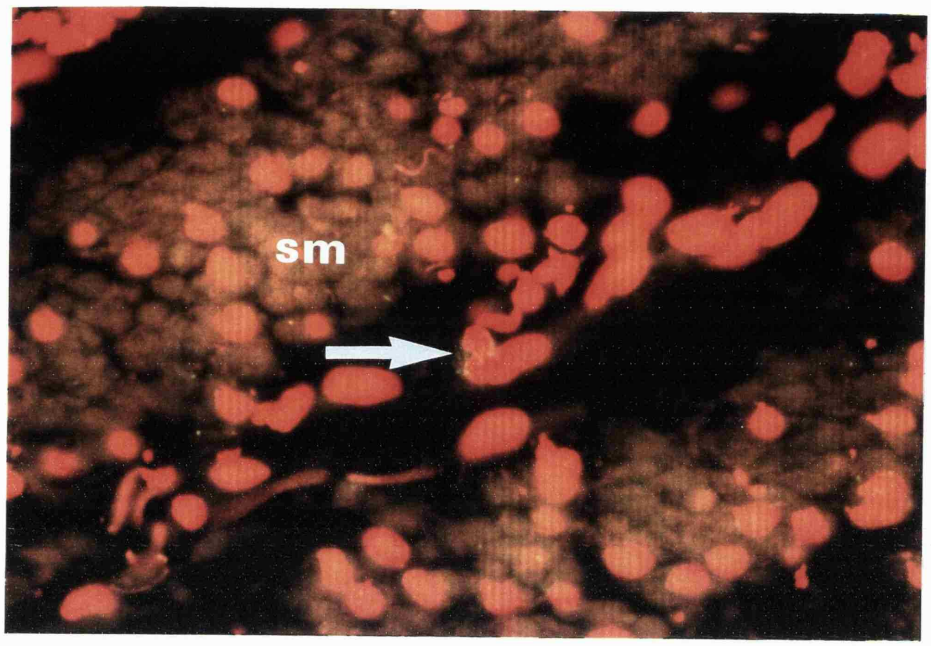

ng
ng
ng

**Figure 2.15**

Section showing muscle at the deep margin of invasion of a moderately differentiated carcinoma of the colon. The tissue has been incubated with antiserum which recognizes **only** the $M_r$ 95,000 form of gelatinase. A solitary polymorphonuclear leucocyte (arrowed) is seen containing fluorescent granules marking the presence of the enzyme. None of the surrounding fibroblast-like cells are stained (original magnification x500).

**Figure 2.16**

An adjacent section showing the same portion of tissue as seen in Figure 2.15 only this time incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase. In contrast to Figure 2.15 at least one fibroblast-like cell (arrowed) demonstrates intracellular fluorescent granules marking the presence of the enzyme. Taken in conjunction with Figure 2.15 the findings suggest that fibroblast-like cells synthesize $M_r$ 72,000 gelatinase and not $M_r$ 95,000 gelatinase which appears confined to polymorphonuclear cells and other mononuclear cells.


sm

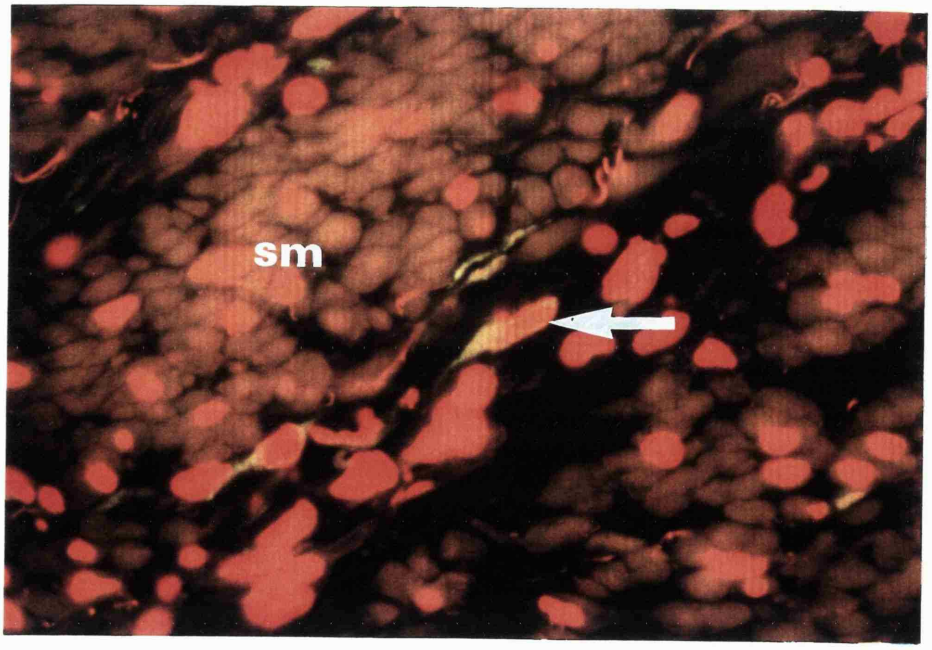
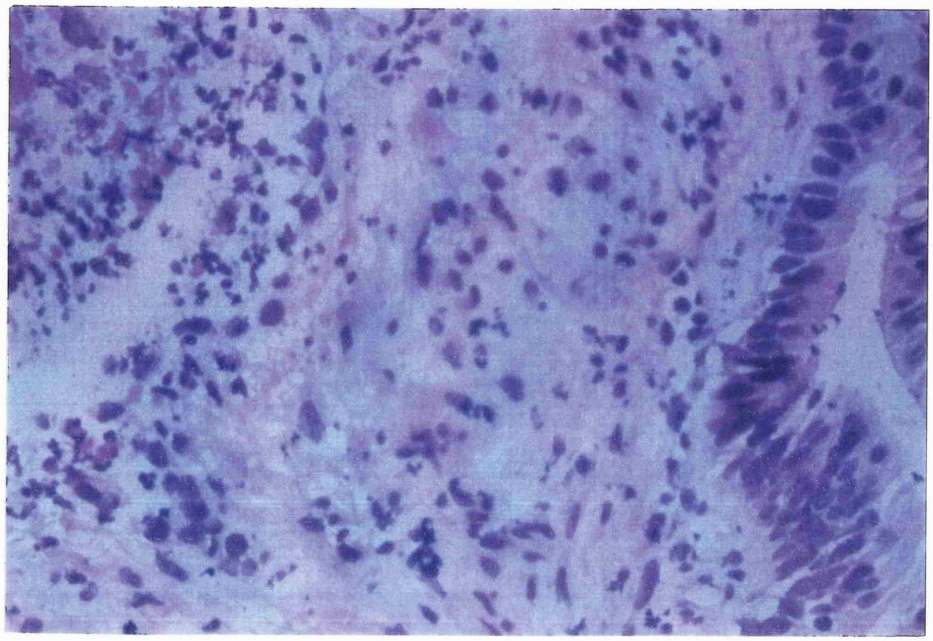

sm

**Figure 2.17**

Figures 2.17 to 2.21 show the relative expressions of gelatinase, collagenase and TIMP-1 in the same portion of a Dukes' B moderately differentiated carcinoma of the rectum as viewed on adjacent sections. The original biopsy was taken from the tumour/mucosal junction and Figure 2.17 shows the relevant area stained with haematoxylin and eosin (original magnification x400).

**Figure 2.18**

Section incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase. The arrows show the presence of intracellular fluorescence in both fibroblast-like cells and mononuclear cells marking the presence of the enzyme (original magnification x500).



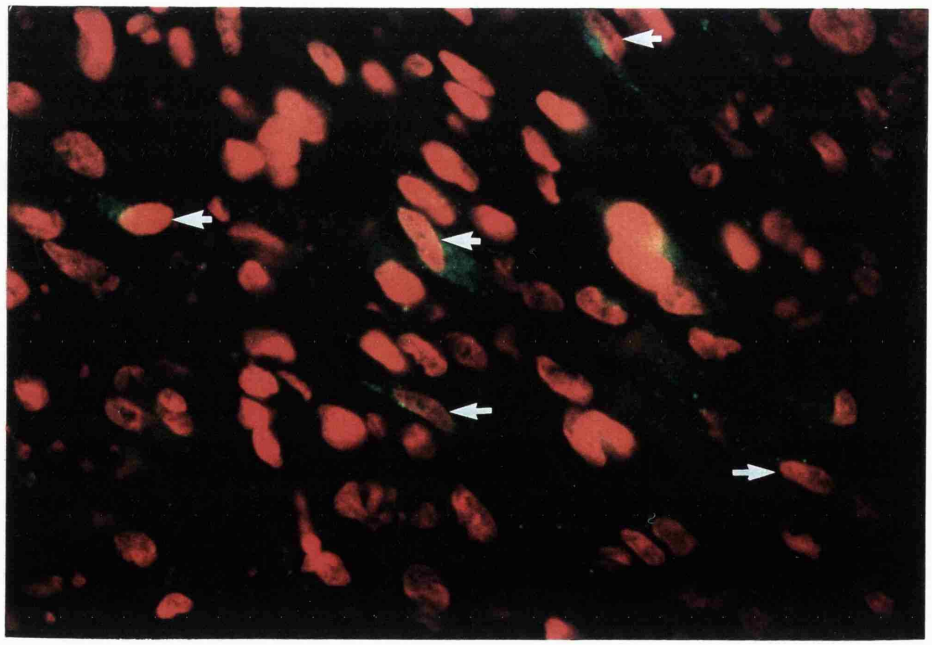
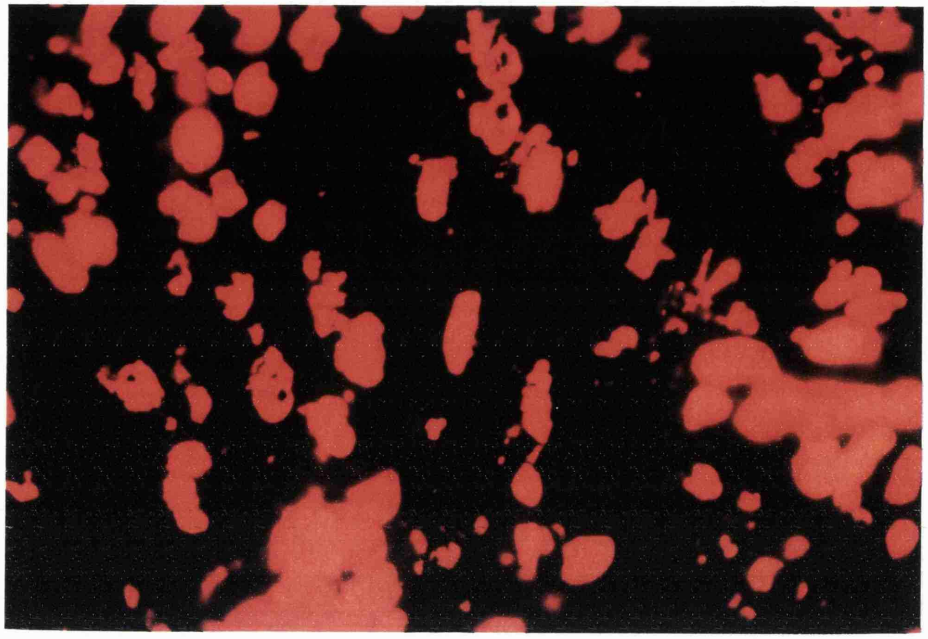


**Figure 2.19**

Section incubated with the antiserum to $M_r$ 95,000 gelatinase. No fluorescence is seen and the staining visualized in Figure 2.18 is presumed to be $M_r$ 72,000 gelatinase (original magnification x500).

**Figure 2.20**

Section incubated with the antiserum to interstitial collagenase. No staining is observed and this enzyme is therefore considered to be absent from this area of tumour (original magnification x500).



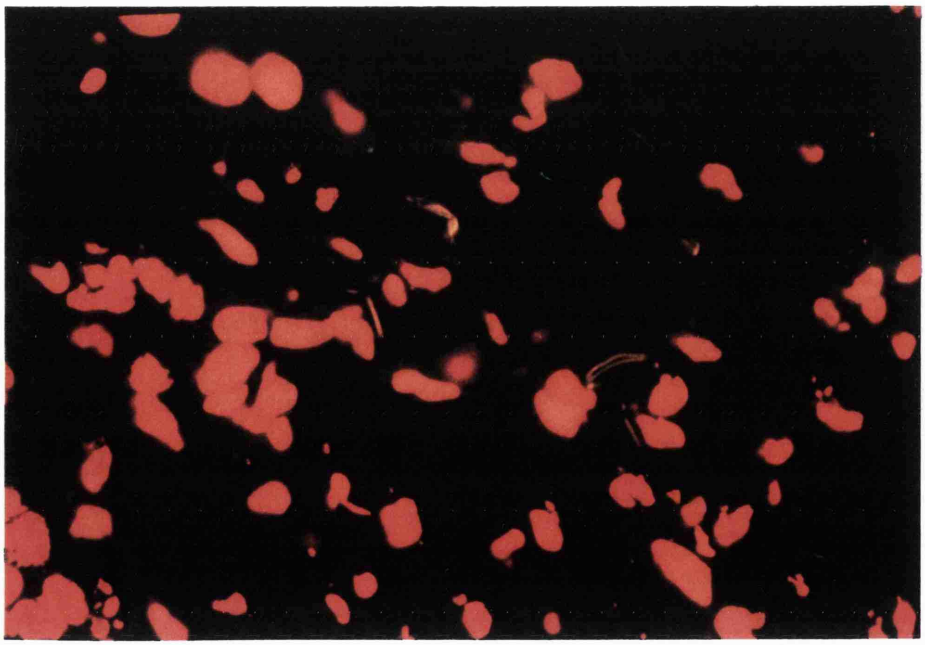
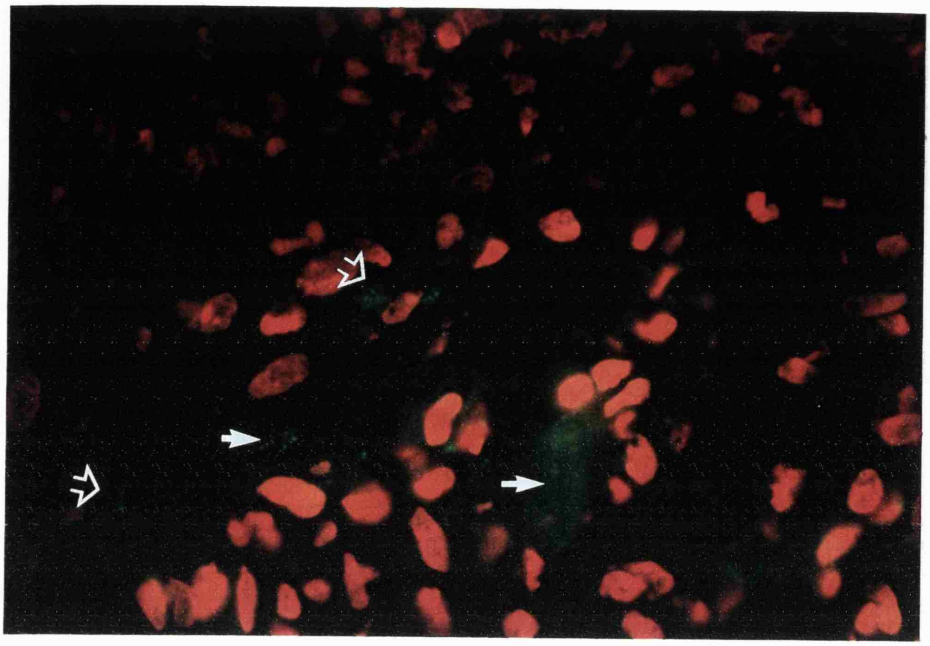


**Figure 2.21**

Section incubated with the antiserum to TIMP-1. Both intracellular (open arrows) and extracellular (closed arrows) TIMP-1 is present. The extracellular staining observed is likely to represent either free TIMP-1 or a complex between TIMP-1 and gelatinase or stromelysin. TIMP-1 alone is unable to bind to collagen fibrils. In this instance a complex between TIMP-1 and $M_r$ 72,000 gelatinase seems possible as this enzyme is being synthesized by cells in this area (Figure 2.18) (original magnification x500).

**Figure 2.22**

Two views from adjacent sections of the same submucosal blood vessel. In (1) the section has been incubated with antiserum to TIMP-1; a rim of fluorescence (arrows) marking the presence of TIMP-1 is seen associated with the endothelial cells lining the vessel lumen. By contrast no such fluorescence is seen in (2) a control section incubated with non-immune sheep serum. Elastic fibres present in the vessel wall autofluoresce and appear yellow in both (1) and (2) (original magnification x400).



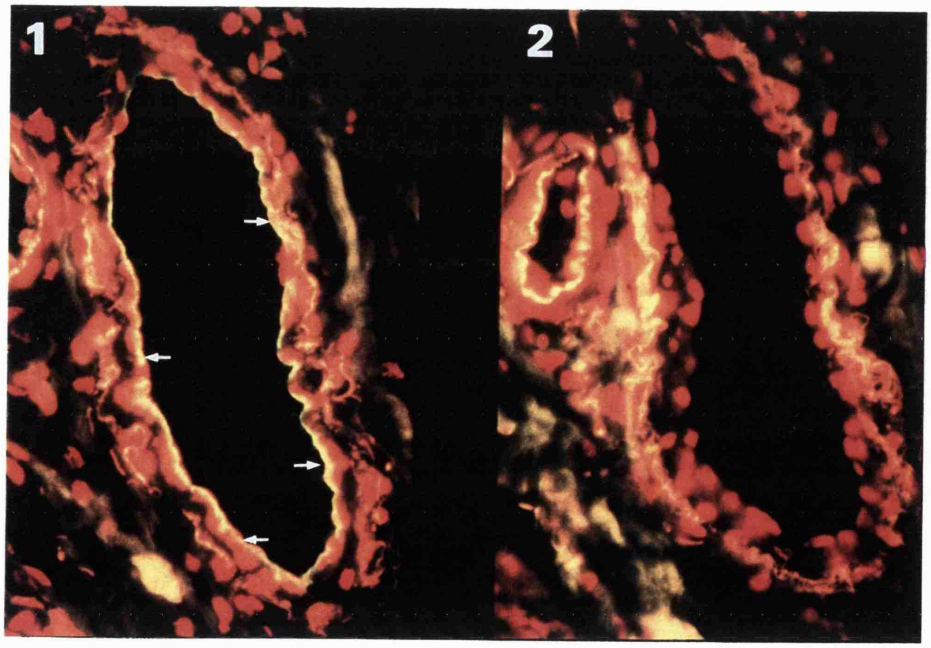

1
2

**Figure 2.23**

Section showing blood vessels stained with the anti-TIMP-1 antibody. TIMP-1 is seen in the endothelial cells lining the vessels.

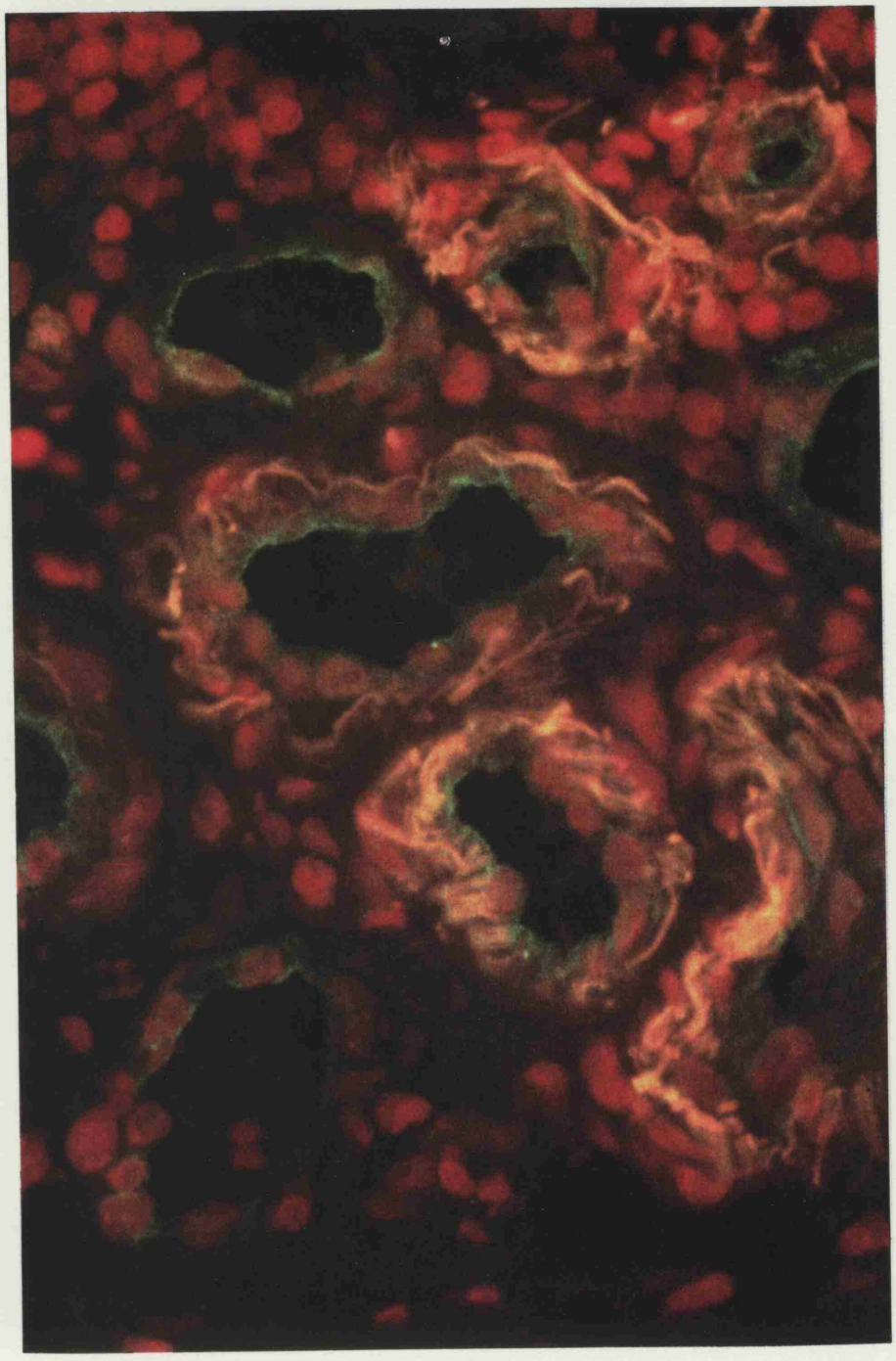
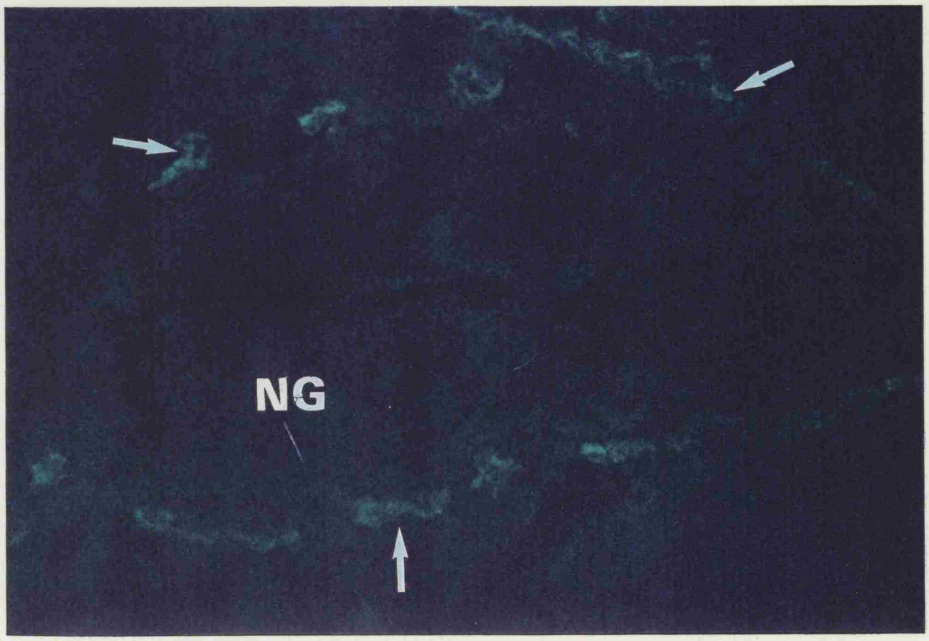


**Figure 2.24**

Section showing a neoplastic gland (NG) from a moderately differentiated carcinoma of the colon. The tissue has been incubated with antiserum to TIMP-1 and viewed using a narrow band FITC filter. The gland is seen to be ringed by areas of fluorescence (arrowed) which mark the presence of TIMP-1 in surrounding capillaries (original magnification x400).

**Figure 2.25**

Figures 2.25 to 2.31 represent the expression of TIMP-1 and the metalloproteinases as seen around the same malignant gland (G) viewed on adjacent sections. The gland in question was present in a moderately differentiated colonic adenocarcinoma and in this figure is seen stained with haematoxylin and eosin (original magnification x500).


NG

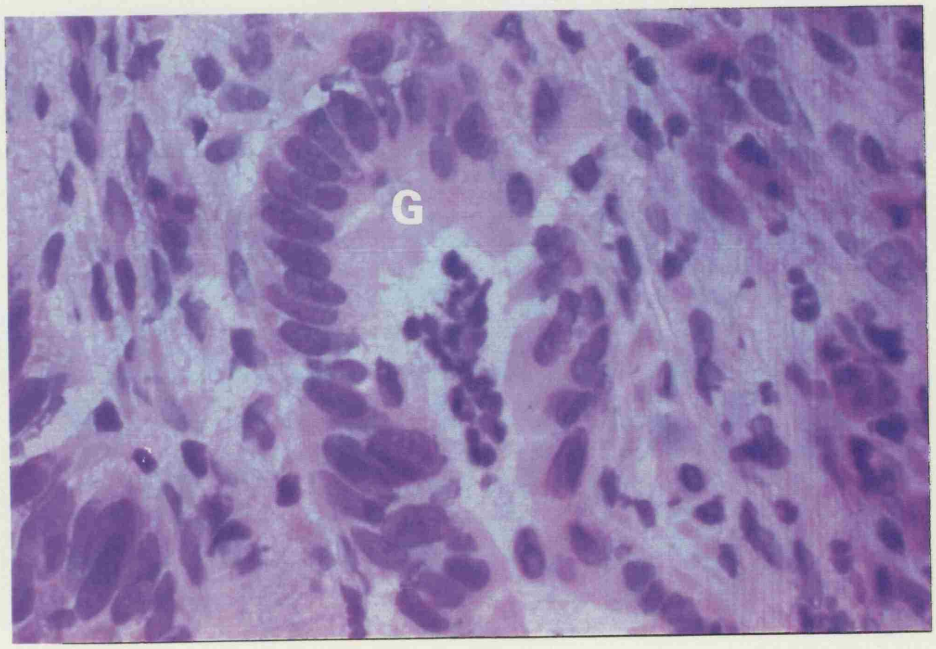
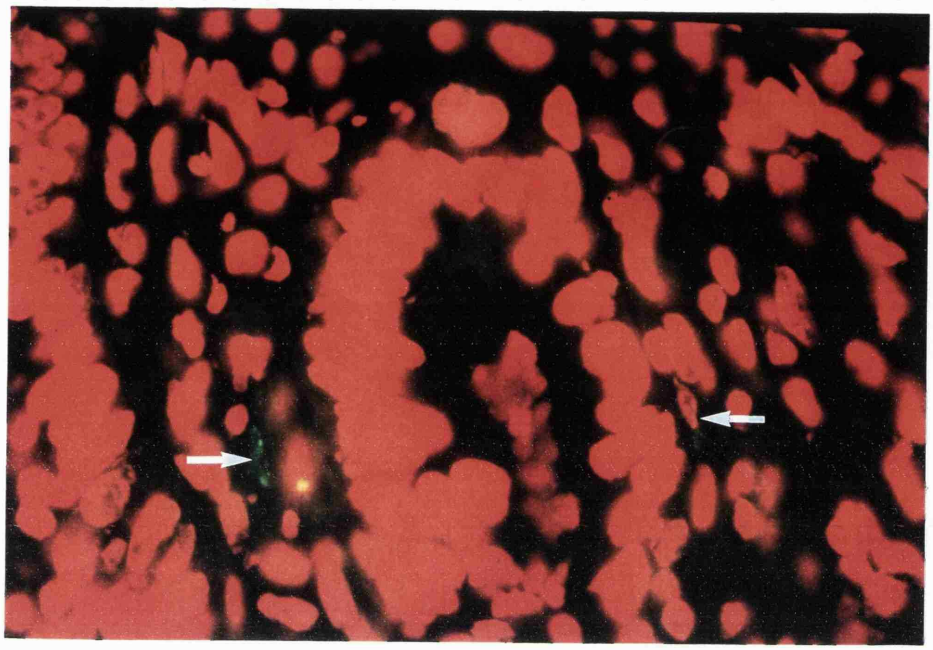

G

**Figure 2.26**

Section stained with the antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase and showing the gland identified in Figure 2.25. Fluorescent staining (arrows) marking the presence of the enzyme is seen associated with fibroblast-like cells surrounding the periphery of the gland (original magnification x500).

**Figure 2.27**

Section incubated with the antiserum to $M_r$ 95,000 gelatinase. Fluorescence within a solitary cell (arrowed) in the lumen of the gland is seen. This cell is likely to be inflammatory in origin, possibly a polymorphonuclear leucocyte containing intracellular granules of $M_r$ 95,000 gelatinase. By comparison with Figure 2.26 no staining is seen associated with the fibroblast-like cells around the periphery of the gland (original magnification x500).



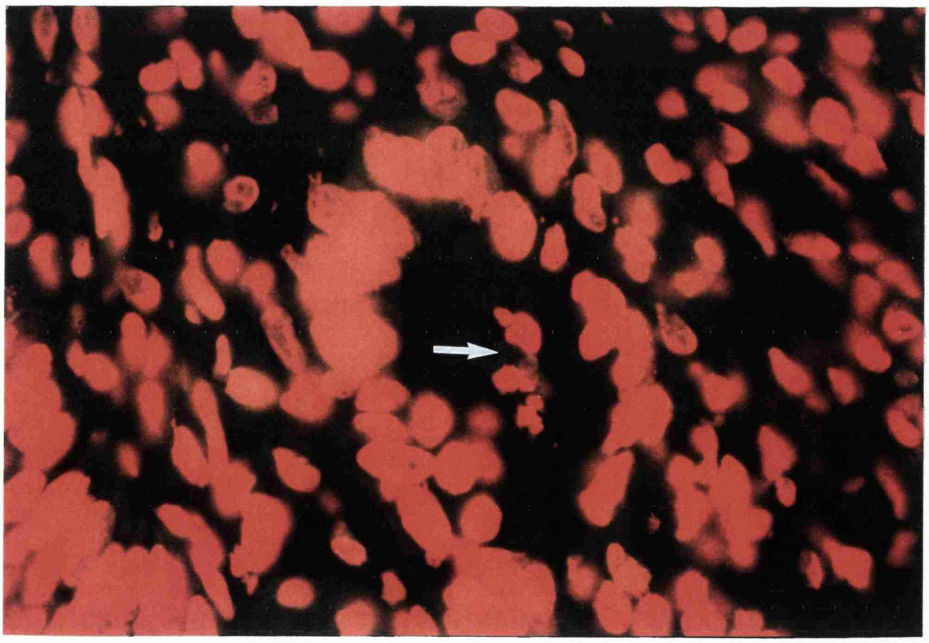
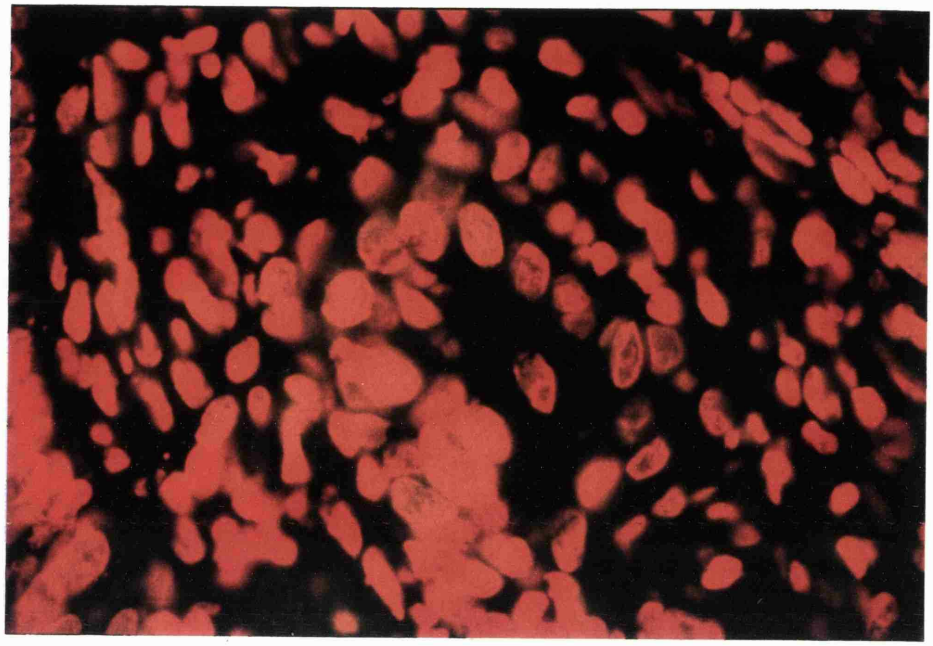


**Figure 2.28**

Section incubated with the antiserum to stromelysin. No fluorescence to indicate the presence of this enzyme is seen original magnification x500).

**Figure 2.29**

Control section incubated with non-immune sheep serum. No staining is present other than faint autofluorescence from collagen fibrils in the extracellular matrix (original magnification x500).



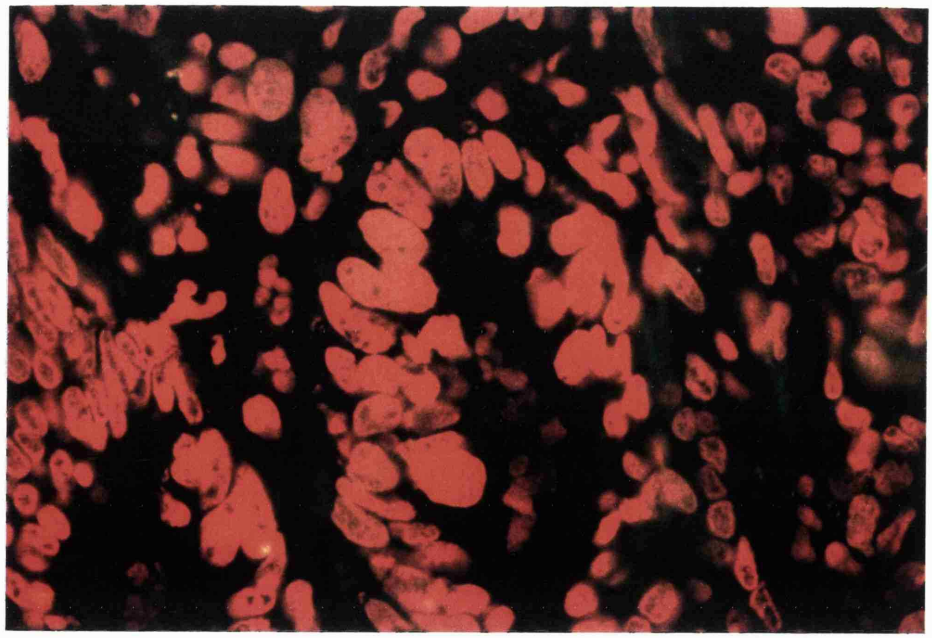
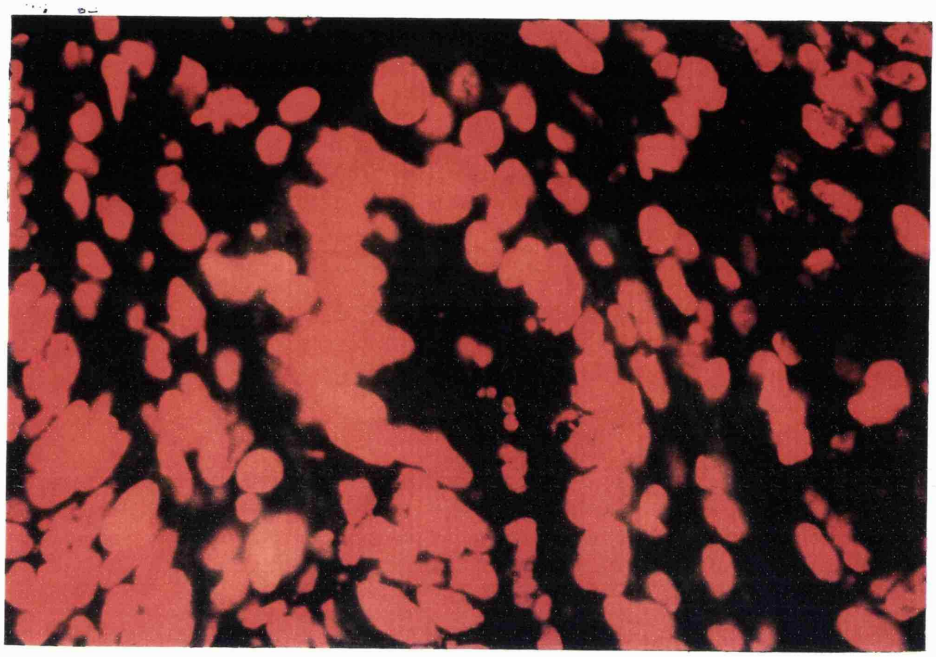


**Figure 2.30**

Section incubated with the antiserum to collagenase. No fluorescence is seen to indicate the presence of this enzyme (original magnification x500).

**Figure 2.31**

Section incubated with the antiserum to TIMP-1. Both extracellular staining (arrows) and intracellular staining (triangle) are present to indicate the presence of the inhibitor TIMP-1 (original magnification x500).



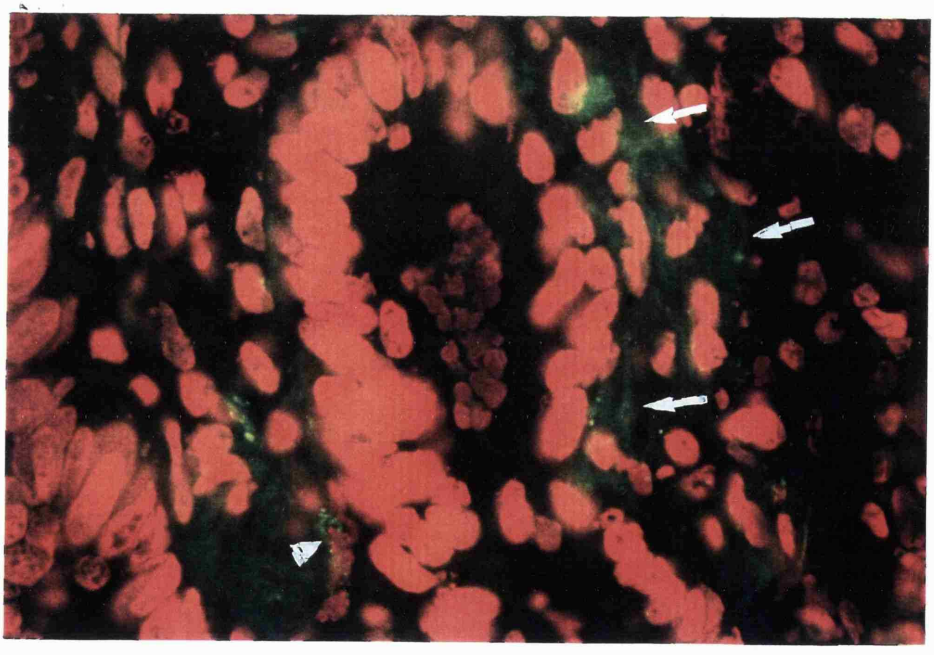
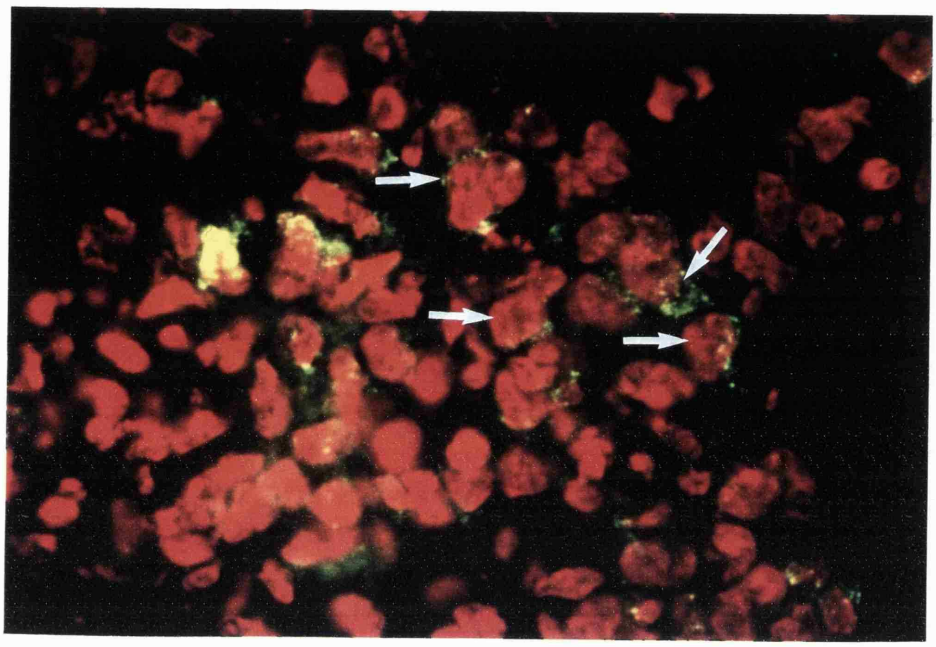


**Figure 2.32**

Section from a poorly differentiated Dukes' C adenocarcinoma of the rectum. Prior to incubation with the antiserum to stromelysin the biopsy had been subjected to tissue culture for a period of six hours with monensin. The arrows indicate intracellular fluorescence marking the presence of the enzyme, which is thought to reside in mononuclear stromal cells (see figure 2.33)(original magnification x500).

Figure 2.33

The section seen in Figure 2.32 was restained with the anticytokeratin antibody 8.13 using an indirect immunoperoxidase technique. The same four cells identified in Figure 2.32 are indicated by arrows. No staining to suggest the presence of cytokeratins is seen. Since cytokeratins are constitutively expressed by epithelial cells the absence of staining may be taken as supportive evidence that the cells showing intracellular immunofluorescence in Figure 2.32 are not epithelial derived tumour cells (original magnification x400).



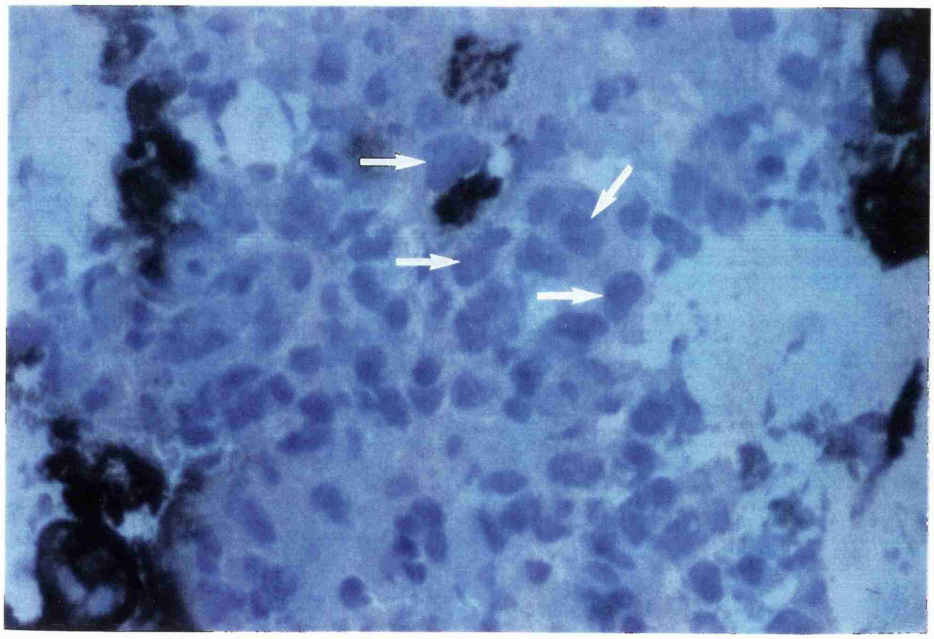


**Figure 2.34**

Section from a well differentiated Dukes' B carcinoma of the rectum incubated with the antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase and viewed using a narrow band FITC filter. A solitary cell (arrowed) demonstrating intracellular fluorescence is seen adjacent to a neoplastic gland (NG) (original magnification x500).

**Figure 2.35**

The same section as seen in Figure 2.34 was restained with the anticytokeratin antibody 8.13 using an indirect immunofluorescent technique. Epithelial cells which express cytokeratins are seen to fluoresce red and are clearly identified forming the neoplastic gland (NG). The cell previously identified in Figure 2.34 as synthesizing gelatinase is not stained by the anticytokeratin antibody and is therefore not seen in this Figure. This may be taken as supportive evidence that the cell was not a tumour derived epithelial cell (original magnification x500).


NG

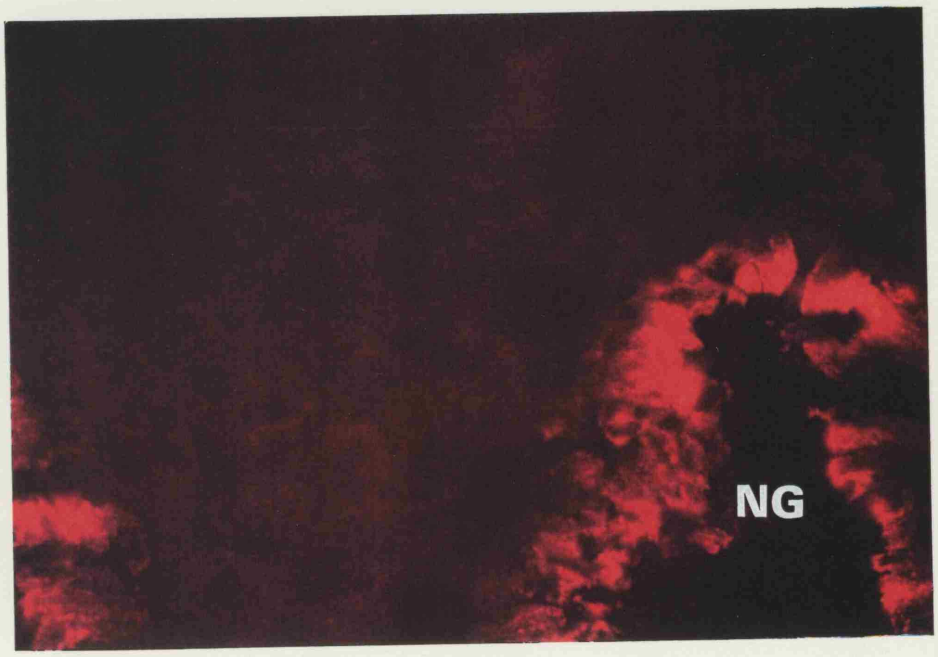
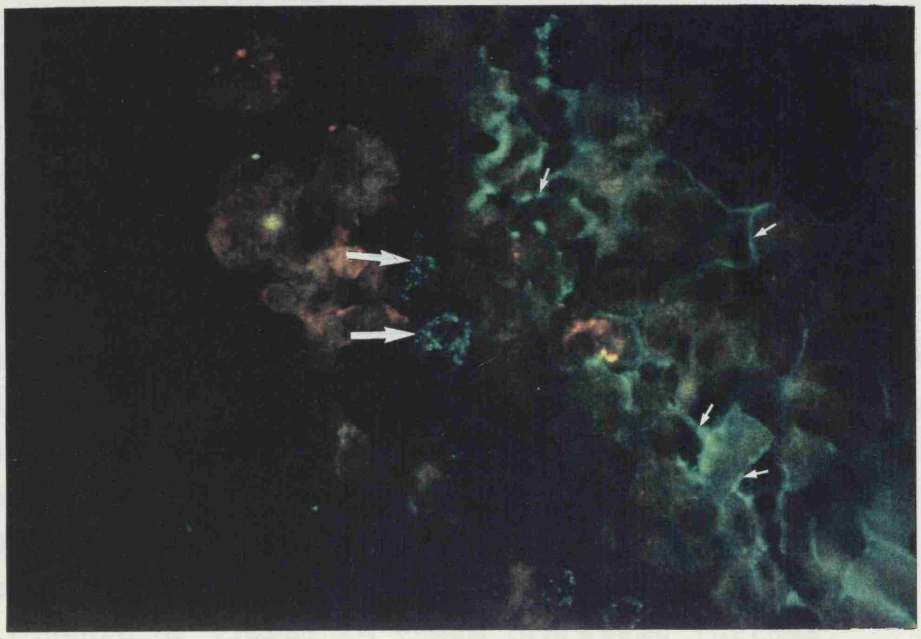

NG

**Figure 2.36**

Section prepared from a biopsy of a well differentiated adenocarcinoma which had been cultured for six hours with monensin. The section has been incubated with antiserum which recognizes both the $M_r$ 72,000 and $M_r$ 95,000 forms of gelatinase, and viewed using a narrow band FITC filter. Fluorescence marking the presence of both intracellular (large arrows) and extracellular (small arrows) gelatinase is evident (original magnification x500).



# CHAPTER 3

# THE IDENTIFICATION OF TIMP-1 IN COLONIC MUCUS

## 3.1 AIM

To determine whether or not TIMP-1 is present as a component of colonic mucus.

## 3.2 INTRODUCTION

The immunohistochemical study described in Chapter 2 demonstrated that the expression of TIMP-1 at the protein level occurred more frequently in malignant than in normal tissues. This observation supported the concept that TIMP-1 may have a role to play both in modulating tumour invasiveness (Ponton et al, 1991) and in angiogenesis (Bicknell and Harris, 1991). However, to explore the relationship of TIMP-1 to these processes further, quantitative measurements of its expression are required in different pathological situations.

TIMP-1 has been measured in homogenates of colorectal tissue using an enzyme linked immunoabsorbent assay (ELISA), and levels found to be elevated in tumour tissue (Lu et al, 1991). However, unless the origin of the measured levels of TIMP-1 are known, spurious conclusions might be drawn as to their significance. The immunohistochemical study previously described goes some way to resolving this question by suggesting that at least some of the differences between tumours may be due to variations in the expression of TIMP-1 by their vasculatures. It is also possible that disparities are the result of differing levels of TIMP-1 in associated body fluids particularly plasma and colonic mucus. While TIMP has been detected in human plasma (Cawston and Noble, 1986) there has, however, been no previous examination of its presence in colonic mucus.

Mucus is an epithelial secretion of concentrated glycoproteins, which are otherwise known as mucins. Typically they have molecular weights ranging from 200 kDa to 15,000 kDa, with carbohydrates responsible for 70% of

their weight (Allen, 1981). TIMP-1 is also a glycoprotein, but with a comparatively low molecular weight of 28 kDa of which only about 20% is due to carbohydrate (Cawston, 1986). In addition, the carbohydrate portion of TIMP-1 contains the sugar mannose (Kishi and Hayakawa, 1982), which although characteristic of serum glycoproteins is virtually absent from mucus glycoproteins.

Despite the differences between them TIMP-1 could become associated with colonic mucins in one of three ways; i) it could simply be present as a contaminant contained in sloughed epithelial cells, bacteria, digested food or digestive enzymes, ii) like albumin, TIMP's presence could be the result of passive transudation across the mucosa from the plasma (Creeth, 1978) and iii) TIMP-1 could be actively secreted as a separate constituent of mucus in a way analogous to the secretion of other non-mucus glycoproteins such as the vitamin $B_{12}$-binding proteins in gastric secretions or secretory IgA (Clamp, 1977). Both of these proteins are similar to TIMP-1 in so far as they contain substantially less carbohydrate than do mucins. The strong negative charge associated with mucus glycoproteins may allow secreted non-mucus glycoproteins to become intimately bound with them, a relationship likely to be enhanced by the additional formation of disulphide bridges.

Variations in the composition of colonic mucins in association with adenocarcinomas are already well described (Boland and Deshmukh, 1990) and have been used to predict the development of local recurrence following tumour resection (Dawson et al, 1987). While these observations refer to alterations in the mucus glycoproteins themselves, it is also conceivable that changes might also occur in the associated non-mucus glycoproteins.

In bronchial and cervical mucus a low molecular weight inhibitor termed human mucus proteinase inhibitor (MPI) has been identified, which controls the activity of trypsin and leucocyte elastase (Meckelein et al, 1990). TIMP-1, if it exists in colonic mucus, may have an analogous role to that

of MPI, in regulating MMPs activity and controlling connective tissue turnover.

Although the immunohistochemical study found TIMP-1 to be absent from both the mucus layer and the cells lining the colonic crypts it is possible that detection was hampered by both its biochemistry and the limitations of the technique used. The opportunity to detect TIMP-1 would be reduced if it were secreted rather than stored by the cells responsible for its synthesis, while tissue fixatives and detergents used in histochemistry may have disrupted the mucus layer possibly obliterating the presence of TIMP-1 within it.

As a preliminary step to its measurement this study therefore sought to confirm or refute the presence of TIMP-1 in colonic mucus by alternative means, namely the use of polyacrylamide gel electrophoresis and Western blotting.

## 3.3 MATERIALS and METHODS

### 3.3.1 *Mucus collection*

Mucus was obtained from colectomy specimens, within one hour of their being resected, by gently scraping the mucosa with a microscope slide. In those instances where heavy faecal contamination was present, the mucosa was gently washed beforehand under running water. To prevent proteolytic degradation, the collected mucus was added to 10 ml of a pre-chilled solution of protease inhibitors in 0.5M Tris-HCl solution with 0.02% sodium azide at pH 8 (Appendix 3.1), and immediately stored at -20°C until required. Mucus was collected from ten colectomy specimens. As the aim of the study was to detect TIMP within mucus rather than map differences in its distribution, no specific attempt was made to gather mucus from different areas of the resected colons, although direct scraping of the tumour was avoided. Details of the patients studied and their pathological status are provided in Table 3.1.

| Patient | Age | Sex | Site | Diff | Dukes' stage | Lane(s) in Exp 3 |
|---|---|---|---|---|---|---|
| DG | 70 | M | Caec | Mod | C | Exp 1 |
| HF | 72 | M | Desc colon | Poor | C | Exp 2 |
| JF | 89 | M | Rectum | Mod | B | h |
| WL | 68 | M | Rectum | Well | B | i |
| KW[3] | 40 | F | Crohns | | | c & j |
| JL | 67 | M | Desc colon | Mod | C | d & k |
| EF | 86 | F | Rectum | Poor | C | l |
| MS | 74 | M | Ascen colon | Mod | C | e & m |
| ES | 91 | M | Rectum | Mod | B | f |
| LT[4] | 66 | M | Rectum | Mod | C | g |

Table 3.1: Details of patients together with their pathological status, from whom colonic mucus was collected. Samples from the first two patients were used in Experiment 1 and Experiment 2 respectively. Mucus from the remaining patients was analysed in Experiment 3 and the letters of the lanes in which the samples were run correspond to those on Figure 3.5. Where two letters are present these refer respectively to fractionated and unfractionated samples of mucus. Diff, differentiation.

[3]This patient was not part of the immunohistochemical study into TIMP-1 and therefore the TIMP-1 status of any tissue is not known.

[4] This patient does not form part of the immunohistochemical study reported in Chapter 2, although the immunohistochemical status of biopsy material has been determined.

## 3.4 EXPERIMENT 1

**Analysis of crude mucus by polyacrylamide gel electrophoresis.**

Unless otherwise stated all chemicals were from Merck, Poole, UK.

In the initial investigation one of the mucus scrapings (patient DG) was selected for examination. The sample was homogenized in a hand held Douce homogenizer, centrifuged at 30,000g for 30 minutes to remove particulate matter and the supernatant retained.

Aliquots of the supernatant were analyzed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Twenty five and 50 µl samples were run under reducing conditions on 10% discontinuous polyacrylamide gels according to the method of Laemmli and Favre (1973). Prior to loading, the samples were mixed with an equal volume of sample buffer containing: 62.5 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.004% bromophenol blue, and boiled for 5 minutes. Two gels were run at 10 mA each, overnight, at 6$^{0}$C in an LKB - Pharmacia Vertical Electrophoretic System (Pharmacia, Milton Keynes, UK). Each gel was also loaded with standard protein markers covering molecular weights between 94 to 14 kDa (Pharmacia, Milton Keynes, UK). At the end of the running period, one gel was fixed and then silver stained, while a Western blot was prepared from the other.

### 3.4.1 *Western blots*

Western blots were prepared by electroblotting proteins from the gel onto nitrocellulose paper as described by Towbin et al (1979). An LKB Pharmacia Multiphor II, semi-dry blotting apparatus was used and blots effected with a current of 0.8 mA/cm$^{2}$ run for one hour.

### 3.4.2 *Immunoblotting*

The blot was initially washed in three changes of Tris-buffered saline (TBS), pH 7.4. The nitrocellulose strip containing the marker proteins was then separated and developed using a 0.5% solution of Indian ink. The remainder of the blot was incubated overnight, at 4°C, in a blocking buffer of TBS with 2.5% milk fat protein, 5% bovine serum albumin and 0.05% Tween 20 (all from Sigma, Poole, UK). At the end of this period the blot was washed in TBS, the control lane separated, and the remainder incubated with the anti-TIMP-1 antibody (as used in Chapter 2) at a dilution of 1:60 in TBS, for two hours at room temperature. After further washing, the blot was incubated with the second antibody, a rabbit anti-sheep antibody conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc, supplied by Bio-nuclear Services, Bude, UK) used at a dilution of 1:2000, for one hour at room temperature. After three washes the blot was developed using a solution in 50 mM Tris-HCl, pH 7.4, of DAB (0.5 mg/ml: see appendix 4.1), 4-chloro-1-naphthol (0.3 mg/ml) and 0.01% hydrogen peroxide. The control strip was treated in an identical fashion except that the primary anti-TIMP-1 antibody was replaced with non-immune sheep serum, 28 mg/ml (Sigma, Poole, UK).

### 3.4.3 *Results*

Figure 3.1 shows both the silver stained gel and the immunostained Western blot. The silver stained gel reveals an extremely heavy concentration of proteins of varying molecular weights. On the immunostained Western blot a faint but distinct band corresponding to a molecular weight of approximately 30 kDa and compatible with the presence of TIMP-1, was present in each sample strip. In addition, the band associated with the strip (b) derived from loading 50 µl of crude mucus onto the gel was more prominent than that obtained with the 25 µl (c).

**EXPERIMENT 2**

**Purification of mucus glycoproteins**

To further characterize the presence of TIMP-1 in mucus it was necessary to purify the mucus glycoproteins from the crude scrapings. This was achieved through the use of caesium chloride density gradient centrifugation according to the method described by Creeth and Denborough (1970).

As before the crude mucus was first gently homogenized using a hand held Douce homogenizer and the samples centrifuged at 30,000g for 30 minutes to remove particulate matter. The supernatant was then retained and ultra-pure grade caesium chloride added at a weight fraction of 0.42, i.e. 0.42g caesium chloride : 0.58 ml of supernatant, to give a starting density of 1.45 g/ml. The samples were centrifuged in a Beckman 50 Ti rotor (Beckman, High Wycombe, U.K.) at 150,000g for 48 hours at 12°C. At the end of this period the density gradient is established, and varies from approximately 1.38 g/ml at the top of the tube to 1.56 g/ml at the base. Glycoproteins, with their higher buoyant density, will be found towards the bottom of the tube, while pure proteins, which are lighter, collect at the top. By piercing the base of the tube, 6 fractions of different density were collected for the sample and dialysed against 62.5mM Tris. HCl, pH 6.8 to remove the caesium.

To confirm that a density gradient had been established the protein concentration of each fraction collected was checked using the Bio-Rad Protein Assay (BioRad, Watford, Herts, U.K.). This is based on the colour change of Coomassiie Brilliant Blue G-250 dye in response to various concentrations of protein. The colour change is quantified using a spectrophotometer to measure the solution's absorbance of light at a wavelength of 595 nm. The protein concentrations of the collected fractions were obtained by reference to a standard curve, constructed from the absorbances of known concentrations of bovine serum albumin (BSA).

Duplicate 100 µl samples of each fraction were combined with 100 µl of sample buffer, and analyzed on SDS-PAGE followed by silver staining of one gel and Western blotting of the other as described above.

### 3.5.1 *Results*

The absorbances at a wavelength of 595 nm of standard solutions of (BSA) and the fractionated samples of mucus are presented in Table 3.2 and 3.3 respectively. The protein concentrations of each of the six fractions as well as that of an unfractionated sample were calculated from these results and are also presented in Table 3.3. They confirm that a gradient had been established by density gradient centrifugation, with the highest and lowest protein concentrations observed in fractions from the top (fraction 6) and bottom (fraction 1) of the sample tube respectively.

The appearance of the silver stained gel (Fig 3.2) provided further confirmation of the success of the density gradient centrifugation, with detectable protein virtually absent from fraction 1 (lane c) while being abundant in fraction 6 (lane h).

The immunostained Western blot obtained from the other gel is shown in Fig 3.3. This revealed a distinct double band in fraction 5 (tract g) corresponding to the presence of a protein of approximately 30 kDa molecular weight and therefore compatible with an identity as TIMP-1. No such double band was seen in fraction 6 (lane h), but a faint band, also at 30 kDa, was present in the unfractionated sample (lane i). No positive bands were seen on the control strip (lane b) on which an unfractionated sample of mucus had been run.

**Table 3.2**

| BSA (μg) | $Abs_{595}$ |
|---|---|
| 0 | 0.0004 |
| 5 | 0.1819 |
| 10 | 0.2470 |
| 20 | 0.4715 |
| 30 | 0.6453 |
| 40 | 0.8535 |

The absorbance of light at a wavelength of 595 nm ($Abs_{595}$) of solutions containing known quantities of bovine serum albumin (BSA). The absorbance values represent the mean of two readings.

**Table 3.3**

| Sample | Volume assayed (μl) | $Abs_{595}$ | Protein (μg/ml) |
|---|---|---|---|
| Unfractionated | 25 | 0.7724 | 1400 |
| Fraction 1 | 500 | 0.0176 | 1 |
| Fraction 2 | 500 | 0.176 | 10 |
| Fraction 3 | 500 | 0.667 | 60 |
| Fraction 4 | 250 | 0.808 | 140 |
| Fraction 5 | 50 | 0.515 | 500 |
| Fraction 6 | 5 | 0.697 | 7000 |

Absorbances at 595 nm ($Abs_{595}$) of samples of mucus before (unfractionated) and after caesium chloride density gradient centrifugation. The buoyant density of the fractionated samples is highest for fraction 1 (bottom of tube) and lowest for fraction 6 (top of tube). The final column gives the approximate protein content of 1 ml of the fractionated samples. The greatest concentration of proteins, which have a lighter buoyant density compared to glycoproteins, is seen to be present in fraction 6.

## 3.6 EXPERIMENT 3

**Comparison of unfractionated with fractionated samples of mucus.**

In the final experiment a sample of crude mucus from each of the eight specimens collected, together with three different fraction 5 samples derived from the caesium chloride density gradient centrifugation of the mucus collected from patients, KW, JL and MS, were analyzed on SDS-PAGE followed by Western blotting. For this experiment, and for this experiment only, recombinant TIMP-1 (330 µg/ml) was available, kindly provided by Dr G. Murphy of Strangeways Research Laboratory, Cambridge. By loading samples (10 µl) of the recombinant TIMP-1 onto the gels, to act as positive controls, a further check on the identity of any protein bands revealed after immunostaining the Western blot was made possible. The running conditions for the gel were as described previously.

### 3.6.1 *Results*

The silver stained gel is shown in Fig 3.4. The protein bands for each of the mucus samples are flanked by lanes containing the protein molecular weight markers (lanes a and o). As before a qualitative difference was distinguishable between the protein bands from the three fraction 5 samples (left hand side of gel, lanes c,d and e) and those from the unfractionated mucus (lanes f,g,h,i,j,k,l,m). A distinct band is present in each of the two lanes (b and n) containing the recombinant TIMP-1 adjacent to the 30 kDa molecular weight marker.

The immunostained Western blot is shown in Fig 3.4. The recombinant TIMP-1 in lane n is clearly revealed at a position, compatible with its correct molecular weight of 28 kDa, just below the 30 kDa marker. Adjacent to the recombinant TIMP-1 band running right to left across the gel, a faint but distinct band is observed in each of the

sample lanes. This band is most obvious in the three lanes containing the fraction 5 samples (lanes c,d and e) and should be compared to the weaker staining obtained in each of the corresponding unfractionated samples on lanes j,k and m respectively. The stronger signal obtained in the fraction 5 samples could be taken to indicate that the concentration of TIMP-1 in these samples had been enhanced by density gradient centrifugation over that present in the unfractionated samples. Although "streaking" is present in each lane a second prominent band is also present lying between the 67 kDa and 43 kDa markers. Disappointingly, the control lane, lane b (not shown), separated from the main blot and containing recombinant TIMP-1, also produced a weak signal despite substitution of the anti-TIMP-1 antibody with non-immune sheep serum. This weak signal on the control lane may have been the result of overloading the recombinant TIMP-1.

## 3.7 DISCUSSION

The three experiments described above represent a preliminary investigation into the presence of TIMP-1 in colonic mucus. Although each of the three experiments will be discussed separately they are complementary to one another.

The immunoblot derived from the first experiment, conducted on crude mucus, provided preliminary evidence for the presence of TIMP-1 by revealing a band adjacent to the 30 kDa molecular weight marker. In addition, the intensity of staining of this band was increased when the sample load was doubled from 25 µl to 50 µl indicating a crude dose response effect. By suggesting its presence, this experiment provided the impetus for a more critical look at TIMP-1 in colonic mucus. To do this the heterogeneous composition of the crude mucus had to be resolved into its component parts, principally glycoproteins and proteins.

In Experiment 2 this was achieved using caesium chloride density gradient centrifugation. The concentrations of protein in the various fractions obtained (Table 3.2) together with the relative absence of proteins from the those fractions with a higher specific gravity as revealed on the silver stained gel (Fig 3.2), confirmed that fractionation of the mucus had been successful.

Subsequent immunoblotting of the fractionated samples revealed a double band adjacent to the 30 kDa molecular weight marker in fraction 5. The detection of TIMP-1 in fraction 5 was compatible with its structure as a glycoprotein in which the carbohydrate portion accounts for approximately 20% of its molecular weight. This would make it sufficiently dense to exclude it from the fraction containing the highest concentration of proteins, fraction 6 (top of tube) and yet sufficiently light to avoid deposition in the densest fractions (fractions 1 and 2, bottom of tube) where mucus glycoproteins, with their much higher carbohydrate content (70%) would be expected. The reason for the double band is not clear but it may be the result of heterogeneity in the glycosylation of the of the TIMP molecule (Clark et al, 1991).

The specificity of the anti-TIMP-1 antibody (discussed in Chapter 2) coupled with the appropriateness of the staining observed in fraction 5 provide support for the presence of TIMP-1 in mucus. However, it was also noted in the immunoblot from Experiment 2 (Fig 3.3) that, despite soaking in blocking buffer, what appears to be non-specific staining has occurred in lanes h and i representing fraction 6 and unfractionated mucus respectively.

In the third experiment (the last that circumstances permitted) both crude and fraction 5 samples of mucus were run alongside recombinant TIMP-1, which acted as a positive control. In each of the sample lanes a band was observed, which corresponded in position to that seen in the recombinant TIMP-1 lane (lane n). The staining of the fraction 5 samples (lanes c,d and e) was also noted to be

more intense than their unfractionated counterparts, indicating a crude dose response as seen in Experiment 1. On their own these observations might have been sufficient to confirm the presence of a glycoprotein with an identity compatible with that of TIMP-1. However, features of the immunoblot raise doubts about the validity of these observations. As in Experiment 2, further bands were also visible on the immunoblot. In particular staining was observed in each lane at a position lying between the 67 kDa and 43 kDa markers. This staining was in fact more pronounced than that seen adjacent to the 30 kDa marker, and was most marked for the unfractionated samples. Since a signal was also obtained from the recombinant TIMP-1 lane (lane b, not shown) which was incubated with non-immune sheep serum to act as a control, it is possible that this higher molecular weight band was due to non-specific binding of the antisera to other proteins on the immunoblot. However, multiple bands with molecular weights of 56 kDa and 70 kDa were observed by Cawston et al (1986) when preparations of TIMP purified from human plasma were run on SDS-PAGE. These additional bands were considered to represent aggregates of TIMP-1 which had formed during storage. Similar bands (56 kDa and 64 kDa) have also been observed in human synovial fluid (Mercer et al, 1985) and protein extracts from human colonic tumours (Lu et al, 1991). If such aggregates were present in the mucus samples analyzed, they too would be detected and in a position compatible with that of the second band, i.e. between the 67 kDa and 43 kDa molecular weight markers.

In none of the three experiments was a band visualized corresponding to a molecular weight greater than 67 kDa. This would imply that any TIMP-1 present was not complexed to MMPs since the molecular masses of such complexes would exceed 67 kDa (Ward et al, 1991).

Because some of the staining on the Western blots may be non-specific, the evidence from the three experiments presented above is insufficient to definitely confirm TIMP-1

as a component of colonic mucus. More work is required and principally the immunostaining method refined to eliminate non-specific binding of the antisera. It may also be possible to further purify mucus samples using an affinity chromatography column labelled with the anti-TIMP-1 antibody. Such a column would remove TIMP-1 from the sample while allowing other proteins to pass through; TIMP-1 could then be eluted from the column as a purified preparation. This together with the use of positive and negative control lanes should help to resolve the identity of the bands obtained in this study.

If TIMP-1 were to be confirmed as a component of colonic mucus then it would be possible to quantify its presence using an enzyme linked immunoabsorbent assay (ELISA) (Cooksley et al, 1990). Measurements of TIMP-1 in a variety of human body fluids have been performed, including; serum, plasma, amniotic fluid, cerebrospinal fluid and saliva (Clark et al, 1991). Similar measurements in colonic mucus would permit comparisons of TIMP-1 expression to be made in mucus from different parts of the colon, e.g. normal compared to malignant mucosa, as well as between tumours of different stages and differentiation.

Subsequent to the experiments detailed in this chapter the unfractionated mucus samples from the ten patients in this study have been analyzed using a TIMP-1 ELISA (performed by Dr G Murphy, Strangeways Research Laboratory, Cambridge, U.K.). The results are presented in Table 3.4. and show that TIMP-1 is detectable in colonic mucus.

Changes in MMPs expression and the level of enzyme-inhibitor complexes in neoplastic disease could also be followed with the appropriate immunoassays (Cooksley et al, 1990). However, as mentioned previously, before quantitative differences in the TIMP-1 content of mucus (or MMPs) are related to tumour behaviour, its origin must be determined. All of the mucus samples examined in this study were obtained by scraping normal colonic mucosa. It is therefore of interest that while each sample examined gave an

indication that it contained TIMP-1, only one of the normal mucosal biopsies from the same patients disclosed its presence in the immunohistochemical study (patient HF). It is possible that TIMP-1 in mucus is simply there as a result of passive transudation from the plasma. Levels of TIMP-1 in mucus would then be similar to those in plasma and less

| Patient | Age | Sex | Site | Diff | Dukes' stage | TIMP ng/ml |
|---|---|---|---|---|---|---|
| DG | 70 | M | Caec | Mod | C | 860 |
| HF | 72 | M | Desc colon | Poor | C | 350 |
| JF | 89 | M | Rectum | Mod | B | 270 |
| WL | 68 | M | Rectum | Well | B | 160 |
| KW | 40 | F | Crohns | | | 370 |
| JL | 67 | M | Desc colon | Mod | C | 330 |
| EF | 86 | F | Rectum | Poor | C | 165 |
| MS | 74 | M | Ascen colon | Mod | C | 100 |
| ES | 91 | M | Rectum | Mod | B | 250 |
| LT | 66 | M | Rectum | Mod | C | 1305 |

**Table 3.4:** The results of using an ELISA to measure the level of TIMP-1 in unfractionated samples of mucus. Diff, differentiation (results courtesy of Dr G. Murphy, Strangeways Research Laboratory, Cambridge).

likely to reflect the pathological status of the colon. Alternatively, TIMP-1 might be produced as a specific component of mucus by cells within the epithelial crypts. Under these circumstances changes in the TIMP-1 content of mucus would be more likely to reflect the processes of malignant transformation and tumour progression. If secreted rapidly into the crypt lumen its presence might be very difficult to detect, even immunohistochemically, particularly in normal mucosa where levels of synthesis are likely to be low. In patients with small bowel Crohns disease TIMP-1 has been localized to cells at the base of

mucosal crypts (Bailey et al, 1990). Although the immunolocalization study reported in Chapter 2 failed to confirm this finding for colonic tissue a repeat study using a longer period of tissue culture with monensin to block the secretion of TIMP-1 might prove more successful.

**Figure 3.1**

The silver stained gel (left) and immunostained Western blot (right) from Experiment 1.

On the silver stained gel lane a represents a run of standard molecular weight markers, and lanes b and c the run of 25µl and 50µl samples of mucus, respectively. The mucus samples are seen to contain a heavy concentration of proteins of varying molecular weights.

On the Western blot which has been stained using the antiserum to TIMP-1 a faint but discrete band (arrow) is present in both lanes b and c marking the presence of TIMP-1 adjacent to the 30 kDa molecular weight marker. The band in lane b (50µl of mucus) appears more prominent than the band in lane c (25µl of mucus) suggesting a crude dose response.

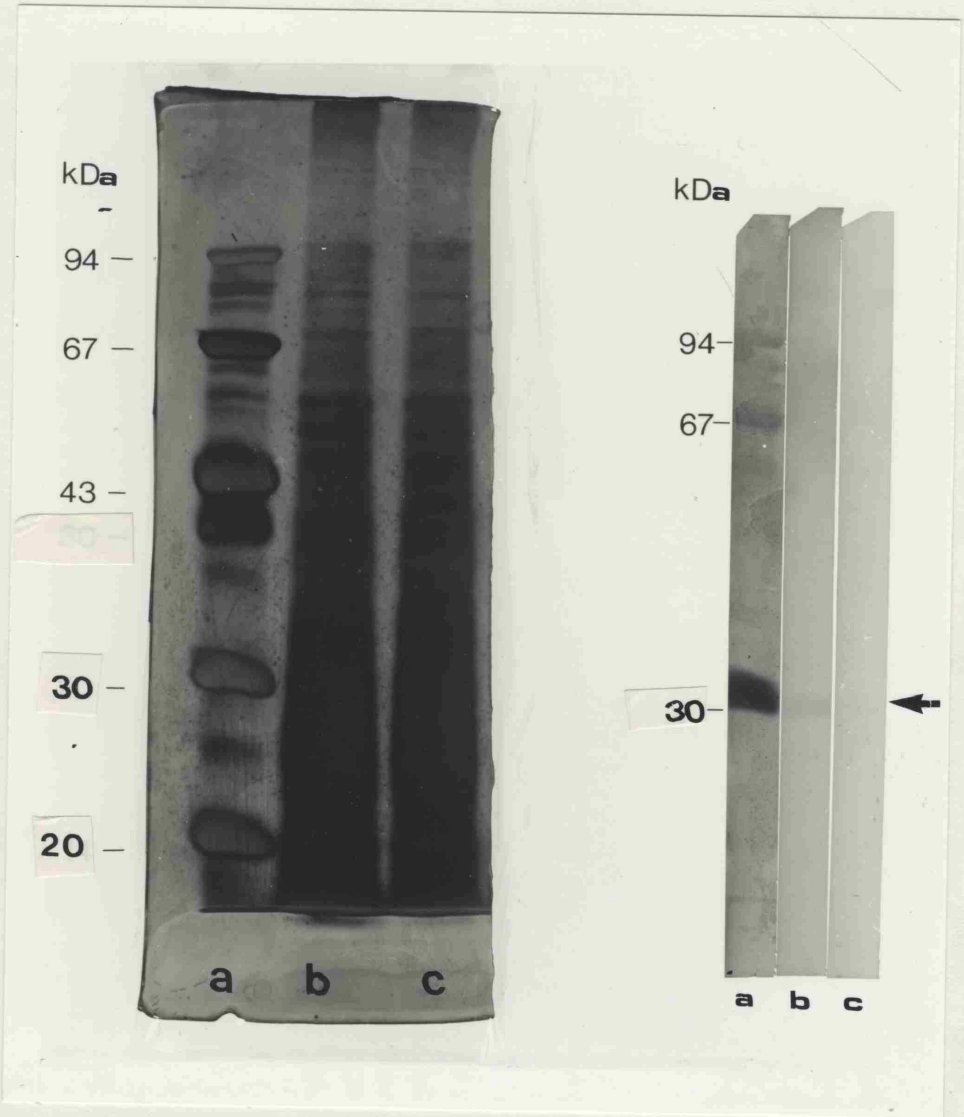
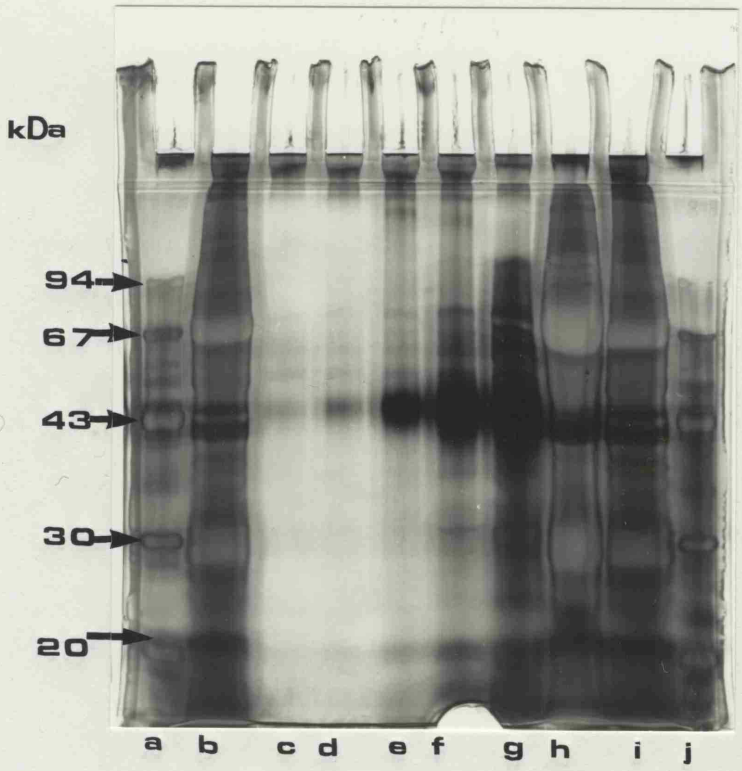

kDa
94
67
43
30
20
a
b
c
kDa
94
67
30
a
b
c

**Figure 3.2**

This shows the appearance of the silver stained gel from experiment 2. Lanes a and j contain standard molecular weight markers, lanes b and i unfractionated mucus, and lanes c to h fractions obtained following caesium chloride density gradient centrifugation of crude mucus. Proteins, represented by the presence of bands within each lane, are seen to be virtually absent in lane c and abundant in lane h, with intermediate concentrations of protein between the two. The fraction of mucus run in lane c (fraction 1) was obtained from the bottom of the centrifugation tube and therefore has a higher buoyant density than the fraction (fraction 6) run in lane h which was obtained from the top. Proteins with their lighter buoyant density tend to accumulate at the top of the tube (lane h) while glycoproteins with their greater buoyant density collect at the bottom.

**Figure 3.3**

The Western blot, corresponding to the silver stained gel seen in Figure 3.2, and immunostained with antiserum to TIMP-1. The presence of TIMP is suggested by a doublet band (arrow) at a molecular weight of approximately 30 kDa in lane g (fraction 5) and faintly in lane i (unfractionated mucus). The detection of TIMP-1 in fraction 5 would be compatible with its structure as a glycoprotein in which the carbohydrate portion accounts for 20% of its molecular weight. Lanes a and j, molecular weight markers; lane b, control incubated with non-immune sheep serum; lane g, fraction 5 mucus; lane h, fraction 6 mucus; lane i, unfractionated mucus.


kDa
94
67
43
30
20
a b c d e f g h i j

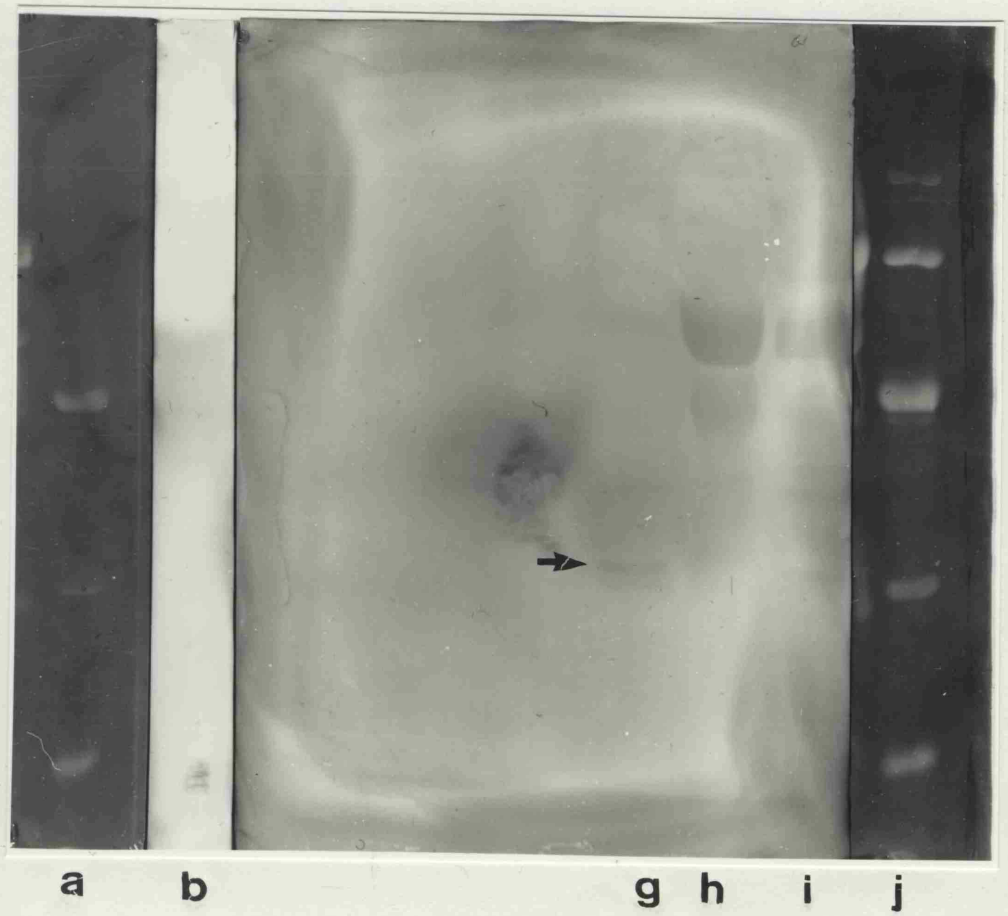
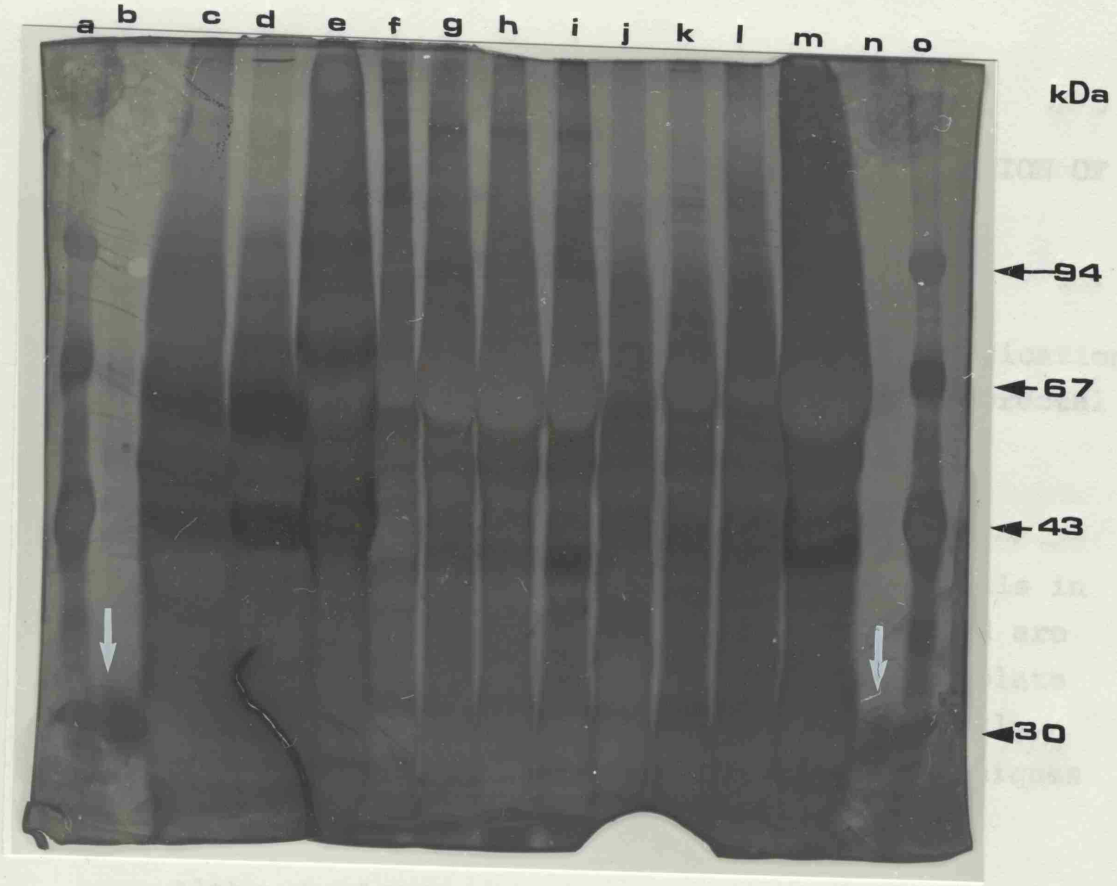

a b g h i j

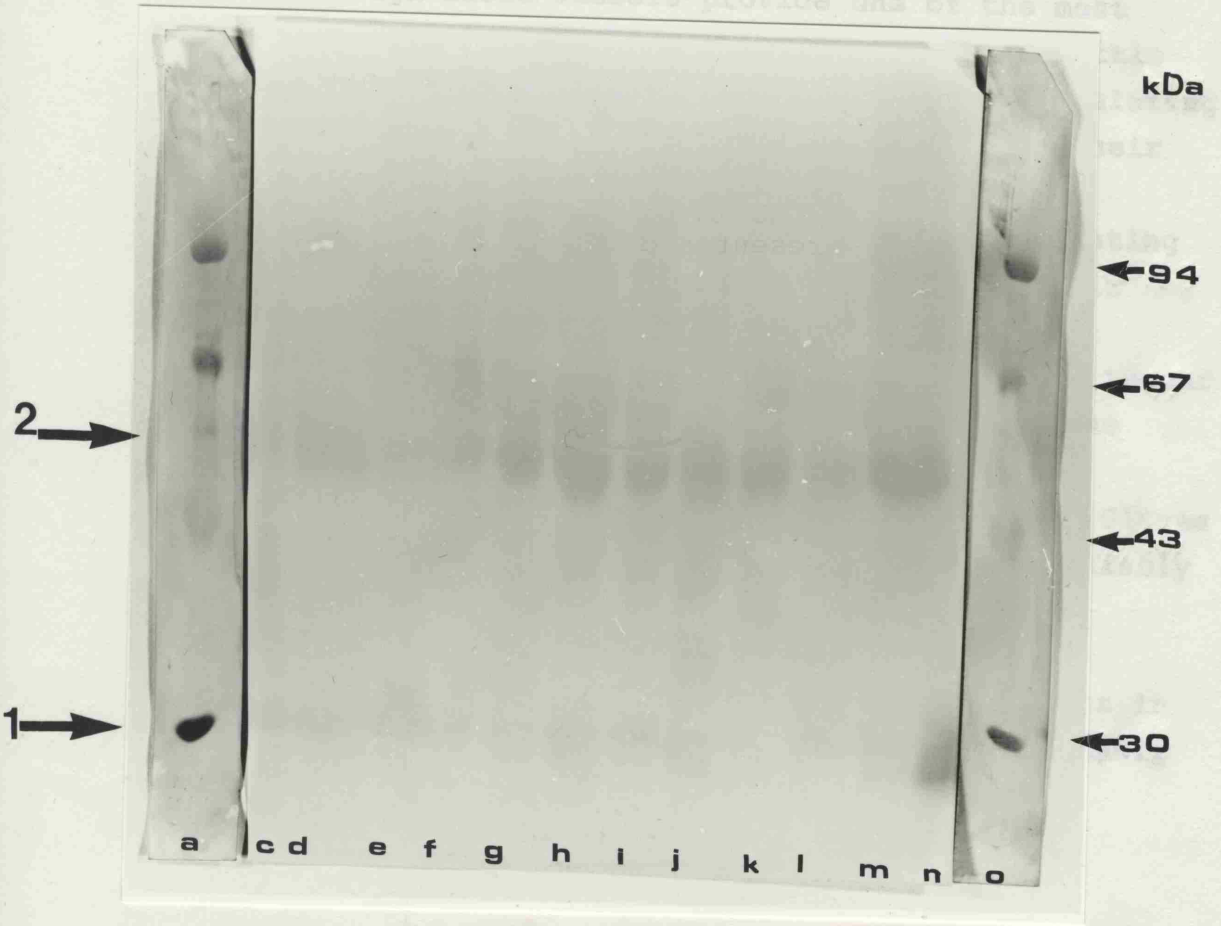
**Figure 3.4**

The silver stained gel from Experiment 3 in which the protein content of unfractionated mucus was compared to that of fraction 5 samples following caesium chloride density gradient centrifugation of crude mucus. A qualitative difference is clearly seen between the protein bands obtained from the fractionated samples of mucus (lanes c, d and e) and those present in the non-fractionated samples (lanes f,g,h,i,j,k,l,m). Lanes b and n contain a sample of recombinant TIMP-1, which appears in each as a band (arrowed) adjacent to the 30 kDa molecular weight marker. Standard molecular weight markers have been run in lanes a and o.

**Figure 3.5**

The immunostained Western blot corresponding to the silver stained gel seen in Figure 3.4; the content of each lane is as described for figure 3.4. Recombinant TIMP-1 in lane n is clearly revealed at a position compatible with its correct molecular weight of 28 kDa. Adjacent to the TIMP-1 band and appearing in each of the sample lanes (c to m) is a faint band (arrow 1) which may indicate the presence of TIMP-1. A second band (arrow 2) is also present in each of the sample lanes at a position between the 67 kDa and 43 kDa molecular weight markers and may represent a dimer of TIMP-1 (see text).


a b c d e f g h i j k l m n o
kDa
94
67
43
30


kDa
94
67
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1
a c d e f g h i j k l m n o

# CHAPTER 4

# A METHOD FOR THE IDENTIFICATION AND QUANTIFICATION OF CIRCULATING TUMOUR CELLS.

## 4.1 AIM

To develop a method for the detection and quantification of circulating tumour cells in patients with colorectal cancer.

## 4.2 INTRODUCTION

Many of the published accounts of circulating cells in human cancers stem from the 1950's and 1960's and are subject to criticism over the methods used to isolate and identify tumour cells. It is only more recently, with the introduction of improved isolation techniques and more reliable methods of identification that interest has been rekindled.

Although blood vessels provide one of the most important conduits for tumour spread, there is little reliable information regarding the number of circulating tumour cells in human cancers (Glaves, 1986) or their prognostic significance. The aim of this study, therefore, was to develop a method whereby circulating tumour cells could be reliably identified and their concentration estimated.

To estimate the concentration of circulating tumour cells account must be taken of two problems: i) the number of tumour cells is likely to be small in comparison with the number of normal blood cells (Glaves et al, 1988) and, ii) the tumour cells must be reliably distinguished from cells of haemopoetic origin.

The techniques chosen for the collection, identification and enumeration of circulating cells in this study are summarised below, with details of their validation described in the subsequent sections.

**Collection:** density gradient centrifugation

**Identification:** immunochemically by an antibody directed against the epithelial cell specific, cytoskeletal intermediate filament proteins known as the keratins.

**Enumeration:** From the ratio of tumour cells to mononuclear cells on prepared cytospins.

These methods were validated against a system in which cells from a malignant colorectal cell line were seeded into aliquots of whole blood.

## 4.3 SELECTION OF CELL LINE AND ANTIBODY

### *4.3.1 Materials and Methods*

It was an important aspect of the experimental system that as many of the seeded tumour cells as possible should be detectable by the immunocytochemical method used. Initial experiments were therefore conducted to determine the relative abilities of two antibodies to recognize cells from different colorectal cell lines.

Of the two antibodies selected, one recognized a cellular membrane antigen, epithelial membrane antigen, and the other, intracellular proteins forming part of the cytoskeleton, known as the cytokeratins. The antibodies used were an anti-epithelial membrane antigen antibody (anti-EMA Ab; a gift from Dr M O'Hare, Institute of Cancer Research, Sutton, Surrey, UK) (Imrie et al, 1990) and the monoclonal anti-cytokeratin 8.13 antibody (Sigma, Poole, UK). The anti-cytokeratin antibody 8.13 binds to a wide variety of epithelial tissues and cultured epithelial cells and recognizes a large number of human cytokeratins (Gigi et al, 1982). These two antibodies were directed against four colorectal cancer cell lines: COLO 205, COLO 320, SW-480 and LoVo.

### *4.3.2 Immunocytochemistry*

A two-step indirect immunoperoxidase technique was used to detect binding of the chosen antibodies to the cells. In this technique incubation with an antibody against the antigen of interest is followed by incubation with a second antibody raised to the gamma-globulin of the species which provided the first antibody. The enzyme peroxidase is conjugated to the second antibody and the reaction between this enzyme and its substrate, hydrogen peroxide, is used to convert a colourless chromogen into a coloured end product. This histochemical reaction allows binding of the second antibody, and hence the primary antibody, to be visualized in histological preparations.

Eight smears, including four which would act as controls, were made for each cell line by allowing a drop of cell suspension in Tris-buffered saline (TBS) (Appendix 4.1) to dry on a microscope slide. Once dry, the cell smears were fixed in acetone for 5 minutes at 4°C and then washed for 5 minutes in each of two changes of TBS. To separate slides of each cell line the two antibodies were applied at the following dilutions in TBS; the anti-EMA Ab at 1:500 and the anti-cytokeratin 8.13 Ab at 1:50. The slides were incubated with their antibodies for one hour at room temperature in an humidity chamber, and then washed twice in TBS.

Binding of the primary antibody was revealed by incubating the slides with a second antibody conjugated to horseradish peroxidase. For slides incubated with the anti-cytokeratin Ab, a rabbit anti-mouse antibody (Dako, High Wycombe, UK) was used, and for those with the anti-EMA Ab, an anti-rat Ab (Dako, High Wycombe, UK) was used. Both secondary antibodies were used at a dilution of 1:100 in TBS and the incubation time was 45 minutes at room temperature. At the end of this period two further washes were carried out in TBS before placing the slides in a solution of the chromogen 3,3 diaminobenzidine tetrahydrochloride (DAB) (Aldrich,

Gillingham, UK) for 10 minutes (Appendix 4.1). On contact with the horseradish peroxidase bound to the second antibody, DAB becomes oxidized to produce a brown coloured product. The slides were then washed in tap water and counterstained in Harris' Haemalum (Merck, Poole, UK) for 20 seconds. After a further wash in tap water the slides were dipped in Scott's tap water (Appendix 4.1) before being sequentially dehydrated in 70% alcohol and 74 OP industrial methylated spirit. Slides were then dipped in CNP 30 (Penetone, Cramlington, UK) and mounted in DPX (Merck, Poole, UK). The control slides were treated exactly as described, except that either the primary or secondary antibody was omitted.

The slides were examined on an Olympus BH2 microscope (Olympus, Tokyo, Japan). Two observers, working independently, assessed the slides using a semi-quantitative scale to express the percentage of cells stained. The experiment was repeated on two separate occasions.

### *4.3.3 Results*

For each of the two experiments the observations of the two assessors agreed with one another completely and are presented in Table 4.1. In each case the corresponding control slides were negative.

| | | Cell Line | | | |
|---|---|---|---|---|---|
| | | COLO 205 | COLO 320 | LoVo | SW-480 |
| | Anti-EMA Ab | - | - | - | - |
| Antibody | | | | | |
| | 8.13 Ab | +++ | +++ | +++ | ++++ |
| | | | | | |

Table 4.1: The percentage of cells stained when an anti-EMA and an anti-cytokeratin Ab were used against four different cell lines. -, no staining; +, ≤ 25% cells stained; ++, > 25% but ≤ 50% staining; +++, > 50% but ≤ 100% cells stained; ++++, 100% cells stained.

### *4.3.4 Discussion*

These results indicated that the best test system was likely to be made using cells from the SW-480 cell line which could then be detected with the anti-cytokeratin 8.13 antibody.

It was disappointing that the antibody directed against EMA failed to recognize cells from any of the cell lines. It is possible, that being a membrane component, EMA may have been lost or sufficiently altered under the conditions of cell culture to render it unrecognizable by the anti-EMA antibody used.

## 4.4 ISOLATION OF SEEDED TUMOUR CELLS FROM WHOLE BLOOD.

There are few reliable quantitative estimates of circulating human cancer cells. Of those available, perhaps the best, is a study of the haematogenous dissemination of cells from 10 human renal adenocarcinomas (Glaves et al, 1988). This study found

that concentrations of tumour cell emboli ranged from 14 to 7000 emboli per ml, with a mean of 887 emboli per ml Such low numbers of tumour cells may be missed unless their presence is amplified.

By exploiting differences in cell density, density gradient centrifugation can enhance the presence of a given population of cells by removing others of no interest. The density gradient medium chosen for investigation in this study was Histopaque-1077 (Sigma, Poole, UK) which removes red blood cells and the majority of polymorphonuclear leucocytes to leave a suspension rich in lymphocytes and monocytes.

### *4.4.1 Materials and Methods*

Density gradients were constructed in 30 ml universal tubes (Bibby Sterilin, Stone, UK) and consisted of 5 ml of tissue culture medium (Minimal Essential Medium, Department of Zoology, University College London) underlaid with 5 ml of Histopaque-1077 (Sigma, Poole, Dorset). To the top of the gradient 5 ml of whole blood, containing seeded malignant cells at a concentration of $10^5$ $ml^{-1}$, was layered drop-wise using a Pasteur pipette. The blood mixes with the media to give two layers. Four such gradients were constructed together with a control gradient in which the added blood contained no malignant cells. The gradients were then centrifuged at 403g for 30 minutes in a Mistral 2000 centrifuge (MSE, Loughborough, UK). Cells located at the interface layer were harvested and washed twice in culture medium by centrifugation at 200g for 5 minutes. After the final wash the supernatant was tipped off and the cell pellet resuspended in 1 ml of tissue culture medium. The concentration of cells in this suspension was determined using a haemocytometer (Neubauer Chamber; Merck, Poole, UK) and then adjusted to $10^6$ $ml^{-1}$.

The cellular composition of the suspension was examined by making and staining cytospin preparations. For each gradient 6 cytospins were produced using a Mark

1 cytospin machine (Shandon Elliot, Runcorn, UK) and stained using a modification of the indirect immunoperoxidase technique already described in section 4.3.2.. The modification was required to block endogenous leucocyte peroxidase. If left, endogenous peroxidase would produce false positives amongst the leucocytes which would stain brown when exposed to the chromogen DAB. Endogenous peroxidase was eliminated by placing the slides in 300 ml of methanol containing 300 µl of hydrogen peroxide for 30 minutes. This took place prior to the addition of the primary antibody and was followed by washing in tap water for 5 minutes, distilled water for 2 minutes and finally TBS for 5 minutes.

### *4.4.2 Results*

The predominant cell types in the stained cytospins from each of the test gradients were the seeded SW-480 tumour cells and mononuclear blood cells (lymphocytes and monocytes) (Fig 4.1). A few polymorphonuclear leucocytes were also present. Positive immunostaining was absent on cytospins from the control gradients.

### *4.4.3 Discussion*

This experiment confirmed the ability of density gradient centrifugation, using the medium Histopaque-1077, to extract cells of the SW-480 cell line from whole blood with the exclusion of red blood cells and the majority of polymorphonuclear leucocytes. Furthermore, the SW-480 cells could be identified using the anti-cytokeratin 8.13 Ab without false positives occurring amongst the remaining leucocytes.

## 4.5 QUANTIFICATION OF TUMOUR CELLS SEEDED INTO WHOLE BLOOD: THE EFFICIENCY OF COLLECTION.

In devising a method for the enumeration of circulating tumour cells the assumption was made that both tumour cells and mononuclear cells were deposited with equal efficiency in the interface layer following density gradient centrifugation. If this were true, then the ratio of tumour cells to mononuclear cells (lymphocytes and monocytes), in the interface layer, should not differ significantly from their ratio in whole blood. This in turn would allow the concentration of tumour cells to be determined from the ratio of tumour cells to mononuclear cells observed on prepared cytospins; the method of calculation is set out in Equation 1.

Equation 1

$$[T] = \frac{[M]\ T_c}{M_c}$$

Where:

[T] = the concentration of tumour cells $ml^{-1}$

[M] = monocyte concentration $ml^{-1}$

$T_c$ = tumour cell count on a cytospin(s)

$M_c$ = monocyte count on a cytospin(s)

The validity of this approach was examined in two ways: i) the efficiencies with which tumour cells and mononuclear cells were collected following centrifugation were compared; ii) for known numbers of tumour cells, cytospin counts of tumour and mononuclear cells were made, the values substituted in Equation 1 and the result compared with the original tumour cell concentration.

### *4.5.1 Materials and methods*

**Tumour cells**

Five density gradients were prepared as described above. To each of them 5 ml of phosphate buffered saline (PBS), containing $10^6$ SW-480 tumour cells (2 x $10^5$ cells $ml^{-1}$), was added. Prior to seeding the tumour cells were stained with the supra-vital dye Neutral Red O to accentuate their presence at the interface following centrifugation (Fig 4.2). The gradients were then centrifuged as before, the cells at the interface harvested, washed and resuspended in 1 ml of PBS. The number of cells in the suspension was counted using a haemocytometer.

Efficiency was represented as the number of tumour cells harvested expressed as a percentage of the number of tumour cells added.

**Mononuclear cells**

The efficiency of collection for mononuclear cells was determined in an identical fashion to the above, except that the suspension of tumour cells in PBS was replaced with 5 ml of whole blood, the mononuclear cell content of which was known from a full blood count.

The efficiencies of collection for tumour cells and monocytes were compared using the Mann - Whitney U test.

### *4.5.2 Results*

The results are presented in Tables 4.2 and 4.3. The mean number of SW-480 tumour cells and mononuclear cells recovered from the interface was 1.914 x $10^5$ and 2.546 x $10^5$ respectively. There was no significant difference in the efficiency with which tumour cells and mononuclear cells were deposited in the interface layer (Mann Whitney test, $U=5$, $p>0.05$).

| Number of cells seeded | $10^6$ | |
|---|---|---|
| Gradient | Number of cells recovered $x10^5$ | Efficiency of recovery (%) |
| 1 | 1.59 | 16 |
| 2 | 2.02 | 20.2 |
| 3 | 2.17 | 21.7 |
| 4 | 2.24 | 22.4 |
| 5 | 1.55 | 15.5 |
| Mean | 1.91 | 19 |

*Table 4.2: The efficiency of collection for SW-480 tumour cells.*

| Number of cells seeded | $10.206\ x10^6$ | |
|---|---|---|
| Gradient | Number of cells recovered $x10^6$ | Efficiency of recovery (%) |
| 1 | 2 | 19.5 |
| 2 | 2.74 | 26.8 |
| 3 | 2.79 | 27.3 |
| 4 | 2.2 | 21.5 |
| 5 | 3 | 29.3 |
| Mean | 2.55 | 24.9 |

*Table 4.3: The efficiency of collection for mononuclear cells.*

### *4.5.3 Discussion*

The lack of a significant difference between the collection efficiencies of tumour cells and mononuclear cells gives an indication that both are handled in the same way by the Histopaque density gradient. It also indicates, but does not prove, that the ratio of tumour cells to mononuclear cells present in whole blood will be preserved at the interface.

The range of collection efficiencies observed for each test was wide, being 16% to 22.4% for tumour cells and 19.5% to 27.3% for mononuclear cells. This variation may be explained by differences in the completeness with which cells at the interface layer were harvested. If cells are left behind during this process, even though they may have been deposited at the interface, then estimates of the efficiency with which they are deposited will be lower than expected. However, in a mixture of tumour cells and mononuclear cells deficiencies in harvesting the interface layer should affect both equally. Other factors which may reduce the yield of cells include, failure of cells to be deposited at the interface and loss of cells during the washes.

## 4.6 COMPARISON OF WHOLE BLOOD AND CYTOSPIN RATIOS OF TUMOUR CELLS TO MONONUCLEAR CELLS.

### *4.6.1 Materials and methods*

Five density gradients were constructed as described in Section 4.4.1. From a human volunteer, not known to be suffering from an epithelial malignancy, 30 ml of blood were collected into heparinised tubes (Vacutainer, Meylan Cedex, France). The blood sample was divided into six 5 ml aliquots, one of which was sent for a full blood count (courtesy of the Department of Haematology, UCMSM) to determine the concentration of mononuclear cells (lymphocyte count + monocyte count). To four of the remaining five samples SW-480 tumour cells were

added so that the concentration of tumour cells within each was respectively; $10^1$ $ml^{-1}$, $10^2$ $ml^{-1}$, $10^3$ $ml^{-1}$ and $10^4$ $ml^{-1}$. No tumour cells were added to the fifth sample, which acted as a control. Each of the gradients was then centrifuged, the cells at the interface layer harvested and washed, and cytospins made as described above. For each of the interface layers 24 cytospin preparations were made.

The indirect immunoperoxidase method with the anti-cytokeratin 8.13 antibody was used to identify tumour cells on the cytospins as previously described.

This experiment was conducted five times in all. On each of the subsequent occasions blood from a different volunteer, and hence with a different mononuclear cell count, was used.

### *4.6.2 Method of counting cells on the cytospins*

For each of the control gradients, processed with blood devoid of tumour cells, no cell was identified which had stained positively with the anti-cytokeratin 8.13 antibody. This confirmed again that the anti-cytokeratin antibody 8.13 did not bind to cells of haemopoietic origin.

Examination of the cytospins prepared from the other gradients revealed three distinct populations of cells: tumour cells, stained brown by the immunoperoxidase technique; mononuclear leucocytes, and a few polymorphonuclear leucocytes identifiable by their multi-lobed nuclei. The polymorphonuclear leucocytes were excluded from all cell counts performed.

The number of tumour cells appearing on any one cytospin was relatively small, enabling them to be counted in their entirety. However, the same was not true of the mononuclear cells, which were sufficiently numerous to make a total count impractical. The number of mononuclear cells per cytospin was therefore estimated from a sample count.

### *4.6.3 Sampling method*

To determine the sampling method 6 of the cytospins prepared in section 4.4.1. were selected at random. Each cytospin was examined under a x20 objective with a 1 mm index square graticule (Graticules Ltd, Tonbridge, UK) inserted into a x10 eyepiece, giving a total magnification x200. At this magnification the area of the graticule, superimposed on the cytospin, represented one field.

To determine how many fields should be counted the cells were assumed to be distributed randomly and independently on the cytospin. The Poisson distribution could then be applied, and the standard error of the cell count would be given by Equation 2.

Equation 2.

$$s.e = \sqrt{n}$$

Where:
s.e. = standard error
n = number of cells counted

The number of mononuclear cells on each cytospin ($M_c$) was calculated by multiplying the mean number of cells per field by the number of fields on the cytospin (F), as set out in Equation 3.

Equation 3

$$M_c = \frac{n_f F}{f}$$

Where:
$M_c$ = number of mononuclear cells on the cytospin
$n_f$ = number of mononuclear cells counted in f fields
F = number of graticule fields on the cytospin
f = number of fields counted

The standard error of the number of mononuclear cells on the cytospin ($M_c$) is determined by the standard error of the number of cells counted ($n_f$) (Equation 4).

Equation 4

$$s.e.\ M_c = \frac{\sqrt{n_f}\ F}{f}$$

A consideration of all 6 cytospins studied (Table 4.4) revealed the mean number of cells per field to be approximately 200 and the average number of fields per cytospin about 80.

| Field | Cytospin 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| 1 | 68 | 151 | 157 | 360 | 111 | 153 |
| 2 | 136 | 151 | 160 | 414 | 106 | 313 |
| 3 | 259 | 177 | 173 | 244 | 169 | 348 |
| 4 | 360 | 193 | 220 | 177 | 191 | 306 |
| 5 | 276 | 201 | 217 | 278 | 199 | 276 |
| 6 | 225 | 192 | 307 | 431 | 149 | 255 |
| 7 | 273 | 191 | 190 | 454 | 186 | 301 |
| 8 | 262 | 142 | 173 | 421 | 148 | 268 |
| 9 | 219 | 113 | 141 | 372 | 222 | 245 |
| 10 | 162 | 134 | 155 | 357 | 191 | 207 |
| 11 | 146 | 103 | 108 | 152 | 159 | 232 |
| 12 | 92 | 29 | 27 | 71 | 95 | 143 |
| MEAN | 206 | 148 | 169 | 311 | 106 | 254 |

Table 4.4: Cell counts made on six cytospins. Fields spanning a full diameter of the cytospin were counted. Field numbers 1 and 12 mark the periphery of the cytospin and numbers 6 and 7 the centre. The mean number of cells per field for each cytospin is also given.

If these values are used in Equations 3 and 4 the standard errors for different cell counts can be calculated. These are presented in Table 4.5.

| $n_f$ | f | $M_c$ | s.e. | % error in $M_c$ ($s.e./M_c$) |
|---|---|---|---|---|
| 200 | 1 | 16,000 | 1131 | 7 |
| 400 | 2 | 16,000 | 800 | 5 |
| 600 | 3 | 16,000 | 653 | 4 |
| 800 | 4 | 16,000 | 565 | 3.5 |
| 1000 | 5 | 16,000 | 505 | 3 |

Table 4.5: Variation in the percentage error of the estimated cytospin cell count according to the number of cells counted. Mean number of fields per cytospin = 80.

From these results the sample size chosen was 3 fields. This was considered manageable to count, and would allow the number of cells on each cytospin to be estimated with a standard error of 4%.

In deriving the field sample size it was assumed that the cells were dispersed randomly on the cytospin. In reality this was not strictly true, as an examination of the field counts for the six cytospins will confirm (Table 4.4).

Cytospin production relies on cells being propelled from a chamber onto a slide by centrifugal force; the cells hit the middle of the slide and then spread outwards. This results in the greatest density of cells being found centrally (Fields 6 & 7, in Table 4.4), with the least dense areas at the periphery (Fields 1 & 12, in Table 4.4). If the three sample fields were selected from either of these extremes the total cell count for the cytospin would be either under or over estimated. To compensate for the spatial heterogeneity of cells each cytospin was divided into 3 concentric, equal sub-areas (like an archery target). In terms of graticule fields the width of the three areas was 1 field for the outermost, 1.5 fields for the middle area and 3.5 fields for the central area. Within each sub-area the cells were considered to be approximately uniformly distributed. One field was then chosen at random from each sub-area and the cells within it counted. The <u>total</u> of the three counts was then used in Equation 3 to calculate the number of mononuclear cells on the cytospin.

### *4.6.4 Summary of counting method*

Six cytospins were examined from each of the gradients prepared in section 4.4.1.. For each cytospin *all* the tumour cells were counted while the number of mononuclear leucocytes was estimated from a sample count. The sample consisted of three graticule fields (viewed at a magnification of x200), one selected from

each of three sub-areas in which the cells were considered to be evenly distributed. A count was also made of the number of fields required to cover each cytospin. The total number of mononuclear cells on the six cytospins was calculated using Equation 3 and the concentration of tumour cells from Equation 1. The calculated concentrations of tumour cells were then compared with the concentrations originally present in whole blood using the chi-squared test.

### *4.6.5 Error in the calculated tumour cell concentration.*

From Equation 1 the calculated tumour cell concentration depends on the ratio of the tumour cell count to the mononuclear cell count. Of the two values in this ratio the tumour cell count is much the smaller and therefore errors in this count will have the greater influence on the final calculation of the tumour cell concentration. In Table 4.6 the 95% confidence interval for the tumour cell concentration has been calculated by substituting the upper and lower values for the 95% confidence intervals for the tumour cell counts into Equation 1. The 95% confidence interval for each tumour cell count was obtained by reference to tables for the Poisson distribution (Gardner and Altman, 1989). An example from Table 4.6 will illustrate the method.

The observed ratio of the tumour cell count to the mononuclear cell count in sample 1 when the concentration of seeded tumour cells was $10^2$ $ml^{-1}$ was 5:131370. Using Equation 1 the concentration of tumour cells (T) may be calculated from this ratio:

$$T = \frac{5 \times 3402000}{131370} = 129$$

From tables the 95% confidence interval for a count of 5 is 1.62 - 11.62. Substituting each of these values in turn into Equation 1 gives the 95% confidence interval for the tumour cell concentration as:

$$\frac{1.62 \times 3402000}{131370} - \frac{11.62 \times 3402000}{131370}$$

$$= 41 - 300$$

The error in the mononuclear cell count will have a much smaller influence on the tumour cell concentration. The approximate upper ($x_U$) and lower ($x_L$) limits for confidence intervals for counts (x) greater than 100 are given by:

$$x_L = \left(\frac{N_{1-\frac{\alpha}{2}}}{2} - \sqrt{x}\right)^2$$

$$x_U = \left(\frac{N_{1-\frac{\alpha}{2}}}{2} + \sqrt{x+1}\right)^2$$

For 95% confidence intervals

$$N_{1-\frac{\alpha}{2}} = 1.96$$

In the example chosen the 95% confidence interval for a mononuclear cell count of 131370 will be 130660 to 132082. Substitution of these values in Equation 1 has a negligible influence on the tumour cell concentration calculated (130 cells $ml^{-1}$, 128 cells $ml^{-1}$ respectively).

### *4.6.6 Results*

The results for all five experiments are presented in Table 4.6 and the statistical analysis in Table 4.7. Table 4.7 shows that the observed tumour to mononuclear cell ratio was significantly different to the expected ratio in two instances, both of which occurred when $10^4$ cells $ml^{-1}$ had been seeded into whole blood. In a further two samples, occurring when $10^1$ cells $ml^{-1}$ had been seeded, no tumour cells were detected after six cytospins had been examined for each. The expected ratio of tumour cells to mononuclear cells had therefore been preserved in 16 of the 20 samples examined.

| $[T]_s$ $ml^{-1}$ | | $10^1$ $ml^{-1}$ | | $10^2$ $ml^{-2}$ | | $10^3$ $ml^{-1}$ | | $10^4$ $ml^{-1}$ | |
|---|---|---|---|---|---|---|---|---|---|
| SAMPLE | [M] $ml^{-1}$ | OBS T:M | [T] $ml^{-1}$ (95% CI) | OBS T:M | [T] $ml^{-1}$ (95% CI) | OBS T:M | [T] $ml^{-1}$ (95% CI) | OBS T:M | [T] $ml^{-1}$ (95% CI) |
| 1 | 3402000 | 1:54895 | 62 (1.54-345) | 5:131370 | 129 (41-300) | 16:56092 | 970 (554-1575) | 207:88091 | 8004 (6912-9152) |
| 2 | 2004300 | 1:90484 | 22 (0.6-123) | 11:132238 | 166 (83-298) | 60:129934 | 925 (706-1191) | 510:80799 | 12650 (11584-13792) |
| 3 | 1303800 | no tumour cells | 0 (0-29) | 10:154681 | 84 (40-155) | 124:145008 | 1114 (926-1321) | 1894:256029 | 9644 (9217-10088) |
| 4 | 2298400 | 2:110619 | 41 (5-150) | 3:66757 | 103 (21-301) | 83:166828 | 1143 (910-1417) | 346:88250 | 9001 (8074-10001) |
| 5 | 4102000 | no tumour cells | 0 (0-85) | 10:212828 | 192 (92-354) | 40:163084 | 1066 (718-1370) | 732:330860 | 9075 (8430-9757) |

Table 4.6: Calculated tumour cell concentrations [T] derived from the observed tumour to mononuclear cell ratio (OBS T:M) for different concentrations of tumour cells $[T]_s$ seeded into whole blood samples. The 95% confidence interval (95% CI) for each [T] is also given.

| $[T]_s$ $ml^{-1}$ | | $10^1$ | | | $10^2$ | | | $10^3$ | | | $10^4$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SAMPLE | [M] $ml^{-1}$ | OBS T:M | EXP T:M | $X^2$, p | OBS T:M | EXP T:M | $X^2$, P | OBS T:M | EXP T:M | $X^2$, P | OBS T:M | EXP T:M | $X^2$, P |
| 1 | 3402000 | 1:54895 | 0.2:54895 | 0.022, p=0.88 | 5:131370 | 4:131370 | 0.00, p=1 | 16:56092 | 16:56092 | 0.0, p=1 | 207:88091 | 259:88091 | 5.79, p=0.01 |
| 2 | 2004300 | 1:90484 | 0.45:90484 | 0.14, p=0.71 | 11:132238 | 7:132238 | 0.45, p=0.51 | 60:129934 | 64:129934 | 0.12, p=0.72 | 510:80799 | 403:80799 | 12.47, p=0.0008 |
| 3 | 1303800 | 0:163297 | 1.3:163297 | 0.05, p=0.82 | 10:154681 | 12:154681 | 0.18, p=0.67 | 124:145008 | 111:145008 | 0.72, p=0.60 | 1894:256029 | 1969:256029 | 1.445, p=0.23 |
| 4 | 2298400 | 2:110619 | 0.5:110619 | 0.109, p=0.74 | 3:66757 | 3:66757 | 0.0, p=1 | 83:166828 | 73:166828 | 0.64, p=0.57 | 346:88250 | 385:88250 | 2.07, p=0.15 |
| 5 | 4102000 | 0:176432 | 0.4:176432 | 0.75, 0.61 | 10:212828 | 5:212828 | 1.06, p=0.30 | 40:163084 | 40:163084 | 0.0, p=1 | 732:330860 | 806:330860 | 0.3.55 p=0.056 |

Table 4.7: Comparison between the observed (OBS) tumour (T) to mononuclear cell (M) ratio and the expected (EXP) ratio, for different concentrations of tumour cells $[T]_s$ seeded into whole blood, using the chi-squared test ($X^2$). Shaded cells indicate those observed ratios which were significantly different from the expected ratios.

## 4.7 DISCUSSION

This study produced four principal findings: i) an enriched sample of SW-480 tumour cells, which have been seeded into whole blood, can be obtained by the use of the density gradient medium Histopaque-1077; ii) SW-480 tumour cells can be reliably distinguished from haemopoietic cells by the use of an antibody directed against their content of keratins; iii) when seeded into whole blood the ratio of SW-480 cells to mononuclear cells is reasonably preserved following density gradient centrifugation; iv) the ratio of tumour cells to mononuclear cells on prepared cytospins allowed an estimate to be made of the concentration of tumour cells seeded into whole blood.

The use of anti-cytokeratin antibodies to identify tumour cells is discussed in Chapter 5. In the context of this study the anti-cytokeratin 8.13 antibody was successfully used to identify seeded, epithelial derived, tumour cells on the basis of their keratin content. No positive staining was observed in the five control blood samples from which tumour cells were absent; although it is appreciated that this number of controls is too small to confirm the specificity of the 8.13 antibody for epithelial cells alone.

In this study an antibody to epithelial membrane antigen was also evaluated; tested against four different human colorectal cancer cell lines it failed to recognize tumour cells from any of them. This failure was almost certainly the result of using cultured cells, the relevant membrane antigen having been altered by the trypsinization required for repeated passage. The anti-EMA antibody had been shown to work on tissue sections of colorectal tumours at the dilutions used in this study.

Initial attempts to study tumour cells in the circulation were hampered by failure to balance a reasonable

chance of recovery with the preservation of cell morphology. A number of methods were devised to enrich the presence of tumour cells, including the destruction of red blood cells with saponin (Engel, 1955), lysis of red blood cells and polymorphonuclear cells with streptolysin (Malmgren et al, 1958) and the removal of polymorphs with a magnet after allowing them to ingest iron (Kuper et al, 1961). However, the use of lytic methods was prone to damage the tumour cells, further complicating their identification by cytology. The use of differential centrifugation provided a way of isolating tumour cells without causing them undue damage.

The technique was founded on earlier demonstrations that cells of different populations could be separated according to variations in their density (Vallee et al, 1947); tumour cells could be separated from erythrocytes or polymorphs by using solutions of the appropriate specific gravity. As each cell type reaches an interface of equal or higher density, its effective mass is reduced to zero, and it is deposited at that interface (Agranoff et al, 1954).

Differential centrifugation was first used to isolate cancer cells from the circulating blood in 1958 (Roberts et al, 1958), using density gradients constructed from solutions of albumin. These solutions have now been replaced by synthetic media such as Percoll (Percoll, Pharmacia, Sweden), a polymer coated silica colloid, or mixtures of polysaccharides and radiopaque contrast media. The medium used in this study, Histopaque-1077, is of this latter type, comprising a mixture of ficoll and the radiopaque contrast medium sodium diatrizoate. Histopaque-1077 has a specific gravity of 1.077g $ml^{-1}$ and was designed principally for the separation of lymphocytes and mononuclear cells from blood.

A study of human malignant effusions, using Percoll to create a discontinuous gradient, has shown that 90 per cent of tumour cells (including those derived from colonic

adenocarcinomas) have a density less than 1.056 g $ml^{-1}$, while 98 per cent of lymphocytes have a density between 1.067-1.077 g $ml^{-1}$ (Hamburger et al, 1985). These figures suggested that tumour cells and lymphocytes should be deposited together when differential centrifugation was performed with Histopaque-1077. Based on this premise an empirical examination of the suitability of Histopaque-1077 for the enrichment of circulating tumour cells was made.

The study confirmed that the predominant cell types deposited at the interface, following centrifugation with Histopaque-1077, were lymphocytes and tumour cells, and that there was no significant difference in the efficiency with which either was isolated. Between 70 and 80 per cent of the cells were, however, lost during the process, a high figure, but nevertheless comparable to the 70% loss observed in a similar study using Percoll (Glaves et al, 1988). The multi-step process of differential centrifugation predisposes to cell loss particularly during the washing stages after their recovery from the interface. Also, even within a given population of cells, there is bound to be variation in densities so that not all will be deposited at the interface under the centrifugation conditions employed. Histopaque-1077 cannot isolate tumour cells as a pure suspension in the same way that a discontinuous gradient of Percoll can. However, unlike Percoll, it does not require dilution to a solution of the appropriate specific gravity prior to construction of the density gradient.

A potential weakness in the validation of Histopaque-1077 presented here is that cultured tumour cells may not behave in the same way as cells shed from a tumour *in vivo*. An alternative method, therefore, would have been to use suspensions of cancer cells produced from enzymatically dissociated tumour tissue. However, such suspensions would have inevitably been contaminated by other cell types, e.g. connective tissue cells, making it difficult to produce

accurate counts of cancer cells alone, prior to seeding. There is also some evidence to suggest that proteolytic digestion may alter the dry mass of some cancer cells (Weiss, 1958). If true, this would change their behaviour during density gradient centrifugation. Since the use of enzymes is common to the preparation of both cultured cells and the dissociation of tumour tissue the advantage gained by using the latter in recovery experiments is debatable.

As well as identifying tumour cells in blood this study has also attempted to determine their concentration. Several approaches to this problem, employing a variety of methods, have been made in the past and are discussed in Chapter 1, examples include: direct cytology of tumour cells trapped by a polycarbonate filter (Glaves, 1983b; Kleinerman and Liotta, 1977), the *in vitro* uptake of [$^3$H]thymidine by tumour cells isolated from whole blood (Schirrmacher and Waller, 1982), flow cytometry (Giraldi et al, 1984) and bioassay (Lee et al, 1976). Apart from direct cytology, however, few of these techniques have found an application in the investigation of human cancers.

The quantification method used in this study was based on the concept that the ratio of tumour cells to mononuclear cells in whole blood would be maintained following centrifugation with Histopaque-1077. This theory was tested, and in 16 of the 20 samples examined (Table 4.7) found to be true.

In two of the four failures, both when $10^1$ cells $ml^{-1}$ were seeded, no tumour cells were detected on the six cytospins examined. At these low concentrations this is perhaps not surprising. If the original ratio of tumour cells to mononuclear cells in whole blood is, e.g. 1:410200, as in sample 5, and if there are between 10,000 and 30,000 cells on a cytospin, then approximately 14 to 40 cytospins would have to be examined to find one tumour cell. Although not part of the original experiment this was confirmed by

finding tumour cells when additional cytospins were stained and inspected. It follows that if very low concentrations of tumour cells ($\approx 10^1$ $ml^{-1}$) are not to be missed, then a minimum number of cells (approximately equal to one tenth of the mononuclear cell concentration) must be examined.

The remaining two failures occurred when $10^4$ cells $ml^{-1}$ were seeded. The reason for these failures is not clear. One possible explanation is that at high concentrations the tumour cells tend to clump together, affecting the accuracy with which they can be counted. This would result in distortion of the observed tumour to mononuclear cell ratios. Although these two failures were disappointing, it is noteworthy that the estimated concentrations were still of the right order of magnitude namely, 8004 $ml^{-1}$ and 12650 $ml^{-1}$,

The use of density gradient centrifugation and anti-cytokeratin antibodies to enumerate circulating tumour cells has also been employed by Glaves et al (1988). In their study, however, an attempt was made to separate the tumour cells as a pure suspension using a Percoll gradient. Known volumes of the suspension were collected onto polycarbonate filters, the tumour cells stained and counted, and their concentration calculated. The principal draw back of this method is that an allowance has to be made for the recovery loss in calculating the final concentration of tumour cells. While the study indicates that this recovery loss can be estimated to within plus or minus 2.2% for tumour cell concentrations down to $10^2$ $ml^{-1}$, it is not clear how consistent the efficiency of the system is at lower concentrations. This raises doubts about the accuracy of the observations made in five of the ten patients studied, in whom tumour cells were either absent (2) or present at concentrations less than $10^2$ $ml^{-1}$ (3).

The wide 95% confidence intervals for each of the calculated tumour cell concentrations in this study

emphasizes the difficulty attached to quantifying such a small population of cells. Even when the concentration of tumour cells is 10,000 $ml^{-1}$ they still only represent approximately 0.1% of the total number of circulating cells Glaves has emphasised that given the drawbacks of various techniques absolute precision may not be feasible. It should, however, be possible to determine the order of magnitude with which tumour cells are disseminated into the circulation (Glaves, 1986). The technique described in this study would appear to be capable of doing that.

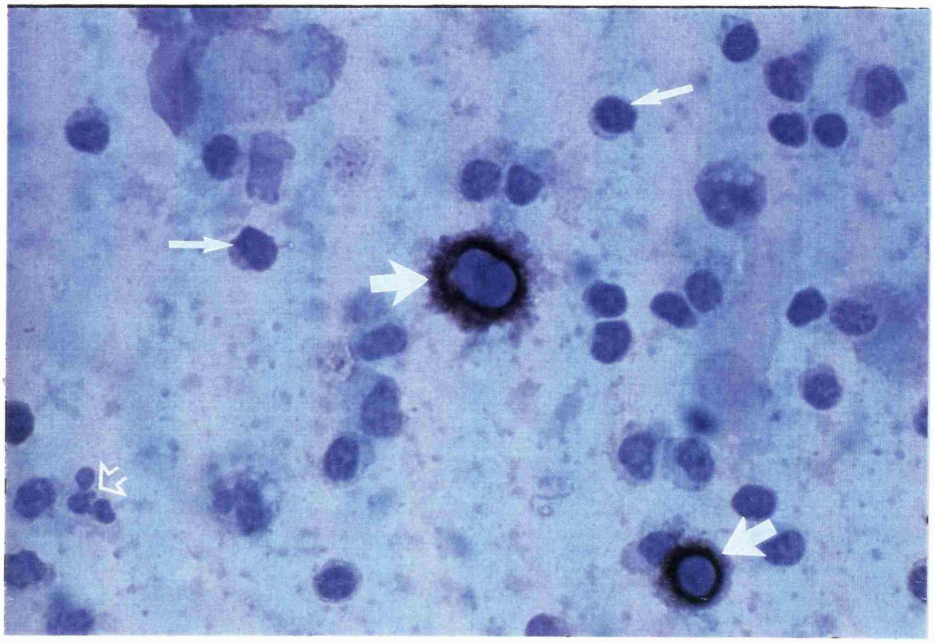
However, while the detection and enumeration of circulating tumour cells is undoubtedly important to an understanding of the metastatic process, their relevance as indicators of prognosis is less certain. Before improvements in accuracy are pursued it is this latter question which requires more investigation. In Chapter 5 the methods of identification and enumeration described in this study are used to examine the haematogenous dissemination of tumour cells in patients with colorectal cancer.

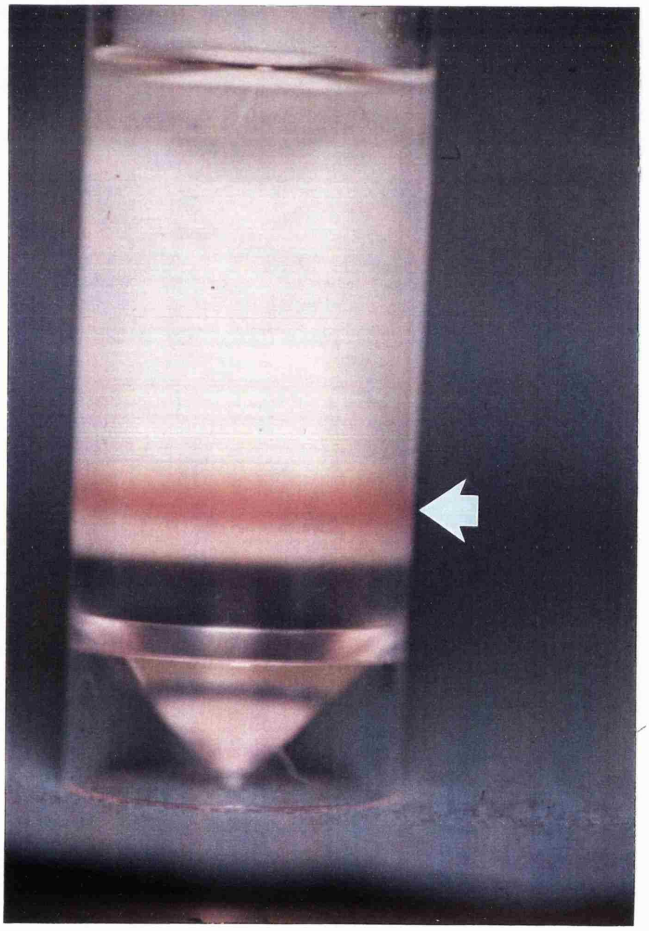
**Figure 4.1**

Cytospin prepared from the interface layer obtained by density gradient centrifugation of whole blood seeded with SW-480 tumour cells. The cytospin has been stained using an indirect immunoperoxidase technique with the anticytokeratin antibody 8.13. SW-480 tumour cells (large arrows) which are of epithelial origin are clearly identified as containing cytokeratins by the brown colour which is seen in their cytoplasm. By contrast cells of haemopoietic origin such as monocytes (small arrows) and polymorphonuclear leucocytes (open arrow) are not stained (original magnification x250).

**Figure 4.2**

Photograph showing a density gradient after centrifugation. SW-480 tumour cells, which were previously stained with the supra-vital dye Neutral Red O, are seen to form a discrete band (arrow). Prior staining of the tumour cells in this way facilitated their accurate aspiration.





# CHAPTER 5

# THE IDENTIFICATION AND ENUMERATION OF CIRCULATING TUMOUR CELLS IN PATIENTS WITH COLORECTAL CANCER.

## 5.1 AIMS

i) To identify and determine the concentration of circulating tumour cells in patients with colorectal adenocarcinomas.

ii) To examine the relation between circulating tumour cells, tumour size and vessel density.

## 5.2 MATERIALS and METHODS

### 5.2.1 *Patients*

Sixteen patients were studied and these are identified in Table 5.1.

### 5.2.2 *Samples*

For each patient 10 ml of blood was taken from the following sites: i) an antecubital fossa vein (sample taken preoperatively without a tourniquet); ii) an internal jugular vein (sample from a central catheter placed routinely by the anaesthetist); iii) samples from the mesenteric artery and vein serving the tumour.

The amount of dissection and handling of the tumour was minimised in order to reduce the iatrogenic embolization of tumour cells. However, a formal no-touch technique as advocated by Turnbull was not used (Turnbull et al, 1967). All blood samples removed peroperatively were taken after mobilization of the tumour; those from the internal jugular vein and mesenteric vessels were taken as close together in time as possible to allow comparisons to be made between their tumour cell contents. The mesenteric vein was sampled first, followed by the artery and then the internal jugular vein. Each sample was collected using a 10 ml syringe and 21

gauge butterfly needle (Abbot, Sligo, Republic of Ireland) and 5 ml decanted immediately into each of two heparinised tubes (Vacutainer PST, Becton Dickinson, UK). One of these samples was then sent for a full blood count to determine the concentration of monocytes.

With minimal delay the other blood samples were diluted 1:1 with tissue culture medium, layered onto the density gradient medium Histopaque-1077, centrifuged and cytospins made of those cells harvested from the interface layer, as described in Chapter 4. Prepared cytospins were wrapped in foil and stored at -20°C until required for staining.

For each blood sample taken six cytospins were stained using the indirect immunoperoxidase technique with the anti-cytokeratin 8.13 antibody already described in Section 4.3.2.. In addition a further six cytospins were stained for conventional cytology using the May-Grunwald-Giemsa technique. Both sets of slides were examined by a consultant cytologist.

### 5.2.3 *Assessment of stained cytospins*

The two sets of slides were examined on separate occasions and without knowledge of the patients' identities or clinical details. With this "blind" method of assessment information from the conventional cytology slides could not be used to reinforce or help with the interpretation of the immunostained slides and vice versa.

The criteria used to identify a tumour cell on conventional cytology were: i) a large hyperchromatic nucleus, ii) mottled chromatin within the nucleus, iii) a large nuclear cytoplasmic ratio, iv) abnormal mitoses, v) a prominent nucleolus. If all five of these criteria were satisfied then the cell was considered to be definitely malignant. A cell whose appearance had some but not all of the five features was regarded as suspicious of malignancy;

detection of these latter cells was signalled by the sign "+/-".

On cytospins stained by the immunoperoxidase technique tumour cells were identified by their brown colour (Fig 5.1

In addition to the cytological assessment, the concentration of tumour cells (when identified) was established according to the method set out in Chapter 4.

Also, to determine if cytokeratins were being expressed by each of the tumours examined, frozen sections, prepared from biopsies of each of the tumours, were also stained with the anti-cytokeratin 8.13 antibody.

### 5.2.4 *Tumour size*

To investigate the relationship between tumour size and the presence of circulating tumour cells the dimension of each tumour was recorded prior to the specimen being fixed. The longest linear dimension of colorectal tumours has been found to correlate with tumour volume (Miller et al, 1985) and therefore this measurement was used to compare tumours associated with tumour cells in the blood with those without.

### 5.2.5 *Vessel density*

For each tumour, paraffin sections, which were prepared for the purposes of routine histopathology, were selected so that tissue from the invading margin of the tumour was represented. This region was considered for two reasons; i) it should represent the site of new vessel formation necessary for tumour growth (Folkman, 1985), ii) capillaries at the periphery of a tumour may be composed solely of endothelial cells rendering them particularly prone to tumour invasion (Warren, 1979).

In a pilot study, two methods of identifying blood vessels by staining their endothelial cells were examined; an indirect immunoperoxidase technique using an antibody to

factor VIII (Rabbit anti-human VIII related antigen, Dakopatts, Denmark) was compared with the staining obtained using lectin histochemistry with the lectin *Ulex europaeus* I (UEA1) (Vector Laboratories, Peterborough, UK). The intensity of staining was greater and more vessels were found to stain positively with UEA1 than with the anti-human factor VIII antibody. UEA1 was therefore used in the definitive study according to the following protocol, which utilized the avidin-biotin-peroxidase complex technique. Examples of the staining obtained using UEA1 are given in Figures 5.2 to 5.4.

**5.2.6 *Lectin histochemistry for endothelial cells.***

Lectins may be defined as sugar-binding proteins or glycoproteins of non-immune origin (Leathem, 1986). As naturally occurring receptors for carbohydrates they are capable of recognizing cells on the basis of their oligosaccharide expression. In this study the binding of lectin *Ulex europaeus* I has been chosen to identify vascular endothelium.

Paraffin sections were dewaxed and rehydrated by successive treatment with CNP 30, 70% alcohol and distilled water. The sections were then washed in three changes of PBS before blocking endogenous peroxidase with 3% hydrogen peroxide in PBS for 25 minutes. Three further washes in PBS were followed by application of a 1:20 solution of normal rabbit serum in PBS containing 0.1% bovine serum albumin in order to block non-specific binding. After 10 minutes excess serum was drawn off with a tissue. Each section was then incubated with biotinylated UEA1 at a dilution of 1:200 in PBS. At the end of one hour the slides were washed again in PBS before incubating them with the avidin-biotin complex at room temperature. The avidin-biotin-peroxidase complex (AB) was made up 30 minutes before use in PBS in the ratio 1:1:100, A:B:PBS, to allow the complex to form. At the end

of 30 minutes the slides were washed and then placed in DAB for 7 minutes as previously described in Chapter 2. The sections were then washed in tap water, counterstained with haematoxylin, dehydrated and mounted in DPX. For each section studied a control section was also prepared in an identical fashion except UEA1 was omitted.

### 5.2.7 *Assessment of blood vessel density.*

Each section was coded by a third party in order to disguise its identity before submission to two observers. To maximise the vessel count the sections were initially scanned to determine the area with the greatest density of vessels. The vessels within three fields from this area were then counted. Each field comprised the view obtained at a magnification of 200x. The average number of vessels per field for each tumour was then calculated. A measure of the agreement or disagreement between the counts of the two observers was obtained by linear regression.

## 5.3 RESULTS

### 5.3.1 *Patients*

Sixteen patients were studied all of whom had a colorectal resection. In 14 patients surgery was performed for colorectal cancer and in the remaining two, for Crohns disease and chronic constipation respectively. The clinical and histopathology details of the 14 patients with colorectal cancer are given in Table 5.1. Only one of these patients (PD) had evidence of liver metastases as assessed by preoperative ultrasound and palpation of the liver at the time of operation.

| Patient | Age | Sex | Site | Diff | Stage | VI |
|---|---|---|---|---|---|---|
| DW | 48 | M | SIG | MOD | C | + |
| WT | 68 | M | LC | WELL | A | - |
| RN | 52 | M | RECT | MOD | C | + |
| LS | 64 | M | RECT | MOD | B | + |
| CM | 81 | F | RECT | MOD | A | - |
| PD | 61 | F | RECT | MOD | B | + |
| AA | 61 | F | CAEC | CAEC | B | - |
| EH | 72 | F | RC | RECT | C | + |
| SP | 57 | F | CAEC | MOD | C | + |
| EN | 68 | F | RECT | N/R | | |
| ZE | 74 | F | RECT | POOR | B | - |
| KA | 61 | M | CAEC | POOR | C | - |
| MP | 67 | F | CAEC | POOR | C | + |
| AG | 60 | F | RECT | MOD | B | - |

Table 5.1: Clinical and histopathological details of patients and tumours in the circulating cell study. N/R, not resected; VI +, extramural vascular invasion present; VI -, extramural vascular invasion absent.

### 5.3.2 *Detection of tumour cells*

Table 5.2 allows a comparison to be made between the results of conventional cytology and immunocytochemistry for each of the blood samples examined. No tumour cells were detected, either by conventional cytology or immunocytochemistry, in samples from the two patients who underwent colonic resection for benign disease.

| Sample site | AC | | IJ | | MV | | MA | |
|---|---|---|---|---|---|---|---|---|
| Patient | CC | I | CC | I | CC | I | CC | I |
| DW | - | - | - | - | - | - | - | - |
| WT | - | - | - | - | - | - | NS | NS |
| RN | - | - | - | - | - | - | - | - |
| LS | - | - | - | - | - | - | - | - |
| CM | +/- | - | - | - | - | - | - | - |
| PD | - | - | - | - | - | - | - | - |
| AA | - | - | - | - | - | - | - | - |
| EH | +/- | - | - | - | +/- | - | NS | NS |
| SP | + | + | + | + | +/- | + | - | + |
| EN | + | + | NS | NS | Not resected | | | |
| ZE | +/- | + | - | - | - | + | - | - |
| KA | +/- | - | - | - | - | - | - | - |
| MP | - | - | - | - | - | - | - | - |
| AG | - | - | - | - | + | + | - | - |

Table 5.2: Conventional histology versus immunocytochemistry in the detection of circulating tumour cells. Positive results (+) and equivocal results (+/-) have been picked out by shading. (-), no tumour cells identified; AC, ante-cubital fossa vein; IJ, internal jugular vein; MV, mesenteric vein; MA, mesenteric artery; NS, sample not obtained.

Based on the immunocytochemistry results alone, 4 patients were identified has having circulating tumour cells in one or more of the samples tested.

| | | CC | | |
|---|---|---|---|---|
| | | + | - | +/- |
| I | + | 4 | 2 | 2 |
| | - | 0 | 39 | 4 |

Table 5.3: Agreement and disagreement between immunocytochemistry (I) and conventional cytology (CC) in the identification of tumour cells. +, positive result; -, negative result; +/-, equivocal result.

Table 5.3 indicates the number of samples in which agreement and disagreement occurred when conventional cytology was compared with immunocytochemistry. Unequivocal evidence of malignant cells was present in four of the samples examined by conventional cytology and in each of these the result was confirmed by immunocytochemistry. In six samples, however, conventional cytology identified cells whose appearance was suggestive of but not conclusive for malignancy (+/-). Of these six samples immunocytochemistry confirmed the presence of malignant cells in two. In a further two samples immunocytochemistry identified tumour cells which had otherwise been missed by conventional cytology.

| | + | - | +/- |
|---|---|---|---|
| CC | 4 | 41 | 6 |
| I | 8 | 43 | 0 |

Table 5.4: Comparison between immunocytochemistry (I) and conventional cytology (CC) for the identification of tumour cells. +, positive result; -, negative result; +/-, equivocal result ($X^2$=7.381, p=0.025)

In Table 5.4 the results of conventional cytology and immunohistochemistry are compared directly with one another using the chi-squared test; the results were found to differ significantly from one another ($X^2$=7.381, p=0.025).

For technical reasons it was not always possible to obtain the full 5 ml of blood, or indeed any blood, from each sample site. This meant that in some cases relatively sparse cytospins were prepared. Under these conditions the number of cells appearing on six cytospins might be insufficient to exclude tumour cells present at concentrations of $10^1$ $ml^{-1}$ or less (see Chapter 4). To counter this, in those cases initially recorded as negative by both conventional cytology and immunohistochemistry, further cytospins were stained immunocytochemically and examined for tumour cells. There was no instance in which tumour cells were seen when they had not already been identified on the initial six cytospins.

### 5.3.3 *Quantification of tumour cells*

For the four patients in whom tumour cells were identified by immunocytochemistry the calculated tumour cell concentrations together with the 95% confidence intervals for each, are presented in Table 5.5. The concentration of tumour cells ranged from 40 $ml^{-1}$ to 954 $ml^{-1}$.

All of the tumour biopsies examined stained positively with the anti-cytokeratin 8.13 antibody.

| Patient | Sample | White cell count $10^6$ $ml^{-1}$ | Lym & Mono $ml^{-1}$ | Tumour: Lym/Mono Ratio | Tumour cells $ml^{-1}$ (95% CI) |
|---|---|---|---|---|---|
| SP | AC (preop) | 3.5 | 1155000 | 6:35179 | 197<br>72-428 |
| | IJ | 3.2 | 864000 | 18:135238 | 114<br>68-181 |
| | MV | 3.8 | 1908000 | 26:52020 | 954<br>622-1397 |
| | MA | 5.3 | 1254000 | 33:53277 | 777<br>534-1091 |
| | | | | | |
| EN | AC (preop) | 4.5 | 1980000 | 2:97731 | 40<br>5-146 |
| | | | | | |
| ZE | AC (preop) | 7.7 | 1925000 | 2:58690 | 65<br>8-237 |
| | MV | 8.4 | 1402500 | 2:30452 | 92<br>11-332 |
| | | | | | |
| AG | MV | 10.5 | 6090000 | 1:78746 | 77<br>2-431 |

Table 5.5: Tumour cell concentrations in those samples in which tumour cells were detected by immunocytochemistry. AC, ante-cubital fossa vein; IJ, internal jugular vein; MV, mesenteric vein; MA, mesenteric artery.

### 5.3.4 Tumour size

The longest linear dimension of each tumour in the study is presented in Table 5.6. No significant difference was noted between patients positive for cells in their blood and those negative.

| | Longest linear dimension of the tumour (cm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| - | 8 | 1.5 | 5 | 3.5 | 5 | 5 | 8 | 10 | 2.5 | 11 |
| + | 4 | 5 | 4.5 | | | | | | | |

Table 5.6: Table comparing the longest linear dimension of those tumours with (+) and without (-) circulating tumour cells. (Mann-Whitney test, $p>0.05$)

### 5.3.5 *Tumour vessel density*

The average vessel count per 200x field, for each tumour examined, as obtained by each observer is recorded in Table 5.7: values ranged from 14.8 to 106 vesels per field. There was good agreement between the two observers, with a correlation coefficient $r = 0.82$, $p=0.0008$. In Table 5.7 the tumours have been ranked according to vessel density as ascertained by combining the mean counts of both observers. Those patients in whom circulating tumour cells were identified are indicated by shading.

No relationship was seen between tumour cells in the blood and vessel density; the 3 tumours associated with circulating cells had vessel densities less than the mean (56.8 vessels per 200x field) for the group. There was also no significant difference in vessel densities when tumours of Dukes' stage A and B were compared with those of Dukes' stage C (Mann Whitney test, $p>0.05$); i.e. when tumours which had already demonstrated the capacity to metastasize at the time of resection, were compared to those which had not. (In

assessing the relationship between Dukes' stage and vessel density the patient PD although having a Dukes' B tumour was grouped with those of Dukes' C because of the presence of liver metastases at the time of operation.)

| Patient | Stage | Size (cm) | Mean vessels per field Obs 1 | Mean vessels per field Obs 2 | Mean vessels per field combining Obs 1 & Obs 2 |
|---|---|---|---|---|---|
| LS | B | 3.5 | 11.3 | 18.3 | 14.8 |
| CM | A | 5 | 24.3 | 13.3 | 18.8 |
| AG | B | 4.5 | 32.6 | 28.3 | 30.5 |
| MP | C | 11 | 24.3 | 38.3 | 31.3 |
| SP | C | 4 | 46.6 | 35.6 | 41.2 |
| PD | B | 5 | 47.6 | 35 | 41.3 |
| ZE | B | 5 | 75 | 37 | 56.2 |
| DW | C | 8 | 67.3 | 50.3 | 58.8 |
| RN | C | 5 | 65.3 | 64 | 64.6 |
| WT | A | 1.5 | 81 | 85 | 83 |
| KA | C | 3.5 | 88 | 95 | 91.8 |
| AA | B | 8 | 75.3 | 126 | 100.6 |
| EH | C | 10 | 106 | 106 | 106 |

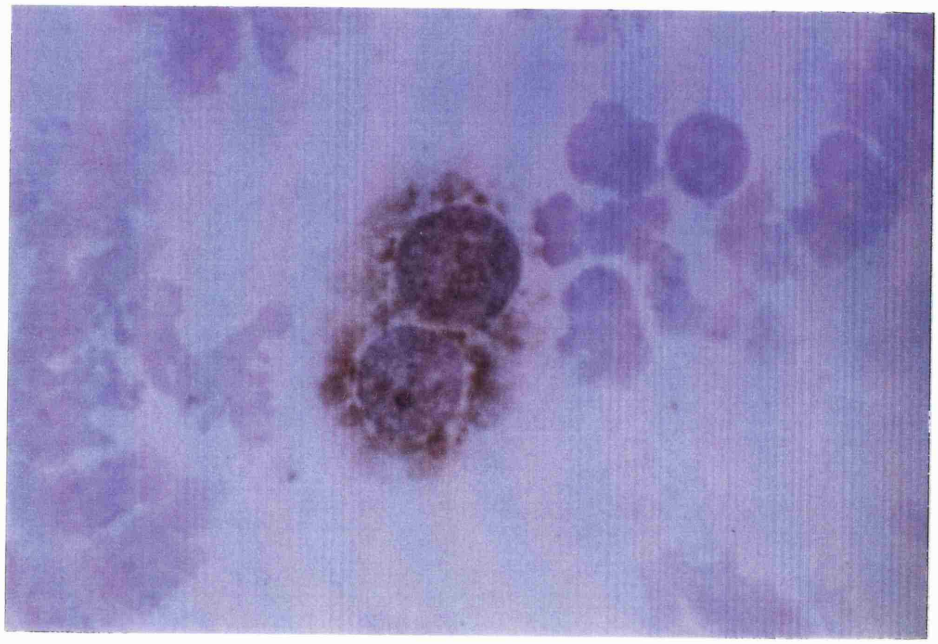
Table 5.7 :The relation between tumour size (as determined by its longest diameter), Dukes' stage and mean vessel density per field. Each field was the area of the section viewed at a magnification of 200x. Each observer counted the vessels in 3 fields. Shadeding indicates patients with circulating tumour cells. Obs 1, observer 1; Obs 2, observer 2; Combined results of observer 1 and observer 2.

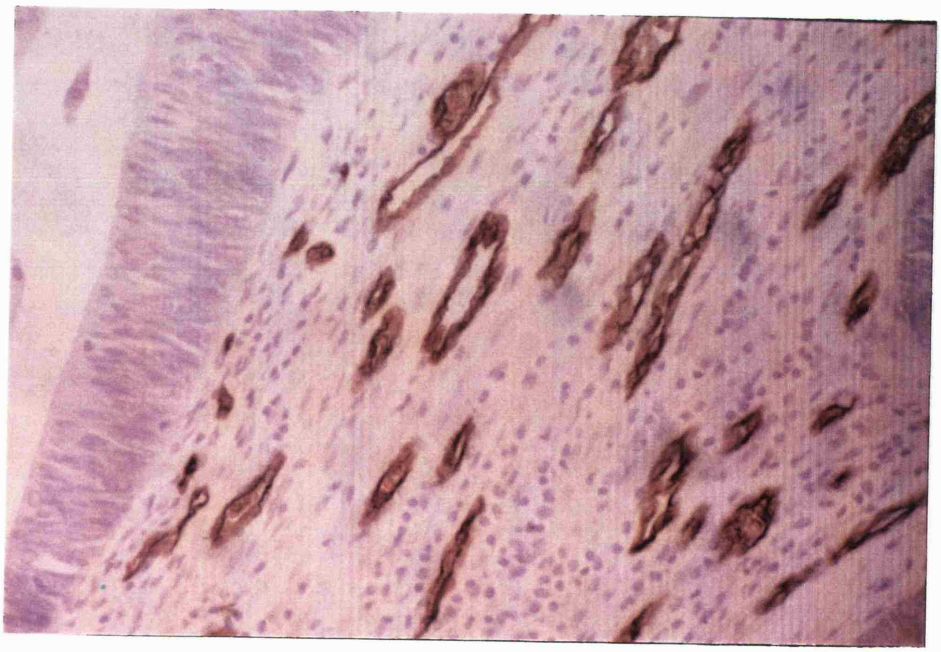
**Figure 5.1**

Cytospin of cells obtained by density gradient centrifugation of blood from a patient (SP) with a moderately differentiated Dukes' C adenocarcinoma of the colon. The cytospin has been stained with the anticytokeratin antibody 8.13 and two tumour cells are clearly identified by the brown staining within their cytoplasm which marks the presence of cytokeratin proteins (original magnification x1000).

**Figure 5.2**

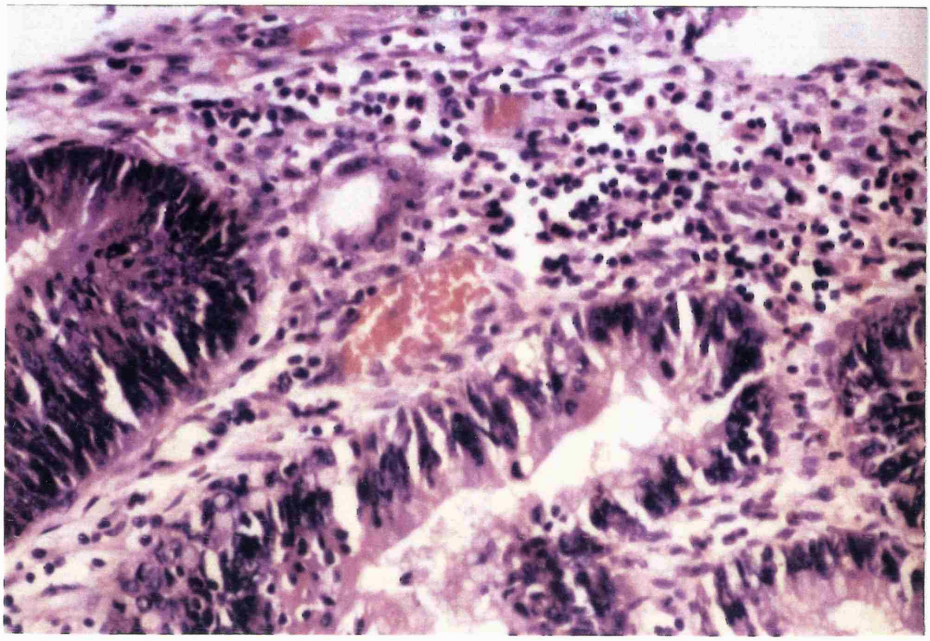
Section from a moderately differentiated adenocarcinoma of the colon stained with the lectin *Ulex europaeus I*. Blood vessels are clearly identified by the brown staining which marks the presence of the lectin in their endothelial cells (original magnification x250).





**Figures 5.3 and Figures 5.4**

These two figures show the same portion of tissue in two adjacent sections. Figure 5.3 has been stained with haematoxylin and eosin and Figure 5.4 with *Ulex europaeus* I. The ability of the lectin stain to accentuate the presence of blood vessels within the tissue is clearly evident (original magnification x250).



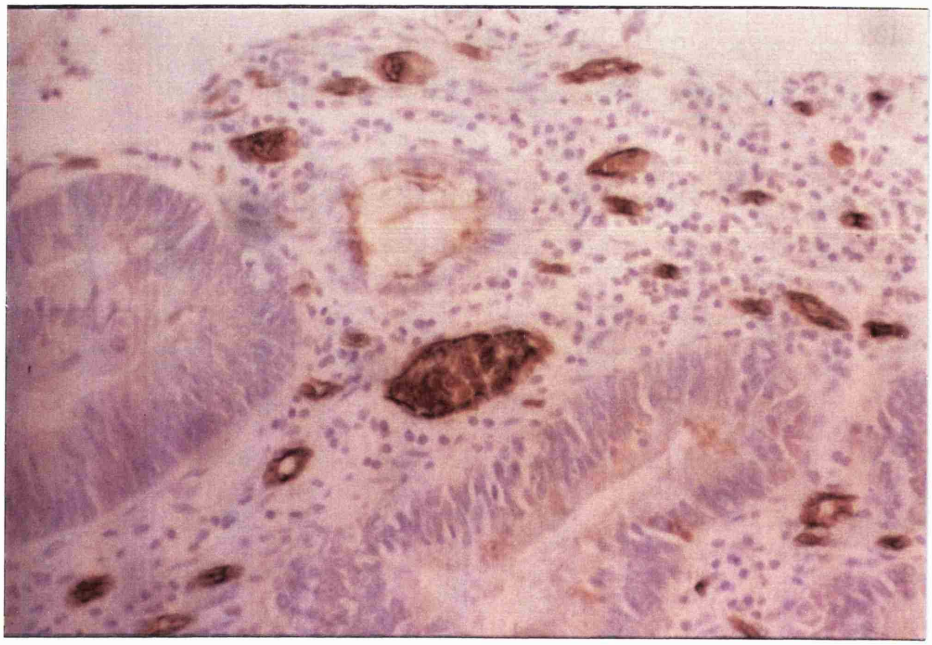
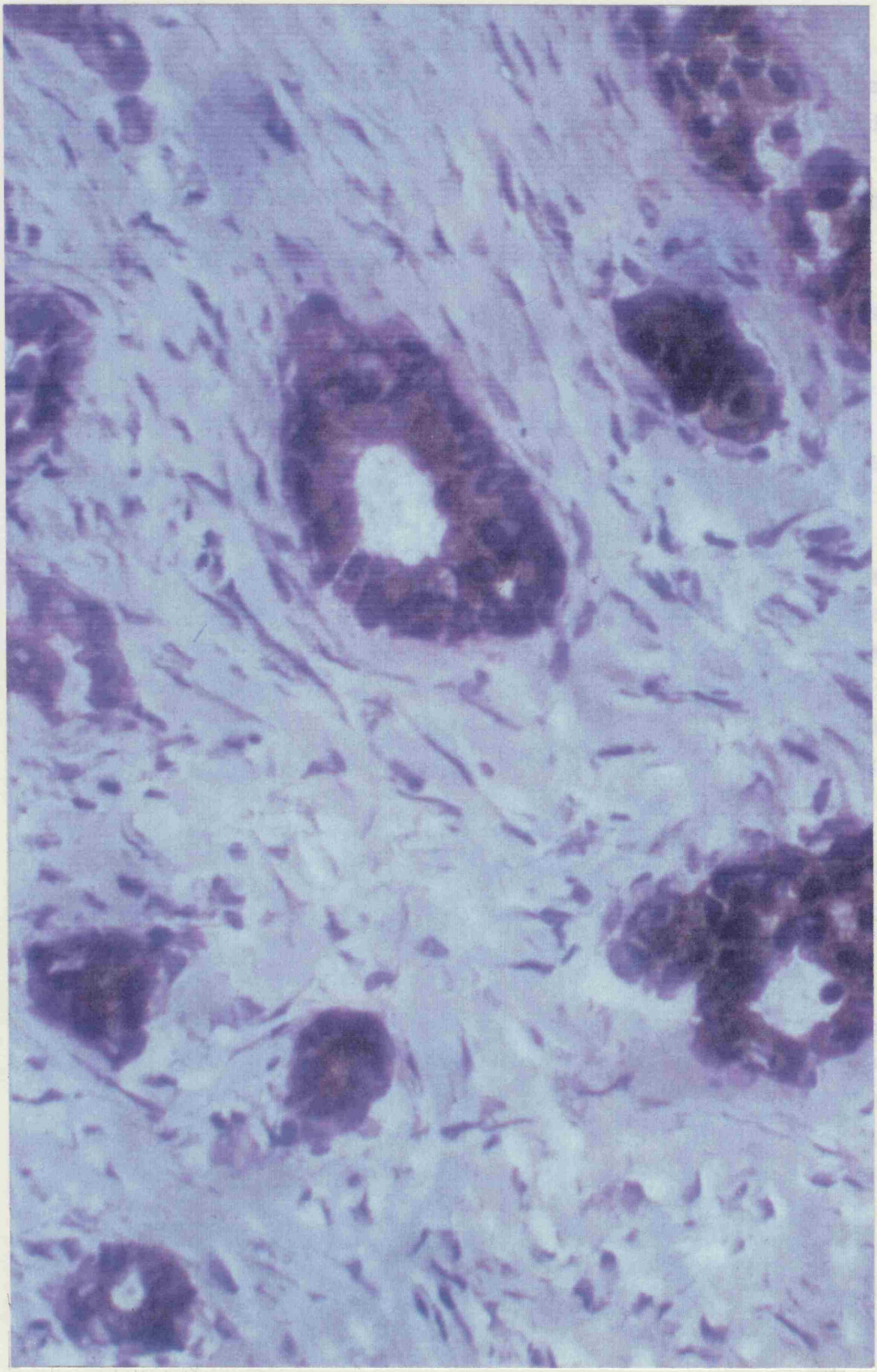


Figure 5.5

Section of a moderately differentiated carcinoma of the colon stained with the anti-cytokeratin antibody 8.13.



## 5.4 DISCUSSION

This study has sought to identify and enumerate circulating tumour cells in patients with colorectal cancer. In addition it has allowed the relative abilities of conventional cytology and immunohistochemistry in identifying tumour cells to be compared.

### 5.4.1 *Incidence and concentration of circulating tumour cells in colorectal cancer.*

Based on the results of immunocytochemistry alone, blood borne tumour cells were detected in four of the 14 patients (28%) in this study. In 3 of these 4 patients tumour cells were detected in blood taken from the mesenteric vein; in the fourth, no sample was available from this site owing to the inoperability of the tumour. The incidence of tumour cells in mesenteric venous blood was therefore 3/13 or 23%. Blood taken preoperatively also revealed tumour cells in 3 out of the four patients, and, in one patient only, cells were detected in blood taken from the mesenteric artery.

The reported incidence of tumour cells in the mesenteric circulation varies between 4% and 67% (Roberts et al, 1958; Salsbury et al, 1965). Wide fluctuations in the incidence of circulating tumour cells have also been observed for other tumours, (Gazet, 1966) and probably reflect the many factors which influence their detection. These factors can broadly be divided between those relating to methodology, those influenced by tumour pathology, and those linked to the pathophysiology of circulating cells.

### 5.4.2 *Methodology and circulating tumour cells.*

Three aspects of methodology are of importance: i) the ability of the technique to recover cells from the blood; ii) the accuracy with which tumour cells can be identified; iii) the method of sampling.

In relation to this study density gradient centrifugation with the medium Histopaque-1077 was used to recover tumour cells from the blood, and this technique has been discussed in Chapter 4. The only other study to use density gradient centrifugation in the isolation of haematogenous colorectal cancer cells was that of Roberts et al (1958). The methods used in the other studies relied instead on combinations of cell lysis, sedimentation and centrifugation (Engell, 1955; Fisher and Turnbull, 1955; Salsbury et al, 1965). With what should be the superior technique, the very low incidence of tumour cells detected by Roberts et al (4%) is difficult to explain. Possibilities include, the inadvertent loss of tumour cells through the use of fibrinogen to eliminate erythrocytes by inducing rouleaux formation and failure to recognize tumour cells on cytology.

That tumour cells should be reliably distinguished from those of haemopoietic origin is clearly of fundamental importance to any study of circulating tumour cells. In the past cell morphology was used, but this was often unreliable, with blood cell precursors and cells of atypical appearance likely to be confused with malignant cells. Furthermore these difficulties are enhanced in patients with cancer, in whom atypical cells tend to occur more frequently (Griffiths and Salsbury, 1965). Alexander and Spriggs (1960) and Scheinin and Koivuniemi (1962), in a detailed study of white cell concentrates prepared from patients with cancer, identified several cell types which could be mistaken for

malignant cells, including, plasma cells, megakaryocytes and erythropoietic cells such as normoblasts.

More recently it has been possible to circumvent these morphological ambiguities by utilizing the restricted expression of intermediate filament types to identify cells of different origin. Intermediate filaments, so called because their characteristic diameter of 100Å lies between that of microfilaments (60Å) and the larger microtubules (250Å), form a substantial portion of the cytoskeleton on which the integrity of eukaryotic cells depend. Five intermediate filament categories are recognized, each characteristic of a particular cell type. Epithelial, mesenchymal, myogenic, neural and glial cells can all be identified by the use of antibodies directed against their specific intermediate filament proteins, respectively: keratin, vimentin, desmin, the neurofilament proteins and the glial fibrillary acidic proteins (Lazarides, 1979).

The use of anti-keratin antibodies to identify circulating, epithelial derived tumour cells is based on three assumptions: firstly that the expression of keratins is specific for cells of epithelial origin; secondly, that keratin expression continues after malignant transformation; and thirdly, that epithelial cells in the blood stream must be derived from a malignancy.

Despite a recent study in which cytokeratins were found to be expressed by a subpopulation of reticulum cells in normal lymph nodes and the spleen (Doglioni et al (1990), there is good evidence to support their continued use as markers for epithelial cells. In particular, no cross-reactivity was observed between anti-cytokeratin antibodies and cells of haemopoietic origin in a study of bone marrow aspirates taken from 102 patients without malignant disease (Schlimok et al, 1990). Although two is too small a number to draw conclusions from, samples from the two patients in this study who did not have a malignancy were also negative.

Circulating cancer cells might also evade detection if keratins were no longer expressed by the primary tumour. To counter this possibility, biopsies from all the tumours resected were stained with the anti-cytokeratin 8.13 antibody. In each case continued expression of cytokeratins was confirmed, with between 90 to 100 per cent of tumour cells present reacting positively (Fig 5.5). In addition the expression of cytokeratins, even by poorly differentiated colonic adenocarcinomas, has also been demonstrated by Brown et al, (1983).

Another potential marker for epithelial cells is epithelial membrane antigen (Sloane and Ormerod, 1981). The use of an antibody to epithelial membrane antigen was excluded in this study because of its inability to recognize cultured epithelial cells (Chapter 4). Although this result almost certainly owed more to the artificial conditions imposed by cell culture than to shortcomings in the antibody, there is evidence to suggest that epithelial membrane antigen is less effective as a tumour marker than keratins. In a variety of neoplasms, including 48 adenocarcinomas, keratins were found to be superior to epithelial membrane antigen as tumour cell markers in one third of the cases studied (Pinkus et al, 1986). It is, however, appreciated that the results of such studies are dependent on the quality of the antibody and the staining technique used. Epithelial membrane antigen is also expressed by human lymphoid cells including reactive and neoplastic plasma cells (Delsol et al, 1984), a feature which could give rise to confusion were it to be used as a marker for circulating epithelial derived tumour cells.

If the immunohistochemical demonstration of cytokeratins is accepted as the "gold standard" for the diagnosis of circulating, epithelial derived, tumour cells then the difficulties faced by previous investigators, reliant on conventional cytology, are highlighted by this

study. Twice as many samples (8 vs. 4) were considered to contain tumour cells when assessed by immunocytochemistry as compared to conventional cytology. In addition, 6 samples could have been erroneously labelled as containing tumour cells (those labelled +/-) were it not for the clarification afforded by the absence of cytokeratin staining. In the study by Engell (1955) the definition of a positive result included those blood samples which contained "atypical tumour-like cells". Had immunocytochemistry been available then, it is possible that many of these would not have classified as malignant.

The success or failure with which tumour cells are detected in the circulation will also depend on the method of sampling. In particular, the timing and frequency of samples, the site from which they are removed and the volume of blood taken, may all be important.

The process of tumour cell intravasation will only be reflected accurately if blood samples are taken from appropriate sites in the circulation. Animal studies, using tumour cells labelled with [$^{125}$I]-iododeoxyuridine, indicate that the majority of tumour cells released into the circulation are trapped in the microvasculature of the organ of first encounter (Fidler, 1970; Murphy et al, 1986). For colorectal cancers, seeding cells into the portal circulation, tumour cells are initially arrested in the liver. Here, the majority of them are killed, so that only a small percentage are delivered in a viable state to the next organ of encounter, the lungs (Weiss et al, 1983), where the process of entrapment and cell death is repeated. It follows that blood samples taken from peripheral sites, such as ante-cubital fossa veins, are less likely to contain circulating cells than those removed from centrally placed vessels. This was certainly the experience of Engell (1955) who found that only 4% of peripheral samples contained tumour cells, and of Moore and Sako (1959) who were unable

to detect tumour cells in any of 40 resectable cases of colorectal cancer. However, Long et al (1960) in a study of the peripheral blood of patients with a variety of cancers found cancer cells in 25% of 59 patients with curable lesions and 39% of 119 patients with an incurable malignancy. The reliability of this latter study is questionable, though, as 5 out of 26 patients with benign disease were also thought to have cells which appeared malignant. In the present study, tumour cells were identified in 3 out of 14 patients in blood taken preoperatively from an antecubital fossa vein, an incidence which may be attributable to the superior recovery of tumour cells with density gradient centrifugation. In this respect it is noteworthy that, based on the average volume of blood taken for all his positive samples (from any site), Engell found 0.3 tumour cells $ml^{-1}$ of blood examined (195 cells observed in 645 ml) compared with 2.25 cells $ml^{-1}$ in the present study (90 cells observed in a total of 40 ml).

The volume of blood removed for each sample will also dictate the success with which tumour cells can be identified. The low concentration of tumour cells detected in 3 of the 4 patients in this study (40-92 cells $ml^{-1}$), suggests that larger samples might be more effective by compensating for the inevitable cell loss associated with their isolation. However, the clinical setting of this, and other the studies imposes a limit on the volume of blood which can be taken. The sample volume used in this study (5 ml) is comparable to that used by others (Engell, 1955; Salsbury et al; 1965; Moore and Sako; 1959) but almost certainly less than that obtained by Fisher and Turnbull (1965) who additionally irrigated the mesenteric vein after releasing and collecting the blood within it.

Experiments with the Lewis lung carcinoma and B16 melanoma transplanted into mice suggest that the liberation of tumour cells into the circulation occurs sporadically

rather than as a continuous process (Glaves, 1983a; Glaves et al, 1986). If this is also true of human tumours, then multiple rather than single blood samples may improve the chances of detecting circulating cells. Sellwood (1965) was able to demonstrate that the number of patients identified as having tumour cells in their blood increased if 3 or more samples were taken. In the present study, only single samples were removed from each site, which may have adversely affected the results. However, unless an indwelling catheter had been inserted into the mesenteric vessels further samples would have been technically difficult to obtain. For the future, a more feasible study might be to take multiple samples from peripheral sites pre and postoperatively. Analysis of these might then provide further information on the dynamics of cell shedding.

The timing of sampling can also influence detection. In particular much discussion has centred on whether or not manipulation of the tumour during surgery increases the volume of circulating cell traffic (Knox, 1922; Salsbury, 1965; Sellwood, 1965; Turnbull et al, 1967). In this study we have been concerned to limit the production of iatrogenic tumour emboli, so that the observations reflect the natural process of intravasation. Although the surgeons involved were asked to handle the tumour as little and as gently as possible, dislodgement of cells into the circulation during the operation cannot be excluded. The significantly increased cell traffic observed in the iliac veins following ligation of the inferior mesenteric vessels offers an example of how the incidence of circulating tumour cells can be affected by operative technique (Salsbury et al, 1965). Whether or not there is an increased frequency of tumour cells in the mesenteric circulation following operative manipulation is open to debate. Their presence following mobilization of the tumour has certainly been observed but data to suggest that it occurs more frequently than would be

expected by chance are lacking. In the study by Engell (1955) 47 patients with colorectal cancer had mesenteric vein samples taken before and after manipulation. An analysis of these 47 patients (Table 5.8) shows no significant disparity in the frequency with which tumour cells were detected in either sample (McNemar's test, $X^2$ = 1.23, $p > 0.25$). The information contained in other studies of colorectal cancer is largely anecdotal. Thus, Moore and Sako (1959) formed the impression that the number of positive samples after surgical manipulation was not increased but that the number of cells released was greater. In this study tumour cells, at concentrations ranging from 40 $ml^{-1}$ to 197 $ml^{-1}$ were detected in blood samples removed preoperatively. These figures indicate that, given the attrition caused by their circulation through the liver and lungs, significant numbers of cells must be shed into the circulation prior to tumour manipulation. If these observations are representative then any additional increase in tumour cell traffic produced at operation would appear unlikely to have a significant effect on prognosis. This would argue against the adoption of the "no-touch isolation technique" advocated by Turnbull et al (1967). However, the two- to five-year survival of patients in whom showers of cancer cells were detected during the course of their operation has been reported to be approximately half that of patients with negative blood (Roberts et al, 1961). Also, Wiggers et al (1988) in a carefully controlled study, were able to show that the number of liver metastases developing in patients whose tumours were shown to have vascular invasion was reduced in those operated on using the no-touch technique. The no-touch technique also produced a reduction in the incidence of liver metastases in patients with a carcinoma of the sigmoid colon. While these results appear to favour the no-touch technique it is possible that differences in the pre-operative numbers of circulating

tumour cells could have influenced the results. Patients who fared favourably with the no-touch technique might have had fewer tumour cells circulating preoperatively than their counterparts in the control group. Alternatively, the success of the no-touch technique may lie in its ability to reduce circulating cell traffic at a time when the patient is particularly vulnerable to metastasis formation. The stress of the operation (Buinauskas et al, 1958), blood transfusion (Beynon et al, 1989), or changes in the coaguability or fibrinolytic activity of blood (Dvorak, 1987; Newstead et al, 1976) may all prejudice the patient towards metastasis formation.

| | | AFTER | |
|---|---|---|---|
| | | + | - |
| BEFORE | + | 24 | 9 |
| | - | 4 | 10 |

Table 5.8: Comparison of the number of mesenteric blood samples containing tumour cells before and after mobilization of the tumour. +, cells present; -, cells absent (from Engell, 1955).

### 5.4.3 *Tumour related factors in the detection of circulating cancer cells.*

It is self evident that for tumour cells to be detected in the blood they must first enter the circulation. This study has sought to establish, for colorectal cancer, whether or not any relation exists between tumour vessel density and metastasis, be it in the form of tumour cells in the blood stream or deposits in regional lymph nodes.

Perhaps not surprisingly, given the small number of patients and the sampling problems already discussed, no relationship was found to exist between vessel density and

circulating cancer cells. This investigation did, however, demonstrate considerable heterogeneity in vessel density amongst the tumours examined; a finding recently confirmed by a resin cast study of the microvasculature of human colon tumours (Skinner and O'Brian; 1991). It may be that for colorectal cancer the shedding of cells into the circulation is not so much related to the number of vessels within it but to the relative size of those vessels. As already discussed in the introduction Liotta et al (1974) found that the relationship between the concentration of tumour cells and vessel density was strongest when only vessels $\geq$ 30 $\mu$m were considered.

If vessel density is an important determinant of the metastatic capability of colorectal cancers, then tumours which have already demonstrated the capacity to metastasize might be expected to have greater vessel densities than those which have not. Although there was no evidence in this study to support such a relationship, it is worth noting that the mean vessel density of Dukes' C and "D" tumours (62 vessels per 200x field; SD 27.8 vessels) was greater than the mean vessel density of Dukes' A and B tumours (50 vessels per 200x field; SD 35.4 vessels). With a larger series this difference might prove to be significant; a calculation of the sample size necessary (see Kirkwood, 1989), based on the observed means and standard deviations for the two groups, suggests that approximately 180 tumours would be required to produce a significant result at the 5% level.

The relationship between circulating tumour cells and tumour stage is also an uncertain one. In the study by Fisher and Turnbull (1955) the majority (6/8) of positive findings occurred in Dukes' B tumours, and Moore and Sako (1959) found that four out of their six patients with positive findings had no lymph node involvement. Of the 26 patients studied by Song et al (1971), five of the six

peripheral blood samples, positive for tumour cells, also occurred in Dukes' A or B tumours. Griffiths et al (1973), however, found no significant difference in the incidence of tumour cells in the peripheral blood between Dukes' B and C tumours (6/22 vs.7/22 respectively). In the present study, two of the four positive cases were seen in Dukes' B tumours, one of which was moderately differentiated and the other poorly differentiated; the third patient had a moderately differentiated Dukes' C carcinoma and, in the remaining case the tumour was unresectable.

One patient in the series, WT, deserves particular mention. The "Dukes' A" tumour resected from this patient's left colon had an unexpectedly high vessel density. In fact this tumour represented a recurrence from a previous excision of a Dukes' B carcinoma and the vessel density observed may reflect the more advanced stage of the original tumour.

It might also be thought that the presence of lymph node metastases in Dukes' C tumours might enhance the haematogenous dissemination of tumour cells through the existence of lymphaticovenous communications (Weiss, 1985a). However, in a rat foot-pad mammary carcinoma model, the tumour cell traffic associated with the hilar blood vessels of involved popliteal nodes was found to make an insignificant contribution to the overall number of cells in the blood stream (Weiss and Ward, 1990). This study did indicate, though, that tumour cells within "fixed" lymph nodes are more likely to enter the circulation.

Poorly differentiated tumours are associated with a reduced probability of survival (Phillips et al, 1984), but apart from Engell's study (1955) there is scant information on the relation between tumour grade or differentiation and circulating tumour cells. In that study a positive correlation was established between the presence of circulating tumour cells and deteriorating tumour grade

according to Broders classification (Broders, 1926). The present study is too small to confirm or refute these observations.

Malmgren (1968) observed a higher occurrence of cancer cells in the blood of patients with a variety of cancers in the presence of tumour necrosis. It is therefore noteworthy, that in the present study, the 3 patients with resectable tumours and circulating cells also had tumours which were ulcerated. Necrotic tissue may act as a diffusible source of enzymes facilitating the processes of cell detachment and intravasation particularly at the tumour periphery (Weiss, 1977).

In tumour animal models both Liotta et al (1974) and Glaves (1983a) were able to observe an increase in the number of tumour cells shed into the circulation as tumours grew in size. No significant relationship between the size of the primary tumour, based on its longest diameter, and the presence of circulating tumour cells was observed in this study. An analysis of those cases of colorectal cancer, reported by Engell (1955), for which precise tumour dimensions are given, also fails to show an association between tumour size and tumour cells in the blood. These observations are in accord with the view that, unlike other tumours, e.g. breast cancer, size is not a prognostic determinant for colorectal cancer (Miller et al, 1985; Wolmark et al, 1984).

### 5.4.4 *Quantification and pathophysiology of circulating tumour cells in colorectal cancer.*

Moore and Sako (1959) estimated that there were between 16 and 50 tumour cells per 5 ml of blood in their positive samples, and Engell (1955) calculated that 2 ml of blood would have to be examined for every tumour cell found in samples from the mesenteric veins. These figures, however,

take no account of tumour cell loss during the preparation of the specimens, and cannot be regarded as accurately reflecting the volume of tumour cell traffic. In the present study the problem of cell loss has been obviated by using the ratio of tumour cells to mononuclear cells to calculate tumour cell concentration, as explained in Chapter 4. The estimated concentrations should therefore reflect the process of intravasation more accurately.

When detected, the concentration of tumour cells in peripheral venous blood samples varied between 40 cells $ml^{-1}$ to 197 $ml^{-1}$ and in samples from the mesenteric vein, between 77 cells $ml^{-1}$ and 954 $ml^{-1}$. These figures are within the range of concentrations (0 - 7309 emboli $ml^{-1}$, median 160 $ml^{-1}$) observed by Glaves et al (1988) in their study of the haematogenous dissemination of tumour cells from human renal adenocarcinomas. Although Liotta et al (1976) have emphasised the importance of cell clusters in initiating metastases, none were detected in this study. It is possible that, if present, they were disrupted during the preparation of the cytospins.

In only one patient (SP) were tumour cells detected in samples from all four sites. This patient, with a caecal carcinoma, although clinically free of metastatic deposits at the time of operation, subsequently (after 10 months) developed widely disseminated disease including pulmonary and hepatic secondaries. The presence of tumour cells in the mesenteric artery strongly suggests that pulmonary metastases were already present at the time of operation. In accordance with the cascade process described by Viadana et al (1978), these pulmonary metastases would have acted as generalizing sites, shedding cells into the arterial circulation. The presence of arterial borne tumour cells also explains the relatively high concentrations of cells seen in the peripheral and internal jugular vein samples of this patient.

The tumour cell concentrations observed in this study may also be used to estimate the inefficiency of metastasis in colorectal cancer. Based on a blood flow of 20 ml $min^{-1}$ 100 $g^{-1}$ (Lundgren, 1989) and a weight of 700g the total blood flow to the large intestine is approximately 140 ml per minute. If the assumption is made that this flow is distributed evenly between the superior and inferior mesenteric arteries then the return flow in the inferior mesenteric vein will be approximately 70 ml $min^{-1}$. With a concentration of 77 tumour cells $ml^{-1}$ (patient AG) nearly 8 million cells per day would be delivered to the liver if cells were continuously released into the blood stream. The fact that no metastases have so far developed in this patient indicates that the vast majority of these cells must be destroyed.

The present study is too small and the follow-up too short to draw conclusions about the prognostic significance of circulating tumour cells. Although one patient, with high concentrations of tumour cells, has developed metastatic disease, the other two patients, resected for cure, remain well at nine months and one year respectively. The remaining patient with circulating cells, whose tumour could not be resected, has been lost to follow-up. All the other patients in whom no tumour cell emboli were detected remain alive and well up to one year after surgery. Engell (1959), in a follow-up of the colorectal cancer patients reported in his 1955 paper, concluded that tumour cells in the blood were not prognostically significant. If the cases of rectal carcinoma are analyzed separately, though, the proportion of patients dying 5 to 9 years later is significantly greater, for those in whom cancer cells were found, than in those with negative blood (29/41 vs. 13/35 respectively; $X^2=8.62$, $p=0.003$). When the same analysis is applied to colonic cancers no significant difference in survival is found between the two groups. Griffiths et al (1973) found no

correlation between tumour cells in peripheral blood samples and subsequent 5-year survival, and this was also the case in a study of 86 patients with various cancers, including colorectal cancer, reported by Roberts et al, (1961).

Given the many fates that can befall circulating tumour cells, as well as the problems associated with their sampling, it is perhaps not surprising that no correlation exists between their presence and prognosis: many patients, in whom cells are present undoubtedly do well while others, who do badly, may have cells which have gone undetected. It is not inconceivable that with more sensitive techniques for their detection, and more efficient sampling, blood borne tumour cells might be identified in the majority of patients with malignant disease. Indeed the recently described use of reverse transcriptase and the polymerase chain reaction for the detection of melanoma cells in peripheral blood (Smith et al, 1991) combined with the use of bone marrow aspirates rather than blood specimens, offers an opportunity for such refinement. By sequestering circulating tumour cells, the bone marrow may act as a sump in which cells are more liable to be detected. Tumour cell emboli, particularly from rectal carcinomas, may also reach the marrow of the axial skeleton in greater numbers than are likely to be found in peripheral blood samples, by entering tributaries of Batson's plexus of vertebral veins (Batson, 1940) thus evading destruction in the lungs.

If circulating tumour cells are ultimately found in the majority of patients with malignant disease then this would further dilute their prognostic value. The position might well come to mirror that seen in the examination of lymph nodes for the presence of "occult" nodal metastases. In a recent study of regional lymph nodes in breast carcinoma, the increased diagnostic yield of metastases obtained using monoclonal antibodies was not translated into an obvious clinical advantage (Galea et al, 1991).

More information is therefore undoubtedly required, not just about the presence of circulating tumour cells, but also about the characteristics of the cells themselves. The numbers in which the cells circulate may well be important, with high concentrations affording an advantage over the inefficiency of the metastatic process as a whole. Also required are details of phenotypic differences which may influence their intravasation and extravasation, their ability to survive host defences and their capacity to form new growths. Ideas for further research into circulating tumour cells are discussed in Chapter 6.

## 5.5 CONCLUSIONS

This study has shown that the use of anti-cytokeratin antibodies is superior to conventional cytology in the identification of circulating tumour cells. In addition, the concentrations of circulating tumour cells have been calculated, allowing the inefficiency of the metastatic process to be illustrated in colorectal cancer. Within the limitations of the small sample of patients studied, no correlation was found between tumour vessel density and the presence of circulating tumour cells or tumour vessel density and Dukes' stage. However, considerable heterogeneity in the vascular density of tumours was observed suggesting that a further study using a larger sample of patients might be worthwhile.

# CHAPTER 6

## OVERVIEW AND FUTURE DIRECTIONS

The studies detailed in the preceding chapters have been concerned with elucidating aspects of metastasis as they apply to colorectal tumours. Particular attention has been focused on the role played by a family of enzymes known as the matrix metalloproteinases in the process of tissue invasion, and on the detection and quantification of blood borne tumour cells in both regional and peripheral blood samples. The techniques of immunohistochemistry have allowed *in vivo* information to be gathered on both these issues.

In Chapter 2 evidence was presented to suggest that two enzymes, stromelysin and $M_r$ 72,000 gelatinase, were particularly responsible for the remodelling of the connective tissue matrix which accompanies tumour invasion. Furthermore, the predominant appearance of these enzymes in the stromal tissues intimated that the process of tumour invasion may rely an interplay between tumour cells and those of the host connective tissues. Tumour cells might for example induce members of the stromal cell population, such as fibroblasts, to increase their synthesis and secretion of matrix degrading enzymes. It is also possible to speculate that the process of carcinogenesis itself may be linked to disturbances in the normal relationship between epithelial cells and stromal cells. Such interactions are clearly of importance during morphogenesis in the embryo and again in the maintenance of normal function in adult organs (Cunha et al, 1985). It is perhaps not surprising then that the development of cancer is likely to involve a loss of co-ordination between epithelial cells and their underlying stroma of which the control of matrix metalloproteinase activity may form a part. If this hypothesis proves correct then it may be possible in the future to monitor the malignant potential of colorectal epithelium by determining

the level of metalloproteinase activity within it. The recent development of enzyme linked immunoabsorbent assays for these enzymes (Clark et al, 1991) would facilitate this process by providing the necessary quantitative data on the presence of these enzymes.

A similar argument may be put forward for TIMP-1. The ultimate expression of matrix metalloproteinase activity within the microenvironment of the tumour is linked to the presence or absence of this inhibitor. That TIMP-1 may have an important role in mediating tumour invasion is implied by the increased incidence with which it was found at the invading edge of the tumour compared to either the tumour centre or normal mucosa. The use of TIMP-1 or synthetic analogues may provide a novel therapeutic approach to modifying, or indeed inhibiting, the invasive and metastatic potential of malignant tumours. Some experimental work already exists to support this therapeutic concept as d9iscussed on page 49 (Alvarez et al, 1990). However, a full assessment of such agents must be founded on a proper understanding of the distribution of TIMP-1 in both normal and pathological situations. Although further confirmation is required, evidence to suggest that TIMP-1 is present in colonic mucus is given in Chapter 3. In so doing this study has provided the basis for the subsequent quantification and evaluation of TIMP-1 in mucus derived from neoplastic colonic mucosa. Given in vitro evidence that the TIMP-1 gene may act as a tumour suppressor (Khokha et al, 1991), direct measurement of the gene product in mucus could provide important information on tumour development and progression in the colon. It would also be the aim of a future study of colonic neoplasia to determine whether TIMP-1 levels in mucus could be correlated with the position of the sample relative to the tumour; it may then be possible to equate changes in TIMP-1 with the underlying histology of the mucosa. The use of such an approach in the examination of

used criteria for assessing the likelihood of tumour recurrence.

Chapters 4 and 5 of this thesis were concerned with the detection and quantification of circulating tumour cells in colorectal cancer. Much of the previous work on this subject was conducted in the 1950's and 1960's and relied exclusively on the parameters of conventional cytology to identify the tumour cells. By using an anticytokeratin antibody to distinguish epithelial derived cells from those of the haemopoietic system, uncertainties in the reliability of earlier observations on the incidence of circulating tumour cells have been revealed. In chapter 5 data were presented showing disagreement between the results of conventional cytology and immunocytochemistry in the identification of blood borne tumour cells; 6 blood samples could have been erroneously labelled as containing tumour cells were it not for the clarification afforded by the absence of cytokeratin staining. The ability to identify blood borne tumour cells accurately may be seen as an essential first step in gathering more information about their characteristics.

As discussed in the introduction to this thesis the metastatic process can be regarded as a series of sequential steps, beginning with the invasion of adjacent tissues and the subsequent intravasation and circulation of cancer cells. That metastases might arise as a consequence of phenotypically distinct subpopulations of cancer cells within the primary tumour has been advocated by Leighton (1965). His concept was later reinforced by the experiments of Poste and Fidler (1980) who showed that cells isolated from lung metastases had an increased ability to metastasize compared to those of the parent cell line. If metastases do arise as a result of the behaviour of a selected group of cells it would be of potential prognostic value if the number of such cells in a primary colorectal cancer could be

estimated. Such information would be useful, for example, in predicting those patients most likely to develop liver metastases and hence most liable to benefit from adjuvant therapies. However, the identification of metastatic subclones within primary colorectal tumours has so far proved difficult. For instance Jass et al (1989) using the technique of flow cytometry failed to demonstrate any appreciable difference between the DNA content of primary tumours and their metastases in the majority of cases examined. It is possible, however, that important phenotypic differences may be expressed only transiently at specific points in the metastatic process. By comparing the cells of the primary tumour with those of its metastasis these intermediate differences may be missed. An examination of the characteristics of circulating tumour cells, therefore, has the potential to identify features which have enabled these cells to at least complete the initial steps of the metastatic process. One example would be to determine the expression of laminin receptors and other cell adhesion molecules by such cells, which may reflect their ability to bind to exposed basement membranes and capillary endothelium.

The quantitative data presented in Chapter 5 has confirmed the inefficiency of the metastatic process in colorectal cancer. In addition the detection of tumour cells in peripheral venous samples obtained preoperatively suggests that substantial numbers of cells must have been capable of traversing the capillary beds of both the organ of first encounter (the liver) and the lungs. It would be of interest to know if these cells differed in some way from those destined to arrest in the liver. That circulating cells were detectable in peripheral blood samples also suggests that their presence might be used to augment current methods for the detection of recurrent disease. Any attempt to do so, however, would have to contend with the

dual problems of the small number of cells likely to be present in any given specimen, and the necessity to take multiple blood samples if false negative results are to be avoided.

The study presented in Chapter 5 was concerned only with the detection of blood borne tumour cells and not with their viability. The percentage of cancer cells in the blood stream which are viable and tumourgenic remains unknown, but clearly will have an important bearing on their prognostic significance. Once again the small numbers of cells present in blood samples of 5 or 10 ml will make determination of this information difficult. To the author's knowledge only one previous study has attempted to culture tumour cells obtained from the blood of patients with colorectal cancer: in 1961 McDonald and Cole confirmed the viability of cancer cells obtained from the blood in two out of twenty patients with colorectal tumours by successfully growing them in culture medium. Whether or not the viability of such cells in culture equates with their ability to form metastases remains unknown. Experiments on the capacity of isolated tumour cells to form metastases following inoculation into nude mice might be useful, although their relevance to events in an immune competent patient can be questioned.

In conclusion the studies presented in this thesis may provide the basis for the development of newer prognostic indices in colorectal cancer. These indices would supplement traditional pathological parameters such as the depth of invasion, involvement of draining lymph nodes, vascular spread and surgical clearance deep to the tumour. By concentrating on the mechanisms which lie behind the events recorded in current staging systems a more direct approach to predicting the biological aggressiveness of tumours might emerge.

**Appendix 2.1**

**Hank's Balanced Salt Solution** - with N-2-hydroxyethyl piperazine N'-2-ethanesulphonic acid (HEPES) and antibiotics.

| **Salt** | **grams/litre** |
|---|---|
| NaCl | 8 |
| KCl | 0.4 |
| $CaCl_2.2H_2O$ | 0.185 |
| $MgSO_4.7H_2O$ | 0.1 |
| $MgCl.6H_2O$ | 0.1 |
| $Na_2HPO_4$. anhydrous | 0.048 |
| $KH_2PO_4$ | 0.06 |
| $NaHCO_3$ | 0.35 |
| Glucose | 1 |
| HEPES | 4.766 |
| penicillin 1x | 0.06 |
| penicillin 10x | 0.6 |
| streptomycin 1x | 0.1 |
| streptomycin 10x | 1.0 |
| amphotericin 1x | 0.0025 |
| amphotericin 10x | 0.025 |

**Phosphate-buffered saline (PBS) (5x concentration)**

| | |
|---|---|
| NaCl | 106.25 grams |
| $Na_2HPO_4.2H_2O$ | 40.2 grams |
| $NaH_2PO_4.2H_2O$ | 1.95 grams |
| Distilled water | 2.5 litres |
| pH | 7.35 |

Diluted to 1x when required

**4% Paraformaldehyde**

Paraformaldehyde 4grams in 50 ml PBS with 4-5 drops of 5M NaOH. Heat until dissolved, cool, make up to 100 ml with PBS and then pH with acetic acid to 7.4.

**4-Chloro-1-naphthol**

Dissolve 54 mg of 4-chloro-1-naphthol in 18 ml of AnalaR grade methanol, and add to 90 ml PBS. Immediately before use add 30 µl of hydrogen peroxide.

**Appendix 2.2**

**Additives to medium used for tissue culture**

Dulbecco's modification of Eagles medium was used containing HEPES (ICN Flow, High Wycombe, UK). To 500 ml of medium were added:

| | |
|---|---|
| Penicillin | 30 mg |
| Streptomycin | 50 mg |
| Amphotercin | 1.25 mg |
| Glutamine | 0.146 g |
| $NaHCO_3$ | 0.25 g |
| Acid treated fetal calf serum | 5 ml |

The fetal calf serum is treated with 5M HCl to pH 3 and left for one hour at room temperature, after which time the acid is neutralised with NaOH.

**Appendix 3.1**

**Composition of the protease inhibitor solution used to collect mucus**

All chemicals are from Sigma, Poole, Dorset, UK.

| | |
|---|---|
| 4 mM | phenylmethyl- sulphonyl fluoride |
| 4 mM | N-ethyl maleimide |
| 10 mM | ethylene diamine tetra-acetic acid |
| 1 mg/l | Soyabean Trypsin Inhibitor |

- made up in 0.5 M Tris-HCl, pH 8

**Appendix 4.1**

**0.05 M Tris-buffer**

Prepare 1M Tris (121.14 g/l) and 1M HCl (87.2 ml/l).
Use 50ml Tris and 38.4ml HCl and make upto 1 litre and pH to 7.6.

For Tris-buffered saline add 8.5g NaCl to each litre of the above.

**3,3 diaminobenzidine tetrahydrochloride (DAB)**

75 mg DAB dissolved in 300 ml Tris-buffer, pH 7.6. Just prior to use add 300 µl hydrogen peroxide.

Scotts Tap Water

| | |
|---|---|
| Sodium bicarbonate ($NaHCO_3$) | 7g |
| Magnesium Sulphate ($MgSO_4$) | 40g |
| Tap Water | 2 litres |

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ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| BSA | Bovine serum albumin |
| C | Centigrade |
| CL | Interstitial collagenase |
| cm | centimetre |
| $cm^2$ | square centimetre |
| DAB | 3,3 diaminobenzidine hydrochloride |
| FITC | Fluorescein isothiocyanate |
| g | gravity constant |
| GL | Gelatinase |
| HEPES | N-2-hydroxyethyl piperazine N'-2-ethanesulphonic acid |
| IgG | Gamma globulin |
| kDa | kilo Dalton |
| mA | milliampere |
| ml | millilitre |
| mM | millimole |
| MMPs | matrix metalloproteinase |
| Mr | molecular weight |
| mRNA | messenger RNA |
| MPI | mucus proteinase inhibitor |
| NM | Normal mucosa |
| nM | nanomole |
| PBS | phosphate buffered saline |
| SL | Stromelysin |
| T | Tumour |
| TBS | Tris-buffered-saline |
| TC | Tumour centre |
| TIMP-1 | Tissue inhibitor of metalloproteinases-1 |
| TIMP-2 | Tissue inhibitor of metalloproteinases-2 |
| TMJ | Tumour mucosal junction |
| µg | microgram |
| µm | micron |
| $X^2$ | chi-squared test |