# INTERACTIONS BETWEEN COAGULATION, SURGERY AND THE METASTATIC PROCESS

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I would like to dedicate this thesis to my parents for their unerring support both emotional and frequently financial, over many years. I would also like to thank my wife Clare for her patience and reluctant understanding during the first year of our marriage.

## SUMMARY

Evidence from clinical and animal studies indicates an interaction between cancer and the haemostatic system. Human malignancy disrupts the normal haemostatic balance in favour of a hypercoagulable state while studies on the effects of anticoagulant/antiplatelet agents in animal models of metastasis indicate a role for coagulation in the metastatic process. Recent work has demonstrated that Streptokinase inhibits pulmonary tumour seeding in an animal model of metastasising breast carcinoma. The purpose of the first half of this thesis was to investigate the mechanism of pulmonary tumour inhibition by Streptokinase. Validation of the experimental tumour model was first performed to confirm the dose, time of administration and effect of Streptokinase on pulmonary tumour seeding. The results of these experiments confirmed that the inhibitory effect of Streptokinase on pulmonary tumour nodule formation was reproducible.

It was hypothesised that the effect of tumour inhibition by Streptokinase was secondary to dissolution of the fibrin clot surrounding tumour cells entrapped within the pulmonary microcirculation. In order to confirm fibrin clot lysis, a study was undertaken to measure fibrinolytic activity during Streptokinase treatment by estimation of plasma fibrin degradation product levels (FDP). The results showed a tendency to increased FDP levels in animals receiving

Streptokinase although this failed to reach statistical significance possibly due to the low level fibrinolytic activity exhibited by untreated controls.

In order to provide conclusive evidence that the antitumour effect of Streptokinase was due to fibrin clot breakdown, a further study was performed to compare the effect of Streptokinase and a second fibrinolytic agent, human recombinant tissue Plasminogen Activator (rt-PA), on pulmonary tumour seeding. The results showed a comparable twofold reduction in pulmonary tumour seeding by both these fibrinolytic agents. The demonstration that two structurally distinct compounds, unrelated except for their ability to induce fibrin clot lysis, produce a similar antitumour effect, suggests that this effect is likely to be secondary to fibrin clot lysis.

Platelets are also an integral component of thrombi surrounding tumour cells arrested in the microvasculature. Further animal studies were therefore performed to establish whether a dual assault on fibrin clot formation and platelet deposition had an additive inhibitory effect on pulmonary tumour seeding. Since tumour cell-induced platelet activation may occur via a number of pathways including the generation of thrombin and activation by ADP, this study used two anti-platelet agents with separate mechanisms of action. Although there was a trend towards inhibition of pulmonary tumour seeding with aspirin, this failed to reach a significant level suggesting that platelet

activation by the Mtln3 cell line may be independent of the cyclo-oxygenase pathway. In contrast, ticlopidine significantly inhibited pulmonary tumour seeding suggesting that tumour cell-induced platelet aggregation by the Mtln3 cell line may be dependent on the generation of ADP. The combination of ticlopidine and Streptokinase on inhibition of pulmonary tumour seeding was no more effective than Streptokinase treatment alone indicating that in this animal model, fibrin may play a more important role than platelets in the intravascular phase of the metastatic process. These animal studies suggest that fibrinolytic therapy may have a role in reducing metastatic progression in patients with malignant disease.

The second half of this thesis was designed to identify cancer patients who might benefit from such antimetastatic therapy. Animal studies have demonstrated that manipulation of a primary tumour facilitates tumour cell dissemination via the bloodstream and may promote metastasis. Surgery and anaesthesia may further enhance the metastatic potential of circulating tumour cells shed during operative manipulation by inducing a state of relative immune suppression and facilitating tumour cell seeding in target organs in a more fertile, hypercoagulable blood environment. Fibrinolytic therapy is most likely to be effective if administered during such tumour cell dissemination. The studies of Turnbull using the "no-touch" isolation technique in patients with colon

cancer have provided the only clinical evidence that surgical manipulation of primary tumours may facilitate metastasis. Evidence of intra-operative tumour cell dissemination might indicate a role for peri-operative antimetastatic therapy. It was therefore hypothesised that surgical manipulation during resection of a primary malignant tumour enhanced metastasis by facilitating tumour cell shedding into the bloodstream. Initial studies using two colour flow cytometry to detect circulating tumour cells in patients with breast cancer during surgery were unsuccessful due to inadequate sensitivity at low tumour cell concentrations. Further studies used reverse transcriptase PCR to amplify two genes encoding structural epithelial proteins, cytokeratin 18 and the breast carcinoma-associated antigen DF3. The absence of epithelial cells in normal peripheral blood suggested that these genes might be suitable targets for the detection of circulating malignant breast epithelium. Our initial results however demonstrated expression of the cytokeratin 18 gene in the peripheral blood of healthy volunteers possibly due to low level transcription of the CK 18 gene in non-epithelial tissues. In contrast, DF3 was consistently expressed in cells derived from primary malignant breast carcinomas but was absent in all normal peripheral blood samples. An assessment of sensitivity demonstrated that reverse transcriptase PCR could detect 10 tumour cells per 5 ml of blood. A preliminary clinical study of nine patients

undergoing surgery for primary breast carcinoma demonstrated the presence of DF3 in the blood of one patient pre-operatively and four patients during surgery. DF3 was absent in all post-operative samples and in all samples obtained from patients undergoing surgery for benign disease. The presence of DF3 in patients undergoing surgery for malignant disease but not benign disease suggests that this represents circulating malignant epithelial cells. Furthermore, the presence of DF3 in four of nine patients during surgery suggests that surgical manipulation of malignant breast tumours may enhance haematogenous tumour cell dissemination. These findings suggest that antimetastatic therapy may have a role in patients undergoing surgery for malignancy.

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## LIST OF ABREVIATIONS

| | |
|---|---|
| ADP | Adenosine Diphosphate |
| CAM | Cell Adhesion Molecule |
| cDNA | Complementary Deoxyribonucleic Acid |
| CK 18 | Cytokeratin 18 |
| EMA | Epithelial Membrane Antigen |
| FCS | Foetal Calf Serum |
| FDP | Fibrin(ogen) Degradation Products |
| FITC | Fluorescein Isothiocyanate |
| FSC | Forward Scatter |
| F 344 | Fischer 344 |
| F10/DMEM | 1:1 mixture of Hams' F10 and Dulbecco's Modified Eagles' Medium |
| HMFG | Human Milk Fat Globule Membrane |
| HMW-kininogen | High Molecular Weight-kininogen |
| LFA-I | Leucocyte Function Antigen (Integrin alpha-chain) |
| MAB | Monoclonal Antibody |
| mRNA | Messenger Ribonucleic Acid |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PE | Phycoerythrin |
| PEM | Polymorphic Epithelial Mucin |
| rt | Reverse Transcriptase |
| rt-PA | Recombinant Tissue Plasminogen Activator |
| SSC | Side Scatter |

| | |
|---|---|
| TBE | Tris-Borate Buffer |
| TCIPA | Tumour Cell-Induced Platelet Aggregation |
| u-PA | Urokinase-type Plasminogen Activator |

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Finally I would like to thank Professor WD George for giving me the opportunity to perform these studies

**DECLARATION**

I declare that this thesis has been composed by myself, and that the work described herein was performed by me, or by myself in conjunction with others. The laboratory tests for analysis of Fibrin(ogen) Degradation Products, described in Chapter 2, were performed by Mr Barron, Department of Haematology, University School of Vetinary Medicine, Garscube. Statistical analysis of all the work described was supervised by Dr J Love, Department of Statistics, University of Glasgow. All other work described herein was performed entirely by myself. This work has not been presented at any previous application for a degree by myself or others.

The work described in this thesis has been presented at the following meetings of scientific societies:

| | |
|---|---|
| December 1992 | British Association of Surgical Oncology, London |
| December 1992 | Scottish Society for Experimental Medicine, Glasgow |
| January 1993 | Surgical Research Society, London |
| March 1993 | 7th Meeting of the Academic Departments of Surgery in Europe (ADSE), Free University of Amsterdam, The Netherlands |
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Publications:

Inhibition of pulmonary tumour seeding by antiplatelet and fibrinolytic therapy in an animal experimental model. Brown DC, Purushotham AD, George WD. *J Surg Oncol* 1994; **55**: 154-159.

Streptokinase inhibits pulmonary tumour seeding in an animal experimental model. Purushotham AD, Brown DC, McCulloch P, Choy A, George WD. *J Surg Oncol* 1994; **57**: 3-7.

Detection of intra-operative tumour cell dissemination in patients with breast cancer using reverse transcription and the Polymerase Chain Reaction. Brown DC, Purushotham AD, Birnie GD, George WD. *Surgery* 1994; In press

# INTRODUCTION

## 1. THE MECHANISM OF DEVELOPMENT OF METASTATIC DISEASE

### 1.1 Pathogenesis of metastasis

The term "metastases" was coined by the French physician, Joseph Claude Recamier in his 1829 treatise Recherches du Cancer (Recamier, 1829). Previously, surgeons had recognized that malignant tumours could invade locally and spread to draining lymph nodes but distant metastases were thought to be distinct tumours. Recamier provided anatomic evidence that distant metastases arose from cancer cells shed into the circulation by the primary tumour. Halsted (1907) suggested that such haematogenous dissemination was secondary to lymphatic invasion but later pathological studies established that malignant tumours could invade the vascular system directly (Friedel et al, 1965), even in the absence of lymphatic involvement (Salsbury, 1975).

Metastasis is now defined as the ability of a tumour to disseminate via lymphatic or vascular channels to a distant, non-contiguous site and it is the most important criterion distinguishing benign from malignant tumours. The resistance of metastases to conventional therapies explains why the cure rate for the major cancers has remained relatively unchanged

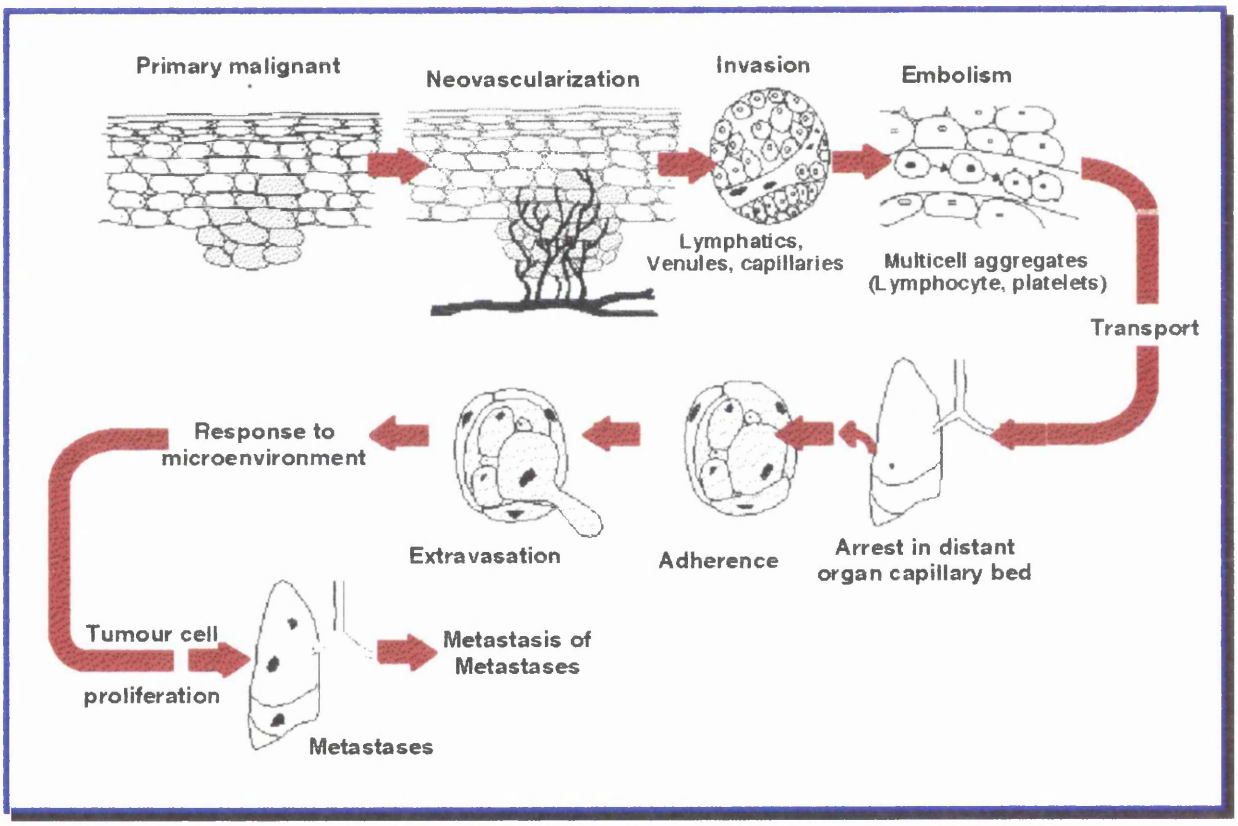
despite a significant improvement in our understanding as to their aetiology, biology and treatment (Fidler et al, 1987). Considerable evidence has established that the metastatic process is a complex sequential pathway which few tumour cells complete successfully (Weiss et al, 1986). Primary tumours possess heterogeneous metastatic properties and only a minor subpopulation of cells have the necessary intrinsic properties to complete all stages of the metastatic process (Fidler et al, 1990). This successful metastatic cell has been likened to a "decathlon champion" (Fidler et al, 1978) which can successfully interact with the host environment and survive many lethal events to eventually grow into a metastasis. The metastatic process can be divided into five stages: 1) invasion of host tissues and penetration of the vascular system 2) dissemination by the circulation and evasion of host defences 3) adhesion to vascular endothelium 4) extravasation into host tissues and 5) secondary tumour growth (Figure 1).

Tumour vascularisation (angiogenesis) is essential for growth and metastasis. Once a tumour reaches a critical diameter of 2-3mm, its nutritional requirements can no longer be met by simple diffusion and further growth requires the development of a vascular system (Folkman et al, 1972). Specific angiogenic molecules released by the tumour or adjacent macrophages induce both vascularisation and lymphatic proliferation (Polverini et al, 1984; Folkman et al,

# Figure 1



## Stages of the Metastatic Process




(Original In Colour)

1989). The prevascular phase may persist for many years and is associated with a paucity of metastases while the vascular phase is associated with rapid tumour growth and dissemination (Brem et al, 1978; Sillman et al, 1981). The intensity of neovascularization has been shown to correlate with the development of both local lymph node and distant metastases suggesting that these immature blood vessels, which lack an organised extracellular matrix may facilitate the entry of tumour cells into the circulation (Blood et al, 1990; Weidner et al, 1991). This process may be enhanced by tissue pressure generated by growth-associated expansion in the parent tumour (Hori et al, 1983). Invasion through intact basement membrane of existing mature blood vessels by enzymatic degradation of the extracellular matrix may also occur. Tumour production of proteolytic enzymes capable of such activity has been shown to correlate with metastatic potential (Nakajima et al, 1983; Wooley, 1984) but the relevance of this process to intravasation as opposed to extravasation in secondary sites is unclear.

The presence of tumour cells in the bloodstream does not always determine that metastasis will occur. Although the majority of cells released from a primary tumour are potentially tumourigenic (Glaves and Mayhew, 1984), less than 1% of tumour cells are viable after 24 hours in the circulation and less than 0.1% survive to produce metastases (Fidler, 1970). Mechanical trauma and nutritional defficiency are

thought to explain the short half-life of the majority of circulating cells (Sato and Suzuki, 1976; Weiss et al, 1985). Antitumour immune activity may also contribute to cell loss and involves the participation of both specific and non-specific immune defence mechanisms including cytotoxic T lymphocytes, natural killer (NK) cells and mononuclear phagocytes (Wood et al, 1975; Fidler et al, 1980; Hanna et al, 1985). Individual tumour cell survival can be enhanced by aggregation with each other, with host cells, including platelets and lymphocytes (Fidler, 1977; Gasic, 1984; Updyke, 1986) or by adhesion to endothelium (Korach et al, 1986). It is thought that tumour cells placed within the central zone of such emboli are better protected from mechanical and immune destruction. Therefore, blood appears to be an extremely hostile environment for tumour cells which being derived from solid tissues, have no particular properties that enhance their survival in this environment. It would seem that tumour cells which minimize their circulation time by encouraging early extravasation may have the greatest metastatic potential.

The arrest of tumour cells has been postulated to be a random process during which mechanical entrapment occurs in the microcirculation of the first organ encountered. This haemodynamic theory was first postulated by Ewing (1928) and has since been confirmed by numerous animal models which have demonstrated that the lungs are the preferential site of metastasis after

intravenous tumour cell injection (Proctor et al, 1976; Becker et al, 1978) and the liver after intraportal injection (Fisher and Fisher, 1965; Vaage, 1973). However, animal studies using radiolabelled tumour cells injected into the arterial system have revealed that certain tumours preferentially metastasise to specific organs independent of vascular anatomy, rate of blood flow and number of tumour cells delivered (Murphy et al, 1988).

Clinical observations describing a non-random pattern of visceral metastasis were first reported for breast cancer by Paget (1889). He concluded that certain tumour cells (the seed) had specific affinity for certain organ environments (the soil). Experimental verification of this "seed-soil" hypothesis has recently been provided by in vitro studies demonstrating preferential adhesion of tumour cells to the preferred site of metastasis - melanoma cells to lung endothelium (Johnson et al, 1991), mammary carcinoma cells to lymphatic endothelium, glioma cells to brain-derived capillary endothelium (Auerbach et al, 1987) and liver-metastatic tumour cells to hepatic sinusoidal endothelium (Roos et al, 1984). Cell adhesion molecules, which encompass four major families, integrins, cadherins, immunoglobulins and selectins, are now thought to mediate the adhesion of tumour cells to target organ capillary endothelium (Honn and Tang, 1992). A correlation has been shown between the expression of specific cell adhesion

molecules and the metastatic potential of various human tumours. Expression of the intercellular adhesion molecule-1 (ICAM 1), the ligand for the integrin receptor lymphocyte function-associated-1 (LFA-1) found on leucocytes, has been implicated in melanoma metastasis (Johnson et al, 1989), ICAM 2, PECAM 1 and CD 44 in lymphoma metastasis (Roos et al, 1991) and ELAM-1 in colon metastasis (Rice et al, 1988). These experimental studies suggest that the site selectivity of bloodborne metastasis is predominantly determined by the pattern of surface adhesion receptors expressed by tumour cells and endothelium.

Extravasation requires penetration of a mature basement membrane and thus is dependent upon the production of hydrolytic enzymes capable of degrading specific matrix molecules (Nakajima et al, 1983; Wooley, 1984). In addition, tumour cell invasion may be enhanced by degraded fragments of the extracellular matrix which are chemotactic, thereby stimulating tumour cell motility (Blood et al, 1988; Albini et al, 1987). Increased motility is frequently observed in highly metastatic tumour cells (Geiger and Raz, 1984; Young et al, 1985). The final stage of secondary tumour growth is dependent on the ability of individual tumour cells to respond selectively to growth modulators in the surrounding microenvironment and to promote angiogenesis.

## 1.2 Metastatic heterogeneity of malignant neoplasms

The inefficiency of the metastatic process led researchers to question whether the development of metastases represented the fortuitous survival and growth of a few tumour cells or whether it simply represented the selective survival of a unique subpopulation of tumour cells possessing special properties. Ewing suggested that the pattern of metastasis was merely a consequence of anatomical and haemodynamic factors (Ewing, 1929). While circulatory anatomy may influence the distribution of metastasis the "seed-soil" hypothesis of Paget demonstrated that the pattern of metastasis was non-random and not due to chance.

The first experimental evidence for pre-existing metastatic heterogeneity of malignant tumours was provided by Fidler and Kripke (1977) with the murine B16 melanoma. Using a modification of the fluctuation assay of Luria and Delbruck (1943), they demonstrated considerable variation in the ability of different tumour cell clones, each derived from an individual cell isolated from the parent tumour, to produce pulmonary metastasis after intravenous administration. Similar findings were subsequently shown for human tumour lines and fresh tumour using nude mice as models for metastasis (Fidler, 1986). The idea that neoplasms contained subpopulations of tumour cells with different metastatic propensities was thus established. The

source of such heterogeneity was however difficult to perceive as the majority of naturally occuring and induced neoplasms were known to be unicellular in origin (Nowell, 1976; Vogelstein, 1985). Clinical and histopathological observations that benign tumours could change over a period of time into malignant tumours led to the concept of neoplastic progression. This was defined as "acquisition of permanent, irreverible qualitative changes in one or more characteristics of a neoplasm" (Foulds, 1975). Studies using virally-transformed murine fibroblasts showed that acquired phenotypic heterogeneity could produce subpopulations of tumour cells with different metastatic potentials at an early stage of development (Fidler, 1981). Furthermore, karyotypic analysis of primary tumours and their metastases showed that tumour progression was associated with increasingly altered karyotype and ploidy of metastatic subpopulations (Nowell, 1989). Comparison of metastatic and non-metastatic cloned lines isolated from different rodent neoplasms established that highly metastatic cells were consistently phenotypically and genotypically less stable than their non-metastatic counterparts (Cifone and Fidler, 1982; Kaden et al, 1989). Thus it appears that genetic instability enables highly metastatic cell subpopulations to undergo rapid phenotypic diversification. Current therapeutic modalities that destroy most, but not all, tumour cell subpopulations may enable these highly metastatic subpopulations to

survive and produce new variants with even greater metastatic properties (Poste, 1986).

## 2 THE HAEMOSTATIC SYSTEM

The mechanism of haemostasis involves a complex interaction between the vascular endothelium, platelets and the coagulation and fibrinolytic pathways. After vascular injury, transient primary haemostasis is achieved by arterial vasoconstriction and the formation of a platelet plug. More permanent haemostasis is achieved by activation of coagulation and formation of a fibrin clot. This secondary phase forms a platform for endothelial cell growth and the release of fibrin degradation products. In addition, plasminogen activation stimulates angiogenesis and repair of the vascular wall while fibrinolysis and collagenase activity remove debris. A complex system of checks ensures that activation of the haemostatic system is balanced and does not progress to either life threatening haemorrhage or disseminated intravascular coagulation. The role of each system is discussed below.

### 2.1 Vascular Endothelium

Vascular endothelium contributes to a normal haemostatic balance in two ways. Firstly, a healthy intact endothelium provides an interface separating

platelets and coagulation factors from thrombogenic components of the vascular subendothelium. Platelets are shielded from activation factors of the subendothelial matrix including von Willebrand factor (vWF), collagen and fibronectin while extrinsic and intrinsic activation of the coagulation pathway is prevented by endothelial shielding of tissue thromboplastin and surface contact activators (Koteliansky et al, 1981; Osterud, 1984; Stel et al, 1985). In addition, vascular endothelial cells contribute to haemostatic balance by synthesizing and secreting both anticoagulants and procoagulants.

The major anticoagulants synthesized by endothelium are prostacyclin (PGI2) and tissue plasminogen activator (t-PA). PGI2 is the major arachidonic acid metabolite of endothelium and is a potent vasodilator and inhibitor of platelet aggregation (Honn et al, 1983). Tissue plasminogen activator is a powerful fibrinolytic agent and has a high affinity and specificity for fibrin-bound plasminogen where it causes enzymatic degradation of the latter into plasmin and consequently fibrinolysis (Collen et al, 1989).

The major procoagulant factors produced by endothelial cells are vWF and fibronectin. These large glycoprotein molecules are stored in endothelial cells and the alpha granules of platelets. During platelet activation they are released and together with circulating vWF and fibronectin preferentially bind to

a specific glycoprotein receptor IIb-IIIa, on the membranes of activated platelets (Plow et al, 1985; Haverstick et al, 1985). At the same time, these adhesive proteins bind to receptors on the extracellular matrix and thus serve as cables and connectors that link activated platelets to each other and to the subendothelial extracellular matrix (Hynes et al, 1987) thereby enhancing platelet aggregation and adhesion.

Several structurally unrelated anticoagulants are also produced by endothelium or activated on endothelial surfaces. This family of naturally occuring anticoagulants act on endothelial surfaces to inhibit terminal stages of the coagulation pathway. The principal components of the endothelial antithrombin system are antithrombin III which inactivates thrombin on the surface of endothelium (Busch et al, 1982), and two vitamin-K dependent enzymes: protein C and protein S which inactivate coagulation factors Va and VIIIa (Stern et al, 1986).

In summary, it is clear that the endothelium has an important role in maintaining haemostatic balance. Loss of endothelial lining as a result of acute vascular injury, chronic endothelial damage by atherosclerosis or hypertension deprives the vessel of the intrinsic anticoagulant protective mechanisms outlined above. Furthermore, exposure of the thrombogenic constituents of the subendothelial matrix, including vWF, fibronectin and tissue thromboplastin,

leads to platelet adhesion, the initial event in platelet plug formation.

## 2.2 Platelets

### Platelet structure

Platelets are discoid fragments of marrow megakaryocytes that normally circulate for about 10 days in numbers ranging from 200 000 to 400 000 platelets/ul. The surface membrane is composed of a typical trilaminar phospholipid structure interspersed with proteins and glycoproteins anchored by hydrophobic roots. The three major groups of membrane glycoproteins, termed glycoproteins I, II and III possess receptor, transport and enzymatic functions. Cytoplasmic granules (alpha, dense and lysosomal) secrete agents essential for platelet adhesion and aggregation including vasoactive amines, adenosine diphosphate (ADP), beta-thromboglobulin and prostaglandins (Tuffin, 1991). Cytoplasmic microtubules composed of the contractile proteins actin and myosin are responsible for clot retraction in addition to shape change and spreading during activation (Gerrard et al, 1976).

**Platelet function**

The mechanism by which platelets participate in the formation of a haemostatic plug involves initial adhesion to exposed subendothelial matrix at the site of vascular injury. This is followed by aggregation of platelets and the formation of a platelet plug. Finally, platelets initiate procoagulant activity and facilitate the generation of thrombin and formation of a fibrin clot.

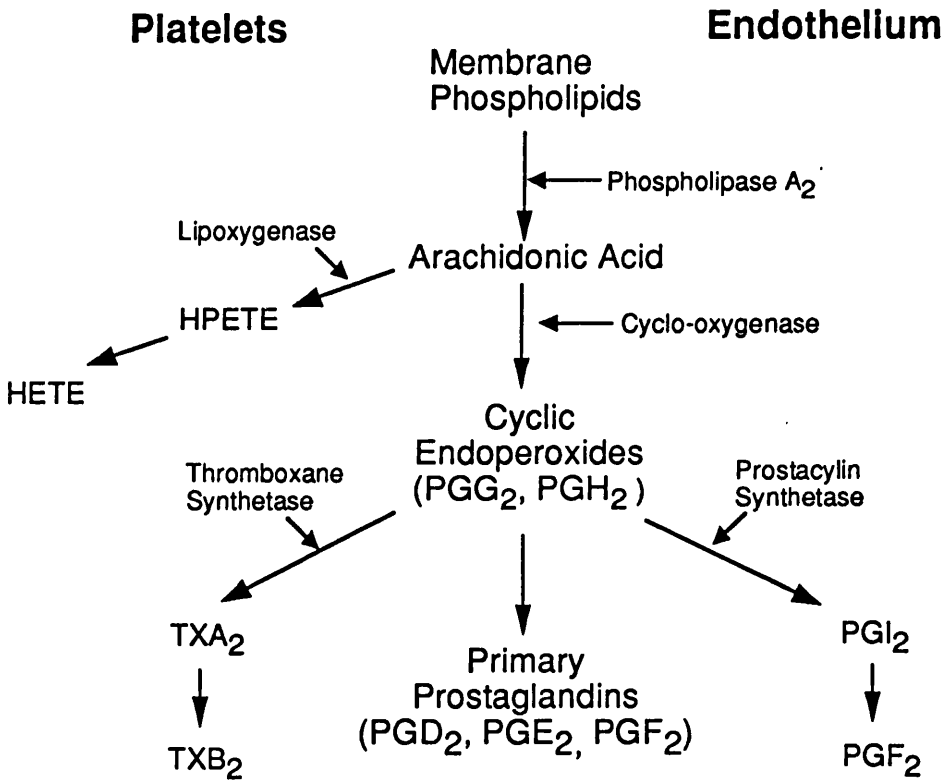
Platelet adhesion and aggregation require the interaction of platelet surface glycoproteins with adhesive proteins (ligands) present in plasma and platelet granules. Two of the major surface glycoproteins, termed IIb and IIIa, exist as a non-covalent 1:1 complex and serve as a common receptor for fibrinogen (Bennett et al, 1983), fibronectin (Gardner and Hynes, 1985), vitronectin (Pytela et al, 1986) and vWF (Ruggeri et al, 1982). The interaction of integrin receptors and ligands is an essential component in cell-matrix and cell-cell adhesion in many physiological mechanisms including haemostasis (Hynes, 1987). vWF and thrombospondin are the major adhesive proteins which facilitate platelet-subendothelial adhesion whereas primary aggregation is predominantly associated with the binding of fibrinogen (Mayer and Baumgartner, 1983; Peerschke, 1986). Exposed subendothelial collagen normally initiates platelet activation but other agonists released by damaged

tissue or previously activated platelets including ADP, thrombin and adrenaline, can stimulate further platelet adhesion or primary aggregation. The second wave of aggregation termed the "release reaction" is characterised by the release of ADP and arachidonic acid and results in additional fibrinogen binding and stabilization of platelet aggregates (Colman, 1986). Platelet activation is also associated with transformation to a discoid shape and surface spreading thereby increasing the surface area for adhesion.

Mechanical and agonist stimulation also liberate arachidonic acid from membrane phospholipid leading to prostaglandin synthesis by activation of cyclo-oxygenase and lipoxygenase pathways (Neufield and Majerus, 1983). The major products of the cyclo-oxygenase pathway are prostacyclin (PGI2) and thromboxane A2 (TXA2) while activation of the lipoxygenase pathway produces the hydroperoxides, hydroperoxyeicosatetraenoic acid (HPETE) and hydroxyeicosatetraenoic acid (HETE) (Figure 2). TXA2 is the predominant product of arachidonic acid metabolism in platelets and is a powerful stimulator of platelet aggregation and arterial vasoconstriction. As described earlier, endothelial cells defend their non-thrombogenic surfaces by secreting PGI2 and normally an equilibrium exists between endothelial PGI2 and platelet-derived TXA2. The increased release of TXA2 during platelet activation leads to an imbalance which favours platelet aggregation.

Figure 2

# Prostaglandin Metabolisim in Platelets and Endothelium




| | |
|---|---|
| $TXA_2$ | - Thromboxane $A_2$ |
| $TXB_2$ | - Thromboxane $B_2$ |
| $PGI_2$ | - Prostacyclin |
| HPETE -12 | - Hydroperoxyeicosatetraenoic Acid |
| HETE | - Hydroeicosatetraenoic Acid |

Adequate haemostasis cannot be achieved by platelet deposition alone. Reinforcement of this relatively weak platelet-subendothelial bond is provided by fibrin and is essential to prevent detachment of the platelet plug by intravascular blood flow. Platelets participate in the formation of fibrin by providing a favourable surface for the binding of co-factors and enzymes essential to the generation of thrombin.

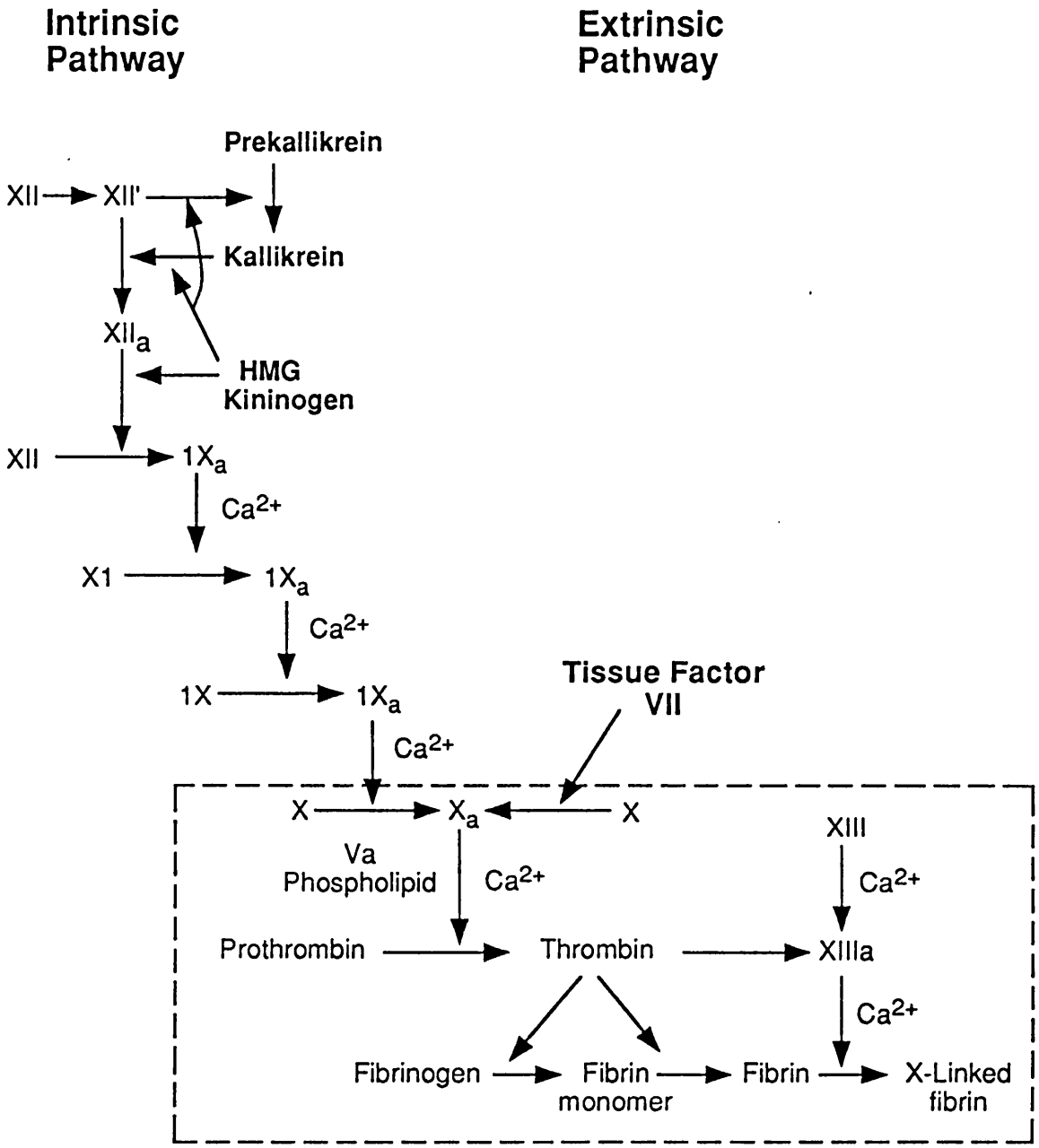
## 2.3 Coagulation pathway

The coagulation pathway culminates in the conversion of the soluble plasma protein fibrinogen, to insoluble fibrin transforming plasma from a solution to a gel. The rate of this conversion is greatly increased by the assembly of co-factors and enzymes in complexes on platelets. This requires a complex series of reactions which convert sequentially inert plasma precursors into their active forms. With the exception of factors V, VII and high molecular weight kininogen, which are peptide co-factors, activated coagulation factors are proteolytic enzymes. Activated factors then activate factors down the line domino fashion by a process of limited proteolysis.

Coagulation is mediated by two distinct pathways which share the final three steps of: activation of factor X, prothrombin to thrombin conversion and fibrin formation (Figure 3).

Figure 3

# Pathways to Blood Coagulation




Note: Broken box denotes shared pathway to Fibrin formation

The extrinsic pathway is initiated by the release of tissue factor (thromboplastin) by vascular endothelial cells, macrophages and neutrophils at the site of vessel disruption. Thromboplastin then interacts with factor VII to form a complex with platelet phospholipid which, in the presence of calcium ions, activates factor VII (VIIa). The subsequent activation of factor X by factor VIIa also occurs in the presence of platelet phospholipid and calcium ions.

The intrinsic pathway is initiated by exposure of plasma to non-endothelial surfaces including basement membrane, collagen and platelet procoagulants. Platelets facilitate factor XII activation by binding factor XII to a receptor that alters its conformation and enhances proteolysis. Factor XII may also be activated by a contact activation complex, composed of high molecular weight kininogen (HMWK) complexed to prekallikrein or factor XI and anchored to an endothelial surface. The interaction of factor XII with this complex creates a positive feedback loop which leads to the generation of factor XIa, IXa and subsequently factor Xa.

The ability of factor Xa to activate prothrombin is accelerated several thousand-fold by 1:1 binding of factor Va and calcium ions to platelet phospholipid forming a prothrombinase complex. Thrombin then catalyses the final stage of plasma fibrinogen conversion to fibrin by limited proteolysis and removal of fibrinopeptides A and B. These fibrin monomers then

polymerize to form double-stranded protofibrils which become covalently crosslinked by factor XIII to form interwoven fibrin polymers (Weisel et al, 1983).

Vitamin K is required for the synthesis (carboxylation) of prothrombin together with factors VII, IX and X by hepatocytes. These carboxyl groups are essential for the binding of calcium ions and the formation of stable complexes with platelet phospholipid (Esmon et al, 1975).

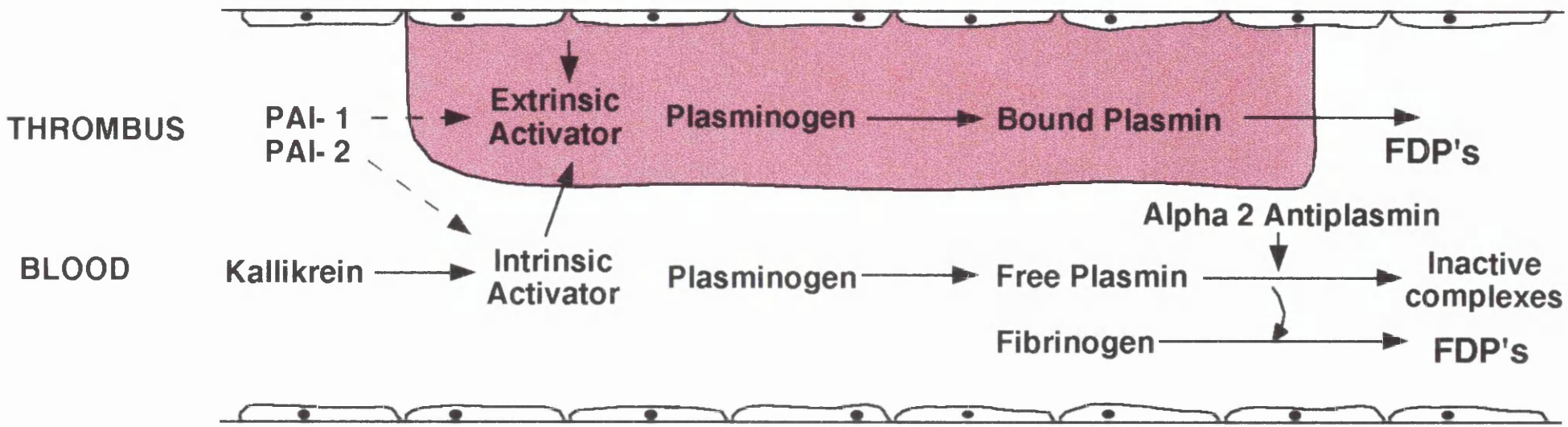
## 2.4 Fibrinolytic pathway

Fibrin clot serves a temporary haemostatic function and simply provide a stop-gap measure until healing can restore an intact endothelium. Fibrinolysis is an integral part of the repair process and ensures complete restoration of blood flow after vessel repair. The fibrinolytic pathway is initiated by enzymes termed plasminogen activators that either are "intrinsic" components of plasma or are released or secreted by "extrinsic" tissue including vascular endothelium or macrophages. These activators then convert the plasma protein plasminogen into the fibrin-splitting protease plasmin (Figure 4).

Surface contact activation of the coagulation pathway is accompanied by the generation of plasmin via the identical enzymatic sequence of proteolysis of factor XII, HMW Kininogen, kallikrein and factor XI (Mandle and Kaplan, 1979).

Figure 4

## Major Reactions of the Intravascular Fibrionolytic Pathway




PAI - Plasminogen Activator Inhibitor
FDP - Fibrin Degradation Product

Note: Broken arrows represent inhibitory influences

(Original In Colour)

Extrinsic tissue plaminogen activator (t-PA) is released from endothelial lysosomes of damaged vessels and together with plasminogen binds to specific receptors on exposed fibrin strands (Tran-Thang et al, 1984). This preferential concentration of t-PA/plasminogen complexes on fibrin facilitates rapid conversion of plasminogen to plasmin and in addition protects plasmin from degradation by alpha 2-antiplasmin. Although some t-PA enters the plasma, systemic activation of fibrinolysis is minimal as a result of the short lifespan of proteolytic activator and the immediate neutralizing effect of alpha 2-antiplasmin.

Fibrin degradation products (FDP) released from the clot by plasmin can impair coagulation by binding to thrombin at its fibrinogen-receptor site or binding to the fibrin clot and weakening its structure. If the macrophage clearing system for FDP is overwhelmed, systemic release can lead to platelet and endothelial dysfunction and increased capillary permeability, in addition to systemic activation of fibrinolysis.

# 3 CANCER AND HAEMOSTASIS

## 3.1) Clinical evidence for altered coagulation in malignant disease.

A high incidence of superficial venous thrombosis in patients with gastric cancer was first noted by Armand Trousseau in 1865 and provided the earliest evidence to suggest an association between the haemostatic system and malignant disease. Over the following century many studies reported numerous thrombotic and haemorrhagic complications in patients with cancer. Pancreatic carcinoma has been associated historically with a high incidence of thomboembolic disease (TED) (Sproule, 1938; Sack et al, 1977) but a wide variety of malignant tumours may disrupt the haemostatic balance causing venous and arterial thrombosis, migratory thromboembolism, pulmonary embolism and non-bacterial thrombotic endocarditis (Goodnight, 1974; Weick, 1978; Sack et al, 1977; Rickles and Edwards, 1983). In particular, mucin-secreting tumours of the ovary, prostate and gastrointestinal tract can cause significant disturbance of haemostasis (Miller et al, 1967; Goodnight, 1974). This has been attributed to the secretion of plasminogen activator (Soong and Miller, 1970) or to the presence of factor X activator in mucus (Pineo et al, 1974). The overall incidence of TED is within the range 1-11% (Nand and Messmore, 1990) but

may increase with therapeutic intervention and chemotherapy (Kasimis and Spiers, 1979; Seifter et al, 1985; Levine et al, 1988). At postmortem, 74% of patients with cancer have evidence of underlying non-bacterial thrombotic endocarditis (NBTE) (Sack, 1977). NBTE is seen most commonly with mucin-producing adenocarcinomas and may lead to limb gangrene and cerebro-vascular occlusion (Rosen and Armstrong,1973).

The susceptibility of the haemostatic system to alteration by malignant disease is such that a hypercoagulable state may indicate occult malignancy (Goldhaber et al 1987, Adamson and Currie, 1993). Pulmonary embolism or deep venous thrombosis may precede the development of malignant neoplasms of the lung, breast, uterus and gastrointestinal tract by up to five years (Gore et al, 1982; Goldberg et al, 1987). Activation of coagulation and fibrinolysis in patients with malignant disease may relate to tumour stage and prognosis (Seitz et al, 1993). The importance of subclinical activation of coagulation as a possible marker for malignancy has led to intensive laboratory investigation of patients with cancer.

### 3.2) Laboratory evidence for altered coagulation in patients with cancer.

Subclinical dysfunction of haemostasis can be identified in up to 92% of patients with cancer (Sun et al, 1979). Quantitative alteration in platelets,

usually thrombocytosis, occurs in up to 60% of patients whereas functional abnormalities are uncommon and may be related to the presence of fibrin degradation products (Rickles and Edwards, 1983).

The most common clotting abnormalities seen are increased levels of fibrin(ogen) degradation products (FDP) and elevated fibrinogen levels (Davis et al, 1969; Sun et al, 1979; Rickles and Edwards, 1983; Van Duijnhoven et al, 1993). Some patients show prolonged prothrombin and activated partial thromboplastin times. Elevated Fibrinopeptide-A (FPA) levels are present in the majority (60-95%) of patients with local or disseminated malignancy (Peuscher et al, 1980; Mombelli et al, 1982). FPA is a short chain peptide cleaved from the alpha chain of fibrinogen by thrombin. Since the plasma half life is less than four minutes, plasma levels reflect ongoing coagulation and therefore the presence of intravascular thrombin. FPA levels appear to reflect the clinical response of patients with cancer and persistent elevation correlates with progressive disease and poor prognosis (Rickles et al, 1983).

Disseminated intravascular coagulation (DIC) is a disorder characterised by the initial deposition of fibrin thrombi in the microcirculation of many organs. Subsequent activation of the fibrinolytic system produces FDP which further stimulate plasmin production by positive feedback. In addition, FDP disrupt platelet function and increase capillary permeability. The

continuous consumption of coagulation factors and platelets eventually progresses to a decompensated, haemorrhagic state. Although subclinical DIC is common in malignancy, only 9-15% of patients develop DIC of clinical significance (Belt et al, 1978; Sun et al, 1979) and this is usually associated with mucin-secreting adenocarcinomas (Belt et al, 1978), gram negative sepsis or liver impairment (Al-Mondhiry, 1975).

### 3.3 Role of fibrin and the fibrinolytic system in the metastatic process

The earliest evidence to suggest a role for the coagulation pathway in the spread of malignant disease came from post mortem studies of Theodore Bilroth in 1878 (Bilroth, 1878). He described the association of human tumour cells with thrombi and suggested that the spread of tumours could be brought about by the circulation of thrombi-containing tumour cells. The studies of O'Meara (1958) demonstrated that primary carcinomas were surrounded by a fibrin mesh and that extracts of these tumours could activate the coagulation pathway (O'Meara and Thornes, 1961). Subsequent studies demonstrated that fibrin deposition occured within hours of tumour implantation and remained throughout the period of tumour growth (Dvorak, 1984). Fibrin is thought to possibly enhance tumour integrity by providing a scaffold for tumour

cell growth, enhancing angiogenesis (Dvorak, 1986) or protecting against immune destruction (Gunji and Gorelik, 1988; Cardinali et al, 1990).

Extravascular fibrin is not a consistent finding and immunohistochemical analysis has demonstrated that many tumours lack a surrounding cocoon of fibrin (Costantini et al, 1991a,b; Wojtukiewicz et al, 1989a). These tumours also vascularise and proliferate indicating that while fibrin may contribute to tumour progression, it is not essential. Furthermore, many tumours including breast, colon and prostate, express urokinase-type plasminogen activator (u-PA) rather than promotors of coagulation (Constantini et al, 1991a,b; Wojtukiewicz et al, 1989b, 1991). Several studies have correlated plasminogen activator (PA) production with invasive and metastatic potential (Ossowski, 1983; Skriver et al, 1984; Mignatti et al, 1986; Reich et al, 1988; Cajot et al, 1989). Since plasmin is capable of digesting many biological barriers either directly or by activation of proenzymes of metalloproteinases (Tryggvason et al, 1987; Murphy et al, 1989), it appears that tumour plasminogen activator (PA) may facilitate tumour growth and invasion by initiating the proteolytic cascade pathway, leading to degradation of extracellular matrix (Reiter et al, 1993).

Ossowski (1991) has demonstrated in a murine model that specific anti-PA antibodies do not prevent metastasis once tumour cells have gained access to the circulation (Ossowski, 1991). In addition, PA

production has recently been shown to correlate with the metastatic potential of melanoma cells after subcutaneous implantation but not after intravenous injection (Quax, 1991). While these findings indicate an early role for PA in the metastatic cascade by enhancement of intravasation, several studies have reported an association between tumour PA production and the metastatic potential of intravenously inoculated tumour cells (Hearing et al, 1988, Axelrod et al, 1989). On balance, the current evidence suggests that tumour PA promotes metastasis by facilitating the penetration of the vascular basement membrane thereby enabling tumour cells to enter the bloodstream (Dano et al, 1985; Liotta, 1986). The role of PA in the later stage of extravasation remains to be clarified.

Evidence supporting fibrin involvement in metastasis has come from microscopy studies which have looked at the fate of intravenously injected tumour cells in numerous animal models of artificial metastasis. Circulating tumour cells arrest in the microcirculation of target organs surrounded by a meshwork of fibrin and platelets (Wood, 1958; Jones et al, 1971; Warren and Vales, 1971; Chew and Wallace, 1976). It is currently believed that fibrin enhances the metastatic process by facilitating the adhesion of tumour cells to vascular endothelium by acting as an intercellular glue (Francis, 1989).

In summary, these studies suggest that fibrin may have opposing effects on the metastatic process. By

forming a protective barrier around the primary tumour, fibrin may prevent penetration of the vascular basement membrane thereby reducing the possibility of haematogenous dissemination. In contrast, fibrin may also promote metastasis by facilitating the entrapment of tumour cells in the microcirculation within a fibrin-platelet mesh (Figure 5). The relative importance of these opposing effects on the metastatic pathway is unknown.

### 3.4 Tumour Procoagulant Activity

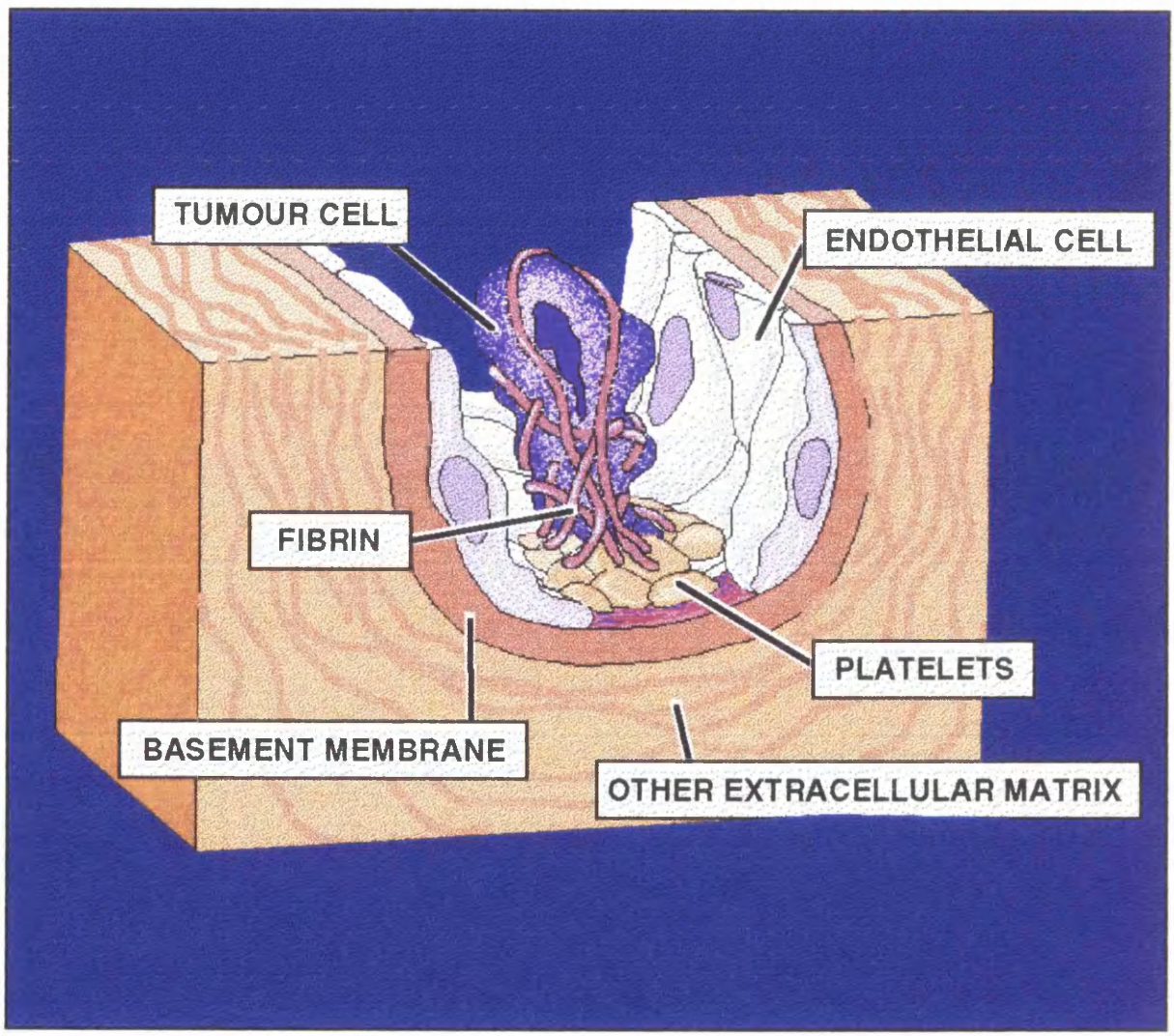
An association between in vitro thrombogenesis and in vivo fibrin formation was first described by Lawrence (1952). Rabbit V2 carcinoma cells demonstrated similar clot-promoting properties to rabbit brain thromboplastin in vitro and were equally effective in the production of thrombosis in vivo after intravenous inoculation. The inhibition of this in vivo effect with heparin and dicoumarol suggests that tumour cell-induced procoagulant activity (PCA) acts via the coagulation pathway. The isolation of tumour PCA in centrifuged cell-free supernatants or cell-free ascitic fluid from various tumour cell lines (Dvorak et al, 1983; Seitz et al, 1993) has now established three subgroups of PCA:

1) Tissue factor (TF) like procoagulants initiate the extrinsic pathway of coagulation by increasing the activity of Factor VIIa which in turn catalyzes the

# Figure 5



## Tumour Cell Arrest - Adhesion to vascular endothelium




(Original In Colour)

conversion of Factor X to Xa (Dvorak et al, 1983). TF was originally identified in buffy coat cells in acute promyelocytic leukaemia (Gralnick and Abrell, 1973) but has since been identified in a variety of malignant tumours including human leukaemias, lymphomas, adenocarcinomas, osteogenic sarcomas and various murine malignancies.

2) Many malignant tumours produce a procoagulant, which unlike tissue factor, does not require factor VII for its activity (Gordon et al, 1975; Curatolo et al, 1979; Hilgard and Whur, 1980). This procoagulant activates factor X directly and a strong positive correlation has been demonstrated between metastatic potential and tumour procoagulant (Gilbert and Gordon, 1983). These single chain cysteine proteases are produced by many human malignancies including breast, lung and colon. While the procoagulant activity of benign melanocytic lesions is dependent on factor VII for activity and can be blocked by concavalin A a tissue factor inhibitor, malignant melanomas exhibit procoagulant activity which is independent of factor VII and unaffected by concavalin A (Gordon et al, 1979; Donati et al, 1986). This suggests that benign tissue produces tissue factor and malignant transformation is associated with tumour procoagulant production.

3) Other tumour-associated procoagulants have been isolated from human and animal tumours, but

contamination with TF-producing macrophages and monocytes have prevented confirmation (Lawrence et al, 1952; Boggust et al, 1963; Svanberg, 1975).

### 3.5 Role of platelets

The first evidence to suggest an association between platelets and metastasis came from the work of Schmidt (1903) who described platelet aggregates surrounding human tumour cells in pulmonary arterioles. Later, electron microscopic studies in animal models demonstrated that after intravenous injection tumour cell emboli arrest in pulmonary capillaries and arterioles surrounded by a meshwork of fibrin and platelets (Jones et al, 1971; Warren and Vales, 1972; Sindelar et al, 1975). Gasic and co-workers (1968) were the first to provide experimental evidence for the role of platelets in the metastatic process by showing a reduction in the metastatic potential of intravenously injected tumour cells in mice rendered thrombocytopenic by neuraminidase (Gasic et al, 1968). A similar antimetastatic effect was shown after thrombocytopenia induced by platelet antiserum which was completely reversed by platelet transfusion (Gasic et al, 1968).

Evidence confirming the importance of tumour cell-induced platelet aggregation (TCIPA) in the metastatic process has come from three separate lines

of investigation. Firstly, experiments have demonstrated a positive correlation between the ability of tumour cells to aggregate platelets in vitro and their metastatic potential in vivo (Gasic et al, 1973; Pearlstein et al, 1980; Tohgo et al, 1986; Mahalingham et al, 1988). Secondly, PGI2 and its analogues such as iloprost and cicaprost possess strong anti-TCIPA properties and have a potent antimetastatic effect in vivo (Honn et al, 1981; Giraldi et al, 1990; Schneider et al, 1990). Finally, recent experiments have shown that synthetic peptides or monoclonal antibodies directed against the common platelet adhesive protein receptor for fibronectin, vWF and fibrinogen, inhibit tumour-platelet adhesion in vitro and metastasis in vivo (Karpatkin et al, 1988; Nierodzik et al, 1991; Sheu et al, 1993). Collectively, these experiments indicate that the ability of tumour cells to activate and aggregate platelets constitute an important aspect of the metastatic process.

The mechanism by which tumour cells trigger the activation of platelets remains to be clarified but current evidence suggests that initial activation involves either the generation of thrombin or ADP by tumour cells (Bastida, 1982, 1988; Honn et al, 1992). Platelet activation subsequently leads to the release of a host of bioactive substances including adhesive glycoproteins (integrins, immunoglobulins and selectins) and arachidonic acid metabolites (TXA2 and 12(S)-HETE). Furthermore, platelet activation leads to

the rapid surface targeting and functional activation of surface adhesion receptors (Honn et al, 1992). These adhesion molecules together with the release products are thought to strengthen platelet-tumour cell bonding and irreversible TCIPA, thereby potentiating tumour metastasis. In support of this theory it has been shown that many tumour cells express the platelet adhesion molecule *a*IIb*B*3 and that antibodies against *a*IIb*B*3 inhibit tumour cell adhesion to platelets in vitro and metastasis in vivo (Karpatkin et al, 1988). Platelet membrane P-selectin may also be involved in tumour cell-platelet interactions as various tumour cells express ligands for P-selectin while antibodies to P-selectin inhibit adhesion of tumour cells to activated platelets (Stone et al, 1993).

Arachidonic acid metabolites are also important mediators of platelet activation. Their role in TCIPA has come from studies which have demonstrated that platelet aggregation induced by some tumour cells correlates positively with production of the cyclo-oxygenase product TXA2 and the degree of malignancy (Pacchiarini et al, 1991). In addition, recent evidence has suggested that lipoxygenase metabolites of arachidonic acid may also have an important role in tumour cell-platelet interactions. Platelets and some tumour cells secrete the lipoxygenase metabolite 12 (S)-HETE which is capable of stimulating both adhesion of tumour cells to endothelium (Liu et al, 1991) and cause reversible endothelial cell retraction (Tang et

al, 1992). It has been suggested that tumour cell-platelet thrombi may produce a microenvironment of high 12 (S)-HETE thereby enhancing tumour cell adhesion to vascular endothelium (Grossi et al, 1989) and accelerating the process of endothelial cell retraction (Honn et al, 1991, 1994).

Several other mechanisms have been proposed to explain the enhancement of metastasis by platelets. Electron microscopy studies demonstrate that platelets are not involved in the initial arrest of tumour cells in the microcirculation (Crissman et al, 1985, 1988). It has been suggested that they may be more important in subsequent stabilization of tumour cell-endothelial adhesion. Platelet-derived factors capable of inducing the expression of receptors involved in tumour cell adhesion to endothelium have been demonstrated (Hawrylowicz et al, 1991; Chopra et al, 1991). In addition, it has been postulated that platelets may physically shield tumour cells within a protective platelet cocoon enabling them to escape immune surveillance (Dvorak et al, 1979; Gasic et al, 1986), encourage tumour survival by providing growth factors (Eastment et al, 1987; Poggi et al, 1988) or enhance tumour cell adhesion to extracellular matrix (Menter et al, 1987).

The experimental observations reviewed above led many workers to conclude that antiplatelet therapy might inhibit the development of metastatic disease. The results in animal models have been variable and

while some studies have reported success with cyclo-oxygenase inhibitors (Gasic et al, 1973; Kolenich et al, 1972; Pollard et al, 1981) and phosphodiesterase inhibitors (Gastpar, 1977; Gordon et al, 1979b) others have failed to show any such effect (Wood and Hilgard, 1972; Hilgard et al, 1976).

In summary, platelets like fibrin, are an integral component of tumour emboli that arrest in the microcirculation. In animals the ability of tumour cells to aggregate platelets in vitro correlates with metastatic potential in vivo. Platelet interaction with tumour cells involves two general types of mediators: membrane-bound molecules (adhesion molecules) and soluble release products (predominately arachidonic acid metabolites). Platelets may contribute to metastasis by stabilizing tumour cell arrest in the vasculature. In animals the antimetastatic effect of antiplatelet agents is inconsistent and the clinical significance is as yet unclear.

## 3.6 Cinical application of anticoagulant and antiplatelet therapy

Pharmacological intervention aimed at modulation of coagulation or platelet activation have been shown to be capable of reducing metastasis in some animal experimental models (Donati et al, 1981; Zacharski et al, 1979, 1982; Yano, 1993). Early clinical trials reporting the application of such treatments in human

malignancy were less convincing and in general were anecdotal pilot studies or at best poorly designed clinical trials (Zacharski, 1981). More recently, prolonged survival has been demonstrated by treatment with the phosphodiesterase inhibitor RA-233, in non-small cell lung cancer (Schneider et al, 1987). Two prospective randomized trials of warfarin and one of heparin have shown significant improvement in tumour response rates and overall survival in patients with small cell carcinoma of the lung (SCCL) (Chahinian et al, 1989; Zacharski et al, 1984; Lebeau et al, 1991). Furthermore, pilot studies with combination chemotherapy and urokinase have shown enhanced survival of patients with disseminated SCCL in comparison with chemotherapy alone (Calvo et al, 1992). While these studies indicate a role for peritumoural fibrin in tumour progression, many tumour types are unresponsive to anticoagulant therapy (Zacharski et al, 1990). By contrast several of these tumours, including non-small cell lung cancer and ovarian cancer, respond to treatment with the protease inhibitor tranexamic acid (Astedt, 1980; Kikuchi et al, 1986; Serdengecti et al, 1988). This suggests the existence of a tumour-associated urokinase-initiated protease pathway in certain human tumour types.

In summary, the heterogeneity of responsiveness to anticoagulant and fibrinolytic agents may be attributable to differences in the mechanisms of interactions of different tumours with the haemostatic

system. The role of antiplatelet agents in the inhibition of metastasis remains to be defined.

### 3.7 Summary and role of future studies

Haemostasis and Cancer

It is evident from clinical and animal studies that malignant disease activates both the coagulation system and platelets. Clinical or subclinical disruption of the normal haemostatic balance in favour of a hypercoagulable state correlates with disease progression. Fibrin is an important component of the malignant process and influences both primary tumour growth and metastasis. The presence of fibrin around primary neoplasms is well documented and while it may enhance primary tumour growth and reduce malignant cell shedding these effects remain to be clarified. The role of fibrin and platelets in the metastatic process is more convincing and considerable evidence from microscopy studies demonstrates that they form aggregates with circulating tumour cells within the microcirculation.

The importance of fibrin and platelets in the intravascular phase of the metastatic pathway has been confirmed by demonstration of the antimetastatic effect of anticoagulant therapy in several animal models. In particular, the coumarin group of anticoagulants has been shown to reduce pulmonary tumour seeding of

intravenously injected tumour cells (Agostino et al, 1966; Ryan et al, 1969). Microscopy studies by Wood (1974) established that the anticoagulant effect was associated with the loss of the fibrin/platelet thrombus surrounding tumour cells adherent to vascular endothelium. Similar antimetastatic effects were demonstrated for heparin (Koike, 1964; Hilgard, 1979) and the defibrinating agent Arvin (Wood and Hilgard, 1973). Thus, it has been postulated that anticoagulants interfere with the mechanism of metastasis by inhibiting clot formation around tumour cells adherent to vascular endothelium (Brown, 1973).

Other workers have suggested that the reduction in metastasis by coumarin anticoagulation might be due to a cytotoxic or cytostatic effect (Ryan et al, 1968b; Ryan, 1969; Hilgard et al, 1977). While some studies have demonstrated such an effect (Lisnell and Melgrin, 1963; Kirsch et al, 1974), the majority have shown little or no reduction in cell viability or rate of division (Brown, 1973; Dolfini et al, 1980; McNeil and Morgan, 1984). Recently, in a rat model of metastasising breast carcinoma, McCulloch and George (1987) confirmed that warfarin inhibits the development of pulmonary metastasis. This effect was not associated with an alteration in the metabolic activity of tumour cells. Furthermore, the administration of the warfarin-dependent factor complex II, IX and X enhanced pulmonary tumour seeding in a similar model (McCulloch and George, 1988). Further studies using radiolabelled

tumour cells showed that this effect of pulmonary tumour enhancement was associated with increased entrapment of tumour cells in the pulmonary microcirculation (Purushotham et al, 1991). Elevation of fibrinopeptide A levels after inoculation of factor complex and tumour cells suggested that this effect was due to activation of coagulation (Purushotham et al, unpublished data). These findings have led to the hypothesis that tumour enhancement by coagulation factor complex is the result of increased entrapment of tumour cells in the microcirculation within a fibrin clot. It was therefore postulated that fibrinolytic therapy might prevent tumour cell entrapment in the microcirculation by dissolution of this fibrin clot, thereby inhibiting metastasis.

Preliminary studies have demonstrated a significant reduction in the metastatic potential of circulating tumour cells after the intravenous administration of the fibrinolytic agent Streptokinase (Purushotham et al, 1994). The mechanism by which Streptokinase produces tumour inhibition requires further investigation.

The aim of this study therefore is to investigate the mechanism of Streptokinase-mediated tumour inhibition in a similar animal model. The following questions require to be answered:

1 Does intravenous Streptokinase inhibit pulmonary tumour seeding by fibrin clot lysis?

The observed effect of tumour inhibition by Streptokinase in this animal model may be directly related to its fibrinolytic properties. It is proposed to investigate whether administration of intravenous Streptokinase is associated with fibrin clot lysis.

2 Do other clot-lysing agents inhibit pulmonary tumour seeding?

If the inhibition of pulmonary tumour seeding by Streptokinase is associated with fibrinolysis, then it is likely that other clot lysing agents may also be antimetastatic. Confirmation that a structurally unrelated fibrinolytic agent has a similar effect on pulmonary tumour seeding will further confirm the mechanism by which fibrinolytic agents inhibit metastases.

3 Does antiplatelet therapy enhance inhibition of pulmonary tumour seeding by Streptokinase?

Platelets also play a role in the intravascular phase of metastasis. It is possible that a dual assault on the fibrin and platelet components of tumour microthrombi in the microcirculation might prove to be effective antimetastatic treatment. It is proposed that a comparison of pulmonary tumour inhibition of antiplatelet and fibrinolytic agents be made.

### 3.8 Clinical application of fibrinolytic therapy

The development of an antimetastatic treatment in an animal model is only relevant if there is evidence for clinical application in patients with cancer. Previous work by White et al (1976) using urokinase to facilitate fibrinolysis in human cancer suggests that prevention of implantation of circulating malignant cells onto vascular endothelium is likely to improve patient survival. Fibrinolytic therapy might therefore be beneficial if administered to patients at risk of haematogenous dissemination from primary tumours.

The inefficiency of the metastatic process suggests that spontaneous haematogenous shedding from primary tumours rarely leads to metastasis (Weiss, 1986). However, the number of metastasis is directly proportional to the number of circulating tumour cells (Fidler, 1973) and therefore any manipulative procedure which facilitates primary tumour cell shedding into the blood may promote metastasis. Fibrinolytic therapy is most likely to be effective in preventing metastases if administered during tumour cell dissemination. The second half of this thesis is designed to investigate whether operative manipulation of malignant tumours promotes tumour cell dissemination via the bloodstream and whether fibrinolytic therapy has a peri-operative role in reducing metastatic progression in patients with cancer.

**SURGERY AND METASTASIS**

## 4.1 Evidence for tumour dissemination by surgical trauma

Tyzzer (1913) demonstrated that manipulation of transplanted breast tumours increased the number of pulmonary metastases in mice. He postulated that this effect was the result of cell shedding from the primary tumour. Ide (1939) provided microscopic evidence to show that tumour manipulation could traumatise friable blood vessels and enable individual tumour cells or tumour fragments to enter the circulation. Cytological studies of the tumour venous effluent in various animal models also demonstrated at least a tenfold increase in cell shedding during tumour manipulation (Cliffton and Agostino, 1960; Romsdahl, 1965; Gazet, 1966; Liotta, 1974, 1976). These findings suggest that manipulation of malignant neoplasms during surgical resection may also promote tumour cell dissemination, but evidence in support of this hypothesis in patients with malignancy is less clear.

In 1934 Pool and Dunlop demonstrated a "hitherto undescribed cell" in the peripheral blood of 17 of 40 patients with advanced malignancy but they were unwilling to label these cells as malignant. Later, Engell (1955) demonstrated what he deemed to be tumour cells in the venous blood draining carcinomas

in 59% of patients during surgery, in 13% of peripheral blood samples during surgery and in 50% of peripheral blood samples in patients with inoperable cancer. Five to ten year survival rates were not significantly worse in patients noted to have tumour cells in the bloodstream at the time of surgery. Following these pioneering studies many attempts were made to estimate circulating tumour cells but the results varied widely due to problems associated with tumour cell isolation and identification (Goldblatt and Nadel, 1965). In the case of advanced, inoperable breast cancer, for instance, the incidence of circulating malignant cells varied from 23 to 66% (Moore et al, 1957; Long et al, 1960; Landell et al, 1963). The morphological characteristics of malignancy known from cytological studies, including increased nuclear cytoplasmic ratio, irregular large nucleoli, nuclear hyperchomatism and irregularity of the nuclear membrane were open to misinterpretation. Many cell types present in the blood of patients without malignant disease, including megakaryocytes, large myeloid cells and mononuclear cells were often confused with tumour cells (Landell et al, 1963; Goldblatt and Nadel, 1965) leading to an overestimation of tumour cell numbers. In addition, many of these studies utilized peripheral venous samples which may have underestimated the number of tumour cells shed since the vast majority would have been removed from the circulation on first passage through the microcirculation of a target organ.

Despite the unreliability of the techniques adopted, a few studies attempted to correlate the presence of tumour cells in the bloodstream peri-operatively with long term survival. The results were inconsistent and while some studies demonstrated reduced survival rates for patients with tumour cells circulating at the time of surgery (Roberts et al, 1956; Watne et al, 1961), others failed to show any such effect (Engell, 1955; Glaves et al, 1988).

The discovery of monoclonal antibodies (Kohler and Milstein, 1975) and the subsequent development of immunocytology led to a significant improvement in the sensitivity and specificity with which circulating tumour cells could be identified in the bloodstream. Using a fluorochrome-labelled anticytokeratin antibody and indirect immunofluorescence, Glaves (1988) reported the presence of epithelial cells in the renal vein of patients undergoing nephrectomy for renal adenocarcinoma. However, no reduction in disease-free survival was demonstrable in patients noted to have epithelial cells shed during surgery, although the malignant nature of these cells was not confirmed by comparison with patients undergoing nephrectomy for benign disease. In addition, patient follow-up was limited to less than three years. More recently, combined immunocytochemistry and conventional cytology has been used to establish the malignant nature of epithelial cells circulating in the bloodstream of patients with colorectal cancer in the peri-operative

period (Leather et al, 1993). Tumour cells were identified in only 4 of 42 patients and no significant association was demonstrated with operative manipulation. However, a preliminary assessment of the sensitivity of the tumour cell separation/staining technique was made using a homogeneous tumour cell line. Since primary tumour cells are phenotypically more heterogeneous, it is highly likely that their behaviour during both density gradient centrifugation and immunocytochemical staining would be more variable. Therefore the low incidence of operative tumour cell shedding found in this study may reflect the insensitivity of the technique to detect circulating primary tumour cells.

Evidence that intra-operative tumour cell shedding may enhance the metastatic process has been provided by the studies of Turnbull (1967). The presence of atypical cells in the portal venous blood of resected cancer- bearing segments of colon (Cole et al, 1954; Fisher and Turnbull, 1955) led Turnbull to advocate a technique for the resection of colorectal tumours wherein the cancer bearing segment was not manipulated until the lymphovascular pedicle and colon had been divided. This "no-touch" isolation technique was based on the principle of preventing tumour cell shedding into the portal circulation at the time of tumour manipulation during surgery. The long term survival for patients thus treated was significantly better than those treated by conventional resection

(Turnbull, 1967). Critics attributed these results to patient selection and the introduction of a new staging system and similar survival rates were claimed for extended resection only (Stearns et al, 1971; Enker et al, 1979). More recently, a study comparing the "no-touch" isolation technique with conventional resection has demonstrated a tendency towards a reduction in hepatic metastases at five years in the "no-touch" isolation group although this did not reach statistical significance (Wiggers et al, 1988). Thus the hypothesis that intra-operative tumour manipulation encourages tumour cell dissemination, thereby facilitating metastasis remains unproven and requires further investigation.

**4.2 The role of surgical stress**

There is some experimental evidence from studies in animal models that enhanced tumour growth can occur following surgery unrelated to the primary tumour (Buinauskas et al, 1958; Lewis et al, 1958), and also after the administration of specific anaesthetic agents (Griffiths et al, 1961; Duncan et al, 1977; Pollard et al, 1977). It is believed that the stress of anaesthesia and surgery can disrupt the host-tumour relationship in favour of tumour growth and considerable evidence suggests that this is due to suppression of host immune defence mechanisms (Slade et al, 1975; Roth and Golub, 1976; Tarpley et al, 1977).

Circulating numbers of all T lymphocyte subpopulations fall during surgery (Lennard et al, 1985) and natural killer (NK) cell activity may be reduced by greater than 50 per cent (Uchida et al, 1982; McCulloch and MacIntyre, 1993). Furthermore, the magnitude and duration of the reduction in cell numbers correlates with the degree of surgical trauma (Jubert et al, 1973; Lennard et al, 1985).

Surgical trauma may also contribute to enhanced tumour cell survival by facilitating the entry of large tumour cell clumps into the bloodstream (Ide, 1939; Liotta, 1976). Such multicellular emboli have been shown to possess greater metastatic potential (Fidler, 1973; Liotta, 1976; Glaves, 1983). Finally, the association between the haemostatic system and metastasis suggests that alteration in the haemostatic balance in favour of a hypercoagulable state, which occurs during surgery (Kambayashi et al, 1990), may enhance the entrapment of circulating tumour cells in the microcirculation of target organs.

In summary, the results of these studies suggest that surgery may have several roles to play in the metastatic process:

1. To increase tumour cell shedding during operative manipulation.
2. To enhance the survival of circulating tumour cells by inducing a state of relative immune suppression.
3. To shield tumour cells within multicellular emboli thereby preventing detection by the immune system.

4. To facilitate the seeding of tumour cells in target organs by inducing a more fertile, hypercoagulable blood environment.

The relative importance of each of these various processes in the human situation is as yet unclear.

### 4.3 Summary and statement of hypothesis

The studies of Turnbull (1967) using the "no-touch" isolation technique have provided the only evidence that manipulation of malignant tumours during surgical resection may facilitate metastasis. It remains to be established whether intra-operative, haematogenous tumour cell dissemination represents a significant prognostic factor in patients undergoing "curative" resection of a primary malignant tumour.

Ninety five per cent of patients who present with breast cancer have local disease only, on staging by conventional methods (Coombes et al, 1980). However, up to a quarter of these patients develop distant metastases within 10 years (Valagussa et al, 1978) and despite considerable improvements in surgical treatment and adjuvant therapy, survival rates have remained relatively unchanged. The advent of immunocytochemistry has greatly enhanced the ability to detect small foci of tumour cells and recent studies have reported bone marrow micrometastases in 16-35% of breast cancer patients undetected by conventional haematological techniques (Cote et al, 1988; Ellis et al, 1989;

Salvadori et al, 1990). It would seem therefore that a proportion of patients with apparent local disease who develop distant metastases may have occult micrometastases undetected at initial presentation. However, it may also be hypothesized that some patients develop metastatic disease as a result of malignant cell shedding during surgical resection of the primary lesion. Confirmation of tumour cells in the circulation peri-operatively would indicate a role for peri-operative adjuvant therapy.

The results of adjuvant therapy in patients with early breast cancer demonstrate that systemic chemotherapy with multiple agents increases overall survival and disease-free survival. In addition significant benefit has been shown for peri-operative chemotherapy and has the potential added advantage of a dual assault on circulating tumour cells and established micrometastases (Early Breast Cancer Trialists Collaborative Group, 1992). However, adjuvant therapy is not without risk and in particular chemotherapy is associated with a significant incidence of systemic toxicity (Early Breast Cancer Trialists Collaborative Group, 1992) while fibrinolytic therapy may theoretically increase the risk of peri-operative haemorrhage. It would therefore seem difficult to justify such therapy in all patients undergoing "curative" resection in whom the majority would not develop metastatic disease. Confirmation of peri-operative tumour cell shedding would identify patients

who might benefit from such therapy. Conversely, if it were shown that surgery did not enhance tumour cell dissemination via the bloodstream then the relevance of developing an antimetastatic regime would be called into question. At present there are no reliable estimates of tumour cell dissemination during surgery and the role of operative manipulation in the metastatic process remains to be defined.

The aim of the second half of this thesis is therefore to investigate the role of operative manipulation in the metastatic process and to answer the question:

Does surgical manipulation of primary malignant tumours enhance tumour cell dissemination via the bloodstream?

It is proposed that blood samples be collected from patients with operable breast cancer in the peri-operative period and that they be analysed for the presence of tumour cells using reliable immunocytochemical and molecular biological techniques. Such a study will potentially identify circulating tumour cells and enable correlation of their presence or absence with clinico-pathological variables such as type of operation, stage of disease and grade of malignancy.

The work contained in this thesis is therefore divided into two closely related investigations: experimental studies using a rat model of metastasising breast carcinoma to investigate the effect of

fibrinolytic and antiplatelet therapy on metastasis and a clinical study of patients with breast cancer to address the problem of identifying tumour cells shed into the circulation during surgical manipulation of the primary tumour.

# CHAPTER 1

# VALIDATION OF PREVIOUS WORK AND THE ANIMAL MODEL OF METASTATIC CARCINOMA

## INTRODUCTION

Animal experimental evidence has been outlined suggesting a beneficial effect of fibrinolytic therapy on the metastatic process. Purushotham et al (1994) have shown that the fibrinolytic agent Streptokinase inhibits pulmonary tumour seeding in an animal model of metastasis. To investigate the mechanism by which fibrinolytic therapy inhibits tumour enhancement, it was essential to use a similar animal model in order to draw valid comparisons with the results of previous workers. It was therefore necessary to initially validate the animal model and to subsequently confirm the inhibitory effect of intravenous Streptokinase on pulmonary tumour seeding. Validation of the tumour model was required because phenotypic drift in the tumour cell line can occur leading to a variation in lung colonizing potential (Neri and Nicolson, 1981). In addition, variation in the mechanism of lung colonization may produce discrepancies between surface tumour deposits and total lung tumour burden.

The tumour model utilized for these experiments was the female Fischer 344 rat in combination with the Mtln3 clone of the 13762NF rat mammary carcinoma cell line. This animal model was originally chosen as it

fulfills the following criteria required for comparison with human cancer:

1. The tumour should metastasise reliably and spontaneously from the primary tumour.
2. It should resemble human carcinoma as closely as possible in terms of its histological origin, morphology and behaviour.
3. It should be possible to grow the tumour cells in vitro.
4. The tumour should be syngeneic with the host animal and should not elicit a significant immune response in the host.
5. It should be possible to effectively evaluate changes in the haemostatic system in the host.
6. Administration of intravenous tumour cells should give measurable and reproducible results.

The Mtln3 cell line resembles human breast cancer both morphologically and in its propensity to metastasise to lung and regional lymph nodes (Neri et al, 1982). In addition, the tumour is syngeneic with the host animal and thus does not elicit a significant humoral or cellular immune response either after intravenous injection or prolonged growth in the host (North and Nicolson, 1985). Finally, the cloned cell line can easily be maintained in tissue culture so that in vitro assessment of cytotoxicity can be performed.

**Metastatic tumour model.**

The animals used in this study were female, Fischer 344 rats, mean weight 140 grams, between 6-8 weeks old (Olac Limited, Bicester, UK). Animals were housed three to a cage and fed a standard laboratory diet (CRM diet, Labsure, Cambridge, UK) and tap water with a chlorine content of 7 mg/ml. All animals were healthy to visual inspection and to the results of routine microbiological testing.

The tumour cell line utilized in these experiments was a clone of rat mammary carcinoma designated Mtln3 (Figure 6), and was obtained from frozen cultures at the Department of Oncology, University of Glasgow. The original clone was derived by Neri et al (1982) from the 7, 12-dimethylbenz (a) anthracene - induced adenocarcinoma 13762 (Segaloff, 1966). The cells were cultured in 75 $cm^2$ tissue culture flasks (Gibco, Paisley, UK) in equal parts of Hams' F10 medium and Dulbecco's modified Eagles' medium (F10/DMEM), with 10% foetal calf serum (FCS) but without antibiotics. Cultures were maintained at $37^0C$ in equilibrium with 2% $CO_2$ in air. Once cultures were confluent passage was carried out by washing with 5 ml of trypsin/EDTA (0.25%/1mM) and incubated for 5 minutes at $37^0C$. Subcultures required the addition of $2 \times 10^6$ viable tumour cells to further 75 $cm^2$ flasks. Cells were passaged a maximum of 6 times between thawing and use to minimize the problems of phenotypic drift and

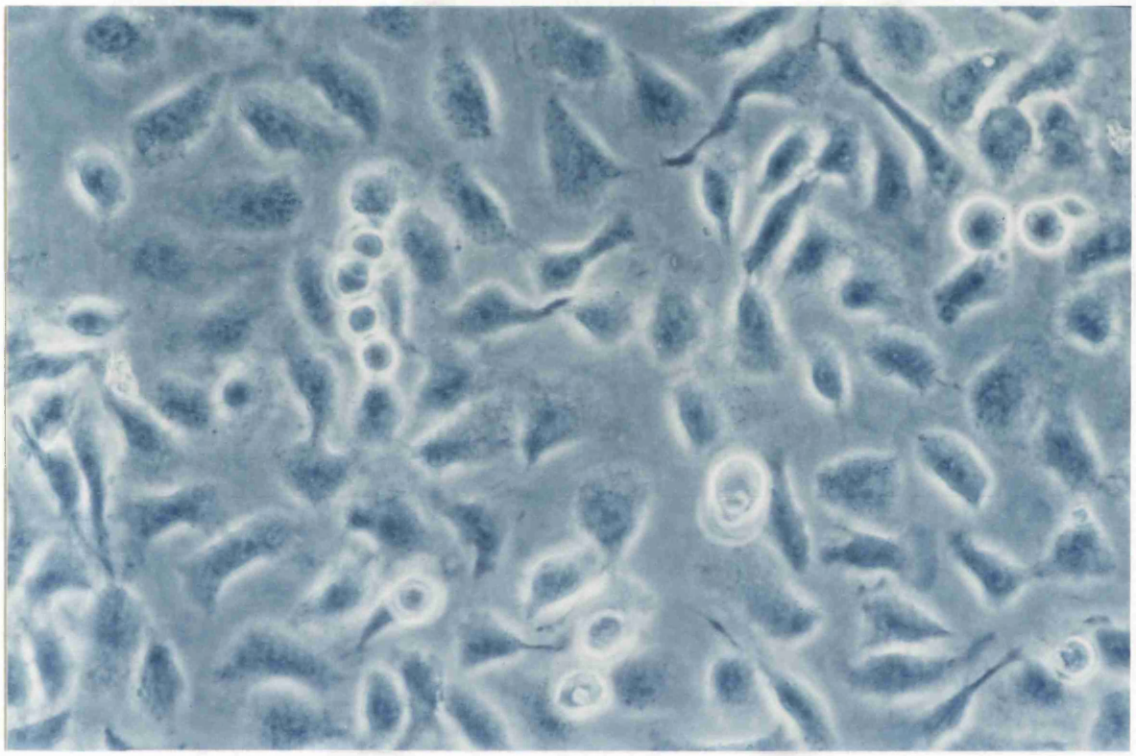


**Figure 6** Morphogy of Mtln3 rat mammary carcinoma cells in culture (x 625 magnification)

reduction in metastatic potential (Neri and Nicolson, 1981). Mycoplasma contamination of cultures was excluded by monthly testing with Hoechst stain 33258.

For the purpose of these studies, tumour cells were prepared for inoculation by trypsinisation, washed twice in F10/DMEM by centrifugation at 200g for 5 minutes, and finally resuspensed in F10/DMEM. Cells were then counted on a Neubauer haemocytometer and resuspended in F10/DMEM at a concentration of $10^5$ cells per 0.5ml of medium. Animals were inoculated intravenously by tail vein injection under general anaesthesia with intraperitoneal pentobarbitone (60mg/kg).

**Validation of Method for Identifying Pulmonary Metastases**

An estimation of pulmonary seeding by serial histological section is time consuming and inappropriate for studies which necessarily involve large numbers of experimental animals. Wexler (1966) described a relatively simple and reliable method for the accurate enumeration of pulmonary tumour nodule formation in other models of metastasis. This well validated technique involves insufflation of the lungs via the trachea with 15% India ink in distilled water. The lungs are then excised and immersed for 48 hours in Fekete's bleaching solution (alcohol, acetic acid and formaldehyde). Pulmonary metastases appear as white

nodules on a black background (Figure 7). The weight of the lungs was used to assess whether surface tumour deposits detected by this method accurately reflected total lung tumour burden.

A pilot study was performed to validate:

a. the dose of Mtln3 cells used in previous experiments ($10^5$)
b. the optimum time of sacrifice after tumour cell injection (17 days)
c. Wexler's method

(McCulloch & George, 1987)

**EXPERIMENT**

Eight female Fischer 344 rats, 6-8 weeks old, were inoculated by tail vein injection with $10^5$ Mtln3 tumour cells in 0.5ml of F10/DMEM, under general anaesthesia. Animals were sacrificed at 17 days after injection and lungs excised and weighed. Surface pulmonary tumour nodules were assessed by the method of Wexler. Statistical correlation between the number of pulmonary tumour nodules and the weight of the lungs was made by Spearman's rank correlation test.

**RESULTS**

Table 1 shows the weight of lungs, expressed as percentage of body weight, and the number of pulmonary tumour nodules detected by the method of Wexler.

**Figure 7** Rat lungs showing subpleural tumour deposits prepared by the method of Wexler

Table 1

## Validation of Wexler's Method

| Number of Pulmonary Metastases | Lung weight (expressed as % bodyweight) |
|---|---|
| 98 | 0.74 |
| 75 | 0.58 |
| 84 | 0.60 |
| 87 | 0.66 |
| 90 | 0.68 |
| 95 | 0.70 |
| 79 | 0.59 |
| 81 | 0.61 |

Spearman's Rank Correlation Coefficient 0.97

Statistical analysis demonstrated that there was a significant correlation between the weight of the lungs and the number of pulmonary tumour deposits as detected by Wexler's method (Spearman's rank correlation coefficient 0.97).

**DISCUSSION**

This preliminary study confirmed that this tumour model was suitable for the investigation of aspects of the metastatic process in an animal model. The intravenous administration of $10^5$ Mtln3 tumour cells was associated with the formation of well circumscribed, easily identifiable, subpleural tumour deposits. In addition, the method for pulmonary tumour nodule formation was successfully validated. Previous work in 8 other animal systems (Wexler, 1966) has shown that excellent correlation exists between the number of surface tumour nodules detected by this method and the total lung tumour burden estimated by more rigorous but laborious methods. A discrepancy between the true and estimated numbers of pulmonary tumour nodules may arise in three ways: tumours may grow in a fashion which prevents accurate identification and counting; there may be a variable relationship between the total lung tumour burden and the surface tumour nodules; artefacts may arise which indicate the presence of a tumour deposit where none exists. These possibilities could be discounted in the experiment described above by observation of the pattern of tumour growth seen. The

tumour formed well-circumscribed deposits after intravenous injection while the proportion of deposits at the pleural surface was consistent, as judged by the weighing of lungs for total tumour burden. Artefact formation did not appear to be a source of serious error as the method of Wexler successfully differentiated tumour-bearing from tumour- free lungs. Thus the correlation between total pulmonary tumour burden as estimated by weighing of lungs, and surface tumour deposits indicates that the method of Wexler is adequate for estimation of major changes in the lung burden of Mtln3 tumour.

**Validation of Inhibitory Effect of Streptokinase on Pulmonary Tumour Seeding.**

The fibrinolytic agent Streptokinase (Hoechst UK Ltd, Hounslow, England) was used in this experiment. Fibrin clot lysis by Streptokinase involves the activation of the intrinsic fibrinolytic system by the formation of a linkage compound between Streptokinase and the proactivator-plasminogen molecule. This activating complex then facilitates the conversion of plasminogen into the fibrinolytic enzyme plasmin.

Previous experiments have shown that an intravenous injection of Streptokinase in a dose of 30 000 Units/Kg, 30 minutes after tumour cell inoculation, inhibits pulmonary tumour seeding in an animal model. (Purushotham et al, 1994)

A pilot study was performed to validate:

a. the dose of Streptokinase previously used (30 000 Units/kg)
b. the optimum time of administration after tumour cell inoculation (30 minutes)

(Purushotham, 1994)

**EXPERIMENT**

Two groups of ten female Fischer 344 rats 6-8 weeks old were used in this experiment. Under general anaesthesia, animals were injected intravenously with a 0.5ml suspension of $10^5$ Mtln3 cells in F10/DMEM. Additional treatment was administered as follows:

Group A: These control animals received no additional treatment
Group B: These animals received an intravenous injection
of Streptokinase 30 minutes after tumour cell injection (t=30).
The dose of Streptokinase administered was 30 000 units/kg body weight. This was based on previous animal experimental work wherein clot lysis was achieved in vivo (Lyden et al, 1990).
Animals were sacrificed after 17 days and pulmonary tumour nodules assessed by the method of Wexler. Comparison of the two groups was made by the Mann-Whitney test.

**RESULTS**

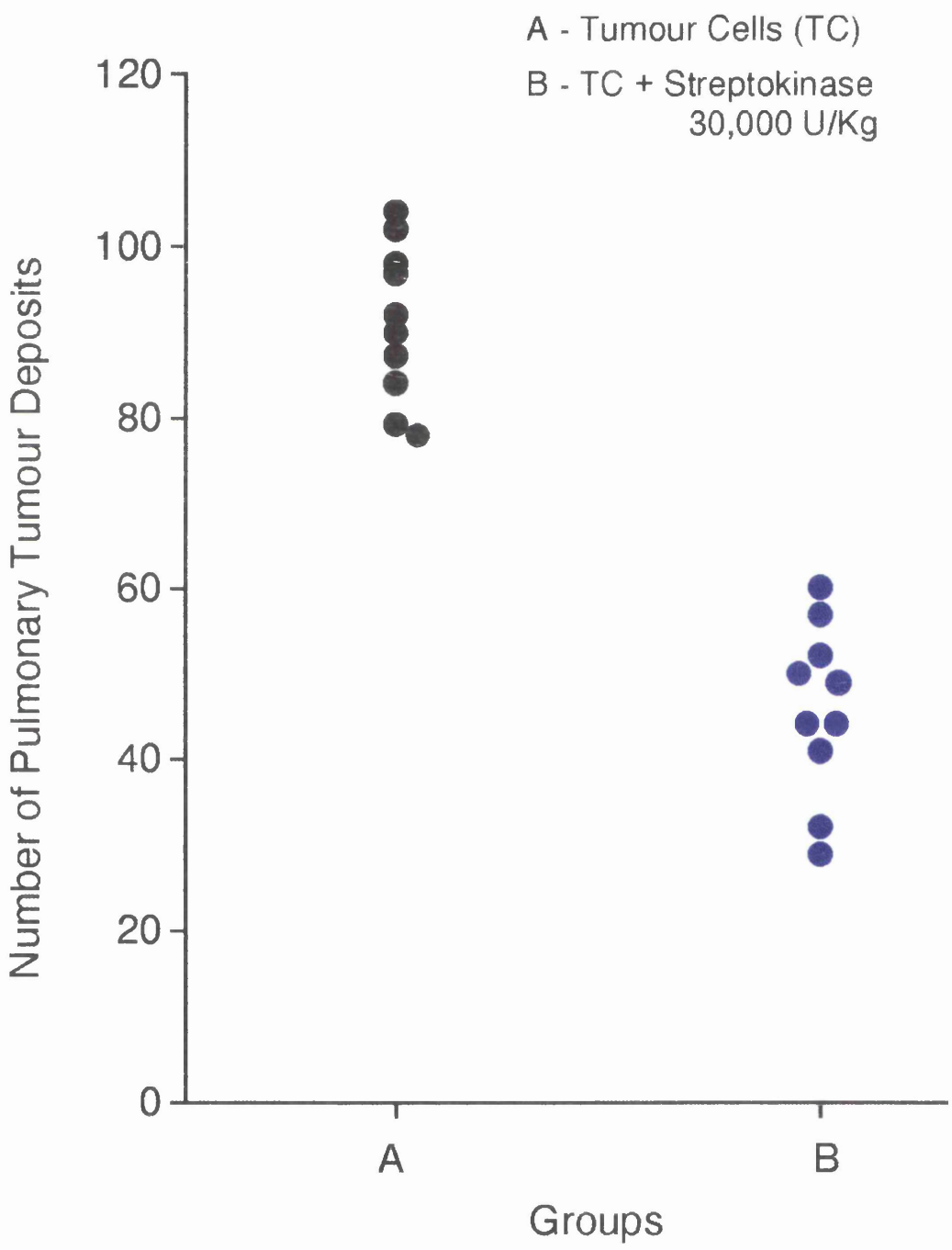
Animals that received intravenous Streptokinase and tumour cells showed a significant reduction in pulmonary tumour seeding (Group B, median = 46.5) when compared with control animals that received tumour cells alone (Group A, median = 91; $p < 0.001$) (Figure 8).

**DISCUSSION**

The results of this experiment demonstrate that Streptokinase produces a significant reduction in pulmonary tumour seeding when administered as a bolus "clot-lysing" dose of 30 000 Units/kg, 30 minutes after tumour cell injection. This confirms the findings of Purushotham et al (1994). The time of administration is critical and is determined by the pharmacodynamics of Streptokinase and the process of intravascular tumour cell arrest. Streptokinase has a very short circulating half-life (Grierson and Bjorson, 1987) and therefore to be effective must be administered at the time of intravascular fibrin formation. Previous studies using radiolabelled tumour cells have demonstrated that there is a sharp rise in the number of cells trapped within the pulmonary microcirculation between 30 and 60 minutes after tumour cell injection - the time of fibrin clot formation (Purushotham et al, 1991). If Streptokinase is administered prior to fibrin clot formation, its rapid disappearance from the circulation

Figure. 8

## Effect of Streptokinase on Pulmonary Tumour Seeding




(Original In Colour)

will result in an absence of any effect on subsequent fibrin clot formation.

The mechanism of action for Streptokinase in this experiment has not been established and while drug-related cytotoxicity for the Mtln3 cell line has previously been excluded (Purushotham et al, 1994), there is no conclusive evidence to demonstrate that its antitumour effect is due to fibrinolysis. It is possible that Streptokinase inhibits pulmonary tumour inhibition via a completely separate mechanism and therefore it is important to establish whether this effect is secondary to fibrin clot lysis. The demonstration of fibrinolysis would indicate the need to perform further work to investigate the role of other fibrinolytic agents with regard to inhibiting tumour metastasis.

# CHAPTER 2

# STUDIES TO DETERMINE WHETHER THE INHIBITION OF PULMONARY TUMOUR SEEDING BY STREPTOKINASE IS ASSOCIATED WITH FIBRIN CLOT LYSIS

## INTRODUCTION

Fibrin is an important component of both primary malignant tumours and metastases. It can be detected at an early stage of tumour growth and is also present within the tumour cell stroma throughout tumour growth (Dvorak et al, 1984). Fibrin also enhances the metastatic potential of circulating tumour cells by forming a latticework around tumour cells trapped within the microcirculation of target organs (Crissman, 1985). This is thought to facilitate the adhesion of tumour cells to vascular endothelium by acting as an "intercellular" glue (Francis, 1989).

The preceding chapter has demonstrated that Streptokinase can reduce the efficiency of the metastatic process in an animal model. The importance of fibrin in the metastatic process would suggest that this antimetastatic effect may be due to fibrin clot lysis but conclusive evidence of an association between fibrin and tumour cells within the microvasculature has not been demonstrated in this model. It is therefore possible that the reduction in pulmonary tumour seeding by Streptokinase in this model may be secondary to a completely different mechanism. Confirmation of fibrinolysis would provide further evidence supporting

an involvement of the coagulation system in the metastatic process.

Fibrin and fibrinogen are progressively split by the enzyme plasmin into a heterogeneous mixture of small peptides known collectively as fibrin(ogen) degradation products (FDP). Plasmin attacks at least 50 cleavage sites in fibrinogen and eventually leads to the release of fragments Y, D and E which can be detected immunologically using a latex agglutination assay. For the purpose of our experiment, fibrin clot breakdown was assessed by estimation of plasma FDP by this technique.

## MATERIALS AND METHODS

### Measurement of Plasma FDP

*Method of blood collection*

In preliminary pilot studies, blood samples were obtained by direct puncture of the abdominal aorta but this technique was found to be unsatisfactory as the trauma of laparotomy was sufficient to activate coagulation and elevate plasma FDP levels. Subsequent studies demonstrated that the less traumatic procedure of direct cardiac puncture was associated with a much lower level of inappropriate activation of coagulation. In addition, in order to minimise the risk of sample clotting, blood was collected into citrate-treated syringes and rapidly transferred to plastic bottles

containing 6% sodium citrate in a ratio of 9:1 v/v. Plasma was prepared by centrifugation of blood samples at 3000 g for 15 minutes at a temperature of $4^0$ C. The fresh rat plasma thus obtained was snap-frozen and stored at $-70^0$C prior to analysis for FDP.

*Time of sampling*

The time interval was determined by a pilot study whereby animals (3 per group) were exsanguinated at 10, 30, 40 and 60 minutes after Streptokinase therapy. Analysis of FDP levels established that maximum fibrin breakdown occured at 10 minutes (Table 2).

*Method of analysis*

Plasma FDP levels were determined using a latex agglutination kit (Diagnostica Stago, Asnieres-Sur-Seine, France). In the presence of the corresponding antigens, the latex particles coated with anti-FDP antibody form macroscopic clumps. A 20 ul plasma sample was mixed with 20 ul of a solution of latex particles coated with mouse monoclonal anti-human FDP antibodies. The presence of agglutination was assessed by comparison with a positive control (20 ul plasma + 20 ul FDP solution) and a negative control (20 ul plasma + 20 ul FDP-free solution). Serial dilution of plasma with glycine buffer enabled semi-quantitative analysis of FDP levels.

Table 2

## Effect of Intravenous Streptokinase on FDP Levels

| Time after Streptokinase therapy (Minutes) | FDP level (μg/ml) |
|---|---|
| 10 | > 120 |
| 30 | 5 - 20 |
| 40 | < 5 |
| 60 | < 5 |

**EXPERIMENT**

Three groups of 10 female Fischer F344 rats, 6-8 weeks old were used for this experiment. All animals received a tail vein injection of $10^5$ Mtln3 tumour cells in 0.5ml of F10/DMEM, under general anaesthesia. Additional treatment was administered as follows:

Group A: These control animals received no additional treatment.

Group B: These animals received an injection of 0.5 ml normal saline 30 minutes after tumour cell inoculation.

Group C: These animals received an injection of Streptokinase (30 000 U/kg) in 0.5ml normal saline 30 minutes after tumour cell inoculation.

*Time of blood sampling*

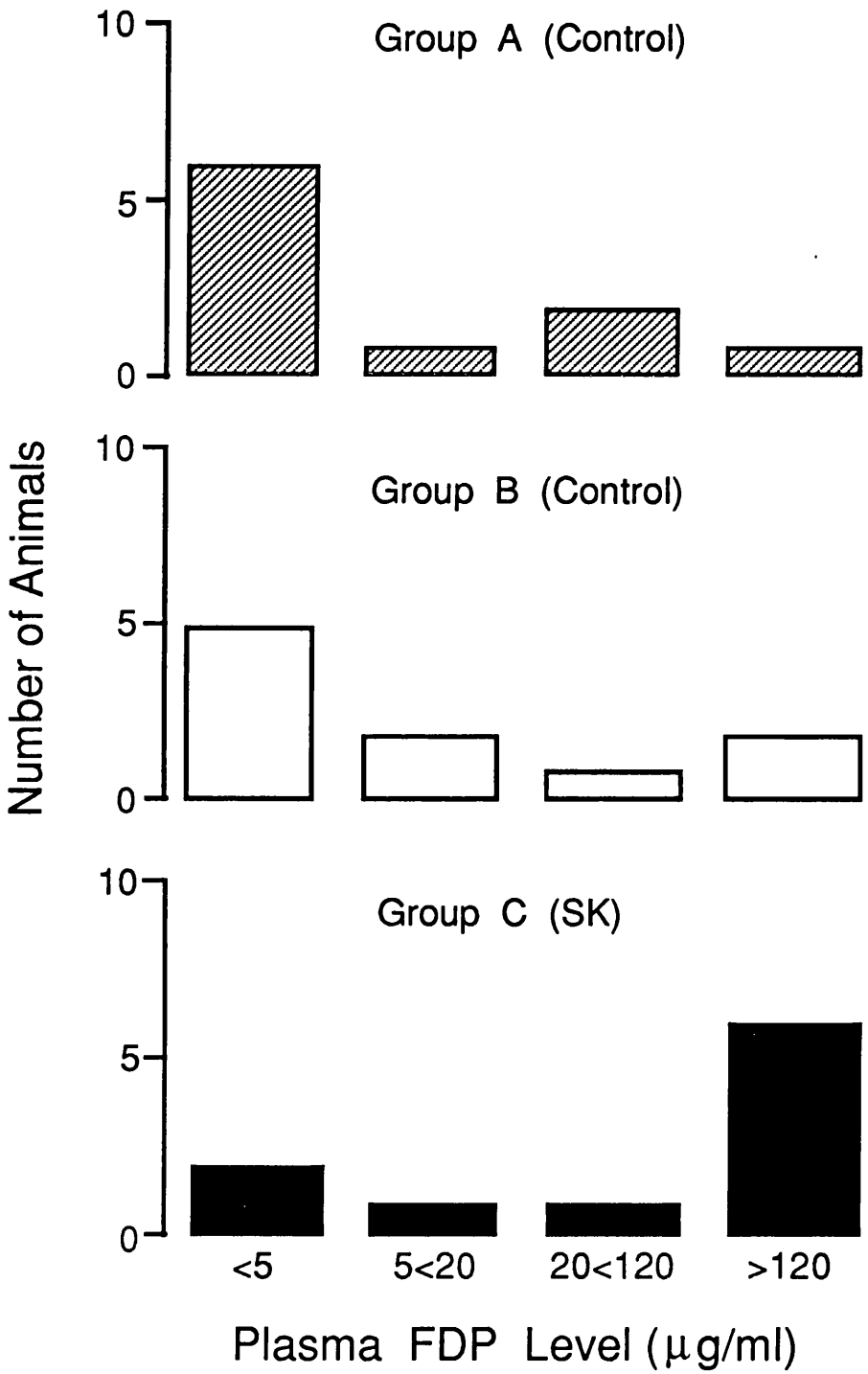
Blood samples were taken 10 minutes after the administration of normal saline or Streptokinase. Samples were then prepared and analysed for FDP estimation as previously described.

**RESULTS**

Semi-quantitative analysis of plasma FDP demonstrated that 6 of 10 animals that received Streptokinase (60%) had FDP levels > 120 ug/ml in comparison with 3 of 10 animals in both control groups (30%) (Figure 9). Statistical analysis by rank testing demonstrated that although there was a trend towards increased FDP

Figure 9

## Semi - Quantitive Measurement of Plasma FDP



production in animals that received Streptokinase, this was not statistically significant (p=0.061).

**DISCUSSION**

The role of fibrin in the metastatic process suggests that the inhibition of pulmonary tumour seeding by Streptokinase might be secondary to fibrinolysis. This experiment has however failed to demonstrate evidence of significant fibrin breakdown with Streptokinase therapy. Information from this experiment provided us only with an index of activity of the fibrinolytic system at one fixed point in time. The preliminary studies suggested that the most suitable time of sampling blood for assay of FDP was 10 minutes after administration of Streptokinase. Estimated FDP values however showed wide variation within the same experimental group. This may partly be explained by the tendency for rat plasma to clot spontaneously despite atraumatic blood collection and adequate anticoagulation (Lewis et al, 1985). FDP levels reflect activity in either the coagulation or fibrinolytic system and their presence in control samples was presumably the result of low grade activation of coagulation. As a result, a significant rise in FDP in the Streptokinase group may have been masked by the detectable FDP levels in controls.

It could also be argued that latex agglutination lacks the sensitivity required to detect the changes in

haemostatic balance which occur in association with the metastatic process. McCulloch and George (1988) have shown, that in a similar tumour/host system, the administration of Arvin, a defibrinating agent, could reduce the fibrinogen level by nearly 70% but had no effect on metastasis formation. This suggests that the amount of fibrin required to form tumour microthrombi may be small and therefore insufficient to produce a marked rise in FDP during fibrinolysis.

Finally, it is possible that the antimetastatic effect of Streptokinase may not be associated with fibrin clot lysis. If this is the case then it is unlikely that a second fibrinolytic agent, unrelated to Streptokinase, will also inhibit metastasis unless there is a shared mechanism involving fibrinolysis. Human recombinant tissue plasminogen activator (rt-PA) is frequently used for thrombolytic therapy in humans and would seem an appropriate agent for comparison with Streptokinase. Confirmation of a similar antitumour effect by rt-PA in a similar animal model will add weight to the evidence that the antimetastatic effect of Streptokinase is secondary to clot lysis.

# CHAPTER 3

# INHIBITION OF PULMONARY TUMOUR SEEDING BY FIBRINOLYTIC THERAPY: A COMPARISON OF STREPTOKINASE AND HUMAN RECOMBINANT TISSUE PLASMINOGEN ACTIVATOR.

## INTRODUCTION

The studies thus far have provided evidence in support of the hypothesis that intravascular fibrin clot formation contributes to the metastatic process. It has been shown that administration of Streptokinase is associated with a reduction in metastatic potential of circulating tumour cells. In addition, elevated FDP levels during Streptokinase therapy suggest that the antitumour effect may be due to fibrin clot lysis. The demonstration that another clot lysing agent, structurally unrelated to Streptokinase could also reduce pulmonary tumour seeding would add further evidence to the above hypothesis. An experiment was therefore designed to study the effect of a second fibrinolytic agent on pulmonary tumour seeding.

The fibrinolytic agent chosen for comparison with Streptokinase was human recombinant tissue Plasminogen Activator (rt-PA) (Boehringer Ingelheim, Ingelheim, FRG). Evidence from randomized controlled trials using fibrinolytic therapy for coronary recanalization during myocardial infarction indicate that rt-PA is a more efficient thrombolytic agent than Streptokinase (Verstraete et al, 1985; Chesebro et al, 1987; Chesebro et al, 1988). In addition, the

fibrinolytic process induced by rt-PA is relatively fibrin-specific and causes only limited systemic plasminogen activation and fibrinolysis.

Preliminary in vitro studies were initially performed to establish whether rt-PA was cytotoxic for the Mtln3 tumour cell line. Subsequently, a further experiment was performed to compare the effects of intravenous Streptokinase and rt-PA on pulmonary tumour seeding.

**EXPERIMENT 1**

**In Vitro Assessment of Cytotoxicity**

No single in vitro test is an ideal measurement of cytotoxicity and each measures a different aspect of the balance between cell division and cell death. For this reason two complementary in vitro techniques were used to determine the cytotoxicity of rt-PA for the Mtln3 cell line. The clonogenic assay assesses the ability of tumour cells to survive and multiply to form individual colonies. It is therefore affected by cytotoxic effects which kill cells and cytostatic effects which inhibit multiplication. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is a chemosensitive assay which is used to estimate surviving cell numbers following exposure to a cytotoxic drug (Plumb et al, 1989). It is influenced by the rate of cell division and the rate of cell death. The assay relies on the ability of live but not dead

cells to reduce the water-soluble yellow dye MTT, to a water-insoluble purple formazan product. MTT-formazan production is measured by spectral analysis at 570 nm and if the MTT concentration is optimal, a linear relationship exists between cell number and MTT-formazan production.

*Clonogenic Assay*

Mtln3 cells were cultured in F10/DMEM with 10% FCS. Cells were then trypsinised, incubated for 5 minutes at $37^0$ C, resuspended in 10 ml medium and plated out at a concentration of $10^6$ cells per 25 $cm^2$ flask (Gibco, Paisley, Scotland). Cells were then incubated in 5mls F10/DMEM + 10% FCS at $37^0$ C in equilibrium with 2% $CO_2$ in air for 2 days. The medium was removed and flasks fed with 5 mls of medium or medium containing drug and incubated for 4 hours. The medium was then removed and the cells trypsinised and resuspended in 10 mls medium at a concentration of 100 cells per ml. Five mls (500 cells) were plated out into each of four Petri dishes (60 cm Nunclon; Gibco, Paisley, Scotland) and incubated at $37^0$C in equilibrium with 2% $CO_2$ in air for 7 days. The medium was then removed and clones washed with PBS, fixed in methanol and stained with crystal violet. Colonies of greater than 50 cells were counted.

The dose of rt-PA and Streptokinase added to flasks (4 flasks per dose) ranged from 0.05 mgs/ml to 1 mg/ml of medium for rt-PA and 250 Units/ml to 5000 Units/ml for Streptokinase.

These concentrations were determined from an estimation of the expected plasma concentration of rt-PA and Streptokinase in the experiment. These estimates were based on knowledge of the size of the vascular compartment in rats. Previous studies have shown the blood volume of the rat to be 5.75 - 8.4 ml/100 gm body weight (Huang and Bondurant, 1956; Jorgenson et al, 1958; Ormond and Rivera-Velez, 1965). Since the mean weight of rats in this experiment was 150 gms the approximate blood volume was 12.6 mls (8.4% body weight, Ormond and Rivera-Velez, 1965). Since the mean pack cell volume of a rat is 35% (Lewis et al, 1985) the plasma volume for a rat weighing 150 gms is 8.19 mls (65% of 12.6 mls). The dose of drug administered to animals was 5 mgs/kg for rt-PA and 30 000 Units/kg for Streptokinase. For animals weighing 150 gms this represents a dose per animal of 0.75mgs of rt-PA and 4500 Units of Streptokinase. Therefore the peak plasma concentration for rt-PA would be 0.092 mgs/ml (0.75mgs/8.19mls) and for Streptokinase would be 549 units/ml (4500 Units/8.19mls).

**RESULTS**

The mean number of colonies in the experimental groups exposed to Streptokinase ranged from 195-212, with 208 colonies in the control group (Table 3). Streptokinase did not exhibit any cytotoxic or cytostatic effect on Mtln3 cells.

The mean number of colonies in the experimental groups exposed to rt-PA at concentrations below 0.5 mg/ml ranged from 190-202, with 191 colonies in the control group. At a concentration of 1 mg/ml the mean number of colonies was significantly reduced in comparison with controls (117 vs 191, $p < 0.001$). rt-PA had no inhibitory effect on the clonogenic potential of Mtln3 cells at concentrations below 0.5 mg/ml.

*MTT Assay*

Mtln3 cells were trypsinised and prepared as above and plated out in 200 ul of F10/DMEM + 10% FCS at a concentration of 500 cells per flat-bottomed well in 96-well microtiter plates (Linbro; Flow Laboratories, Irvine, Scotland). The first and last 8 wells contained medium only. Plates were incubated at $37^0$C in equilibrium with 2% $CO_2$ in air for 2 days. The medium was then removed from the wells and replaced with 200 ul of fresh medium or medium containing drug (8 wells per concentration). Cells were incubated for 4 hours after which medium was replaced by drug-free medium. The medium was replaced at 24 hour intervals for a further 2 days. On the fourth day following drug addition, medium was replaced and 50 ul of MTT dissolved in PBS (5 mg/ml) was added to each well. Plates were wrapped in aluminium foil and incubated for a further 4 hours. Medium and MTT were removed from the wells and the formazan crystals were dissolved in 200 ul of dimethyl sulphoxide (DMSO). Finally, 25 ul of

Sorensen's glycine buffer (0.1M glycine plus 0.1M NaCl equilibrated to pH 10.5 with 0.1M NaOH) was added to each well. The absorbance was recorded in an enzyme-linked immunosorbent assay plate reader (Model 2550 enzyme immunoassay plate reader; Bio-Rad Laboratories, Ltd., Watford, England) at a wavelength of 570 nm. The first and last rows of 8 wells which contained medium and PBS (50 ul) only were used to blank the plate reader. The following drug concentrations were used:

rt-PA - 0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mgs/ml

Streptokinase - 0, 100, 250, 500, 750, 1500, 3000, 4000 and 5000 Units/ml

**RESULTS**

Spectral absorbance at 570 nm ranged from 2.72-2.75 for rt-PA (control 2.70) and from 2.71-2.76 for Streptokinase (control 2.71). MTT-formazan formation was not reduced by exposure to either drug thereby indicating that rt-PA and Streptokinase were not cytotoxic for the Mtln3 cell line (Table 4).

**EXPERIMENT 2**

**Effects of Streptokinase and rt-PA on pulmonary tumour seeding**

Three groups of 10 F344 female rats, 6-8 weeks old were used in this experiment. Cells were prepared as previously described from low-passage in vitro cultures

# Effect of rt-PA and Streptokinase on Tumour Cells

Table 3

**Clonogenic Assay**

| rt-PA Dose (mg/ml) | 0 | 0.05 | 0.1 | 0.5 | 1 |
|---|---|---|---|---|---|
| Number of colonies | 191 (c) | 202 | 194 | 190 | 117 |

| Streptokinase dose (units/ml) | 0 | 250 | 500 | 2500 | 5000 |
|---|---|---|---|---|---|
| Number of colonies | 208 (c) | 205 | 212 | 197 | 195 |

C : Control

Table 4

**MTT Assay**

| rt-PA Dose (mg/ml) | 0 | 0.01 | 0.05 | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
|---|---|---|---|---|---|---|---|---|---|
| Absorbance (570 nm) | 2.70 (c) | 2.73 | 2.72 | 2.74 | 2.72 | 2.72 | 2.74 | 2.75 | 2.74 |

| Streptokinase dose (units/ml) | 0 | 100 | 250 | 500 | 750 | 1500 | 3000 | 4000 | 5000 |
|---|---|---|---|---|---|---|---|---|---|
| Absorbance (570 nm) | 2.71 (c) | 2.74 | 2.72 | 2.71 | 2.74 | 2.72 | 2.75 | 2.76 | 2.75 |

C : Control

and resuspended in F10/DMEM. Animals received an intravenous injection of a 0.5ml suspension of $10^5$ Mtln3 cells under general anaesthesia (intra-peritoneal pentobarbitone 60mgs/kg). Additional treatment was then administered as follows:

Group A: No additional treatment (controls).

Group B: A single tail vein injection of rt-PA, in a dose of 5mgs per kg body weight, 30 minutes after tumour cell inoculation.

Group C: A single tail vein injection of Streptokinase, in a dose of 30 000 Units per kg body weight, 30 minutes after tumour cell inoculation.

The dose of rt-PA administered was based on previous animal experimental work wherein clot lysis was achieved in vivo (Lyden, 1990).
The estimated peak plasma concentrations for rt-PA in a dose of 5mgs/kg, and for Streptokinase in a dose of 30 000 Units/kg, were 0.092 mgs/ml and 549 Units/ml respectively (see materials and methods).
Animals were sacrificed at 17 days after tumour cell injection and the lungs prepared according to the method of Wexler as previously described.
Three animals did not survive the experimental period (one in Group B and two in Group C). Post-mortem

examination did not reveal any evidence of haemorrhagic complications and the cause of death was unknown.

**RESULTS**

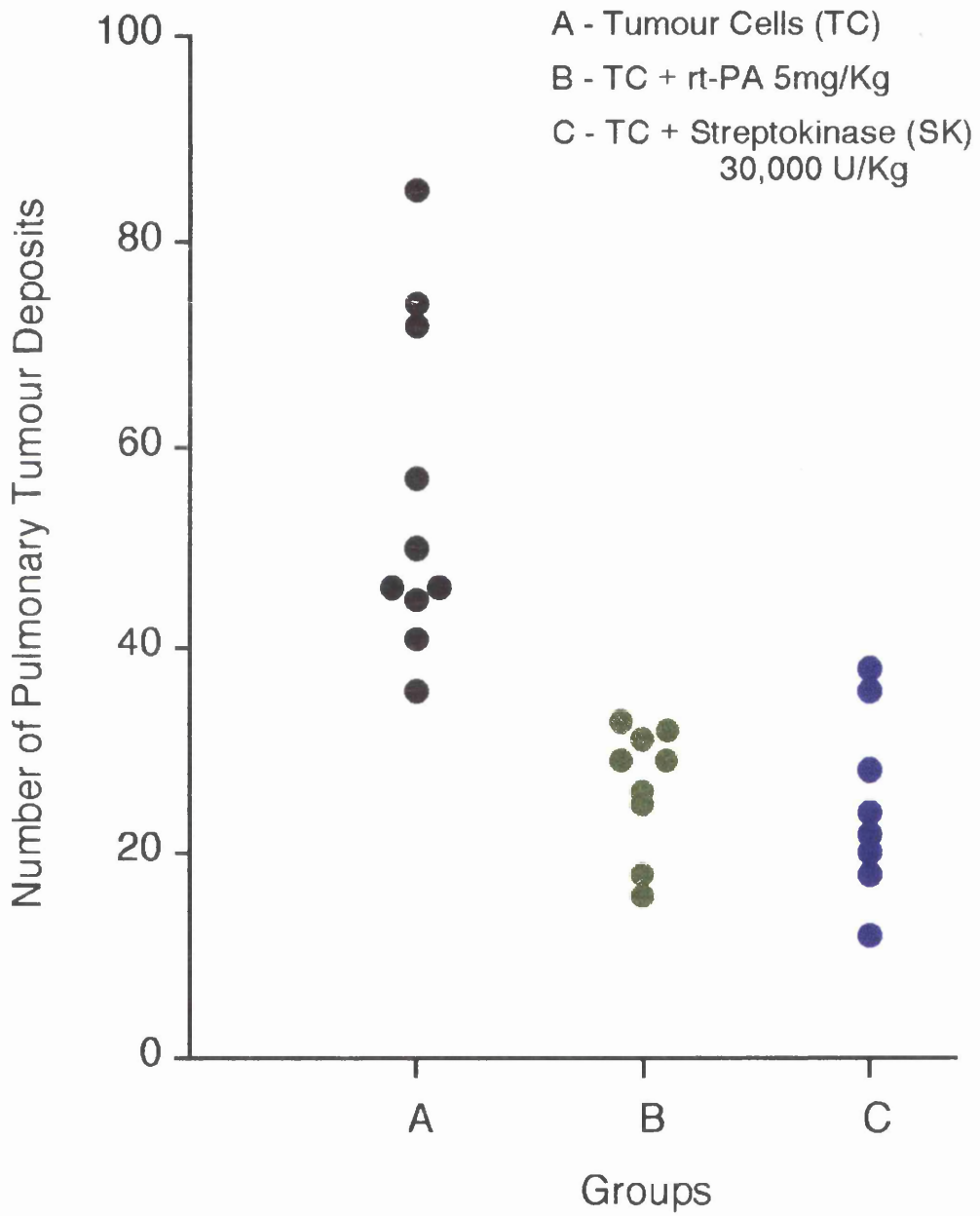
Both Streptokinase and rt-PA caused a significant reduction in pulmonary tumour nodule formation (Figure 10). The mean number of lung nodules in untreated controls was 55 in comparison with 27 in the group administered rt-PA (Group A vs Group B, $p < 0.001$) and 25 in the group administered Streptokinase (Group A vs Group C, $p < 0.01$). There was no significant difference in pulmonary nodule formation between groups treated with rt-PA or Streptokinase (Group B vs Group C, $p = 0.56$).

**DISCUSSION**

The preliminary in vitro studies showed that Streptokinase was not cytotoxic or cytostatic for Mtln3 cells. However rt-PA reduced colony-forming potential at high concentration although this was not associated with a reduction in cell viability as measured by the MTT assay. The sensitivity of the clonogenic assay to factors which disrupt the ability of cells to proliferate suggests that the reduction in the number of colonies may be due to a cytostatic effect. The clonogenic assay requires exposure of cells to drug before plating and the observed effect may be a consequence of cell membrane disruption sufficient to reduce surface adherence and proliferation but not to

Figure 10

**Effect of rt-PA and Streptokinase on Pulmonary Tumour Seeding**



kill cells. It has been assumed that rt-PA disperses evenly throughout the intravascular compartment and is rapidly cleared from the circulation by hepatic degradation (initial half-life < 3 minutes in rats, Harris et al, 1988). Studies in humans however, have shown that fibrin-bound rt-PA may remain pharmacologically active at the clot site for several hours (Verstraete et al, 1985; Eisenberg et al, 1987). Thus, tumour cells trapped within this clot may be exposed to higher drug levels than circulating tumour cells although it is unlikely that this would exceed the concentrations and duration of exposure achieved in the in vitro studies.

This experiment has demonstrated that both rt-PA and Streptokinase inhibit pulmonary tumour seeding. Streptokinase is produced by beta-haemolytic streptococci and its mechanism of action involves activation of the intrinsic fibrinolytic system. rt-PA is produced by bulk fermentation of a Chinese hamster ovary cell line transfected with the cDNA for the naturally occuring human protease enzyme synthesized by the vascular endothelium (Collen et al, 1989). rt-PA has a high affinity and specificity for fibrin-bound plasminogen where it causes enzymatic degradation of the latter into plasmin and consequently thrombolysis. The demonstration that two structurally distinct compounds, unrelated except for their ability to induce fibrin clot lysis, could inhibit pulmonary tumour

formation substantiates the role of fibrin in the metastatic cascade.

In this study both fibrinolytic agents were administered as a bolus injection in contrast to the standard therapeutic regime in man of a loading bolus (usually 10% of the total dose) followed by an infusion over 3 hours. It could therefore be argued that an intravenous infusion might be a more effective regimen for the reduction of pulmonary tumour seeding in this animal model. However, the thrombolytic effect of rt-PA has been found to be sustained for up to 7 hours following its clearance from the circulation in animal models (Agnelli et al, 1985; Clozel et al, 1989) and in patients with myocardial infarction (Eisenberg et al, 1987). These observations are consistent with prolonged fibrinolytic activity of rt-PA bound to fibrin. This suggests that bolus injection may be as effective as infusion therapy. Indeed, equivalent efficacy has been demonstrated in preliminary experiments with bolus rt-PA and infusion in animals (Longridge et al, 1991) and patients with acute myocardial infarction (Tebbe et al, 1989).

These findings raise interesting questions as to whether fibrinolytic therapy can influence the human metastatic cascade and thus play a role in the treatment of patients with malignancy. rt-PA is the more attractive agent for such therapy as it is identical to endogenous t-PA and does not elicit an immune response. In contrast, Streptokinase is

antigenic and immunogenic in humans, allergic reactions occuring in up to 5% of patients. Furthermore, anti-Streptokinase antibody can bind to platelets and induce platelet activation thereby limiting the therapeutic effect (Vaughan et al, 1991). Finally, the fibrin specificity of rt-PA limits activation of circulating plasminogen and as a result bleeding complications are less frequent than with Streptokinase (Verstraete et al, 1985; Magnani et al, 1989).

# CHAPTER 4

# STUDIES TO DETERMINE WHETHER PULMONARY TUMOUR INHIBITION BY FIBRINOLYSIS IS ENHANCED BY ANTIPLATELET THERAPY

## INTRODUCTION

The preceding chapters have investigated the role of fibrin in the metastatic process. The presence of platelets within tumour emboli trapped within the microcirculation of target organs has also attracted considerable attention. Platelets do not appear to influence initial tumour cell arrest in the microvasculature (Crissman et al, 1985, 1988), rather they seem to be more important in stabilization of tumour cell-endothelial adhesion (Hawrylowicz et al, 1991; Chopra et al, 1991). In addition, platelets may enhance metastasis by shielding tumour cells from immune destruction (Dvorak et al, 1979; Gasic et al, 1986), by providing growth factors (Eastment et al, 1987; Poggi et al, 1988), or by facilitating adhesion to the extracellular matrix (Menter et al, 1987).

Our previous experiments have suggested that destruction of the fibrin element within tumour emboli reduces pulmonary tumour seeding. Studies in other animal models have shown that antiplatelet agents also reduce the metastatic potential of circulating tumour cells (Gasic et al, 1973; Kolenich et al, 1972; Pollard et al, 1981). The importance of both fibrin and platelets in the metastatic process suggests that the

combination of fibrinolytic and antiplatelet therapy may have an additive inhibitory effect on metastasis. In addition, platelets are thought to play a major role in reducing the efficiency of thrombolytic therapy. They are an important component of thrombi particularly at their point of attachment to the vessel wall and recent studies have demonstrated that fibrinolytic agents cause marked platelet activation (Fitzgerald et al, 1988, 1989; Vaughan et al, 1991). Consequently, antiplatelet therapy has been advocated during coronary thrombolysis to enhance vessel patency and the combination of Streptokinase and aspirin has been shown to be superior to the use of either agent alone (ISIS-2, 1988). It would therefore seem appropriate to study the antimetastatic effect of a combination of Streptokinase and antiplatelet agents in this animal model to determine whether this combination therapy is superior to fibrinolytic therapy alone.

For the purpose of our experiment, we used two antiplatelet agents with completely separate mechanisms of action. Aspirin (acetyl salicylic acid) causes irreversible acetylation of the enzyme cyclo-oxygenase, leading to a reduction in the synthesis of prostaglandins derived from arachidonic acid. At low dose, aspirin preferentially inhibits the synthesis of the prostaglandin Thromboxane A2 (TXA2), a potent platelet aggregator and vasoconstrictor. Ticlopidine (a thienopyridine derivative) inhibits ADP-induced platelet aggregation and although the precise mechanism

of action has yet to be determined, it appears to prevent ADP-induced exposure of the fibrinogen binding site on the platelet glycoprotein IIb-IIIa complex (McTavish et al, 1990). It is only active after repeated oral administration and is rapidly metabolised with one metabolite more active than the parent drug. In animals, ticlopidine does not reduce thromboxane production and therefore, unlike aspirin, does not appear to inhibit cyclo-oxygenase (Valentin et al, 1988).

**Studies to determine cytotoxicity of antiplatelet agents:**

Prior to proceeding to study the effects of aspirin and ticlopidine on pulmonary tumour seeding in our animal experimental model, it was essential to establish whether either agent had a cytostatic or cytotoxic effect on the Mtln3 tumour cell line. Two in vitro assays, the clonogenic assay and the MTT assay, were performed as previously described in Chapter 3. The estimated plasma concentrations in the animal experiment were 3 mgs/ml for aspirin and 15 mgs/ml for ticlopidine (see methods, Chapter 3).

For the clonogenic assay, drug was added to flasks (4 flasks per dose) giving the following range of concentrations:

Aspirin - 0, 1, 10, 50 and 100 mgs/ml of medium

Ticlopidine - 0, 10, 50, 100 and 200 mgs/ml of medium

For the MTT assay, drug was added to each well (8 wells per concentration) giving the following range of concentrations:

Aspirin - 0, 1, 5, 10, 20, 40, 60, 80 mgs/ml of medium

Ticlopidine - 0, 10, 20, 40, 80, 160, 320 mgs/ml of medium

**RESULTS**

*Clonogenic assay*

The mean number of colonies in the experimental groups exposed to aspirin ranged from 195-207 (controls 196) and for ticlopidine ranged from 192-204 (controls 197) (Table 5). There was no reduction in the clonogenic potential of Mtln3 cells exposed to either aspirin or ticlopidine.

*MTT assay*

Spectral absorbance at 570 nm ranged from 2.78-2.83 for aspirin (control 2.80) and from 2.73-2.77 for ticlopidine (control 2.73) (Table 6). MTT-formazan formation was not reduced by exposure to either aspirin or ticlopidine.

Therefore aspirin and ticlopidine were not cytotoxic or cytostatic for the Mtln3 cell line.

# Effect of Aspirin and Ticlopidine onTumour Cells

Table 5

## Clonogenic Assay

| Aspirin (mg/ml) | 0 | 1 | 10 | 50 | 100 |
|---|---|---|---|---|---|
| Number of Colonies | 196 | 197 | 205 | 195 | 207 |

| Ticlopidine (mg/ml) | 0 | 10 | 50 | 100 | 200 |
|---|---|---|---|---|---|
| Number of Colonies | 197 (c) | 192 | 198 | 204 | 192 |

C : Control

Table 6

## MTT Assay

| Aspirin (mg/ml) | 0 | 1 | 5 | 10 | 20 | 40 | 60 | 80 |
|---|---|---|---|---|---|---|---|---|
| Absorbance (570nm) | 2.80 (c) | 2.78 | 2.78 | 2.79 | 2.79 | 2.83 | 2.80 | 2.81 |

| Ticlopidine (mg/ml) | 0 | 10 | 20 | 40 | 80 | 160 | 320 |
|---|---|---|---|---|---|---|---|
| Absorbance (570nm) | 2.73 (c) | 2.77 | 2.74 | 2.74 | 2.75 | 2.73 | 2.77 |

C : Control

**EXPERIMENT 1: Studies to determine the effect of Streptokinase and aspirin on pulmonary tumour seeding.**

**MATERIALS AND METHODS**

Four groups of female 344 Fischer rats, 8 per group, 6-8 weeks old were used in this experiment. Under general anaesthesia, animals were given tail vein injections of a 0.5 ml suspension of $10^5$ Mtln3 cells in F10/DMEM. Additional treatment was then commenced as follows:

Group A: These animals received no additional treatment.

Group B: These animals received a single intravenous injection of Streptokinase, in a dose of 30 000 U/kg, 30 minutes after tumour cell inoculation.

Group C: These animals received a single intravenous injection of aspirin, in a dose of 20 mgs/kg, 15 minutes before tumour cell inoculation.

Group D: These animals received an intravenous injection of aspirin (20 mgs/kg) 15 minutes before tumour cell inoculation and a further injection of Streptokinase (30 000 U/kg) 30 minutes after tumour cell inoculation.

The dose of aspirin administered was 20 mgs/kg body weight. This was based on previous animal experimental

work which demonstrated inhibition of ex-vivo platelet aggregation (Morikawa et al, 1986). Aspirin (aspisol, Bayer, Leverkusen, FRG) was reconstituted in sterile water and administered intravenously to ensure accuracy of dosage.

The estimated peak plasma concentration for aspirin in a dose of 20 mgs/ml, was 3mgs/ml (see materials and methods).

Animals were sacrificed at 17 days and pulmonary tumour seeding assessed by the method of Wexler as previously described (Chapter 1).

One animal (Group D) did not survive to the end of the experimental period. Post-mortem examination did not reveal any evidence of haemorrhagic complications and the cause of death was unknown.

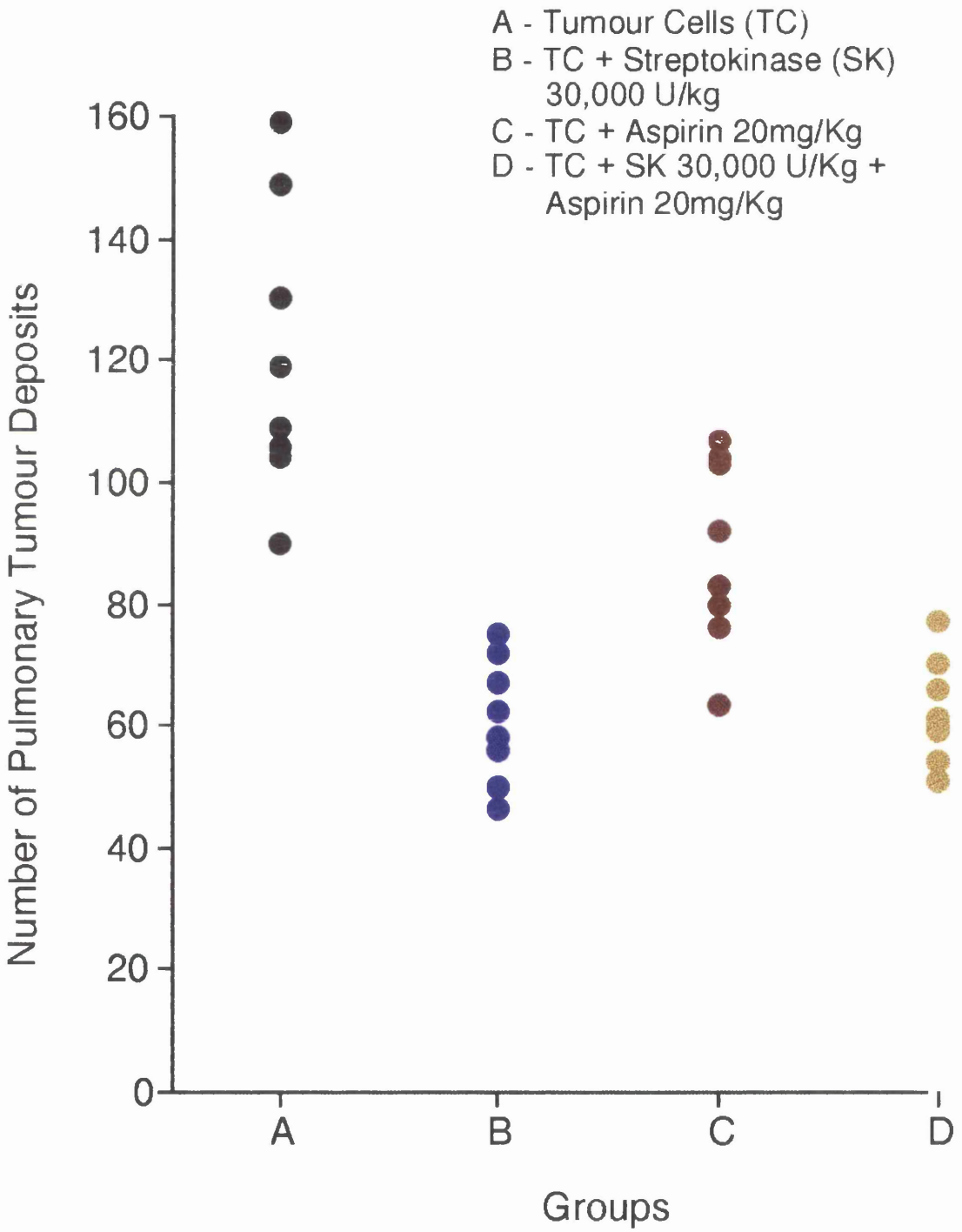
Comparison of the different groups was made by the Mann-Whitney test using Bonferoni's correction for multiple comparisons.

**RESULTS (Figure 11)**

Animals that received Streptokinase (Group B) showed a significant reduction in pulmonary tumour seeding (median = 60) when compared with controls (Group A) that received tumour cells alone (median = 114, $p < 0.01$). There was a reduction in pulmonary tumour seeding in animals that received aspirin (Group C, median = 87.5) although this failed to reach statistical significance when compared with controls (p

Figure 11

## Effect of Streptokinase and Aspirin on Pulmonary Tumour Seeding

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= 0.052). Those animals that received both aspirin and Streptokinase (Group D) also showed a significant reduction in pulmonary tumour seeding (median = 60.5) in comparison with controls, although this effect was not significantly different from the reduction in pulmonary tumour seeding demonstrated in the group treated with Streptokinase alone.

**EXPERIMENT 2: Studies to determine the effects of ticlopidine on pulmonary tumour seeding.**

**MATERIALS AND METHODS**

Four groups of female 344 Fischer rats, 10 per group, 6-8 weeks old were used in this experiment. Under general anaesthesia, animals were given tail vein injections of a 0.5 ml suspension of $10^5$ Mtln3 cells in F10/DMEM. Additional treatment was then commenced as follows:

Group A: These animals received no additional treatment.

Group B: These animals received a single intravenous injection of Streptokinase in a dose of 30 000 U/kg 30 minutes after tumour cell inoculation.

Group C: These animals received oral ticlopidine in a dose of 100 mgs/kg for 3 days prior to tumour cell inoculation.

Group D: These animals received oral ticlopidine in a dose of 100 mgs/kg for 3 days prior to tumour cell inoculation and intravenous Streptokinase in a dose of 30 000 U/kg 30 minutes after tumour cell inoculation.

The estimated peak plasma concentration for ticlopidine in a dose of 100mgs/ml, was 15 mgs/ml (see materials and methods).

Animals were sacrificed and pulmonary tumour seeding assessed by the method of Wexler.

Two animals (one in Group C, one in Group D) did not survive to the end of the experimental period. Post-mortem examination did not reveal any evidence of haemorrhagic complications and the cause of death was unknown.

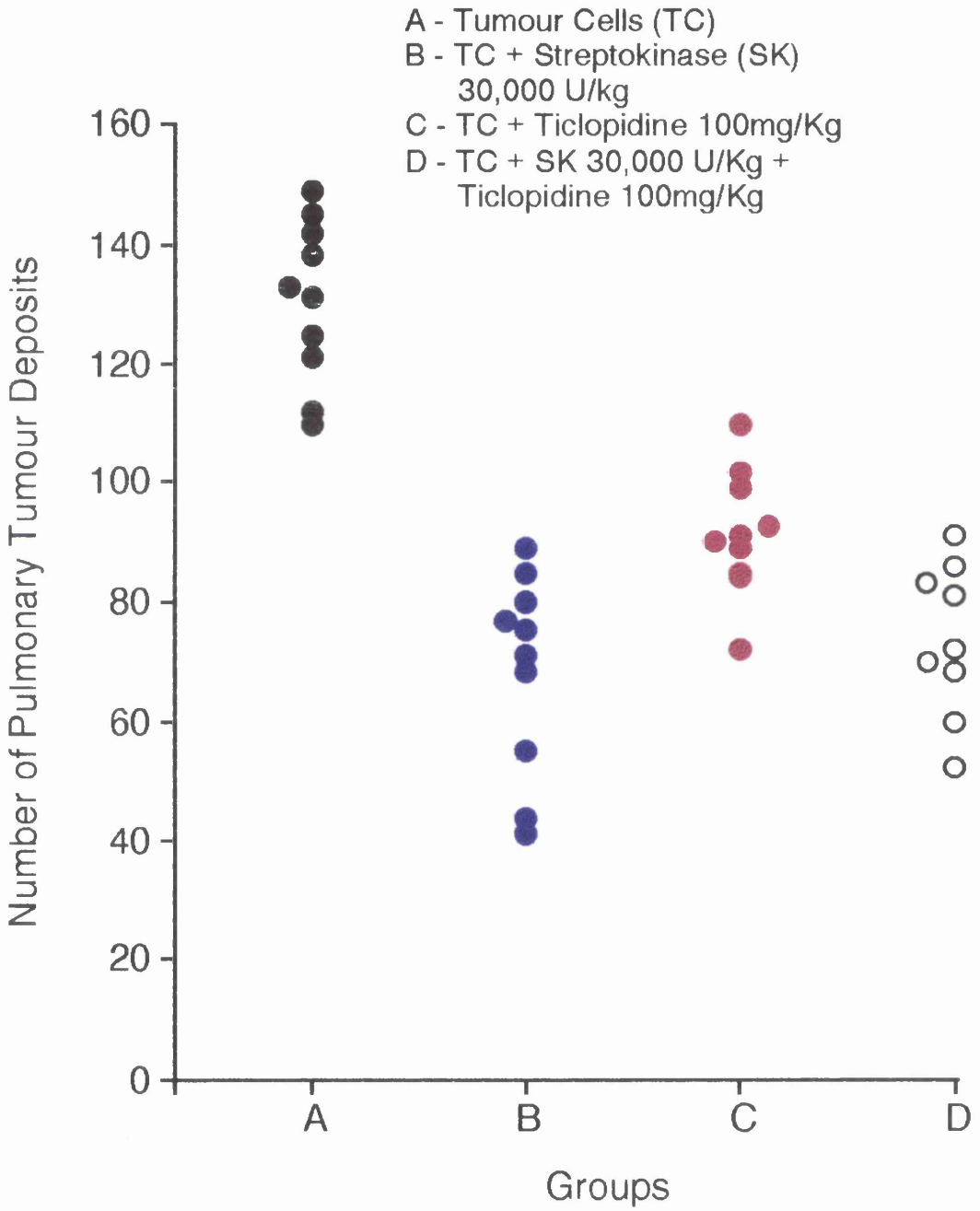
Statistical analysis was made by Mann-Whitney test using Bonferoni's correction for multiple comparisons.

**RESULTS (Figure 12)**

Animals that received Streptokinase (Group B) showed a significant reduction in pulmonary tumour seeding (median = 73) when compared with control animals (Group A) that received tumour cells alone (median = 132, $p < 0.005$). Animals that received ticlopidine (Group C) also demonstrated a significant reduction in pulmonary tumour nodules (median = 92) in comparison with controls ($p < 0.005$). The reduction in pulmonary tumour seeding in animals that received Streptokinase was

Figure 12

## Effect of Streptokinase and Ticlopidine on Pulmonary Tumour Seeding




(Original In Colour)

significantly greater than in those animals that received ticlopidine alone (Group B vs C, $p < 0.01$). However, the reduction in pulmonary tumour seeding in those animals that received both ticlopidine and Streptokinase (Group D) was not significantly different to those that received Streptokinase alone.

**DISCUSSION**

The mean number of pulmonary tumour deposits per group in the two experiments above were higher than the mean number of pulmonary tumour deposits per group in the rt-PA/Streptokinase experiment. This is due to phenotypic drift (Neri and Nicolson, 1981) and is the result of using tumour cell suspensions derived from tumour colonies of different passage number for different experiments. While this prevents comparison between experiments it does not affect comparison between groups within the same experiment.

Platelets, like fibrin, are an integral component of tumour thrombi which become trapped in the microcirculation of target organs. In addition, they are thought to play a major role in reducing the efficiency of thrombolytic therapy by inducing platelet activation (Fitzgerald et al, 1988, 1989; Vaughan et al, 1991). Consequently, antiplatelet therapy has been advocated during coronary thrombolysis to enhance vessel patency and the combination of Streptokinase and

aspirin has been shown to be superior to the use of either agent alone (ISIS-2, 1988). It was therefore hypothesized that combined antiplatelet/fibrinolytic therapy would have a greater inhibitory effect on pulmonary tumour seeding than either treatment alone.

Our results demonstrate that aspirin in a dose of 20 mgs/kg reduced pulmonary tumour seeding although this effect failed to reach a significant level when compared with untreated controls. Furthermore, the combination of aspirin and Streptokinase was no more effective in preventing pulmonary tumour seeding than Streptokinase treatment alone. The negative results with aspirin reported in other animal models (Wood and Hilgard, 1972; Hilgard et al, 1976) have been ascribed to the use of high doses of aspirin. Recognition of the inhibitory effect of aspirin on endothelial cell prostacyclin (PGI2) production (Kelton et al, 1978) has led to the use of low dose aspirin which preferentially inhibits production of the potent platelet aggregator thromboxane A2 by causing irreversible acetylation of the enzyme cyclo-oxygenase. Since tumour cell-induced platelet activation may occur via other pathways such as the generation of thrombin, activation by ADP or a combination of these mechanisms (Bastida et al, 1984; Honn et al, 1987), failure to demonstrate a significant antimetastatic effect in this study suggests that platelet activation by the Mtln3 cell line may be independent of the cyclo-oxygenase pathway. In contrast, ticlopidine significantly inhibited the

development of pulmonary tumour nodules suggesting that tumour cell-induced platelet aggregation by the Mtln3 cell line may be dependent on the generation of ADP. However, like aspirin, the combination of ticlopidine and Streptokinase was no more effective in tumour inhibition than Streptokinase treatment alone. These results indicate that fibrinolytic inhibition of metastasis is not enhanced by antiplatelet therapy and suggest that tumour cell-platelet interactions may be less important than fibrin deposition in the metastatic process.

The preceding four chapters have shown that a single "clot-lysis" dose of Streptokinase or rt-PA can inhibit pulmonary metastasis in an animal model. This effect is likely to be due to destruction of fibrin clot surrounding tumour thrombi trapped in the pulmonary microcirculation. If these results are applicable to the human metastatic process then fibrinolytic agents may have a role in the treatment of patients with malignant disease. Previous clinical studies of anticoagulant therapy have been compromised by a preponderence of patients with advanced disease and established metastasis. However, the study of White et al (1976), using urokinase to facilitate fibrinolysis in human malignancy, has shown that patient survival may be improved by prevention of entrapment of circulating malignant cells in the microvasculature. It is clear that to be effective, antimetastatic therapy should be administered to

patients at risk from haematogenous dissemination in order to prevent seeding of circulating tumour cells in secondary organs. Therefore the development of an antimetastatic treatment in an animal model is only relevant if this therapy can have clinical application in patients with cancer.

Evidence from animal studies has demonstrated that the manipulation of malignant tumours is associated with increased tumour cell shedding into the bloodstream (Cliffton and Agostino, 1960; Romsdahl et al, 1964; Liotta et al, 1974) and may lead to an increase in the development of distant metastases (Tyzzer, 1913; Liotta et al, 1976). These findings suggest that operative manipulation may also enhance the human metastatic process implying that fibrinolytic therapy applied peri-operatively might inhibit this type of metastatic seeding. Although many studies have attempted to identify circulating tumour cells in cancer patients, the results have been unreliable due to the problems associated with the detection of a minor sub-population of tumour cells in blood. In view of the potential risk of peri-operative haemorrhage with fibrinolytic agents it would be difficult to justify a large prospective study of peri-operative fibrinolytic therapy without conclusive evidence of tumour cell shedding during surgery.

The experimental work in the second half of this thesis is designed to establish whether tumour cell dissemination occurs during surgery for malignant

disease. This will determine whether there is a future role for the prescription of peri-operative antimetastatic therapy in these patients.

# CHAPTER 5

# FLOW CYTOMETRIC DETECTION OF TUMOUR CELLS IN BLOOD

## INTRODUCTION

The metastatic process is a complex, cascade process which is initiated by intravasation of malignant cells from the primary tumour into the circulation. The intensity of vascularisation and the presence of vascular invasion in the primary tumour correlates with the development of metastatic disease (Willis, 1930; Weidner et al, 1991). The identification and enumeration of circulating tumour cells within the bloodstream may therefore be used to predict patients at risk from secondary spread.

At the present time, the role of surgery in human cancer dissemination is unclear due to the unreliability of techniques for cancer cell isolation and identification (Goldblat and Nadel, 1965). This has been attributed to difficulties in the interpretation of cellular morphology. As a result large primitive blood cells normally present in blood, including precursors of the erythroid, myeloid and lymphoid series, have frequently been misinterpreted as malignant leading to an overestimation of tumour cell numbers (Landell et al, 1963; Goldblatt and Nadel, 1965).

As reliable estimates of circulating cells are required to assess the role of surgery in the

metastatic process, new techniques have been adopted in an attempt to avoid the difficulties encountered in previous studies. The development of immunocytological techniques has greatly enhanced the specificity and sensitivity by which tumour cells can be detected in bone marrow (Osborne et al, 1991) and blood (Moss and Sanders, 1990). The use of a monoclonal antibody to a tumour-specific antigen should therefore enable the accurate detection of a minor subpopulation of tumour cells in blood. Standard immunocytochemical analysis of blood would necessitate laborious and possibly inaccurate microscopy studies, however the relatively new technique of flow cytometry has the capability to screen individual cells for their light scattering abilities (which provides information on their size and granularity) as well as their fluorescent intensity when labelled with fluorochrome conjugated monoclonal antibody. The emissions from fluorochrome-labelled cells are automatically detected and quantified and converted to digital data thereby simplifying the process of immunocytochemical analysis of blood. The aim of this series of experiments was therefore to investigate whether flow cytometry was a suitable technique to screen large numbers of peripheral blood cells to identify circulating tumour cells prior to its application in patients with malignant disease.

To identify circulating tumour cells in the peripheral blood of patients with breast cancer requires the use of specific monoclonal antibodies that

can distinguish between haemopoetic cells and malignant breast epithelial cells. Transformation to the malignant phenotype is associated with heterogeneity of antigen expression and monoclonal antibodies to epithelial tissues may bind to only some cells in a tumour (Edwards, 1985). Preliminary flow cytometry experiments were therefore designed to compare the binding affinity of five monoclonal antibodies (MNF116, Anti-EMA, HMFG1, SM3, Ber-EP4) that react with different breast epithelial antigens in order to establish which antibody produced optimal fluorescent labelling of tumour cells in peripheral blood. Having established the specificity and sensitivity of the technique, a pilot study of patients with breast cancer was designed to establish whether flow cytometry could identify circulating tumour cells in the bloodstream in the perioperative period. To maximise tumour cell detection and avoid tumour cell loss from filtration in the pulmonary microcirculation, central venous access was achieved and blood samples were obtained.

## 1 PRELIMINARY EXPERIMENTS

## MATERIALS AND METHODS

### Monoclonal Antibodies Reacting With Breast Epithelia

The technique of monoclonal antibody (MAB) production has changed little since the original work of Kohler

and Milstein (1975) and is based on the principle of fusing stimulated B cells (usually from the spleen of a mouse or rat) with drug-selected non-immunoglobulin-secreting murine myeloma cells. The use of monoclonal antibodies specific to breast antigens enables breast epithelial cells circulating in the bloodstream to be distinguished from haemopoetic cells. Although some of these antibodies show preferential reaction with breast cancer tissue, they do not detect cancer specific-antigens; rather, they react with normal or modified tissue antigens which are either preferentially or inappropriately expressed on malignant cells (Tjandra and McKenzie, 1988).

*1 Anticytokeratins*

The antigenic determinants for this class of antibody are the cytokeratin proteins which form the intermediate filaments in the cytoplasm of all epithelial cells. A total of 19 proteins, related to but not identical with epidermal alpha keratins, have been identified with molecular weights varying from 40,000 to 68,000 kilodaltons (Moll et al, 1982). The pattern of cytokeratin expression is different for each epithelium and for normal breast epithelium this includes a broad range of cytokeratins from low to high molecular weight. Although this pattern is altered by malignant transformation Cytokeratins 8, 18 and 19 are consistently expressed by breast carcinoma cells (Jarasch et al, 1988; Tsuburu et al, 1991). Endothelial

cells, stromal cells and cells of haemopoietic origin do not express cytokeratin filaments (Lazarides, 1980).

The anticytokeratin monoclonal antibody utilized, MNF 116 (Dako Ltd., High Wycombe, Bucks, England), was originally derived from a crude extract of splenic cells from a nude mouse engrafted with the breast carcinoma cell line MCF7. The antibody reacts with an epitope which is present on a wide range of Cytokeratins, varying in molecular weight from 45 - 56.5 kilodaltons, including 10, 17 and 18. This IgG1 antibody was obtained conjugated to fluorescein isothiocyanate (FITC) giving a FITC/protein ratio (E495nm/E278nm) of approximately 1.0.

*2 Anti-Polymorphic Epithelial Mucins*

Milk mucins, now termed Polymorphic Epithelial Mucins (PEM), are large, cell surface-associated glycoproteins (50-60% carbohydrate) that are characteristic, but not speciifc to, breast epithelial cells. The sugar side chains are linked to the serines and threonines through the sugar N-acetyl galactosamine. Many of these antibodies were raised by immunization of mice with extracts of the human milk fat globule membranes (HMFG) which are surface membranes that surround milk triacylglycerol droplets secreted from mammary epithelial cells. One of the first and most widely used monoclonal antibody in this class is HMFG1 originally produced by immunizing mice with the HMFG membrane and cultured human milk cells (Taylor-Papadimitriou et al,

1981). Over the years these antigens have become known by the monoclonal antibody-defined epitopes and include B72.3. DF3, NCRC 11 and SM3. The amino acid sequence coding for the core protein of the milk mucin (PEM) has been determined by cDNA cloning (Gendler et al, 1990) and it has been established that the various antibodies recognise an epitope found within a tandem repeat element comprising 20 amino acids in the core protein (Siddiqui et al, 1988). Over 90% of breast cancers react with these antibodies (Berry et al, 1985; Thomas et al, 1987).

The antibodies utilized in this experiment were HMFG1, SM3 and anti-EMA. HMFG1 and SM3 were kindly donated by Dr. J Taylor-Papadimitriou (Institute of Cancer Research, London) in an unconjugated form. Anti-EMA was obtained conjugated to FITC giving a FITC/protein ratio of 1.1 (Dako Ltd., U.K.).

*3 Ber-EP4*

This relatively new murine monoclonal antibody was produced by standard hybridoma techniques using the MCF 7 breast carcinoma cell line. It is directed against two membrane glycoprotein antigens (34 and 49kD mol. wt.) which are distinct from other surface antigens, including EMA and related antigens (Latza et at, 1990). All carcinomas and non-neoplastic epithelial cells express the Ber-EP4 antigen except the superficial layers of squamous epithelia, hepatocytes and parietal cells.

The antibody was obtained conjugated to FITC giving a FITC/protein ratio of 1.1 (Dako Ltd., High Wycombe, U.K.).

*Tumour Cells*

Three cultured breast carcinoma cell lines, ZR75, MCF7 and MDA/MB/231, were utilized for monoclonal antibody labelling studies and were obtained from the Department of Oncology, Beatson Institute, University of Glasgow. The original clones were isolated from malignant pleural effusions in patients with metastatic breast carcinoma as this source provides a large number of tumour cells with high viability and minimal fibroblast contamination (Soule et al, 1973; Cailleau et al, 1974; Engel et al, 1978).

Cells were cultured in 75 $cm^2$ tissue culture flasks (Gibco, Paisley) in RPMI medium, with 10% foetal calf serum (FCS) but without antibiotics. Cultures were maintained at $37^0$ C in equilibrium with 2% $CO_2$ in air. Once cultures were confluent, passage was carried out by washing with 5 ml of trypsin/EDTA (0.25%/1mM) and incubated for 5 minutes at 37 degrees C. Subcultures required the addition of $2 \times 10^6$ viable tumour cells to further 75 $cm^2$ flasks.

**Flow Cytometry**

*FACScan Analyser*

Quantitative analysis of tumour cells in blood was performed by flow cytometry using a FACScan analyser (Becton Dickinson, Oxford, U.K.). This cytometer analyses cell suspensions by utilizing a low energy 15 mW Argon ion gas laser and measures and records up to five parameters on an individual cell. The forward scatter of the laser light (FSC) is related to cell size, like the shadow of a cell, while the 90 degree side scatter (SSC) is the light reflected from the cell and is related to granularity. In addition, the binding of up to three fluorochromes conjugated to monoclonal antibodies can be measured.

The energy level of the Argon ion laser is sufficient to stimulate and cause maximal energy emission from the fluorochromes and the fixed wavelength of 488 nm only excites fluorochromes in the red/green spectrum. The fluorochromes used in this study were fluorescein isothiocyanate (FITC) which emits in the green waveband from 480nm to 600nm and phycoerythrin (PE) which emits in the orange-red waveband from 550nm to 640nm.

*FACScan Analysis*

Sample analysis requires prepared cell suspensions to be placed in cytometer tubes (Falcon 2052) which are then pushed over the sample pipette of the FACScan and

held in place by a rubber grommet to give an airtight seal. Air is then pumped into the tube increasing the pressure and propelling the sample into a conical chamber in the flow cell. Laminar flow is produced by a stream of isotonic sheath fluid driven under pressure, causing the cells to form a single stream as they leave the conical chamber and enter the flow cell, at a rate of 60ul/min, where they are exposed to the Argon ion laser.

The laser beam is first passed through a prismatic refracting beam expander and converging lens before being focused on the stream of cells by a steering plate. The forward scatter is recorded on a silicon photodiode lying in the path of the laser while the side scatter is reflected and collected together with the fluorochrome emissions, and converted into parallel light by a bi-convex lens. The side scatter, green (FL1), orange-red (FL2) and strong red (FL3) fluorescence are separated according to wavelength by a series of mirrors and filters. Separate photoelectric multipliers detect and amplify the side scatter and fluorochrome emissions which are then converted to digital data by an analog/digital converter. The data is collected and stored on the software programme FACScan Research and displayed on a four decade log scale.

*Conjugation of Monoclonal Antibodies with Fluorescein Isothiocyanate*

The immunoglobulins (HMFG1, SM3) were first dialysed for 24hrs in a carbonate buffer (0.25 M sodium carbonate pH 9.0 containing 0.1 M sodium chloride) to give a protein concentration of 10-20 mg/ml. 0.05mg of fluorochrome isothiocyanate (FITC) per mg of protein was added and mixed at $4^0$ C for 12hrs. The mixture was then applied to a Sephadez G25 column (3 x 15cm) and eluted with PBS. Two coloured fractions were visible in the column and the first one was collected (conjugate) while the second was discarded (unbound fluorochrome).

The fluorescein/protein ratio was calculated by measuring the absorbance of the conjugate at 280 and 495nm:

$$\text{Molar ratio} = \frac{2.87 \times A495}{A280 - 0.35 \times A495}$$

The fluorescein/protein ratio for HMFG1 and SM3 was calculated at 0.55.

*Sample Preparation*

Tumour cells were prepared from subconfluent cultures by trypsinization with trypsin/EDTA (0.25%/1mM), washed (x2) in phosphate buffered saline (PBS) by centrifugation at 200g for 5 minutes and finally resuspended in PBS. To enable intracellular monoclonal antibody labelling of cytokeratins by MNF 116, cells were permealized by the addition of 70% alcohol,

incubated on ice for 30 minutes and washed (x2) in PBS. This stage was not necessary for labelling with surface binding monoclonal antibody. Ten ul of the appropriate MAB was then added to $10^6$ cells suspended in 200ul PBS and incubated on ice, in the dark, for 20 minutes. Cells were washed and resuspended in 0.5 ml PBS for flow cytometric analysis.

*Erythrocyte Lysis*

For flow cytometric analysis of whole blood, erythrocytes were first removed using an ammonium chloride erythrocyte lysing solution. The lysing solution was mixed with whole blood in a ratio of 4:1 and incubated at room temperature for 10 minutes with gentle mixing. A pellet of peripheral blood leucocytes was prepared by centrifugation at 200g for 5 minutes, washed (x2) in phosphate buffered saline (PBS) and finally resuspended in PBS.

The osmotic fragility of erythrocytes ensures that this technique is specific for erythrocyte lysis. A preliminary study using a tumour cell suspension confirmed that the lysis solution did not disrupt tumour cell membranes.

**EXPERIMENTS**

## 1 One Colour Flow Cytometric Analysis

### 1.1 Studies to determine monoclonal antibody binding affinity for tumour cells

The homogeneous expression of surface and cytoplasmic antigens in benign breast epithelial cells is altered in the transformation to the malignant phenotype. Malignant breast cells exhibit a broad spectrum of antigenic expression and either surface or intracellular antigens may be preferentially retained. This experiment was designed to establish whether fluorochrome-conjugated monoclonal antibodies reacting with cytokeratins or cell membrane antigens differed in their ability to label a tumour cell population.

Tumour cells were prepared from subconfluent cultures and $10^6$ cells from each cell line (ZR 75, MCF 7, MB/MDA 231) were labelled with 10ul of MAB (FITC conjugated HMFG1, SM 3, EMA, Ber-EP4, MNF 116) as described above. Cell suspensions were analysed by FACScan and 10, 000 events were collected in list mode for multiparametric analysis using the FACScan software programme. The flow cytometer was calibrated for maximal tumour cell detection by adjustment of gain and amplitude settings using an unlabelled tumour cell sample (negative control). The limit of sensitivity of the FACScan was 1000 FITC equivalent molecules per cell

and the resultant range of cellular fluorescence was displayed on a four decade log scale.

**RESULTS**

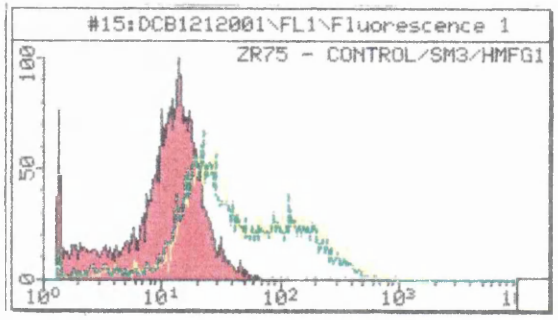
The results of monoclonal antibody labelling with FITC of the three tumour cell lines are shown in Figure 13 and 14. A logarithmic scale of green fluorescence (FL1) is plotted on the horizontal axis and the number of events (cells) is plotted on the vertical axis. The red graph on each histogram represents a control population of unlabelled tumour cells expressing low intensity green fluorescence due to endogenous production of naturally fluorescent molecules from culture medium (autofluorescence). Figure 13a demonstrates that HMFG1 and SM3 produce an identical fluorescent pattern with all three cell lines (green and yellow graph respectively) suggesting that they identify a closely related epitope. Figures 13b, 14a, and 14b represent the results of MAB labelling of tumour cells with anti-EMA, Ber-EP4 and MNF 116 respectively. It can be seen that for all three tumour cell lines, the anticytokeratin antibody, MNF 116, gives the strongest intensity of fluorescence.

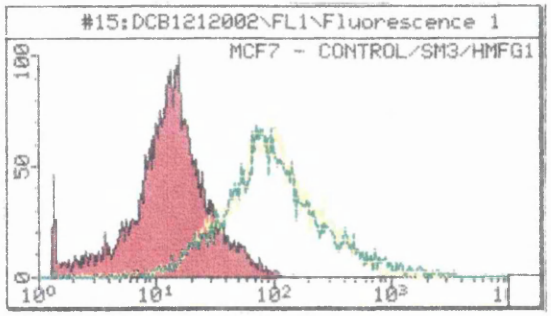
**1.2 Tumour cell/leucocyte separation by one colour flow cytometry**

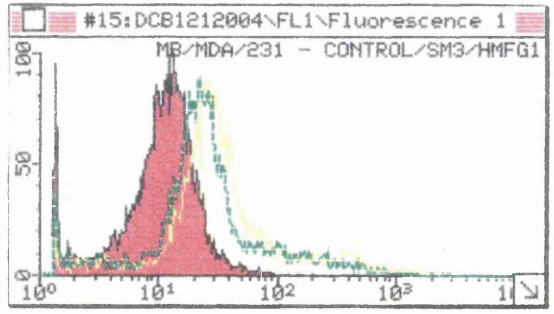
This experiment was designed to assess the ability of one colour flow cytometry to separate tumour cells and

Figure 13.
Analysis of MAB-labelled tumour cells.

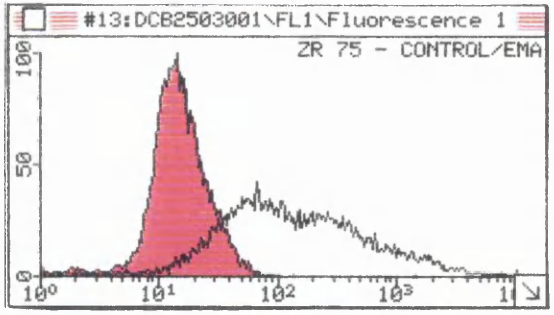
a. SM3 / HMFGI

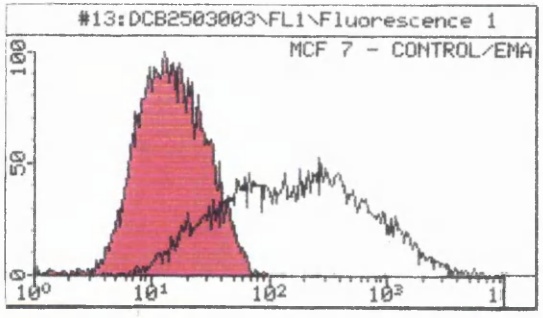


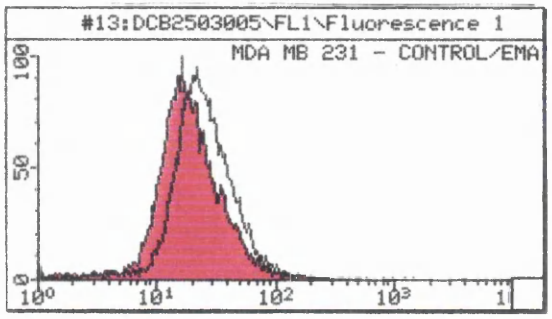




b. EMA



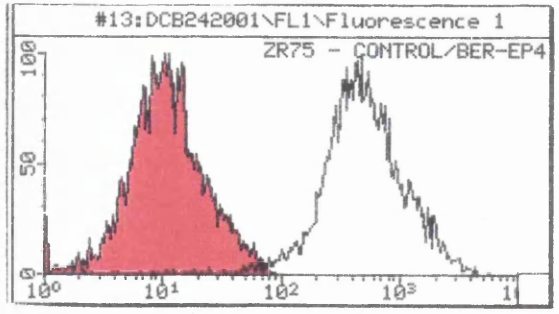



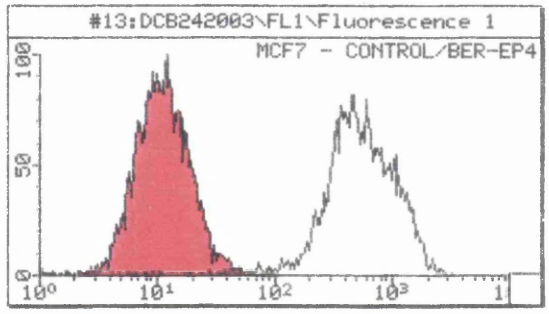



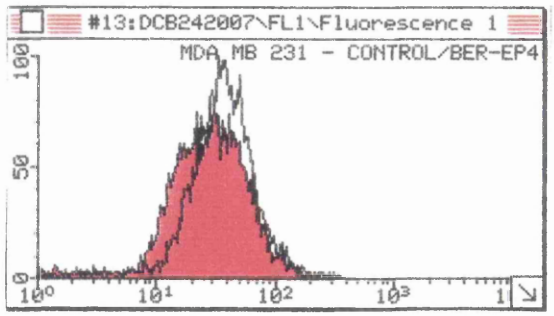

(Original In Colour)

Figure 14.
Analysis of MAB-labelled tumour cells.

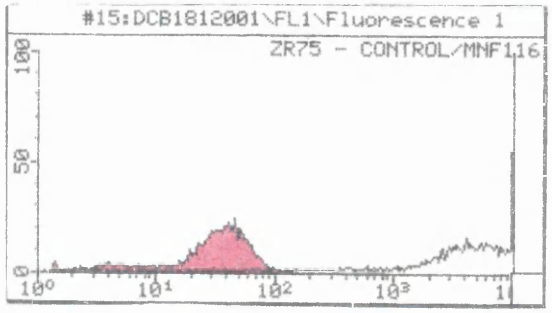
a. BER - EP4

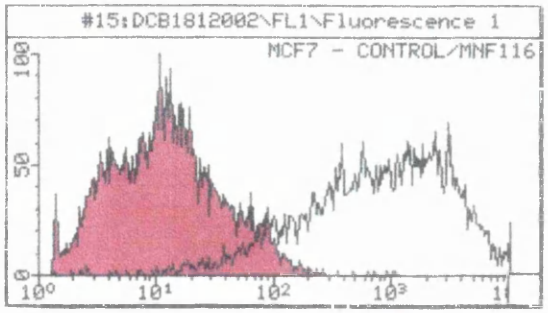


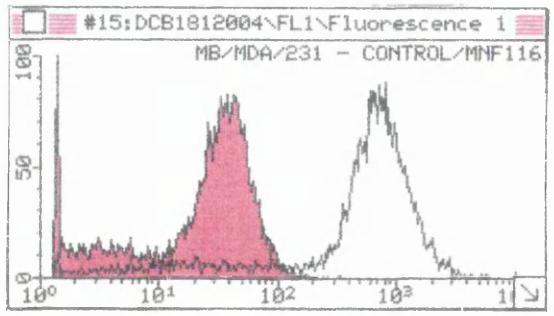




b. MNF 116




(Original In Colour)

leucocytes using a fluorochrome conjugated monoclonal antibody specific for cytokeratin 18.

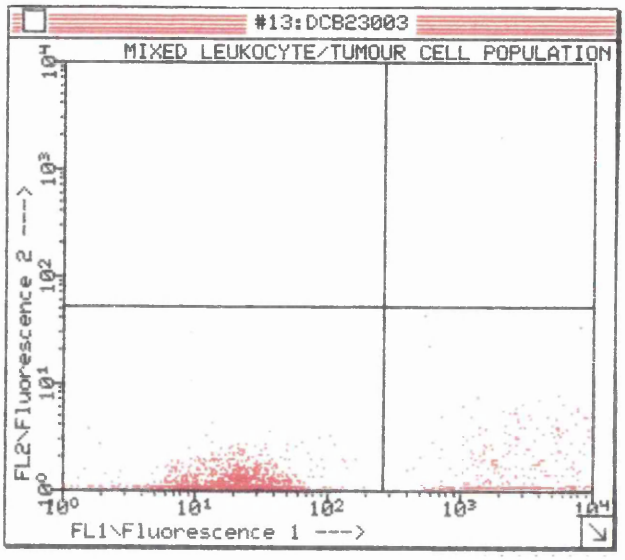
Cultured breast carcinoma cells (ZR75) were seeded into a 1 ml suspension of peripheral blood leucocytes prepared by lysis of whole blood (see methods section, Chapter 5), centrifuged at 200g for 5 minutes and resuspended in 200 ul of PBS prior to labelling with FITC/MNF 116 as previously described. Two control samples were prepared; a suspension of $10^6$ peripheral blood leucocytes served as a negative control while a suspension of $10^6$ ZR 75 tumour cells served as a positive control. Analysis on FACScan was carried out using the gain/amplitude settings of experiment 1.

**RESULTS**

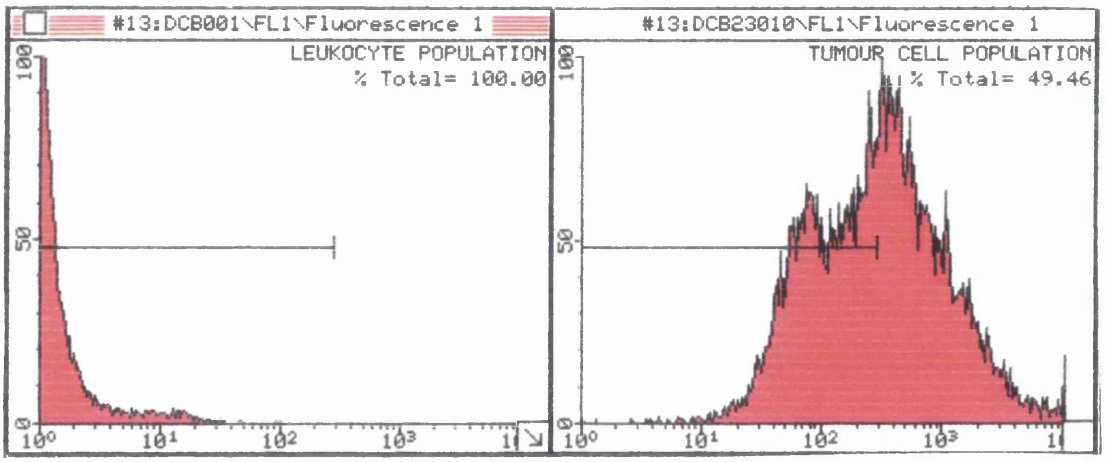
One colour flow cytometric analysis enables separation of tumour cells and peripheral blood leucocytes (Figure 15a). Tumour cells form a distinct population of highly fluorescent cells due to the binding of the fluorochrome-conjugated anticytokeratin antibody. In contrast, peripheral blood leucocytes fail to bind the epithelial-specific antibody and exhibit low intensity autofluorescence. On closer inspection it can be seen that this separation is incomplete and there is an overlap between highly autofluorescent leucocytes and weakly fluorescent tumour cells (Figure 15b). 49.46% of tumour cells express a similar intensity of fluorescence to leucocytes. Therefore the exclusion of

Figure 15.

One colour separation of leucocyte/tumour cell populations.




a.




b.

a. Scattergram of leucocyte tumour/cell populations.
b. Percentage FL1 overlap.

(Original In Colour)

all leucocytes during FACScan analysis of blood for tumour cells by one colour flow cytometry would have been associated with a 50% loss of tumour cells.

## 2 Two Colour Flow Cytometric Analysis

### 2.1 Tumour cell/leucocyte separation by two colour flow cytometry

An attempt was made to enhance the separation of tumour cells and peripheral blood leucocytes using two colour analysis. This required dual labelling of blood samples with tumour and leucocyte specific monoclonal antibodies conjugated to different fluorochromes. Tumour cells labelled with FITC expressed green fluorescence (FL 1) while leucocytes labelled with phycoerythrin (PE) expressed red fluorescence (FL 2).

**Antibodies that react with peripheral blood leucocytes**

*Anti-LFA-1*

This murine monoclonal antibody was produced by standard hybridoma techniques and recognises a cell surface a/b heterodimeric glycoprotein of 180kDa molecular weight within the CD11a cluster. The CD11a antigen is a member of the integrin supergene family of adhesion molecules and binds to ICAM-1, mediates cell-cell adhesion and may participate in lymphocyte activation. All leucocytes, myeloma and neuroectodermal

cells show strong reactivity. 100% activated T cells, 99% peripheral lymphocytes, 97% peripheral monocytes and 97% peripheral granulocytes react positively. This antibody was obtained conjugated to the red fluorochrome Phycoerythrin (PE) (Serotec, Oxford, U.K.).

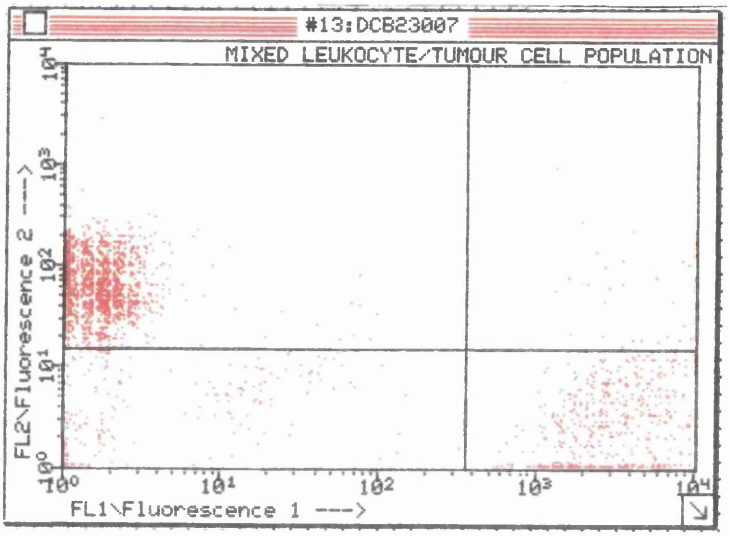
$10^6$ ZR75 cells were diluted in PBS and added to $10^6$ peripheral blood leucocytes. In addition two control samples were prepared; a suspension of $10^6$ peripheral blood leucocytes served as a negative control while a suspension of $10^6$ ZR75 tumour cells served as a positive control. The specimens were resuspended in 100 ul of PBS and permealized with 70% ethanol as previously described. Ten ul of MNF 116 and anti-LFA were added and samples were then incubated for 20 minutes in the dark on ice. After washing with PBS, specimens were resuspended in 0.5 ml for FACScan analysis.

**RESULTS**

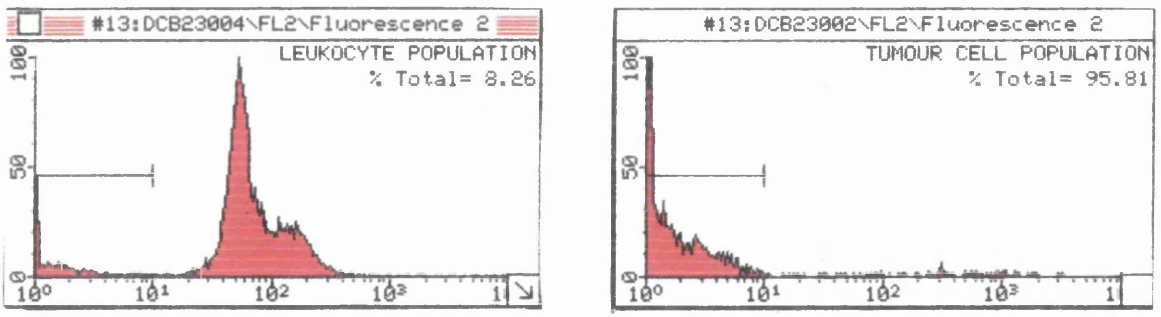
Figure 16a shows the separation of tumour cell and leucocyte populations achieved by two colour FACScan analysis. The majority of PE-labelled leucocytes exhibit high red fluorescence and can easily be distinguished from tumour cells with low red fluorescence. On closer inspection it can be seen that this separation is incomplete and a small proportion of PE-labelled leucocytes (8.26%) express a similar level

Figure 16.

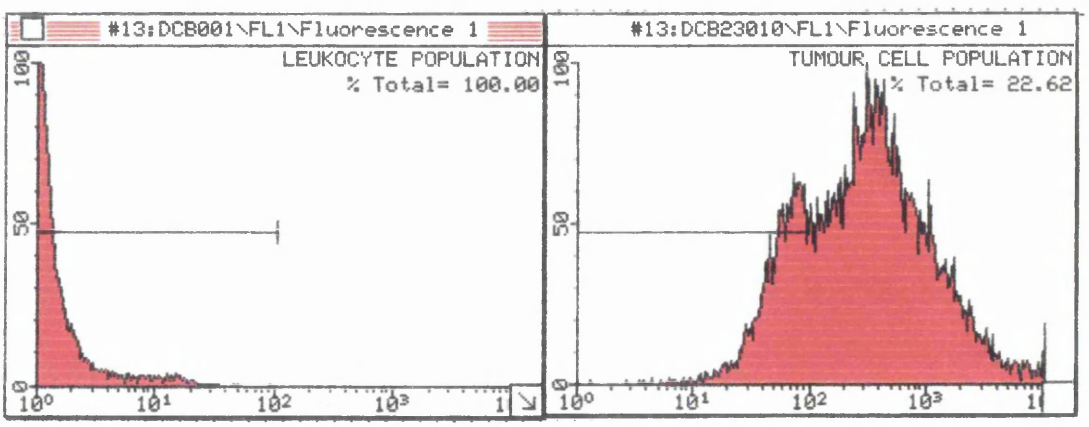
Two colour separation of leucocyte/tumour cell populations.




a.




b.




c.

a. Scattergram of leucocyte tumour/cell populations.

b. Percentage FL2 overlap.

c. Percentage FL1 overlap.

(Original in Colour)

of red fluorescence to tumour cells (figure 16b). The exclusion of those leucocytes with high red fluorescence (FL2 channel) prior to analysis for green fluorescence (FL1 channel, figure 16c), enabled the complete exclusion of leucocytes with green fluorescence in combination with a 22.62% exclusion of tumour cells.

In summary, two colour flow cytometric analysis of blood for tumour cells reduced tumour cell loss to less than 25% after exclusion of all leucocytes.

**2.2 Sensitivity Assay**

The viability of circulating tumour cells is short lived and many may be lost or destroyed during the processing of blood for FACScan analysis. A sensitivity assay was therefore performed to guage the overall efficiency of tumour cell recovery from blood.

ZR75 cells were serially diluted in PBS and added to 1 ml samples of blood. Suspensions of ZR 75 cells at 10, 100, 1000, 10000, and 100000/ml blood were made. Erythrocytes were lysed and samples labelled with MNF 116 and anti-LFA as previously described. Each dilution was repeated 5 times. Gating criteria for FACScan analysis were established using an unseeded leucocyte sample as a negative control.

**RESULTS**

Table 7 shows that the number of cells with high FL1/low FL2 fluorescence increased with an increasing concentration of ZR 75 cells in blood. It can be seen that the efficiency of tumour cell recovery varies according to the number of tumour cells initially seeded in blood. At low tumour cell concentrations (< $10^3$ cells/ml) recovery was inefficient and less than 50% of seeded tumour cells were detected. At high concentrations the efficiency of recovery was greater such that $10^5$ cells could be recovered with an efficiency of 67.3%.

**PATIENT STUDY**

**MATERIALS AND METHODS**

**Blood Sampling Procedure**

Approval for this study was granted by the Western Infirmary Ethical Committee. Fourteen patients with breast cancer and four patients with benign breast disease had a central venous catheter inserted under local anaesthetic, into the antecubital vein in the anticubital fossa on the ipsilateral side of the breast lesion. Under radiological guidance the catheter was advanced into the subclavian vein and the position of the tip ascertained by the injection of radiopaque dye. During sampling a venous tourniquet was placed on

Table 7

# Efficiency of Tumour Cell Recovery from Blood by Two Colour Flow Cytometric Analysis

| No. of ZR 75 cells/ml blood | Sample mean no. of ZR 75 cells | Efficiency of recovery (%) |
|---|---|---|
| 10 | 0.2 | 2.0 |
| 50 | 5.6 | 11.2 |
| $10^2$ | 19.9 | 19.9 |
| $10^3$ | 456.7 | 45.7 |
| $10^4$ | 6454.2 | 64.6 |
| $10^5$ | 67320.8 | 67.3 |

the ipsilateral upper arm to reduce the dilutional effect of blood returning from the upper limb.

A 5 ml pre-operative sample was taken immediately after insertion of the catheter. A further 5 ml intra-operative sample was taken at the point of maximal tumour manipulation. For "lumpectomy" this coincided with grasping of the tumour by forceps prior to excision, and for mastectomy coincided with lateral stripping of the breast from the chest wall. A post-operative 5 ml sample was taken 24 hours after completion of the surgical procedure. All samples were immediately placed in EDTA anticoagulated tubes.

**Sample Analysis**

All samples were immediately treated with erythrocyte lysing solution and resuspended in PBS for dual antibody labelling with FITC-conjugated MNF 116 and PE-conjugated anti-LCA as previously described. Gating criteria for FACScan analysis were established using an unseeded leucocyte sample, prepared from a healthy female volunteer, as a negative control. The maximum green (FITC) fluorescence on this sample was then used to set the lower limit of the green gate to screen the patient samples for tumour cells.

**RESULTS**

No cells expressing high fluorescence were detected by FACscan analysis in any of the peri-operative blood

samples from patients undergoing surgery for either benign or malignant breast disease.

**DISCUSSION**

The purpose of the preliminary experiments was to determine the specificity and sensitivity of flow cytometry to detect breast cancer cells in blood prior to applying it to a prospective clinical study in patients to determine whether the presence of these cells in the bloodstream peri-operatively is a predictor of poor prognosis. In contrast to immunocytochemical techniques where the malignant nature of an antibody-labelled cell can be confirmed morphologically, the diagnosis of malignancy by flow cytometry is solely determined by the single parameter of fluorescence. Since the number of peripheral blood leucocytes per ml of blood is approximately $10^7$, the inclusion of only 1% of peripheral blood leucocytes would lead to a significant overestimation of tumour cell numbers (approximately $10^5$ false positive events/ml of blood). In these experiments it was therefore essential that the fluorescent threshold for tumour cell detection was sufficiently high to exclude all peripheral blood leucocytes.

The results of the first experiment demonstrated that a monoclonal antibody directed against the epithelial cytoskeleton produced optimal labelling of cultured breast carcinoma cells. The specificity of this antibody is well characterised and it reacts with

the majority of primary breast cancers but not with normal blood elements (Lazarides, 1980).

One colour flow cytometric analysis of a mixed leucocyte/tumour cell population using the anticytokeratin antibody showed incomplete separation of antibody-labelled tumour cells and peripheral blood leucocytes. Consequently, the exclusion of all leucocytes during blood analysis would have resulted in the loss of half of the total number of tumour cells. Two colour analysis enhanced tumour cell detection by enabling the exclusion of all peripheral blood leucocytes with less than a 25% loss in tumour cells. An assessment of sensitivity showed that the efficiency of tumour cell recovery varied according to the number of cells initially seeded into normal blood. The greater efficiency in tumour cell recovery at high cell concentrations emphasizes that the proportionately fewer cells detected after monoclonal antibody labelling of low cancer cell concentrations was due to mechanical and dispersion factors rather than inherent differences in cell fluorescence.

A pilot clinical study of two colour flow cytometry did not demonstrate any circulating breast epithelial cells in patients with benign or malignant breast disease in the peri-operative period. These results are in contrast to the preliminary findings of Nusbaum (1991) who used flow cytometry to identify anticytokeratin-labelled urothelial cells in the blood of patients undergoing urological surgery. This study

demonstrated the presence of circulating tumour cells in the peripheral blood of 7 of 11 patients (64%) although positive events were rare and analysis for more than an hour was frequently required to detect 1 cytokeratin positive cell per $10^6$ leucocytes. However, gating criteria using a negative control were inferior to our study (one colour analysis of $10^4$ leucocytes vs two colour analysis of $10^6$ leucocytes) and thus such a low incidence of positive events after prolonged analysis could represent ungated leucocytes. Furthermore, it should be stressed that while preliminary studies demonstrated strong staining of cell lines with anticytokeratin antibody, the intensity of primary tumour staining was more variable. It is therefore possible that gating criteria may have excluded a significant proportion of primary tumour cells with low fluorescence.

While the results of our study may indicate an absence of tumour cell shedding during operative manipulation, a failure of the sampling technique cannot be discounted. Central venous catheterisation enabled samples to be obtained before the majority of tumour cells were removed by filtration in the pulmonary microcirculation (Fidler, 1970; Glaves, 1980). Direct cannulation of tumour draining veins was unsuccessful, either because the axillary or internal mammary veins were not identified or were too small to enter, and the catheter was thus left in a high flow, wide diameter vein. Animal studies have demonstrated

that cell shedding during manipulation is rapid and short-lived (Cliffton and Agostino, 1961; Romsdahl et al, 1965; Gazet, 1966). Since sampling was performed at a fixed point in time it is possible that if the catheter was not in close proximity to tumour draining veins and sampling did not coincide with maximal tumour manipulation, then tumour emboli could potentially escape detection due to rapid haemodilution and flushing into the systemic circulation. Axial flow, which keeps the cellular components of blood close to the vessel wall while maintaining the catheter within the central lumen, could have enhanced this loss. Continuous or intermittent operative venous sampling might have provided a more fail-safe mechanism to ensure tumour cell detection, but the processing of such a large volume of blood would have not have been practical both from the the point of view of the quantity of antibody required and the duration of analysis by FACScan.

In summary, flow cytometry has failed to demonstrate peri-operative tumour cell shedding in patients with breast cancer. It has not however, provided conclusive evidence that surgical manipulation does not enhance cell shedding, in view of its unreliability in detecting tumour cells at low concentrations as well as the problems encountered with venous sampling. It is clear that a more sensitive technique of tumour cell detection is required in order

to establish the significance of peri-operative tumour manipulation on the metastatic process.

# CHAPTER 6

## REVERSE TRANSCRIPTASE AND POLYMERASE CHAIN REACTION TO DETECT BREAST CARCINOMA CELLS IN PERIPHERAL BLOOD

### INTRODUCTION

The preceding chapter has shown that immunofluorescent analysis of blood by flow cytometry is not a sensitive method to detect circulating tumour cells in patients with breast cancer. It is clear that a more sensitive and specific technique is required to detect tumour cells in blood in order to investigate the hypothesis that intra-operative manipulation of malignant neoplasms facilitates tumour cell dissemination into the bloodstream.

The Polymerase Chain Reaction (PCR) is a newly developed molecular biology technique that has the ability to amplify a particular sequence of either DNA or RNA and can potentially detect one cell in $10^6$ normal cells (Mullis et al, 1986; Saiki et al, 1985; Schochetman et al, 1989). It therefore has the necessary sensitivity to detect small numbers of tumour cells in peripheral blood by amplification of specific DNA sequences. No tumour-specific genes have been identified for breast cancer but since epithelial cells are not normally present in peripheral blood, genes specific to epithelial tissue might be suitable targets for the detection of circulating malignant breast epithelium. To target epithelial-specific gene

transcription requires a technique whereby complementary DNA (cDNA) can be prepared by reverse transcription of blood messenger RNA (mRNA). This ensures that only genes that are being actively expressed are represented in the cDNA. The aim of the following series of experiments was to assess the ability of reverse transcriptase and the polymerase chain reaction to detect circulating malignant breast epithelium.

The level of gene expression varies according to the prevalence of the encoded protein. It was therefore decided to compare two genes encoding structural epithelial proteins for which the genomic organisation and cDNA sequence were known. Cytokeratin 18 (CK 18) is one of the human cytokeratin polypeptides which constitutes the intermediate filaments within the cytoplasm of all epithelial cells (see previous chapter). CK 18 is expressed by the majority of breast carcinomas (Jarasch et al, 1988; Tsubura et al, 1991) and while it is also found in diverse simple epithelium, trachea and transitional epithelium, it is not expressed by haemopoetic and vascular endothelial cells (Moll et al, 1982).

The DF3 human breast carcinoma-associated antigen was prepared against a membrane-enriched fraction of a human breast tumour (Kufe et al, 1984). It is a member of the polymorphic epithelial mucins and is expressed on the apical borders of secretory breast epithelial cells (Kufe et al, 1984). It is found in the

cytosol and on the cell membrane of adenocarcinomas of breast, ovary and lung and mesothelial cells (Szpak et al, 1984). Like CK 18 it is not normally expressed by haemopoetic and vascular endothelial cells.

Preliminary studies were performed to establish the specificity of CK 18 and DF 3 gene expression for breast epithelial cells and the sensitivity of reverse transcriptase PCR in detecting breast epithelial cells in blood.

## MATERIALS AND METHODS

*The Polymerase Chain Reaction (PCR)*

The basic requirements for PCR are oligonucleotide primers, the DNA or RNA sequence to be amplified, DNA polymerase, various buffers and nucleotides necessary for DNA synthesis. The PCR method is based on repeating cycles of: denaturation of double-stranded DNA, annealing of oligonucleotide primers and primer extension using DNA polymerase. Denaturation occurs when the reaction temperature is raised above $90^0$ C. In addition, at this temperature the complementary DNA strands remain separate in solution. Lowering of the reaction temperature to between $55^0$C and $72^0$C enables annealing of oligonucleotide primers to denatured DNA. The primers bind to sequences of DNA flanking the portion of DNA to be amplified and because they are non-complementary they will not anneal to each other

but only to opposite DNA strands. The formation of primer-DNA complexes as opposed to re-annealing of the native DNA strands is enhanced by a vast excess of primer in the reaction mixture. The final step of primer extension is accomplished by using *Taq* DNA Polymerase to bind to the primer DNA complex and synthesise a complementary DNA strand in the 5' to 3' direction. This enzyme, purified from Thermus Aquaticus, is thermostable up to $98^0$C and therefore will not degrade at high denaturation temperatures. Furthermore, the use of *Taq* Polymerase enables the annealing temperature to be raised, thereby increasing the binding of the primer to the desired DNA sequence and reducing non-specific binding to DNA outside the region of interest. In other words, the higher the temperature the more specific the binding will be between the primer and its intended DNA "match".

In each cycle there is a doubling of the DNA product such that in a typical PCR analysis of 20-40 cycles of amplification there will be a million-fold amplification of the desired gene product.

*Design of Oligonucleotide Primers*

Primers were devised from the published gene sequences for human cytokeratin 18 (CK 18) (Kulesh and Oshima, 1988) and the 5' region of the human DF3 antigen (Abe et al, 1989) (Figure 17). Each primer consisted of 20 bases with an average Guanine + Cytosine content of about 50% in order to create a similar annealing

# Primer Design

FIG 17 a

## 5' Region of CK 18 Gene

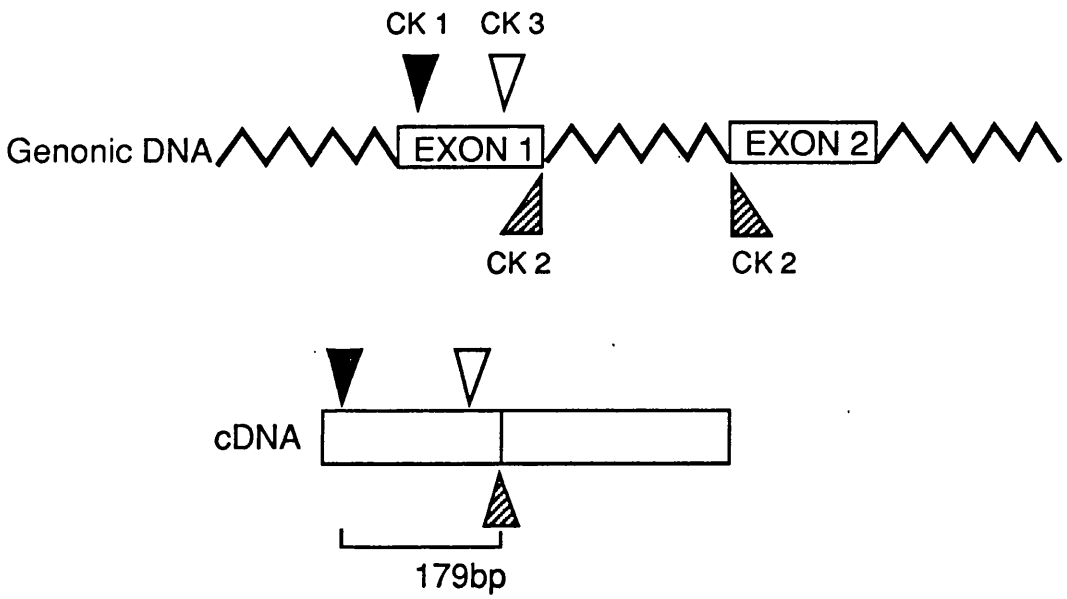
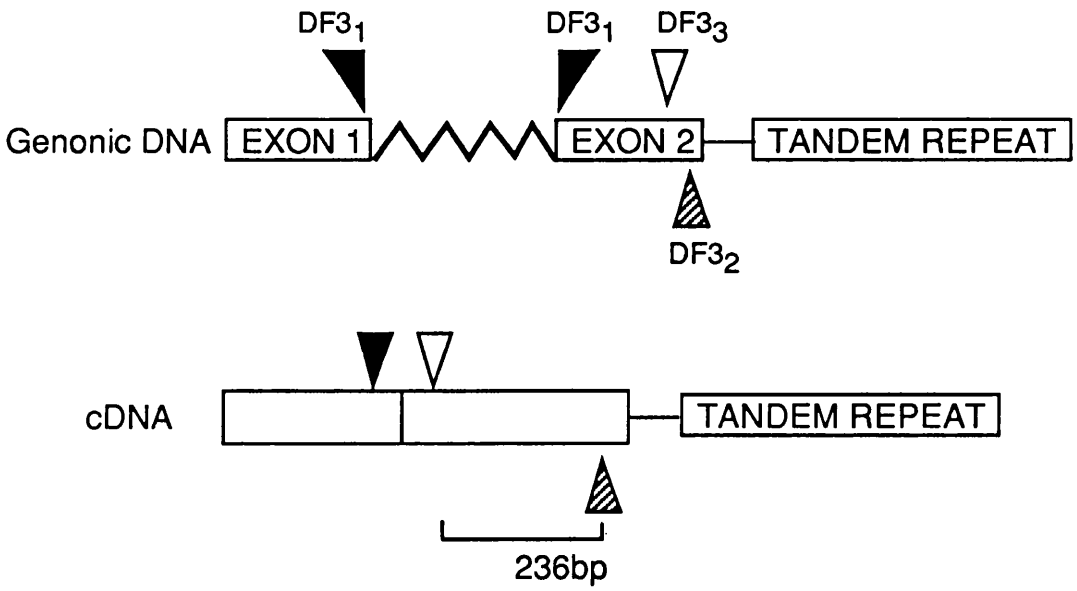



FIG 17 b

## 5' Region of DF 3 Gene




**Primer Base Sequence**

CK 1 - 5' AGACCATGCAAAGCCTGAAC 3'
CK 2 - 5' GAAGATCTGAGCCCTCAGGT 3'
CK 3 - 5' TCTTACCTGGACAGAGTGAG 3'

DK 3$_1$ - 5' CACAGTGCTTACAGCTACCA 3'
DF 3$_2$ - 5' AGGTGGCAGCTGAACCTGAA 3'
DF 3$_3$ - 5' GTTCAGTGCCCAGCTCTACT 3'

temperature between 55 and 80$^0$C. The possibility of mispriming was reduced by avoiding stretches of polypurines, polypyrimidines and palindromic sequences. In addition, complementarity was avoided at the 3' ends of primer pairs to prevent the formation of primer-dimer artifacts which could reduce the yield of desired product.

In order to prevent amplification of genomic DNA, one of the pair of primers for both genes was designed to hybridise to cDNA across an intron site. This ensured that the primer would only recognise and amplify the gene if it was being expressed by transcription into RNA. The size of the DNA fragment defined by these primers was 179 base pairs for CK 18 and 263 for DF3. A third primer (probe) complementary to the central portion of the PCR product was utilized for Southern Hybridization.

*Analysis of PCR product by gel electrophoresis*

Electrophoresis through agarose gels enables the separation and identification of DNA fragments. The location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1-10 ng of DNA can be detected by direct examination of the gel in ultraviolet light. Agarose gels are usually run in a horizontal direction and when an electric field is applied across the gel, DNA, which is negatively charged at neutral pH,

migrates towards the anode. The rate of migration is determined by a number of parameters including the agarose concentration, molecular size of the DNA, confirmation of the DNA, composition of the electrophoresis buffer and the applied voltage. DNA from 200 base pairs to approximately 50 kilobases in length can be separated on agarose gels of various concentrations. For the purpose of these experiments 2% agarose gels were used in Tris-borate (TBE) buffer and run at 5 volts/cm. Photographs of gels were made using transmitted ultraviolet light (wavelength 302 nm) with Polaroid Type 57 film.

*Analysis of PCR Product by Southern Hybridization*

This transfer technique was first described by Southern (1975) and enables the specificity of the PCR product to be confirmed. The DNA on the agarose gel is denatured in situ and transferred by capillary transfer from the gel to a solid support (usually a nitrocellulose filter or nylon membrane). The relative positions of the DNA fragments are maintained during their transfer to the filter. The DNA attached to the filter is hybridized to a radiolabelled oligonucleotide probe and autoradiography is used to locate the positions of bands complementary to the probe.

## PRELIMINARY EXPERIMENTS

### Experiment 1 A comparison of CK 18 and DF 3 gene expression in primary breast carcinomas and peripheral blood.

*1.1 RNAzol™ B Extraction of RNA*

In order to extract RNA from blood, peripheral blood leucocytes were prepared by erythocyte lysis of blood obtained from healthy volunteers (see Chapter 5, materials and methods). Leucocyte suspensions were then sedimented and homogenised with RNAzol™ B solution (0.2 ml/$10^6$ cells) by repeat pipetting. To extract RNA from primary tumour samples, the tissue was first frozen at -70$^0$C and ground to a fine powder before the addition of RNAzol™ B. Chloroform was added (0.2 ml/ml lysate) to the prepared samples which were vortexed briefly before being chilled on ice for 5 minutes. Samples were spun at 12 000 g for 15 minutes at 4$^0$C. After centrifugation, the RNA in the aqueous phase (DNA and proteins in the interphase and phenol phase) was transferred to sterile tubes and precipitated with an equal volume of isopropanol for 15 minutes at 4$^0$C. Samples were then centrifuged for 15 minutes at 12 000 g (4$^0$C). The supernatant was removed and the RNA pellet washed once with 0.8 ml of 75% ethanol by vortexing followed by centrifugation for 8 minutes at 7500 g (4$^0$C). The purified RNA pellet was dried briefly under vacuum for 10-15 minutes and then dissolved in 50 ul of

water. A small volume was removed for spectrophotometric assessment of the concentration. An optical density of 1, at a wavelength of 260 nm, represented an RNA concentration of 40 ug/ml. (To prevent degradation of RNA, water and all containers were autoclaved and treated with diethyl pyrocarbonate (DEPC) to remove Ribonuclease and gloves were worn at all times to avoid contamination with skin RNase).

*1.2 First-Strand cDNA synthesis and PCR Amplification of cDNA Product*

Reverse transcription of RNA and PCR amplification of cDNA was performed in a single reaction tube using a combined RNA PCR kit (GeneAmp RNA PCR Kit, Perkin-Elmer Cetus, Norwalk, Connecticut, USA). Contaminating DNA was first removed prior to reverse transcriptase PCR by adding 10 IU of RNase-free DNase to each sample and incubating at $37^0$C for 30 minutes. The reaction was then stopped by heating to $95^0$C for 5 minutes and then cooled to room temperature.

*1.2.1 Reverse Transcription Protocol*

For reverse transcription, 1ug of RNA (1 ul) was added to 19 ul of a reaction mixture (total volume 20ul) giving final concentrations of: 1 X PCR buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl), 5 mmol/l magnesium chloride, 1 mmol/l each of dATP, dTTP, dGTP and dCTP, 1 unit RNase Inhibitor, 2.5 units maloney murine leukaemia virus reverse transcriptase and 30

pmol "downstream" primer. Each sample was overlaid with 50 ul mineral oil, heated to $42^0$C for 15 minutes, $99^0$C for 5 minutes and soaked at $5^0$C for 5 minutes.

*1.2.2 PCR Protocol*

For amplification, each reverse transcription reaction sample was diluted with 79 ul of a PCR mixture containing final concentrations of: 1 X PCR buffer, magnesium chloride 2 mmol/l and 2.5 units AmpliTaq DNA Polymerase. 30 pmols of "upstream primer" were dispensed into each sample to give a total volume of 100 ul. The PCR reaction consisted of an initial cycle of denaturation at $93^0$C for 5 minutes, primer annealing at $55^0$C for 5 minutes and polymerisation at $73^0$C for 15 minutes. 30 PCR cycles were carried out ( $93^0$C for 1 minute, $55^0$C for 1 minute and $73^0$C for 5 minutes) and completed by extension at $73^0$C for 15 minutes.

*1.3 Gel Electrophoresis of PCR Product*

1/10 of the PCR product was used for analysis on a 2% agarose gel impregnated with ethidium bromide (0.5ug/ml). The gel was run at a voltage of 5 volts/cm for approximately 2-3 hours. A DNA ladder suitable for sizing double-stranded DNA from 72 to 1353 base pairs (PHI X 174 RF DNA/Hae III Fragments, Gibco Brl) was used for comparison. The gel was then photographed under ultraviolet illumination using a Polaroid camera.

**RESULTS (Figure 18a)**

The expected size of the amplified fragment for CK 18 was 179 bp in size. All RNA samples derived from peripheral blood of healthy volunteers and from primary breast carcinomas gave a band of identical size. Comparison with the DNA ladder indicated that this DNA fragment was slightly smaller than 194 base pairs in keeping with the size of the expected PCR product. The expected size of the amplified fragment for DF 3 was 263 bp in size. Amplification of RNA from all primary tumour samples but not from peripheral blood samples of healthy volunteers gave a band of the expected size.

These results suggest that while the CK 18 gene is expressed in both normal peripheral blood and primary breast carcinomas, the DF 3 gene is only expressed by primary breast carcinomas. Confirmation of these findings was performed by Southern hybridization.

**Experiment 2 Southern hybridization of gene products**

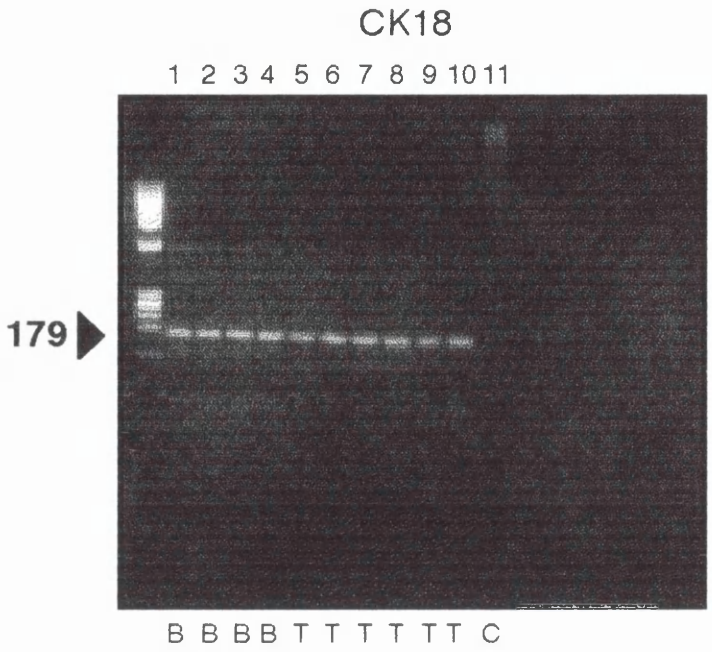
*2.1 Capillary transfer of DNA to nitrocellulose filter*

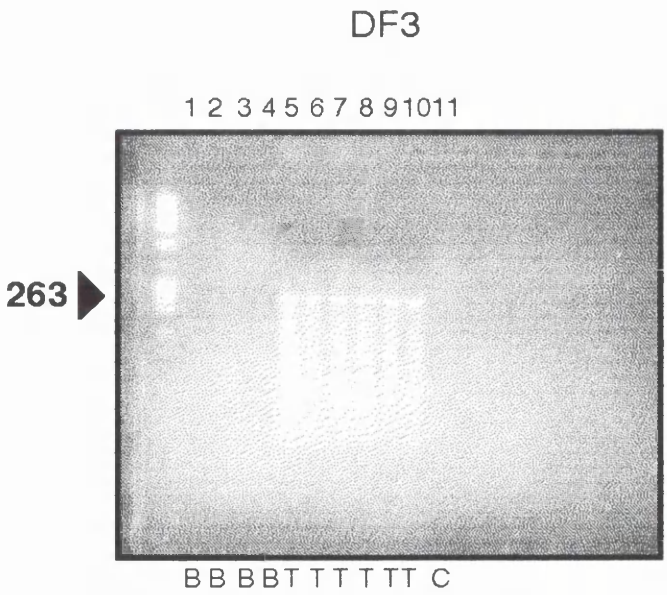
DNA was first denatured by soaking the gel for 30 minutes in 0.5M NaOH and 1.5M NaCl with constant, gentle agitation (x 2), followed by a second gel soak for 30 minutes in 1M Tris HCl (pH 7.5) and 1.5M NaCl (x 2). Denatured DNA was transferred to a nitrocellulose filter (Hybond N+, Amersham, UK) by capillary transfer using a standard protocol (Maniatis et al, 1989).

Figure 18 a

# CK 18 I DF3 expression in Primary Breast Carcinomas and Peripheral Blood

## Electrophoretic Analysis




B Blood
T Primary Breast Carcinomas
C Negative control

*2.2 Hybridization of radiolabelled probes to immobilized DNA*

One hundred nanograms of each oligonucleotide probe was radiolabelled by addition of 2 ul 10x Kinase buffer, 2 ul DTT, $^{32}P$ - ATP and $T_4$ polynucleotide kinase and incubated at $37^0C$ for 30 minutes. The radiolabelled probe was precipitated with 1 ul transfer RNA (2mg/ml), 1 ul 0.2M EDTA (pH 8), 10 ul 7.5M ammonium acetate and 90 ul ethanol on dry ice for 2 hours. Samples were centrifuged for 15 minutes at 12 000 g ($4^0C$) and resuspended in 100 ul of water. Each radiolabelled probe was added to 10 ml of hybridization buffer (7% SDS/0.5M $NaPO_4$, pH 7.4) and sealed in a plastic bag with the appropriate filter. Hybridization was performed at $42^0C$ for 12 hours. Filters were washed in 2x SSC/0.1% SDS at room temperature for 30 minutes followed by 0.1x SSC/0.1% SDS at $42^0$ C for 30 minutes and resealed in plastic bags. Each filter was exposed to x-ray film (Kodak XAR-2 or equivalent) at $-70^0C$ for 12-24 hours to detect DNA fragments.

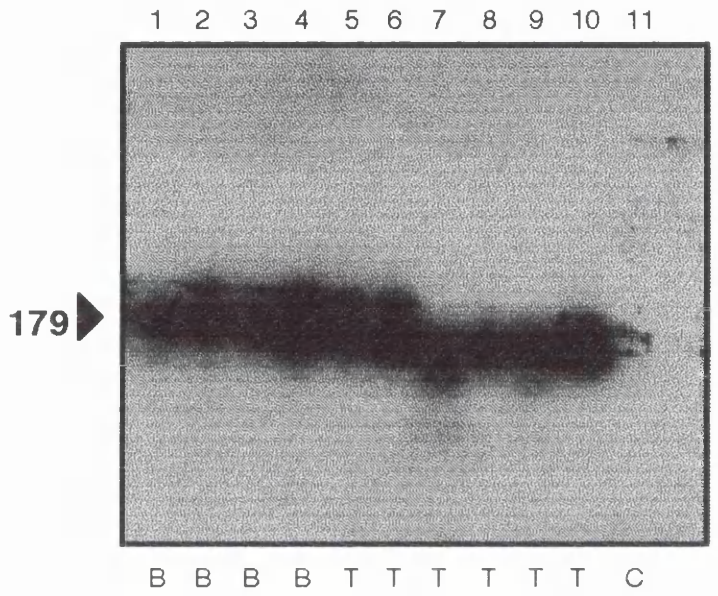
**RESULTS (Figure 18b)**

For CK 18, autoradiography demonstrated a region of localised radiodensity over the site of the amplification product in all samples. This confirmed the PCR product to be the CK 18 gene fragment showing that all primary breast carcinomas and peripheral blood samples of healthy volunteers expressed the CK 18 gene. For DF3, a hot spot coincided with the PCR product in

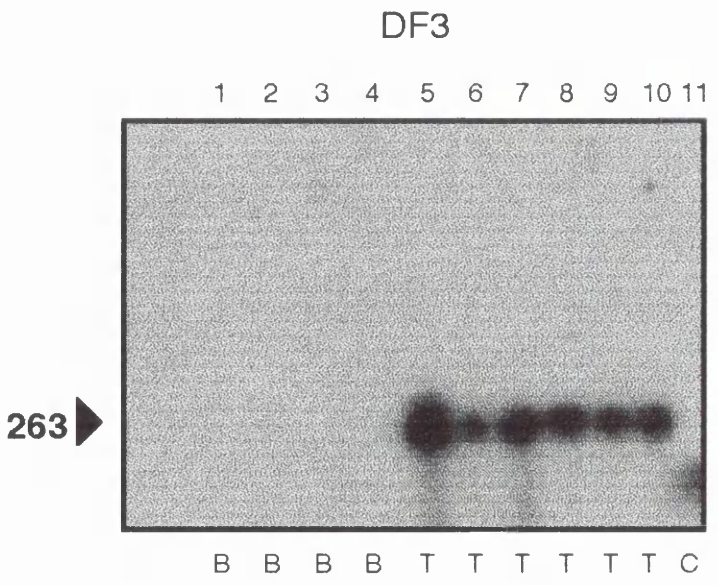
# CK 18 I DF3 expression in Primary Breast Carcinomas and Peripheral Blood

## Autoradiographic Analysis

CK18




DF3




B Blood
T Primary Breast Carcinomas
C Negative control

all primary tumour samples confirming this to be the DF3 gene fragment. There was no evidence of DF3 gene expression in peripheral blood samples of healthy volunteers.

The unexpected presence of the CK 18 gene in normal peripheral blood therefore made it unsuitable as a marker for breast epithelial cells in blood. In contrast, the absence of the DF 3 gene in normal peripheral blood in combination with its universal expression in primary breast carcinomas made it a suitable marker of circulating breast epithelium.

**Experiment 3 Sensitivity assay for reverse transcriptase PCR using the DF3 gene**

A suspension of MCF 7 tumour cells was serially diluted in PBS and 0, 10, $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$ cells added to 5 ml samples of blood. Erythrocyte lysis, RNA extraction, cDNA synthesis and PCR amplification were performed as described previously. Analysis of PCR products required gel electrophoresis on a 2% agarose gel followed by Southern hybridization using a complementary radiolabelled probe. A negative control of blood RNA and a positive control of MCF 7 RNA were also included.

**RESULTS (Figure 19)**

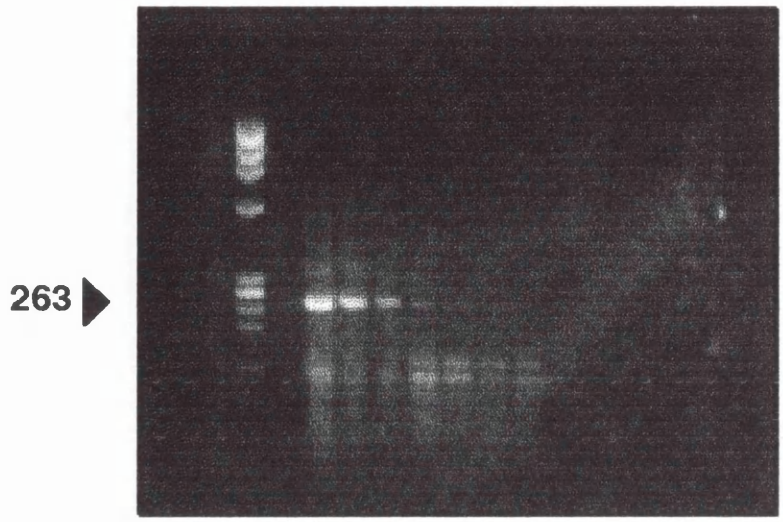
Electrophoretic analysis demonstrated DF 3 gene expression in blood spiked with MCF 7 tumour cells from

Figure 19



# Sensitivity assay for the DF 3 Gene

Electrophoretic analysis




Autoradiography


| | |
|---|---|
| C- | Negative control (Blood) |
| C+ | Positive control (MCF7) |
| 1 | $10^6$ |
| 2 | $10^5$ |
| 3 | $10^4$ |
| 4 | $10^3$ |
| 5 | $10^2$ |
| 6 | 10 |

MCF7 Tumour cells / 5ml blood

a concentration of $10^6$ to $10^3$ cells/5mls of blood. Hybridization with the radiolabelled probe confirmed DF 3 gene expression in spiked blood samples and demonstrated that the intensity of the signal was proportional to the number of tumour cells per blood sample. The sensitivity of autoradiographic analysis was sufficient to detect DF 3 gene expression in blood containing MCF 7 tumour cells at a concentration as low as 10 tumour cells/5ml of blood.

In summary, these preliminary in vitro studies have demonstrated that reverse transcription PCR is a suitable technique for the detection of malignant breast epithelium in blood. Amplification for the DF 3 breast carcinoma-associated gene is specific for breast epithelium since haemopoetic and vascular endothelial cells do not express the gene. Southern hybridization and autoradiography enhance sensitivity thereby enabling the detection of very small numbers of circulating tumour cells.

Having established optimal experimental conditions for the DF3 antigen, reverse transciption PCR was then applied to blood samples collected from patients undergoing surgery for breast cancer. The aim of this study was to investigate whether operative manipulation enhanced tumour cell dissemination via the bloodstream.

**PATIENT STUDY**

Patients undergoing surgery for breast disease were recruited into this study. These were 9 patients with primary operable breast cancer and 3 with benign breast disease. Clinical details are summarised in Table 8. Pre-operative assessment of patients with breast cancer by chest x-ray, liver ultrasound and bone scan excluded those with distant metastases. Approval for this study was granted by the Western Infirmary Ethical Committee.

Five ml peripheral blood samples were obtained 24 hours pre-operatively, intra-operatively and 24 hours post-operatively. Operative samples were taken at the time of maximum surgical manipulation as previously described (Chapter 5). All blood samples were obtained by Vacuutainer puncture and the first 5 mls were discarded to prevent sample contamination from skin. A 5 ml blood sample obtained from a healthy female volunteer and spiked with $10^6$ MCF7 tumour cells served as a positive control. Samples were treated with erythrocyte lysis solution and the residual cell pellet was stored at - $70^0$C. RNA was extracted by the RNAzol™ B technique (0.5 ml RNAzol™ B/5 ml blood) and 1 ug used for reverse transcription PCR using the RNA/PCR kit and DF 3 primers.

PCR products were analysed by electrophoresis on 2% agarose gels with ethidium bromide staining and Southern hybridization.

Table 8

# Clinical and Histological Status of Patients in RT-PCR Study

| Lane No. | Name | Age | Size of Lesion | Histology | Nodal Status | Operation |
|---|---|---|---|---|---|---|
| 2 | FH | 56 | / | Fibrocystic | / | Excision Biopsy |
| 3 | ER | 51 | / | Fibrocystic | / | Excision Biopsy |
| 4 | JM | 45 | / | Fibrocystic | / | Excision Biopsy |
| 5 | LH | 71 | 7cm | Grade III Ductal Carcinoma | +ve | Mastectomy |
| 6 | AY | 68 | 2cm | Grade II Ductal Carcinoma | -ve | Lumpectomy |
| 7 | MB | 57 | 2cm | Grade I Ductal Carcinoma | -ve | Lumpectomy |
| 8 | MM | 53 | 5cm | Grade III Ductal Carcinoma | +ve | Mastectomy |
| 9 | HB | 65 | 2cm | Grade III Ductal Carcinoma | +ve | Mastectomy |
| 10 | MM | 58 | 8mm | Grade I Ductal Carcinoma | +ve | Lumpectomy |
| 11 | MJ | 69 | 2cm | Grade II Ductal Carcinoma | +ve | Lumpectomy |
| 12 | MM | 77 | 7cm | Grade III Ductal Carcinoma | -ve | Mastectomy |
| 13 | MM | 43 | 1cm | Grade I Ductal Carcinoma | -ve | Lumpectomy |

**RESULTS**

*1 Pre-operative (Figure 20a)*

Electrophoretic analysis demonstrated expression of the DF3 gene in the blood sample of the positive control (Lane 1) and in the peripheral blood sample of one patient with primary breast carcinoma (Lane 5). Southern hybridization and autoradiography confirmed the presence of the DF3 gene in the peripheral blood sample of one patient with breast cancer and its absence in all peripheral blood samples of all three patients with benign breast disease.

*2 Operative (Figure 20b)*

In addition to DF3 expression in the peripheral blood sample of the positive control (Lane 1), electrophoretic analysis demonstrated the DF3 gene in the peripheral blood samples of four patients with primary breast carcinoma (Lanes 5, 6, 9 and 11). Southern hybridization and autoradiography further confirmed DF3 expression in the peripheral blood of four patients with breast cancer and its absence in the peripheral blood of all patients with benign breast disease.

*3 Post-operative (Figure 20c)*

Electrophoretic and autoradiographic analysis failed to demonstrate DF3 gene expression 24 hours post-operatively in the peripheral blood of any patient who

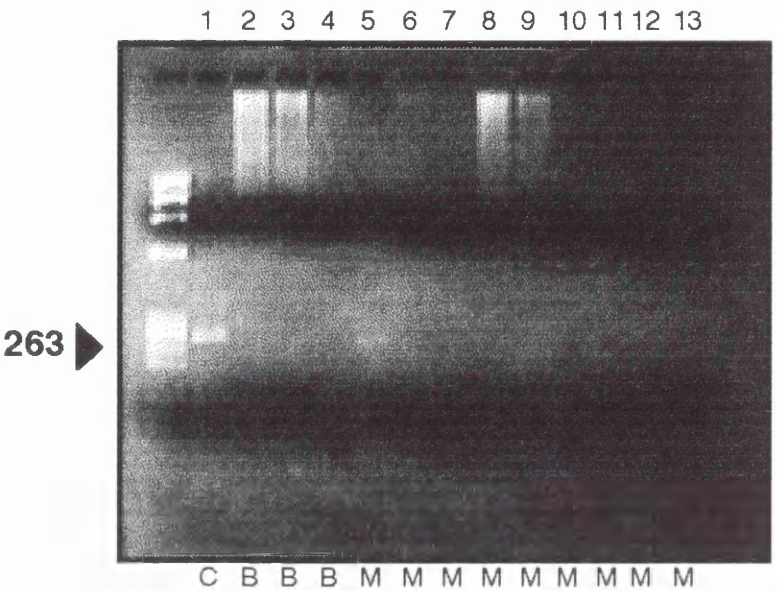
Figure 20a



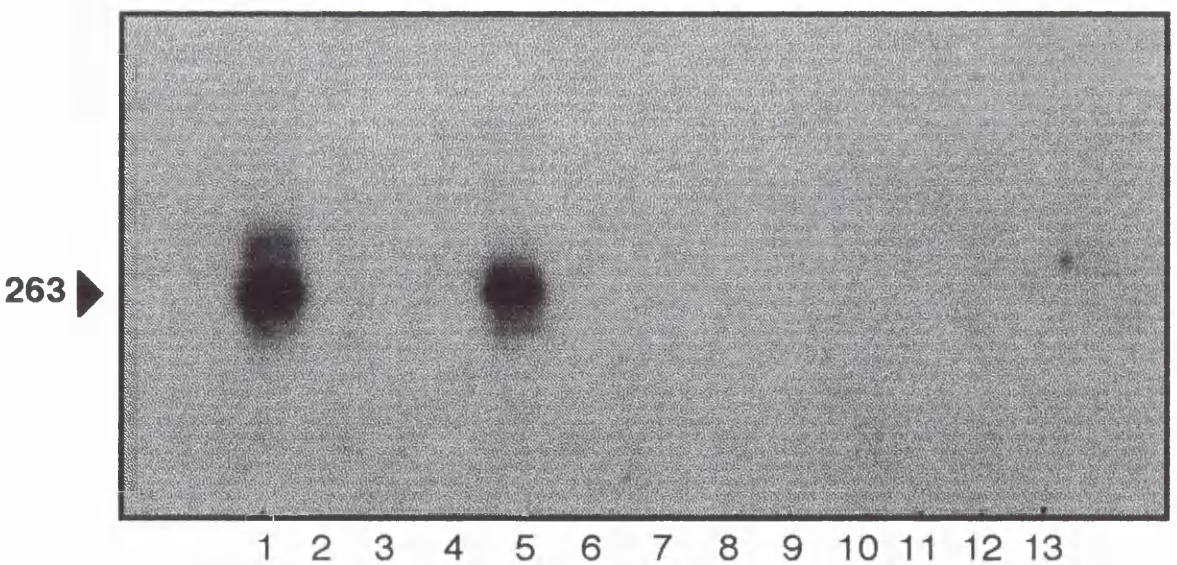
# RT - PCR analysis of peripheral venous blood from patients undergoing surgery for benign and malignant Breast disease

## Pre-Operative

Electrophoretic analysis




Autoradiography



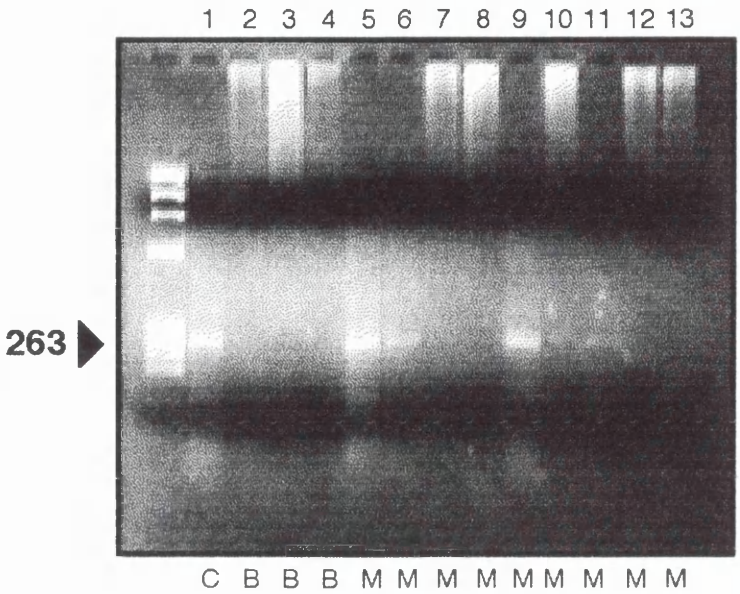

C Positive control
B Benign Breast Disease
M Primary Breast Carcinomas

Figure 20b

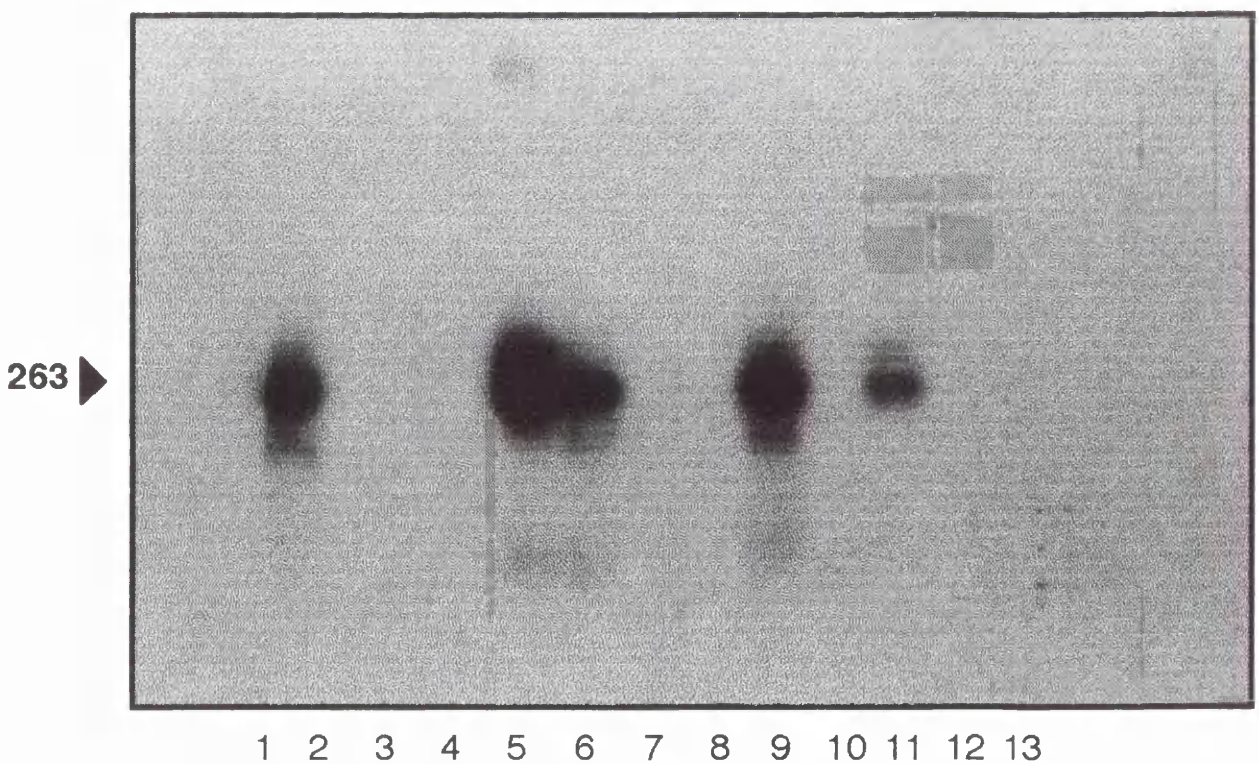


# Operative

Electrophoretic analysis




Autoradiography



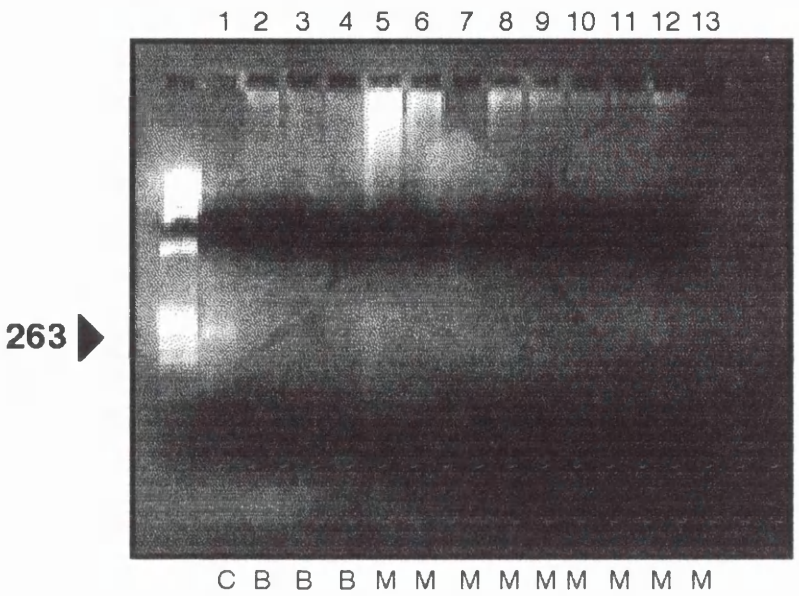

C Positive control
B Benign Breast Disease
M Primary Breast Carcinomas

Figure 20 c

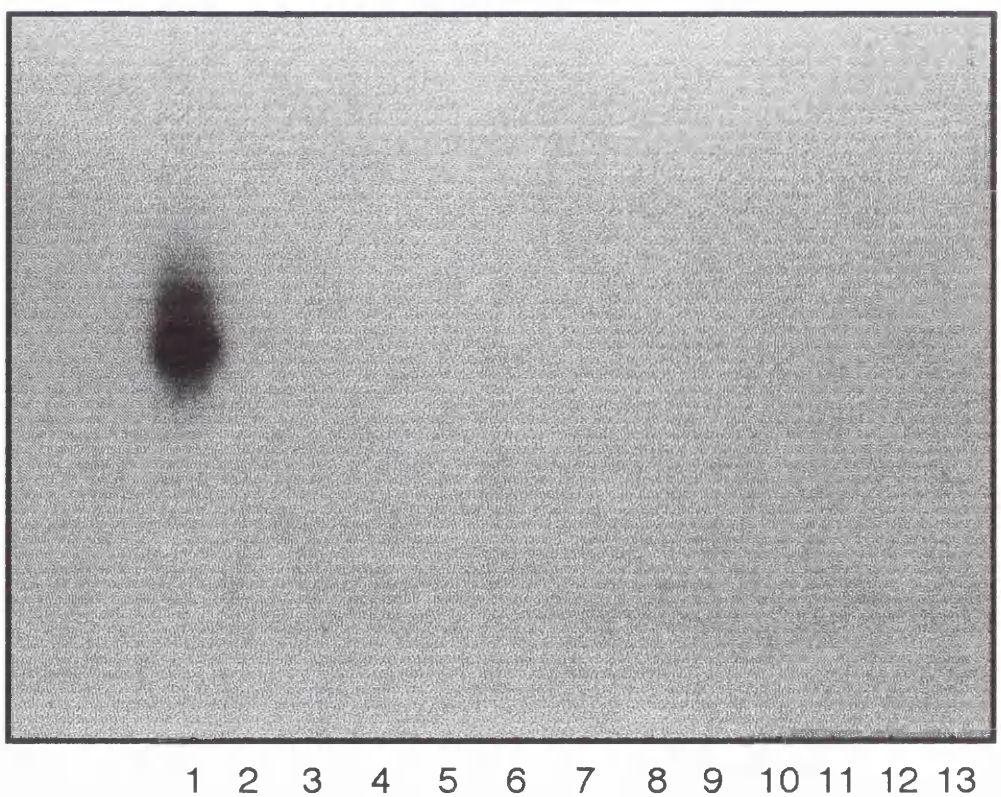


## Post-Operative

Electrophoretic analysis




Autoradiography




C Positive control
B Benign Breast Disease
M Primary Breast Carcinomas

had undergone surgery for either benign or malignant breast disease.

**DISCUSSION**

PCR is a highly sensitive molecular biological technique which is ideally suited to the detection of relatively small numbers of tumour cells in peripheral blood. The specificity is primarily dependent upon the identification of a gene solely expressed by the cell population of interest. At present no genes specific to breast cancer have been identified. Therefore, genes which are expressed by breast epithelium but not by the cellular elements of blood or vascular endothelium were targeted. The identification of the gene encoding CK 18 in normal peripheral blood was therefore totally unexpected. In the absence of circulating epithelial cells these false positive signals were thought to represent sample contamination. Skin is the most likely source of such contamination as it is theoretically possible to introduce a plug of epithelial cells from skin into blood during venesection. However, this risk was minimised by discarding the first 5 mls of blood during collection. In addition immunocytochemical studies indicate that skin does not express CK 18 (Moll et al, 1982). This suggests that the presence of the CK 18 gene in blood may not represent epithelial contamination rather such "illegitimate transcription" may reflect low levels of CK 18 mRNA transcription in

non-epithelial tissues (Chelly et al, 1989), the products of which may not be detectable by conventional immunocytochemical techniques. Evidence of keratin gene expression in cells of haemopoetic origin using PCR has recently been confirmed not only for cytokeratin 18 but also for cytokeratin 8 (Traweek et al, 1993).

DF3 antigen was expressed in breast epithelium, but in contrast to CK 18 it was absent in normal peripheral blood. The epithelial-specific nature of DF3 therefore made it a suitable target for the detection of breast epithelium in blood. The level of gene expression was sufficient to enable malignant breast epithelial cells to be identified at a concentration as low as 10 tumour cells per 5 mls of blood. The universal expression of DF3 in benign and malignant breast tissue is in contrast to conventional immunocytology where the level of expression is significantly lower in benign tissue (Kufe et al, 1984). This may reflect the ability of PCR to detect low levels of DF3 expression in benign breast tissue not detectable by conventional immunocytological methods.

Having established optimal conditions for reverse transcription PCR, a preliminary study of patients with breast cancer was performed to determine whether surgery promoted haematogenous tumour cell dissemination. DF3 gene expression was identified in the peripheral blood of 1 of 9 patients pre-operatively, 4 of 9 patients intra-operatively and in

none of the patients 24 hours post-operatively. None of the patients with benign breast disease demonstrated DF3 expression in the peripheral blood. The presence of the DF3 antigen in the blood of patients undergoing surgery for malignant disease suggested that it was due to the presence of circulating malignant epithelial cells. The presence of malignant cells in the bloodstream of one patient pre-operatively would suggest that spontaneous haematogenous shedding from primary does occur while the presence of malignant cells in four patients during surgery suggests that operative manipulation enhances tumour cell dissemination. The absence of tumour cells in the bloodstream 24 hours folowing surgery confirms previous experimental studies by Fidler (1970) which demonstrated rapid destruction of circulating tumour cells. In view of the size of the study it was not possible to identify any valid associations between the presence of circulating tumour cells and patient clinico-pathological prognostic data.

This preliminary study suggests that operative manipulation of malignant breast neoplasms may promote tumour cell dissemination via the bloodstream. Quantification of tumour cells was not performed as reverse transcription PCR is not primarily designed for this type of analysis, rather it is used as a rapid method for the detection of mRNA at low concentrations as required for the detection of a minor subpopulation of tumour cells in blood. However,

future studies should attempt to incorporate quantification techniques as this will determine whether the degree of tumour cell shedding is related to prognosis and variables such as type of operation, stage of disease and grade of malignancy. The identification of patients at risk from haematogenous dissemination may alter the management of patients with malignant disease.

**FINAL DISCUSSION**

The introduction documented considerable evidence indicating an association between the coagulation system and the intravascular phase of the metastatic process. Microscopy studies in animal models have demonstrated that circulating tumour cells arrest in the microcirculation of target organs surrounded by a meshwork of fibrin and platelets. This is thought to enhance metastasis by facilitating adhesion of tumour cells to vascular endothelium thereby stabilizing tumour cell arrest in the microvasculature. Studies using a rat model of "artificial metastasis" have demonstrated inhibition of metastasis following the administration of coumarin anticoagulants and more recently following the administration of the fibrinolytic agent Streptokinase. Such therapy might therefore form the basis of an effective antimetastatic treatment if administered to patients at risk from haematogenous dissemination from primary tumours. Animal studies have demonstrated that manipulation of primary tumours facilitates tumour cell dissemination via the bloodstream and may promote metastasis. The "no-touch" isolation technique of Turnbull (1967) suggests that surgical manipulation of tumours may enhance the metastatic process by promoting intra-operative haematogenous tumour cell dissemination. This technique has largely been abandoned because of poor reproducibility, but the theory underlying it has

not been entirely refuted. Peri-operative antimetastatic treatment using fibrinolytic therapy might therefore have a role in preventing metastatic progression in patients with cancer.

The work in this thesis was divided into two closely related investigations. The first half of this thesis was designed to investigate the hypothesis that the antitumour effect of Streptokinase was secondary to dissolution of the fibrin clot surrounding tumour cells arrested in the microcirculation. A rat model of metastasising breast carcinoma was therefore used to study the mechanism of Streptokinase-mediated tumour inhibition. The second half of this thesis was designed to investigate whether operative manipulation of malignant tumours facilitates tumour cell dissemination via the bloodstream. A clinical study of patients with operable breast cancer was therefore performed and peri-operative blood samples analysed for the presence of tumour cells using immunocytochemical and molecular biological techniques.

The initial aim of these studies was to validate the animal model of "artificial metastasis" and confirm that Streptokinase inhibits pulmonary tumour seeding in this animal model. The results of these preliminary studies validated the results of previous work by confirming the dose and time of administration of Streptokinase that significantly inhibits pulmonary tumour seeding in this animal model. However, the mechanism of tumour inhibition remained

unclear as conclusive evidence of an association between fibrin and tumour cells within the microvasculature had not been proven in this model. In the absence of histological evidence of intravascular fibrin clot lysis, it was therefore possible that Streptokinase caused this effect of tumour inhibition via a completely different mechanism. The working hypothesis at this stage was that Streptokinase inhibited pulmonary tumour seeding by destroying fibrin clot surrounding tumour cells trapped in the pulmonary microcirculation.

To test this hypothesis, it was important to answer the following question:

Is the effect of Streptokinase in this animal tumour model associated with fibrin clot lysis?

The study designed to answer this question was to assess Plasma Fibrin Degradation Products (FDP), a highly sensitive indicator of fibrinolysis detected by a latex agglutination reaction. Analysis of rat plasma FDP demonstrated that there was a tendency towards increased fibrin clot lysis in animals receiving Streptokinase when compared with controls although this failed to reach statistical significance. The demonstrable rise in FDP in the untreated control group was thought to be due to spontaneous activation of the fibrinolytic system in this group, a phenomenon previously recognised in rodents, in combination with activation resulting from traumatic cardiac puncture and the stress of anaesthesia.

In an attempt to provide further evidence of fibrin clot lysis, the effect of another fibrinolytic agent on pulmonary tumour seeding was studied. Human recombinant tissue plasminogen activator (rt-PA) is different to Streptokinase in relation to both structure and mechanism of action. The results of this study demonstrated that rt-PA was as effective as Streptokinase in inhibiting pulmonary tumour seeding. The demonstration that two pharmacologically unrelated fibrinolytic agents had a similar antitumour effect suggested that this effect was probably secondary to fibrin clot lysis.

These two studies have provided mutually supportive findings as to the mechanism of inhibition of "artificial metastasis" by Streptokinase. While such independent confirmation makes it likely that this effect of Streptokinase is secondary to fibrin clot lysis, microscopic evidence for intravascular dissolution of thrombus has not been demonstrated. This would require extensive microscopic studies of changes in the pulmonary microvasculature under the experimental conditions described earlier. The overall view of the evidence presented makes it likely that the antitumour effect of Streptokinase and rt-PA is related to the fibrinolytic properties of these agents.

Platelets are also an important component of thrombi surrounding tumour cells arrested in the microvasculature. If fibrin clot formation and platelet deposition are required to facilitate increased tumour

cell entrapment, then their destruction should theoretically have an additive inhibitory effect. A further study was therefore performed to answer the following question:

Is the inhibition of pulmonary tumour seeding by Streptokinase potentiated by antiplatelet therapy?

Since tumour cell-induced platelet activation may occur via a number of pathways including the generation of thrombin and activation by ADP, this study used two antiplatelet agents with separate mechanisms of action. Although there was a trend towards a reduction in pulmonary tumour seeding with aspirin this failed to reach a significant level suggesting that platelet activation by the Mtln3 cell line may be independent of the cyclo-oxygenase pathway. In contrast, ticlopidine significantly inhibited pulmonary tumour seeding suggesting that tumour cell-induced platelet aggregation by the Mtln3 cell line may be dependent on the generation of ADP. The combination of ticlopidine and Streptokinase was no more effective than Streptokinase treatment alone indicating that tumour cell-platelet interactions may be less important than fibrin deposition in the metastatic process.

It is important to stress that there are limitations of artificial, intravenous models of metastasis and that the metastatic behaviour of a bolus injection of a cloned tumour cell line may not necessarily reflect spontaneous metastasis from a heterogeneous primary tumour population. However, this

model was simply designed to enable investigation of the intravascular phase of the metastatic process. Further studies are required to investigate whether Streptokinase and rt-PA have a similar effect on the spontaneous metastatic potential of established tumours.

The demonstration that fibrinolytic agents inhibit metastasis raised further questions with regard to the clinical role of such agents in the management of patients with cancer. The introductory section documented evidence to suggest that anticoagulant/fibrinolytic therapy might be beneficial although the results have been compromised by a preponderance of patients with advanced disease in whom the benefits of antimetastatic therapy would be minimal. To be effective it is clear that such treatment should be applied to patients with early cancer but at risk from haematogenous dissemination.

Studies based on animal models indicate that surgical manipulation of malignant tumours promotes tumour cell shedding into the circulation. In addition, surgical stress enhances tumour survival in the bloodstream by inducing a state of relative immune suppression while activation of coagulation encourages tumour cell entrapment in the microcirculation by promoting a fertile, hypercoagulable "soil". If tumour cells that are shed at the time of surgery are prevented from adhering to vascular endothelium and are maintained in circulation, they will eventually die.

Fibrinolytic agents administered in the perioperative period should therefore theoretically prevent tumour seeding in the microcirculation thereby reducing metastasis. However, fibrinolytic therapy is not without risk and in particular allergic reactions to Streptokinase and post-operative haemorrhage must be considered. While rt-PA avoids the former complication, the risk of bleeding, although reduced, still exists. Large prospective studies would therefore only be justifiable if there was conclusive evidence of intra-operative tumour cell dissemination.

Our initial attempts to identify tumour cells in the bloodstream of breast cancer patients during surgery utilized flow cytometry to detect tumour cells labelled with fluorochrome-conjugated antibody. Optimal antibody labelling of tumour cells was achieved using an anticytokeratin antibody MNF 116. However, one colour flow cytometric analysis failed to accurately identify tumour cells in blood due to incomplete separation of cytokeratin labelled-tumour cells and highly autofluorescent leucocytes. Two colour flow cytometry enhanced tumour cell detection by first excluding the majority of leucocytes labelled with a panleucocyte antibody. This technique was then applied to a pilot clinical study of breast cancer patients during surgery but failed to detect peri-operative tumour cell shedding. An assessment of sensitivity however demonstrated that this technique was unreliable to detect tumour cells at low concentrations.

Therefore, the possibility that tumour cell shedding had occured during operative manipulation could not be completely excluded using the technique of flow cytometry.

Gene amplification by reverse transcriptase PCR provided a technique with the necessary sensitivity and specificity to detect a minor subpopulation of breast epithelial cells in blood. Oligonucleotide primers were designed to two genes encoding structural epithelial proteins, cytokeratin 18 and the breast carcinoma associated antigen DF3. The absence of epithelial cells in normal peripheral blood suggested that these genes were suitable targets for circulating malignant breast epithelium. However, amplification for the cytokeratin 18 gene demonstrated that it was expressed in the blood of healthy volunteers. The strength and consistency with which the signal occurred suggested that it was unikely to be due to epithelial cell contamination, rather it was due to low level transcription of the CK 18 gene in non-epithelial tissues. In contrast, DF 3 expression was consistently shown in cells derived from primary malignant breast carcinomas but was absent in all normal peripheral blood samples. An in vitro assessment of sensitivity demonstrated that 30 cycles of PCR using the DF3 primers could detect 10 breast carcinoma cells per 5 ml of blood. A preliminary clinical study of 9 patients undergoing surgery for primary breast carcinomas demonstrated the presence of cells expressing the DF3

gene in the peripheral blood of one patient pre-operatively and four patients during surgery. No DF3 positive cells were identified in post-operative blood samples or in any blood samples obtained from patients undergoing surgery for benign breast disease. The presence of DF3 positive cells in patients undergoing surgery for malignant disease but not benign disease strongly suggests their malignant origin. Furthermore, their presence in the peripheral blood of four of nine patients during surgery suggests that surgical manipulation of malignant breast tumours may enhance haematogenous tumour cell dissemination.

It is important to stress the preliminary nature of this study and a larger prospective study is required to further confirm these findings. Long term follow-up is essential to determine whether peri-operative tumour cell shedding promotes metastasis although interpretation of results may be difficult as some patients may have occult metastases at the time of surgery. Reverse transcription PCR is an extremely sensitive and specific method for the detection of small numbers of tumour cells in blood and it is not primarily designed for quantitative analysis. Since the inefficiency of the the metastatic process determines that the ratio of circulating tumours cells to the number of eventual metastases is high, future studies should incorporate quantitative analysis in order that the degree of tumour cell shedding can be correlated to prognosis. The identification of patients at risk from

haematogenous dissemination during surgery, in combination with the development of a suitable antimetastatic regime, may significantly alter the peri-operative management of patients with malignant disease.

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