UNIVERSITY OF SOUTHAMPTON

SCHOOL OF MEDICINE

Divisions of Cancer Science and
Infection, Inflammation & Repair

The Facilitative Role of the Extracellular Matrix in the Development of Colorectal Cancer Liver Metastases

by John Antony Conti, MRCS

Doctor of Philosophy

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Correction Sheet

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ABSTRACT

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We have investigated the hypothesis that a desmoplastic reaction (DR), characterised by the deposition of collagens I and III produced by activated stromal cells offers Colorectal Cancer (CRC) liver metastases a growth and survival advantage.

Immunohistochemical staining of liver specimens obtained at resection for CRC, demonstrated increased deposition of fibrillar collagens and alterations in collagen IV distribution within the tumour as part of a DR. In addition the deposited fibrillar collagens were closely associated with increased numbers of activated hepatic stellate cells/ myofibroblasts. β1 integrins were highly expressed by both cancer & stromal cells throughout the tumour stroma. However, in poorly differentiated areas of the CRC metastases β1 integrins appeared to be down-regulated, with αv integrin (especially αvβ5) expression upregulated.

Collagen I used as a tissue culture substrate significantly enhanced the growth of the CRC cell lines (HT-29 & KM12 cell lines series) compared to both control (plastic) and collagen IV (normal basement membrane component). Clonogenic (survival) and PARP cleavage (apoptosis) assays, showed that collagen I compared to collagen IV significantly increased the survival and reduced the rate of cellular apoptosis for CRC treated with chemotherapy (5-Fluorouracil). The adhesion and proliferation of CRC cells on collagens I and IV was significantly reduced in a dose dependent manner, after incubation with β1 integrin neutralizing antibodies (5-10μg/ml), compared to IgG controls. In contrast αvβ3 and αvβ5 neutralizing antibodies (5-20μg/ml), had no influence on the CRC cell adhesion, but significantly reduced the rate of proliferation of the CRC cell lines on collagens; especially for the highly metastatic KM12SM cell line. By demonstrating a reduction in proliferation in response to MMP resistant r/r collagen we have further reinforced our αv integrin neutralising antibody experiments, complementing them with a model using a dominant negative ligand.

These results support a role for the desmoplastic reaction in supporting CRC metastases, mediated via β1 and αv integrins. As CRC adopt a more aggressive malignant phenotype, matrix turnover reveals specific binding epitopes which upon ligation by αv integrins plays a key growth regulatory role.

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Abbreviations

ADAMs	A Disintegrin and Metalloprotease
Apaf-1	Apoptotic protease activating factor-1
AIF	Apoptosis Inducing Factor
αSMA	Alpha-Smooth Muscle Actin
$\alpha v\beta_3$	AlphavBeta3
β1	Beta-1
BCA	Bicinchoninic Acid Protein Assay
BM	Basement Membrane
BSA	Bovine Serum Albumin
95% CI	95% Confidence intervals
CI	Collagen I
CIII	Collagen III
CIV	Collagen IV
CRC	Colorectal Cancer
DR	Desmoplastic Reaction
DRPs	Death Receptor Pathways
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
ECM	Extracellular matrix
EMMPRIN (CD147)	Extracellular Matrix Metalloproteinase Inducer
ERK1/ERK2	Extracellular Regulated Kinase 1 and 2
FAK	Focal Adhesion Kinase
FCS	Foetal calf serum

HBSS-Ca^{2+}	Hanks Balanced Salt Solution minus calcium ions
HBSS+Ca^{2+}	Hanks Balanced Salt Solution plus calcium ions
HSCs	Hepatic Stellate Cells
HGF	Hepatocyte Growth Factor
HPV	Hepatic Portal vein
IL-1	Interleukin-1
ILK	Integrin Linked Kinase
IAPs	Inhibitors of Apoptosis Proteins
MAPK	Mitogen Activated Protein Kinase
MEM	Minimum Essential Media
MMPs	Matrix Metalloproteinases
MMPIs	Synthetic Matrix Metalloproteinase Inhibitors
MMR	Mismatch Repair proteins
MSI	Microsatellite instability
MT-MMPs	Transmembrane Matrix Metalloproteinases
PARP	Poly-ADP Ribose Polymerase
PDGF	Platelet Derived Growth Factor
RIPA	Radio-Immunoassay Precipitation Buffer
r/r collagen	Type I r/r collagen
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis
TBS	Tris Buffered Saline
TCP	Tissue Culture Plastic
TIMPs	Tissue Inhibitors of Metalloproteinases
TGF-β1	Transforming Growth Factor Beta 1

TNF (α)	Tumour Necrosis Factor (alpha)
VEGF	Vascular Endothelial Growth Factor
w/t control	Wild type control

Chapter 1

Introduction, Hypothesis and Aims

Section 1.1

Clinical Background

Colorectal Cancer (CRC) is a common neoplastic disease affecting one in every 20 adults in western society (Bodey B et al, 2000). In the United States of America 140,000 patients are diagnosed with CRC and 55,000 patients die annually (Landis SH et al, 1999; Wingo PA et al, 2003). CRC is the 2nd commonest cause of mortality from cancer in the United Kingdom and is responsible for 16,300 deaths annually (Office for National Statistics, 2003). Despite improvements in surgery, and the use of adjuvant chemotherapy, approximately 50% of patients who undergo a curative resection for CRC die within 5 years (Hardingham JE et al, 1995). Even in those patients with early disease Dukes A (Dukes Classification described in Appendix-1a), 30% suffer a relapse and die within 5 years, presumable due to the presence of micrometastatic disease at the time of presentation (Bodey B et al, 2000).

A frequent cause of morbidity and mortality in CRC patients is the development of liver metastases (Hardingham JE et al, 1995; Myers RE et al, 1993) which are frequently incurable by present treatment options, which include hepatic resection and chemotherapy (Primrose JN, 2002). There has therefore been considerable research aimed at finding new treatments for CRC to prevent or treat metastatic disease.

Section 1.2

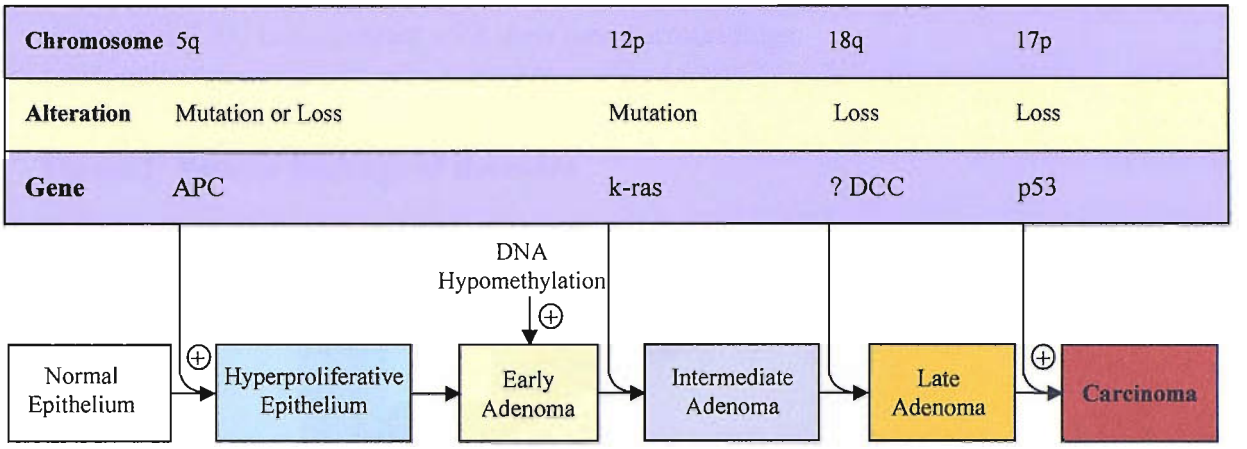
Why do Colorectal Cancers develop?

The precise aetiology of CRC is still not fully understood, however it is known that many develop from existing congenital or acquired colonic polyps (Leslie A et al, 2002), associated with genetic alterations which have become known as the "adenoma – carcinoma" sequence (Fearon ER et al, 1990). Whether all tumours develop in this way is not known. However, it is now well established that point mutations in proto-oncogenes such as *ras*, which when normally functioning play a crucial role in regulating cell growth (Bourne HR et al, 1990), results in the activation of intracellular signaling pathways that enhance the growth of cells (Forrester K et al, 1987, Bos JL, 1989). Another important genetic alteration in the development of CRC, involves the loss of tumour suppressor gene activity; e.g. Adenomatous Polyposis Coli (APC) gene on chromosome 5, which when

normally functioning regulates several other different proteins including β-catenin (Rubinfield B et al, 1996). β-catenins are important due to their ability to activate gene transcription via the T-cell Factor (TCF) family of transcription factors (Molennar M et al, 1996). The loss of APC activity is important in both familial and sporadic colorectal cancers (Jass JR et al, 2002). In the "adenoma – carcinoma" sequence several genetic mutations appear to be important, which by themselves do not lead to the formation of a carcinoma. Instead, the cumulative effect of several different genetic mutations on cell growth results in the formation of a malignant tumour (Fearon ER et al, 1990). An often key mutation in most CRC is the loss of *p*53 suppressor activity, because of its many effector functions, which include DNA repair and regulating cellular apoptosis (May P et al, 1999). (The proposed "adenoma – carcinoma" sequence is shown in Figure 1).

Another alternative pathway of tumorigenesis for a sub-set of colorectal cancers is characterized by the presence of microsatellite instability (MSI) (Tomlinson I et al, 1998, Shitoh K et al, 2000). Microsatellites are tandem repeats of DNA between one and five base pairs, repeated many times. Such errors are normally repaired by Mismatch Repair proteins (MMR), however in the absence of competent MMR function, microsatellite errors accumulate (Wheeler JMD et al, 2000). When cells are MMR deficient it is not only the microsatellites which accumulate, but replication errors may also occur in all nucleotide repeat sequences, including key regulatory genes (Leslie A et al, 2002). Therefore MSI may be interpreted as being a marker for a state of hypermutability or a "mutator phenotype" (Parsons R et al, 1993). MSI is observed in almost all hereditary CRC and 15% of sporadic CRC (Konishi M et al, 1996). There are 5 MMR genes (hMSH2, hMLH1, hPMS1, hPMS2 and MSH6) (Wheeler JMD et al, 2000), with the commonest mutated and therefore non-functioning MMR genes being hMSH2 and hMLH1 (Kinzler KW et al, 1996).

Figure 1: A Genetic Model for Colorectal Carcinogenesis



This figure illustrates a genetic model for colorectal carcinogenesis. This pathway was originally postulated by Fearnon R in Cell, 1990, from which this figure was reproduced. The accumulation rather than the order of changes is most important in tumour development. A recent review by Leslie A, et al in the British Journal of Surgery, 2002, has shown that mutations in the Adenomatous Polyposis Coli (APC) are found in between 40-80% of colorectal cancers (CRC); whereas mutations in k-ras are found in between 35-42% of CRC patients. The deleted in CRC gene (DCC) is found in up to 70% of CRC; however it has now been show that DCC does not have a tumor suppressor role in CRC; other important mutations are found in c-MYC, c-SRC and c-ERB-2. Loss of p53 tumour suppressor activity is found in 50-75% of CRC. Interestingly, several different p53 mutations have been identified in patients with CRC, which results in different down-stream effects on the p53 pathway, thereby resulting in tumors with different malignant phenotypes.

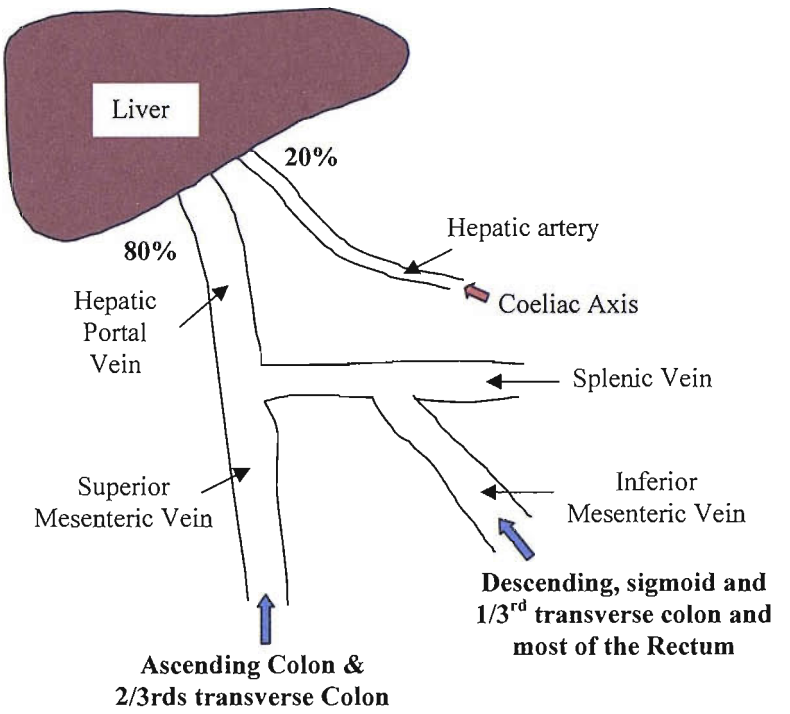
Section 1.3

Where do Colorectal Cancers develop?

The vast majority of CRCs are adenocarcinomas (95%) and are most frequently located in the sigmoid colon and rectum (75%). CRC spread by direct invasion into adjacent tissues or transperitoneally. Metastatic spread occurs via the draining lymphatics and the blood-stream (Burnand KG et al, 1998). Blood-borne spread is very common, with the commonest site of implantation being the liver due to the fact that the majority of the colon, except for the lower 1/3rd of the rectum, drains into the hepatic portal vein (HPV) (McMinn RMH, 1990), which in turn drains into the hepatic sinusoids. Therefore tumour cells released from the primary tumour may lodge in the hepatic sinusoids, invade the normal liver tissue and develop into established metastases. It is now well established using videomicroscopy that tumour cells are released by the primary tumour at an early

stage with only a small number developing into established metastases (Chambers AF et al, 1997). Precisely, why some metastatic tumours grow and others do not, appears related to how the CRC cells interact with their new surroundings.

Figure 2: Venous drainage of the colon



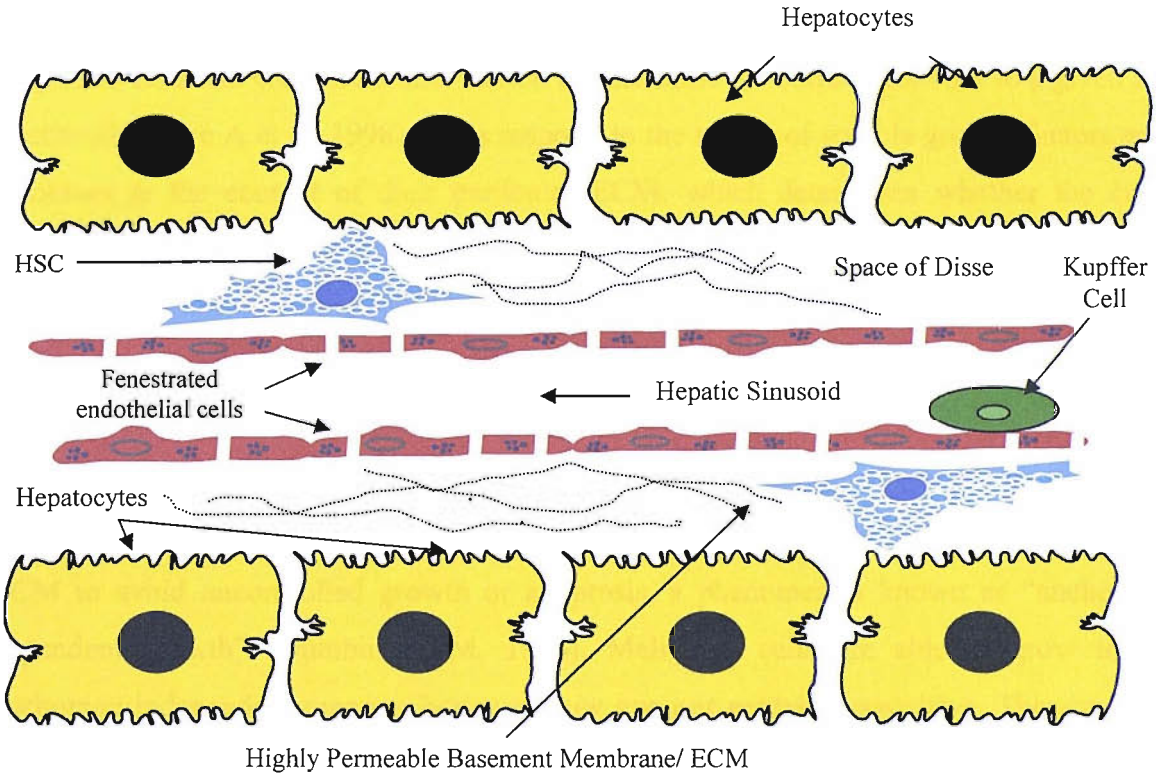
This figure illustrates that the venous drainage of the colon and rectum, is derived from the embryological midgut (Superior Mesenteric) and hindgut (Inferior Mesenteric) vessels. Therefore, the ascending colon and 2/3rds of the transverse colon drain into the Superior Mesenteric Vein (SMV). The remainder of the colon and all but the lower 1/3rd of the rectum are drained by the Inferior Mesenteric Vein (IMV). The lower 1/3rd of the rectum is drained from branches of the Internal Iliac vein. The IMV joins up with the Splenic vein, behind the pancreas. The Splenic vein then joins the SMV and forms the Hepatic Portal Vein (HPV). The HPV carries approximately 80% of the blood which supplies the Liver, with the remaining 20% provided by the Hepatic artery which is a branch of the Coeliac axis (Foregut vessel). The drainage pattern of the colon is ideal, so that the liver can destroy any possible pathogens absorbed from the gut and provides the liver with rapid access to the nutrients it requires to carry out its many physiological functions.

Section 1.4

The Structure of the Liver

The liver has a characteristic structure, consisting of structural units called liver lobules. The epithelial cells called hepatocytes (liver cells) are organized into interconnected sheets, separated by connective tissue, bile ducts, nerves and blood vessels. Between the sheets of hepatocytes lie hepatic sinusoids containing capillary like vessels lined by fenestrated endothelial cells. The hepatocytes sit on a typical basement membrane (BM), beneath which lie stroma and the endothelial cells that line the hepatic sinusoid. This characteristic structure of a hepatic sinusoid is shown in figure 3.

Figure 3: The structure of the Hepatic Sinusoid



This figure illustrates the basic structure of the hepatic sinusoid. As can be seen several different cell types are important. The basement membrane and stroma underlying the hepatocytes is very thin, and highly permeable. This highly permeable ECM and the arrangement of hepatocytes and endothelial cells are critical for normal hepatocyte function. This arrangement bathes the hepatocytes in a rich blood supply to enable the liver to perform its many physiological functions, which include the release of macromolecules into the circulation such as lipoproteins produced in the liver, and the catabolism of many large molecules. NB. The space of Disse is not shown to scale; actually it is only 2μm wide. Therefore, the Hepatic stellate cells (HSC) are tightly wrapped around the endothelial cells.

The composition of the low density basement membrane characteristically consists of collagen type IV, laminin, fibronectin, and proteoglycans. In addition the BM may also contain small amounts of fibrillar collagen and collagen VI. This highly permeable ECM and the arrangement of hepatocytes and endothelial cells are critical for normal hepatocyte function. Within the sub-endothelial space (Space of disse) lie inactive Hepatic stellate cells which upon activation adopt a myofibroblast phenotype and play an important role in matrix turnover. The hepatic sinusoids are lined by phagocytic monocytes called Kupffer cells (Junquiera LC et al, 1989).

Section 1.5.1

The Extracellular Matrix

The ECM is a complex network of macromolecules that provides both architectural support to cells and contextual information to determine the correct response to a given set of stimuli (Howe A et al, 1998). Cells respond to the milleu of soluble growth factors and cytokines in the context of their particular ECM, which determines whether the cells proliferate, differentiate, arrest growth or undergo apoptosis (De Archangelis A et al, 2000, Gustafsson E et al, 2000). In addition, the ECM can act as a reservoir for many mitogens including growth factors and cytokines, which are released from the network after proper activation (Radisky D et al, 2002). The composition of ECM varies widely within and between tissues and changes temporarily as tissues adapt to changing conditions (Streuli C, 1999; Huang S et al, 1999). Normal adherent cells must contact the ECM to avoid uncontrolled growth or apoptosis, a phenomenon known as "anchorage dependent growth" (Gumbiner BM, 1996). Malignant cells are able to grow in an anchorage independent manner; however **they are not matrix insensitive**. Thereby they continue to respond and contribute to the ECM (Radisky D et al, 2002).

Section 1.5.2

Components of the ECM

a) Laminins: Are a family of at least 11 glycoproteins, and are integral scaffolding components of the ECM (Aumailley M et al, 1998). They have a cruciform structure and are composed of heterodimers composed of α, β and γ chain subunits, which are

simultaneously secreted and assembled into cell-associated matrices (Colognato H, et al, 2000). The localized distribution of various laminins conveys information about tissue organization (Virtanen I et al, 2000) and cell polarization (Patton BL et al, 1997), through sub-domains of the laminin protein complex. Complete absence of functioning laminins in transgenic mice results in an embryonic lethal phenotype (Smyth N et al, 1999) and aberrant production of laminin binding integrins may be important in some tumours (Ziober BL et al, 1996).

b) Nidogen/Entactin: Nidogens act to stabilize the connections between the collagen IV network and the laminin network (Radisky D et al, 2002). 2 isoforms are known to exist and they are essential to ensure functional differentiation of mature tissues (Pujuguet P et al, 2000).

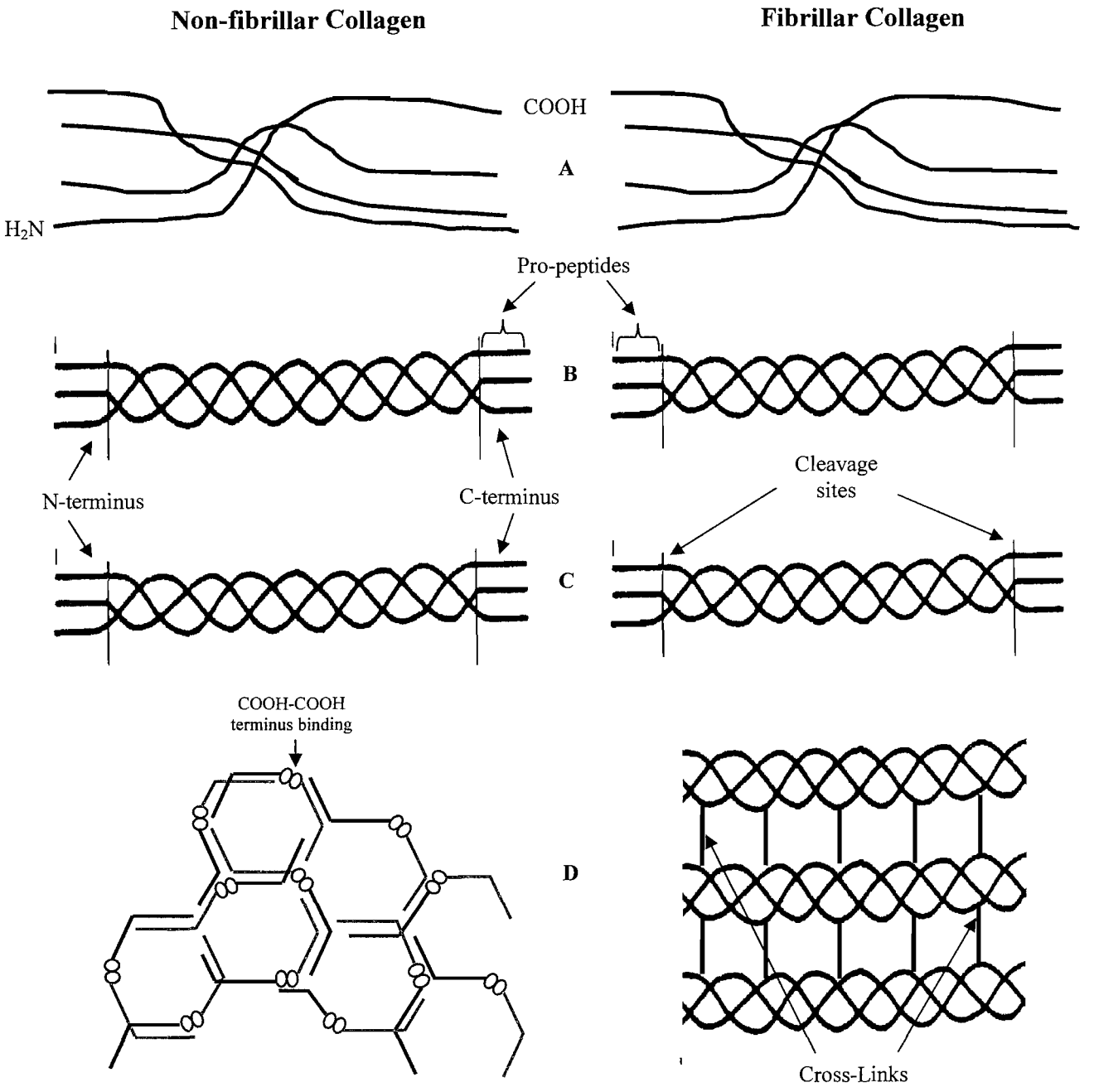
c) Proteoglycans: These are a highly diverse group of glycoproteins, containing glycosaminoglycan side chains (Aumailley M et al, 1998) that play a crucial role in strengthening and maintaining the BMs and in wound repair (Bernfield M et al, 1999). Deficiency in particular forms of proteoglycan are associated with susceptibility of the BM to mechanical stress (Costell M et al, 1999) and others are implicated in regulating growth factor responsiveness (Santra M et al, 2000), which may become altered in cancers (Kleeff J et al, 1998).

d) Fibronectin and Tenascins: Fibronectin is a normal component of the stromal ECM, which comes into contact with epithelial cells when the BM is broken down during remodeling, involution or malignancy (Shoenwalder SM et al, 1999, Sechler JL et al, 1998). Tenascins are anti-adhesive components of the ECM expressed during involution, wound-healing or malignancy (Jones FS et al, 2000). Fibronectin and tenascin cause cell spreading, a necessary component of cell cycle progression (Huang S et al, 1999).

e) Collagens: These are the most abundant structural proteins of the ECM (Aumailley M et al, 1998). The structural motif consists of a trimer of α chains, folded into a coiled triple helix (Huang S et al, 1999). There is a consistent repeated sequence of (Gly-X-Y) of which every third X is proline and every third Y a hydroxyproline (Miller EJ et al,

1985, Prockop DJ et al, 1984). The amino acids glycine and proline allow the triple helix to form, with glycine's small size enabling close packing of the helix; and proline's ring structure stabilizing the helical turns (Wright R et al, 1992). 20 different isoforms of collagen exist, due to the structural motifs present in their α chain subunits, with diversity existing even within collagen sub-types (Radisky D et al, 2002, Prockop DJ et al, 1984, Petticlerc E et al, 2000). Collagen can be split into fibrillar (Collagens I, II, III, V and VI) and non-fibrillar (Collagens IV, VI, VII, VIII and X) sub-types. Collagen I, III, IV and VI are found within the liver (Rojkind M et al, 1979; Schuppan D, 1990), with collagen IV being the principle component of the BM of the subendothelial space (Burt AD et al, 1990; Clement B et al, 1986). Collagen IV can be composed of 6 known α chains, there are therefore 56 possible combinations of the different α chains that may vary between tissues (Petticlerc E et al, 2000). The difference between fibrillar and non-fibrillar collagens can be explained by looking at their production; each collagen is produced as pro-collagens in the cell with pro-peptides on both the C- and N- terminus, with the C-terminal aiding the formation of the triple helix. The helices are secreted from the cell, the fibrillar collagens have their pro-peptides cleaved by proteolytic enzymes, whereas the non-fibrillar variants do not lose their pro-peptides (Prockop DJ et al, 1984). The fibrillar collagens lie in a staggered fashion side-side with crosslink's between them, forming fibrils (Kivirikko KI, et al, 1987). The non-fibrillar collagens bind C-terminus to C-terminus (Siebold B et al, 1988; Yurchenco PD et al, 1990). When viewed under electron microscopy the fibrillar collagens show a striated pattern (Prockop DJ et al, 1984) and non-fibrillar collagens form a lattice structure, due to their C-terminus binding and the distortion in their helixes (Siebold B et al, 1988; Yurchenco PD et al, 1990).

Figure 4: Collagen Structure for fibrillar vs non-fibrillar collagens



This figure illustrates how the different forms of collagens are produced; as procollagens (A), with pro-peptides on both there C- and N-terminus (B). The fibrillar collagens have their pro-peptides cleaved by pro-peptidases (C). The fibrillar collagens lie in a staggered fashion with cross-links between them, whereas in non-fibrillar collagens, the fibrils bind C-terminus to C-terminus (D) and form a meshwork. Reproduced and adapted from Wright's R, Liver and Biliary Disease: Pathophysiology, Diagnosis and Management, 1992.

Section 1.5.3

Collagens identified in the liver

(i) Collagen I & III: Within the normal liver 40% is collagen I, which is predominantly found forming the liver capsule and around the portal tracts and central veins (Geerts A, et al, 1990). Collagen III makes up approximately the same proportion and is found in the peri-portal and centrolobular areas (Geerts A, et al, 1986).

(ii) Collagen IV: This is responsible for the majority of the remaining collagen in the liver and is a major component of the basement membrane (Burt AD et al, 1990; Clement B et al, 1986). The ECM of the space of disse (sub-endothelial space) is particularly rich in this form of collagen. It is now known that Collagen IV possesses many inhibitory sequences to cell growth (Petticlerc E et al, 2000; Maeshima Y et al, 2000). These have been identified on the alpha-3 chain of collagen IV and attempts have been made to use these sequences to inhibit tumour growth in vitro with some success (Maeshima Y et al, 2000). It is now established that the alpha-2 and 6 chains of collagen IV may also contain growth regulatory sequences (Petticlerc E et al, 2000).

Section 1.5.4

Type I r/r Collagen

As described earlier the helical trimeric molecules of type I collagen comprises of two α1 (I) and one α2 (I) chains encoded by the Col1a-1 and Col1a-2 genes respectively (Vuorio E et al, 1990). Degradation of type I collagen requires the action of metalloproteinases (See section 1.11). The peptide bonds formed in the mature collagen I helix, between the Gly_{775} and Ile_{776} of the α1 (I) chain and Gly_{775} and Leu_{776} of the α2 (I) chain, are the only sites in native collagen I molecules known to be cleaved by mammalian collagenases (Liu X et al, 1995). Professor S Krane's group have been able to breed the transgenic type I r/r collagen mouse. This mouse was bred with a simple substitution of a Pro for Ile_{776}, and renders native type I collagen highly resistant to collagenase digestion (Wu H et al, 1990).

Section 1.6

Apoptosis

Apoptosis is a morphologically and biologically distinct form of programmed cell death, where disassembly of the cell comes from within. In contrast, necrosis is usually caused by a direct cell insult, which compromises plasma membrane integrity (Thornberry, 1998). Deregulation of cell death pathways plays an important role in many conditions, including cancer (Arends MJ, 1991). There are believed to be two main apoptosis pathways, although more may exist. These are the Death Receptor Pathways (DRPs) and the mitochondrial pathway (Earnshaw WC, 1999 and Budihardjo I, 1990). Both are characterised by the activation of caspases, which are the effectors of apoptosis (Gupta, 2001). The DRP is activated following ligation of cell surface receptors, such as TNF-α and Fas ligand. The complex formed then binds pro-caspase 8, leading to caspase 8 activation, which in turn can proteolytically activate caspase 3 or can cleave pro-apoptotic bcl-2 family members (Kaufmann SH, 2001).

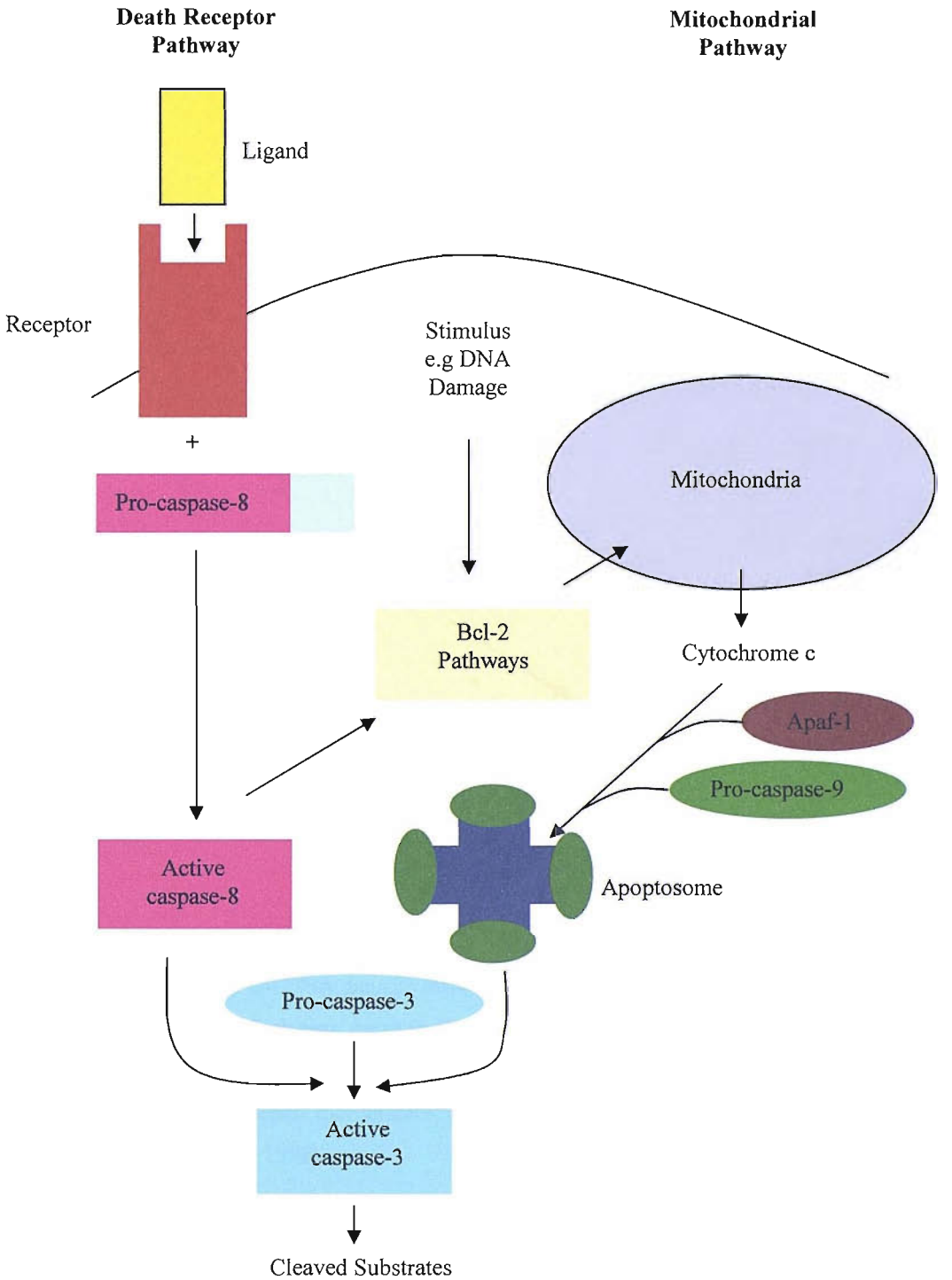
The mitochondrial pathway may be activated by the action of bcl-2 family members in response to many stimuli. Whatever the stimulus, the activation of the mitochondrial pathway results in the release of multiple proteins from mitochondria. One of these, cytochrome c accumulates in the cytoplasm, where it binds to the scaffolding protein apoptotic protease activating factor-1 (Apaf-1). This results in a conformational change in Apaf-1, which when then bound to pro-caspase-9 results in the formation of a complex called an apoptosome (Strasser A, 2000 and Hentgartner MO, 2000), which activates caspase 9, which in turn cleaves pro-caspase 3 resulting in active caspase 3 (Kaufmann SH, 2001). The multiple pathways of apoptosis are illustrated below in figure 5a.

Caspases are members of a group of cysteine peptidases. They are grouped according to function, with caspase 1, 4, 5, 11, 12 and 14, involved in cytokine maturation (Earnshaw WC, 1999). By contrast caspases 2, 3 6, 7, 8, 9 and 10 are implicated in apoptosis. Caspase 3, 6 and 7 are the so-called effector caspases (Strasser A, 2000) they lack intrinsic enzymatic activity but upon proteolytic activation cleave most apoptotic substrates. The initiator capases are pro-caspase 2, 8, 9 and 10. They possess a caspase activation and recruitment domain (CARD), which contributes to the transduction of various signals into

proteolytic activity (Stennicke HR, 1999). The regulation of caspase activity is tightly controlled with bcl-2 family members having an important role.

The bcl-2 family members are the key regulators controlling the release of cytochrome c and other apoptotic factors from mitochondria (Kaufmann SH, 2001). The family can be split up into 3 groups on the basis of their functional and structural criteria. Anti-apoptosis proteins in group 1 include bcl-2, bcl-xl and mcl-1 (Adams JM, 1998). Their mechanism of action is by binding to and sequestering the pro-apoptotic "bcl-2" family members, although other mechanisms based on protein-protein interactions have been proposed (Huang DC, 2000). The pro-apoptotic members include bax & bak (group 2) (Gross A, 1999) and bid & bad (group 3) (Huang DC, 2000). In this system, group 3 members are the sensors of apoptosis, as they respond to a wide variety of pro-apoptotic stimuli. Group 1 are the modulators of the apoptotic pathway and group 2 the output of this integration effort, as their activity is sufficient to result in the release of cytochrome c from mitochondria. In addition to cytochrome c, there are several other polypeptides which play active roles in apoptotic events, such as apoptosis inducing factor (AIF) (Susin SA, 1999). To inhibit apoptosis a group of polypeptides exist called IAPs (inhibitors of apoptosis proteins). They function by binding to active caspases such as 3, 7 and 9 (Deveraux QL, 1999). In cancers another apoptosis inhibitor has been identified called survivin, however it has now been postulated this protein is more involved in regulating mitosis than apoptosis (Okada H, 2004).

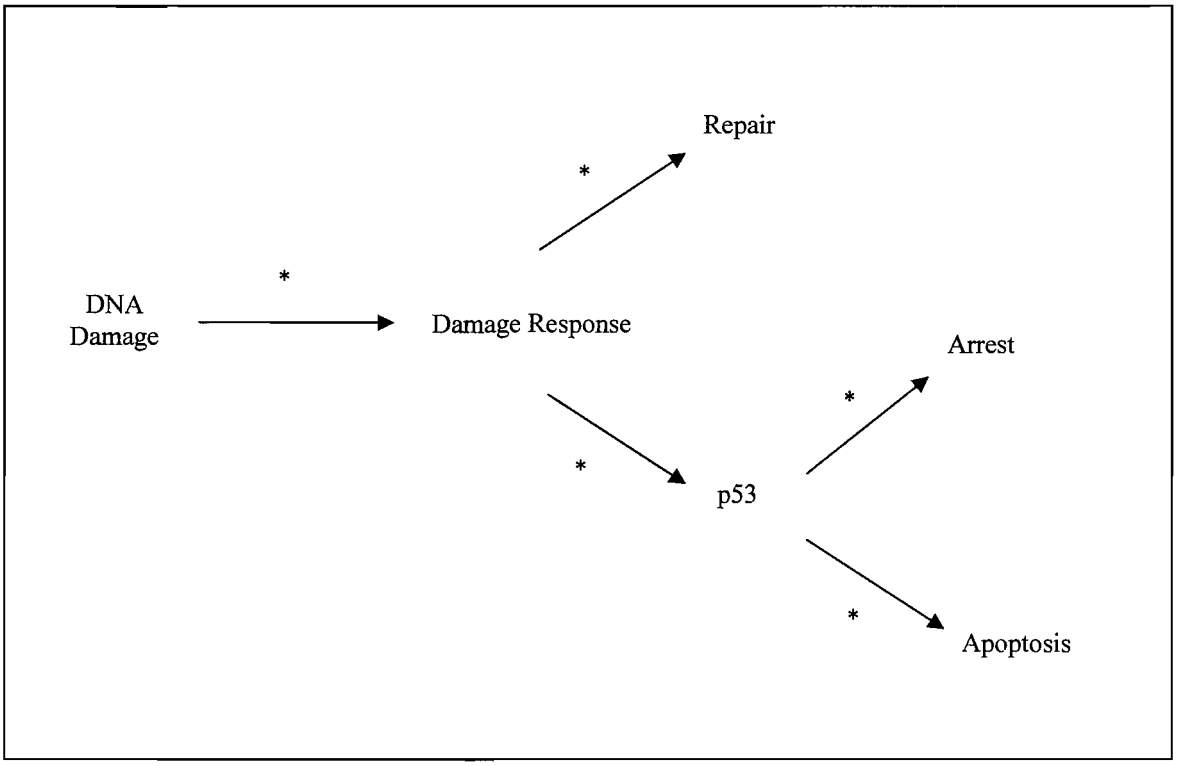
Figure 5a: Apoptosis pathways



This figure illustrates both the death receptor (DRP) and mitochondrial apoptosis pathways and how they may be activated. The final effector for both these mechanisms is active caspase 3. Interestingly, the DRP can also induce apoptosis by activating the mitochondrial pathway. (Adapted from Kaufmann S et al, 2001)

p53, is a tumour suppressor gene, which we know plays an important role in DNA repair and in mediating apoptosis in response to DNA damage and is often mutated in CRC as described earlier. How mutations in genes that respond to DNA damage can effect apoptosis, is illustrated in the figure 5b below.

Figure 5b: The role of p53 in response to DNA damage



The contemporary view of the DNA damage response. There are a variety of active responses that occur following DNA damage, which may result in DNA repair, or result in growth arrest of the cells or the induction of apoptosis. All these pathways may be subverted by mutations (*), which may occur in the DNA damage sensors, the repair mechanisms, p53 itself or the growth arrest and apoptotic pathways. (Adapted from Evan G et al, 1998)

Of interest in recent years is the association between cell proliferation and apoptosis, as many signals that trigger cell proliferation also lead to cell death. This is believed to permit normal tissue development and may be deregulated in cancers. It is postulated that in many cancers inhibition of apoptosis occurs, by inactivating pro-apoptotic pathways such as

those mediated by p53, but there may also be the promotion of genes that promote both proliferation and inhibit apoptosis (Okada H, 2004 and Hipfner DR, 2004).

It is therefore apparent that apoptosis is a complex phenomenon and controlled at several levels. These include the presence of pro-apoptotic and anti-apoptotic proteins and inhibitors of caspsases.

Section 1.7

The role of the desmoplastic reaction in the spread of Cancer

It is now well established that the extracellular matrix which is predominantly produced and regulated by stromal cells can profoundly influence the growth and survival of both normal and malignant cells (Radisky D et al, 2002; Gumbiner BM, 1996). In pancreatic and breast cancer the desmoplastic reaction in the primary tumour, characterized by the degradation of the normal basement membrane, rich in type IV collagen and the accumulation of fibrillar collagens (types I & III collagen), has been shown to be important in the spread of these tumours (Armstrong T et al, 2004; Buchholz M et al, 2003; Meng L et al, 2001). On-going in vitro research at this centre in pancreatic cancer has shown that tumour growth is accelerated on type I collagen compared to that seen on type IV collagen, which normally forms the basement membrane (Armstrong T et al, 2004). Studies in lung cancer have suggested that a desmoplastic reaction may also offer cancer cells a degree of chemoprotection, by reducing the rate of cellular apoptosis in response to chemotherapy. This growth advantage and chemoprotection conferred by being in contact with a desmoplastic matrix in lung cancer was ablated by blocking β_1 integrins (Sethi T et al, 1999). It is therefore postulated that the matrices growth effects are modulated via integrin expression by the cancer and integrin ligand expression by the matrix. Recent studies have demonstrated that a pronounced DR is an independent poor prognostic indicator in terms of shorter survival and the development of metastatic disease for patients with CRC (Nishimura R et al, 1998; Sis B et al, 2005). However, the limited numbers of in vitro studies on the effect of different matrix components on CRC growth have produced contradictory results (Zvibel I et al, 1998; Kouniavsky G et al, 2002).

The expression of integrins by the cancer is under the influence of intercellular signaling pathways, which are linked to several genes (e.g. *ras,* which can be mutated in many cancers) (Miyamoto S et al, 1998; Boudreau NJ et al, 1999). This concept of "**Inside-out**" signaling is postulated to be important in the spread of cancer (Radisky D et al, 2002), with different genetic mutations altering the number of integrin clusters and the class of integrins expressed (Boudreau NJ et al, 1999). Also, the adhesion of integrins to the ECM can be decreased by the activation of Integrin-Linked Kinases (ILK) (Hannigan GE et al, 1996).

The expression of integrin ligands by the matrix is regulated by matrix turnover, with degradation of the matrix revealing different epitopes for integrin binding, e.g. degradation of collagens I and IV reveal specific sequences (i.e. RGD sequences) which can be bound by several different integrins (Davis GE et al, 1992; Xu J et al, 2001). High expression of these particular sequences and their ligation by integrins expressed by the cancer are associated with the spread and growth of several cancers both in vivo and vitro (Erdreich-Epstein A et al, 2000; Petirclerc E, 1999). This concept of "**Outside-In**" signaling is believed to be one of the mechanisms by which the matrix influences the growth of cancers (Radisky D et al, 2002). However, the role of the matrix on cell growth is complex with several other important factors also involved, which the ECM may influence. These include the shape of cells and the expression of growth factors as will be illustrated below.

Section 1.8.1

Adhesion Molecules

Several adhesion proteins have been shown to be important in binding cells to the ECM, such ECM - cell interactions have been shown to regulate many complex cellular functions. In broad terms these adhesive molecules can be split into integrin and non-integrin type receptors.

Section 1.8.2

Non-Integrin Extracellular Matrix Receptors

a) **Syndecans** a family of transmembrane proteoglycans expressed on all adherent cells. They have a role in the formation of focal adhesion and stress fibres and the modulation of integrin function in response to mechanical stresses (Zimmermann P et al, 1999; Woods A et al, 2000).

b) **Dystroglycan** is another receptor, which appears important in ECM signaling and in the assembly of the BM, due to its role as a receptor for laminin. Dystroglycan-Laminin interactions play a critical role in assembling the many proteins that form the BM (Hemler ME, 1999). Loss of syndecans may have a role in tumorigenesis (Alexander CM et al, 2000), whereas loss of dystroglycan results in a non-functional BM (Henry MD et al, 1998).

c) **ADAM proteins** Metalloprotease-disintegrins (ADAMs) are transmembrane proteases which degrade transmembrane proteins and appear important in both normal and malignant cells (Bauvois B et al, 2004; Chang C et al, 2001). ADAM protease activity may release autocrine growth factors from the tumour surface and also degrade ECM proteins, which are important for tumour invasion (Chang C et al, 2001). It has now been established that ADAMs may influence cellular adhesion and migration by the disintegrin region supporting integrin mediated cellular adhesion (Alfandari D et al, 2001; White JM, 2003), and a recent study has suggested ADAMs cysteine rich domain, may interact with syndecan and mediate integrin dependent cellular spreading in vitro (Iba K et al, 2000). It has therefore been postulated that ADAMs play an important role in the spread and growth of cancers, however this is an area requiring further research.

Section 1.8.3

Integrins

Integrins are cell surface molecules named for their ability to integrate the information present in the composition of the ECM and to mediate tissue-specific gene expression (Miyamoto S et al, 1998). Integrins convey signals across the plasma membrane in both directions: association of integrins with ECM ligands can transmit a conformational change on the cytoplasmic face, so called "**Outside – In**" signaling. However, interactions within the cytoplasmic domains can modulate the substrate binding affinity of the extracellular domains, so called "**Inside – Out**" signaling.

Integrins exist as heterodimeric combinations of 17 α subunits and 8 β subunits that interact non-covalently to create more than 20 heterodimeric family members (Plow EF et al, 2000). The specific α/β pairing determines the ligand-binding specificity of the integrin. The subunits consist of larger extracellular ligand binding and transmembrane domains and shorter cytoplasmic tails (Berman AE et al, 2000). Some integrins display specificity for short sequences; the most extensively studied of these is the tripeptide, arginine-glycine-aspartate (RGD), which is present in a variety of ECM proteins (Ruoslathi E et al, 1996). Other integrins recognize conformational structures composed of different Amino Acids (Ruoslathi E et al, 1996). Different cells may express a variety of different integrins, with overlapping specificity and several different integrins may bind to a specific component of the ECM. For example $\alpha_v\beta_3$ binds to laminin, collagen, fibronectin and tenascin (Boudreau NJ et al, 1999). Laminin also associates with $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_4$ integrins (Plow EF et al, 2000; Giancotti FG, 2000). The significance of these findings in unclear, however it is postulated that this allows a rapid response of the ECM to changes in the microenvironment. The integrin family and their ligand specificity are demonstrated in the following table (Table 1).

Table 1: Integrin family and their ligands

Integrin Family		Ligand Selectivity
β1	α1	Laminin, Collagen
	α2	Laminin, Collagen, α3β1
	α3	Laminin, Collagen, Fibronectin, Epiligrin, Entactin, α2β1
	α4	Fibronectin (CS-1), VCAM
	α5	Fibronectin (RGD), L1-CAM, Fibrinogen
	α6	Laminin, Merosin. Kalinin
	α7	Laminin
	α8	Fibronectin
	α9	Tenascin
	αv	Fibronectin, Vitronectin
β2	αL	ICAM-1, ICAM-2, ICAM-3
	αM	iC3b, Fibrinogen, Factor X, ICAM-1
	αX	iC3b, Fibrinogen
αv	β3	αIIb - Fibrinogen, Fibronectin Fibronectin, Vitronectin, Von Willebrands factor, denatured Collagen, Thrombospondin, Del 1, Cyr 61, FISP
	β1	Fibronectin, Vitronectin
	β5	Vitronectin, Osteopontin, Del1, denatured Collagen
	β6	Fibronectin
	β8	Fibronectin
α6β4		Laminin
α4β7		VCAM, MADCAM, Fibronectin (CS-1)

This figure illustrates the substrate specificity, thus far confirmed for the integrin family, adapted from Jin H et al, 2004.

Section 1.8.3-I

Integrin activation

Association of integrins with ECM ligands initiates the assembly of adapter proteins to form focal adhesion complexes that link to the cytoskeleton (Calderwood DA et al, 2000). In cultured adherent cells grown in the presence of serum factors, integrins first associate with talin and α–actinin, then with tensin, vinculin and paxillin to recruit actin filaments (Critchley DR et al, 2000). The re-organization of actin by focal adhesion complexes, in turn causes integrin clustering, which increases the cell-ECM binding by a positive feedback mechanism. This process physically links the cytoskeleton to the ECM, so that perturbations in the ECM are rapidly transduced to the inside of the cell and vice versa (Colognato H et al,1999). Cytosolic enzymes that modulate cytoskeletal structure also effect the organization of the ECM. Thereby, cells in contact with each other are organized into a functional tissue (Radisky D et al, 2002).

Section 1.8.3-II

Intracellular Pathways & Integrin Signalling

Ligation of integrins to ligands expressed by the ECM activates signal transduction pathways through kinases present in focal adhesion complexes. These include tyrosine kinases such as focal adhesion kinase (FAK), integrin linked kinase (ILK) and members of the Src kinase family, as well as serine-threonine kinases of the Abl family.

a) Focal adhesion Kinase (FAK):

FAK is a 120 kDa non-receptor tyrosine kinase. In normal cells, FAK is activated by cell adhesion and rapidly deactivated on cell detachment. FAK signals via many pathways to mediate anchorage dependent cell survival and growth (Renshaw MW et al, 1999). Upon integrin ligation and clustering FAK undergoes rapid tyrosine phosphorylation, which then interacts with docking or adaptor proteins such as paxillin, tensin and Grb2/Son of sevenless ("SOS") (Boudreau NJ et al, 1999). These interactions are not due to FAK phosphorylating these proteins as it is unable to phosphorylate substrates directly. However, when FAK interacts with these docking proteins this in turn activates

downstream signaling mediators, which play an important role in the control of growth including, Src, Ras and Raf (Juliano R, 1996; Calab MB, 1996; Schlaepfer DD, 1994). The importance of FAK is illustrated by the fact that mutations of the tyrosine residues necessary for FAK autophosphorylation, prevent integrin mediated proliferation (Frisch SM et al, 1996). In normal cells, up-regulation of constitutively active FAK leads to cell transformation, anchorage-independent growth and the suppression of apoptosis (Zachary I et al, 1992).

FAK can be activated by certain soluble growth factors, which indicates that integrin and growth factors signaling pathways may converge at FAK (Zachary I et al, 1992). This is reinforced by studies suggesting that following integrin clustering, growth-factor receptors are also recruited into focal adhesion complexes. For example endothelial cells coated with RGD tripeptides have co-aggregation not only of β1 integrins and FAK, but also high affinity receptors for fibroblast growth factor (FGF) in the newly assembled focal adhesions (Plopper GE et al, 1993; Plopper GE et al, 1995). Additional studies have shown that Platelet derived growth factor (PDGF) and Epidermal derived growth factor (EGF), can also interact with integrins via specific growth factor receptors (Miyamoto S et al, 1996). The interactions of growth factors and integrins rely on the functioning of an intact actin cytoskeleton within the cell (den Hartigh JC et al, 1992; Diakonova M et al, 1995). This is illustrated by the action of cytoskeleton inhibitors such as cytochalasin D, which not only inhibit focal adhesion formation but also the activation of growth factor receptors, including PDGF and EGF, resulting in the attenuation of cellular growth as the cell enters G_0 arrest (Defilippi P et al, 1997; Abedi H et al, 1997).

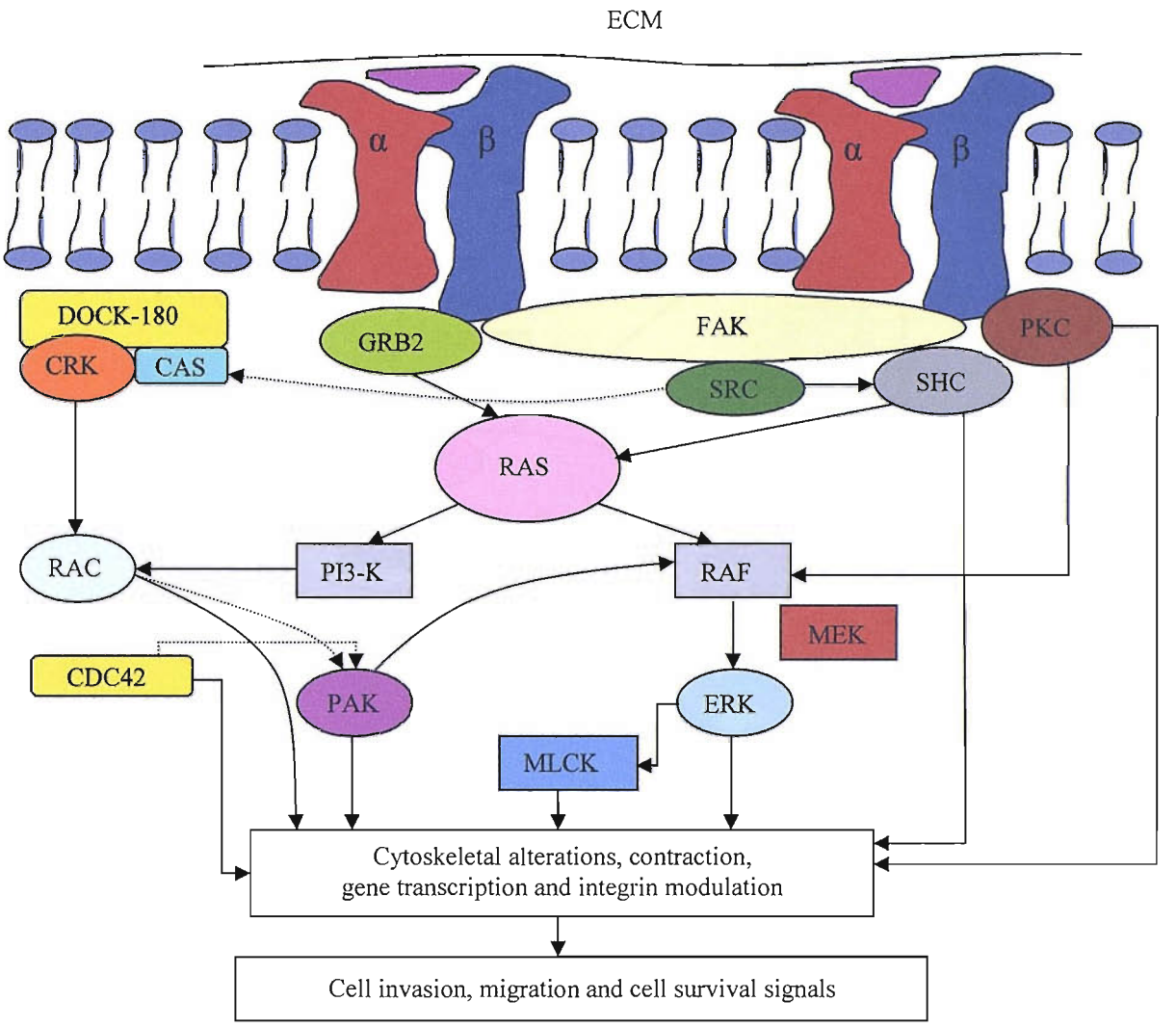
b) <u>Mitogen Activated Phosphokinase (MAPK)</u>

Activation of this pathway provides a common route leading to transcriptional regulation of genes that are crucial for cell growth and differentiation (Boudreau NJ et al, 1999). Family members are activated following transient Ras GTP-binding protein activation, via receptor tyrosine kinase, and include MAPK/ERK kinase (MEK; also known as MAPKK) and ERK1/ERK2 (Hill CS et al, 1995). MEK activation caused by exposure of cells to soluble mitogens leads to phosphorylation of ERK1 and 2, which results in their translocation to the nucleus, where they in turn phosphorylate and activate a number of

transcription factors (Hill CS et al, 1995; Seger R et al, 1995). In addition to growth factors, adhesion of cells to ECM proteins including collagen, via ligation of integrins (e.g. $\alpha v\beta 3$) activates the MAPK pathway (Chen Q et al, 1994; Yokosaki Y et al, 1996). Integrin mediated MAPK activation may act independently of FAK or RAS, implying other pathways may also be important (Lev S et al, 1995; Sasaki H et al, 1995). However the significance of so called "FAK–related proteins" is not known.

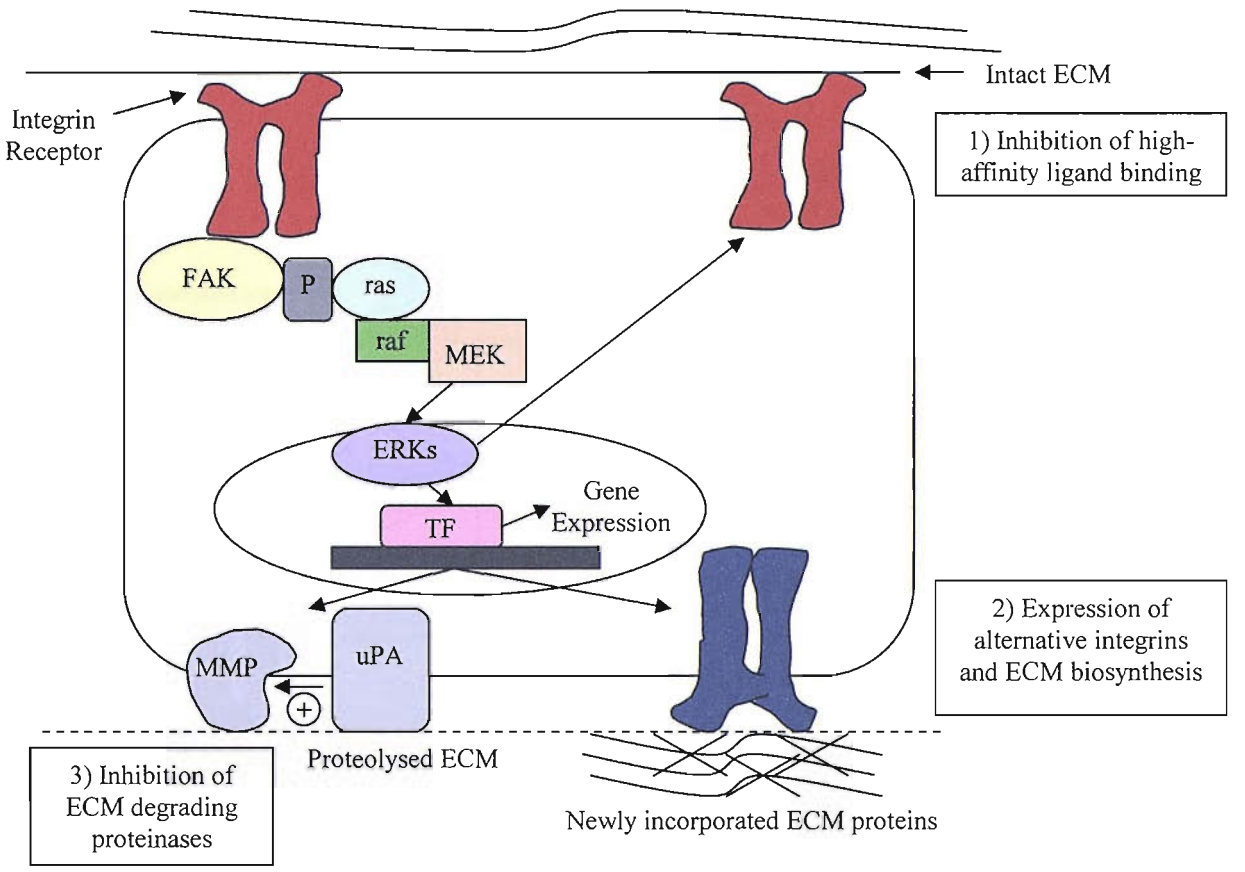
It is unclear if integrin binding initiates signals that pass via the MEK pathway or modulate and/or enhance signals generated by soluble mitogens (i.e. growth factors), due to the intimate association of signals produced by both integrins and growth factors. However, whereas growth factor mediated MAPK activation is Ras dependent, integrin MAPK activation is not (Chen Q et al, 1996). In vitro studies have demonstrated the kinetics of MAPK activation by soluble mitogens and integrin ligation is different (Zhu X et al, 1996). However the cancer cells may produce their own mitogens which can effect genes associated with $G_0 - G_1$ transitions, even in serum free conditions (Hill CS et al, 1995; Zhu X et al, 1996; Rana B et al, 1994), which means these results must be interpreted with care. Therefore, all that can be concluded is that both integrins and MAPKs are essential for eliciting specific cellular responses. What seems likely is that adhesion-mediated regulation of growth factor signaling enables cells to respond properly to the milieu of positive and negative signals from growth factors and cytokines (Boudreau NJ et al, 1999).

Figure 6a: Regulation of intracellular signaling by integrins



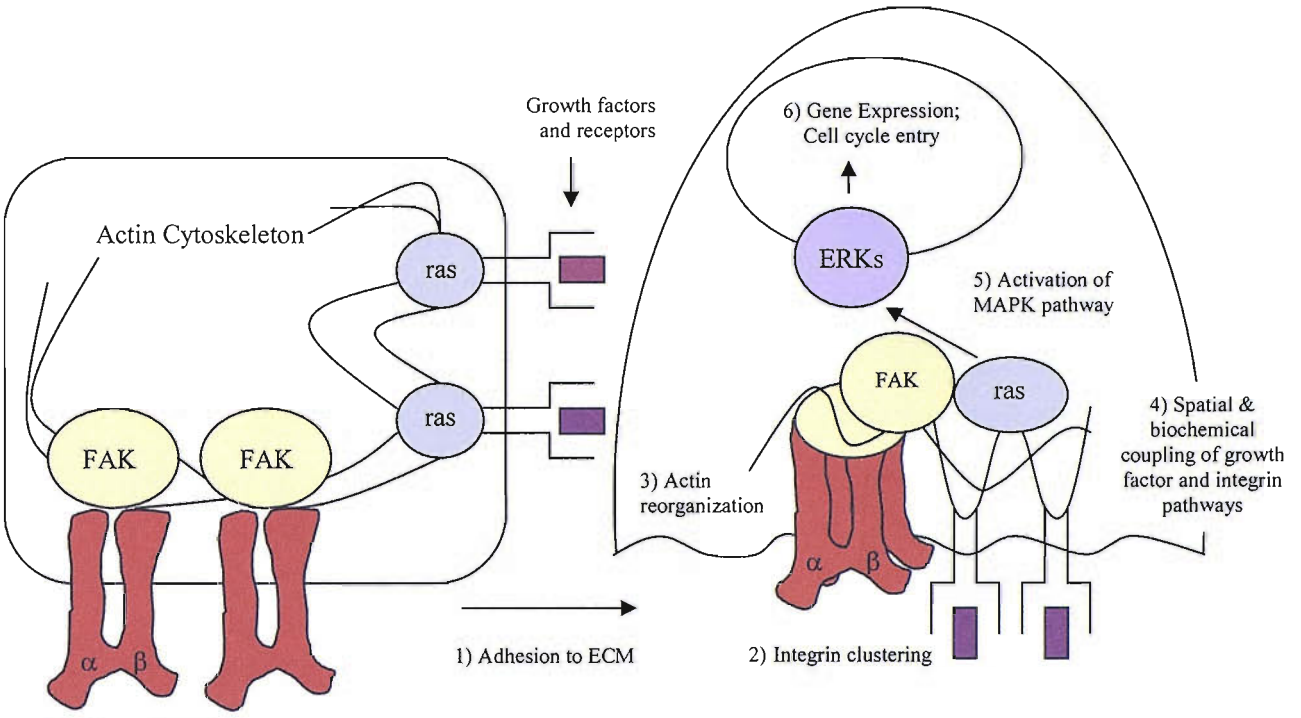
Integrin ligation induces a complex network of signaling pathways to control cell migration. Integrin binding activates Focal Adhesion Kinase (FAK), which in turn binds and activates multiple signaling proteins. FAK autophosphorylation causes it to bind growth-factor receptor bound protein 2 (GRB2) and activate another small G-protein RAS. FAK also promotes SRC dependent phosphorylation of SHC, leading to GRB2 recruitment and RAS activation. Activated RAS recruits RAF to the cytoplasmic membrane, where it can be activated by protein kinases such as SRC, thereby leading to mitogen activated protein kinase (MEK) and extracellular signal regulated kinase (ERK) activation. Once FAK or SHC, activate RAS, this in turn can activate phosphatidinositol 3-kinase (PI3K) and RAF. Activated SRC can autophosphorylate CRK associated substrate (CAS), enabling it to bind CRK and dedicator of cytokinesis 180 (DOCK 180), leading to RAC activation. Activated RAC, in conjunction with activated CDC42, can regulate numerous biochemical pathways, including p21 activated kinase (PAK). PAK in turn interacts with many other pathways including activation of RAF kinase. MEK once activated by RAS and RAF can phosphorylate and activate ERK. ERK activation leads to transcriptional activity, alterations in integrin affinity for ligand and myosin light chain kinase (MLCK) activity. Independent of FAK activation, SHC and PKC are activated by integrin adhesion. Re-produced and adapted from Hood JD et al, 2002.

Figure 6b: Inside-Out Signalling - The functions of FAK and MAPKs



This figure graphically represents how integrin-mediated activation of FAK/MAPK (ERK) signal induction pathway may limit or modify integrin-ECM interactions from inside-out. Activation of the FAK/MAPK pathway leads to inhibition of integrin binding to the ECM in a manner that is independent of de novo transcription. (1) Activation of MAPK pathway may lead to the transcription of target genes that modify cell-ECM interactions. Which, include integrins, ECM proteins (2) and ECM-degrading proteases such as urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs) (3). Abrreviations used; P = Phosphorylation, TF = Transcription factors. **NB.** uPA results in the conversion of plasminogen to plasmin, which in turns activates many MMPs, including MMP-1, 3 and 9. This figure is re-produced and adapted from Boudreau NJ et al, 1999.

Figure 6c; Outside-In signaling, the effects of integrin binding on tyrosine protein kinase pathways and the actin cytoskeleton



This figure graphically represents the influence of integrin dependent changes in cell shape are mediated via the MAPK signaling pathway and downstream cellular functions. Cell adhesion to the ECM via integrins activates FAK, leading to re-organization of the actin cytoskeleton and subsequently changes in cell shape. The cytoskeletal associated proteins, including integrins and growth factor receptors, couple within focal adhesions, thereby interacting and activating the ERK signal transduction pathway. This figure is re-produced and adapted from Boudreau NJ et al, 1999.

c) Integrin mediated effects on Apoptosis

Integrin binding to an appropriate extracellular matrix, via specific ligands prevents cellular apoptosis (Hood JD et al, 2002). The concept of integrin-mediated reliance for survival poses a possible hurdle for neoplastic cell growth. The distinct set of integrin receptors and matrix receptors is tissue and cell specific, therefore if the correct integrin receptor and ligand are not expressed correctly by the cell or matrix, apoptosis ensues via up-regulation of caspase 8 (Frisch SM et al, 1997; Stupack DG et al, 2001). Successful metastatic cells therefore possess a wide variety of integrin receptors and due to the action of proteinases, new ligands which are not normally expressed by the ECM may be revealed. If the cancer cell properly binds these ligands in a particular tissue integrin

mediated pro-survival signals are initiated and the cell survives and can continue to migrate and grow (Matter ML et al, 2001; Frisch SM et al, 1996).

d) Integrin effects on Angiogenesis

The establishment of a tumour mass requires a new blood supply. Several integrins have been implicated as angiogenesis regulators on endothelial cells (Elicieri BP et al, 1999; Elicieri BP et al, 2001). The exact mechanisms by which these angiogenic effects are mediated are still not entirely understood. The αv integrins including αvβ3 and αvβ5 are postulated to be important regulators of angiogenesis. This was illustrated by studies showing that these αv integrins are up-regulated on certain tumour vasculatures (Brooks PC et al, 1994). In addition studies on breast cancer and the eye have shown that by inhibiting αv integrin ligation using monoclonal antibodies that block the RGD tri-peptide sequences recognized by these integrins, was potently anti-angiogenic (Brooks PC et al, 1995; Friedlander M et al, 1995). This therefore suggests that αv integrins are important pro-angiogenic mediators. However, it has now become apparent that these integrins may also possess anti-angiogenic properties via there interactions with alternative ligands expressed by the ECM. Theses include Thrombospondin-1, which when bound by αvβ3, may exert an anti-angiogenic effect (Adams JC, 2001; Jimenez B et al, 2000). αvβ3 integrin ligation, also leads to up-regulation of MMP-2, which although normally is pro-angiogenic, can result in matrix degradation which may reveal anti-angiogenic factors (i.e. tumstatin) (Hynes RO, 2002; Maeshima Y et al, 2002). In addition MMP-2 can cleave itself releasing the anti-angiogenic fragment (PEX) (Brooks PC et al, 1998). Alternatively, thrombospondin and MMPs may have pro-angiogenic effects, for example by up-regulating Vascular Endothelial growth factor (VEGF) levels and low levels of αvβ3 integrins also may be pro-angiogenic as this leads to up-regulation of VEGF receptors (Hynes RO, 2002; Reynolds LE et al, 2002). Therefore, depending on the balance of pro and anti-angiogenic factors mediated via integrins, local control of angiogenesis can be achieved, with αvβ3 integrin playing a role as a balancer of pro-angiogenic and anti-angiogenic effects.

Section 1.8.4-I

The influence of cell shape and mobility on cell growth

The organization of the actin network within cells is controlled by the Rho family of small GTPases, consisting of Rho, Rac and Cdc42, each of which controls different aspects of cytoskeletal structure in cells (Schoenwaelder SM et al, 1999). Rho controls the generation of stress fibres, which are thick bundles of actin filaments which attach to the focal adhesions. Rac controls the formation of thin actin bundles (lamellipodia) that can lift up and fold backwards. Cdc42 controls the formation of thin actin bundles which cause protrusions at the cell surface (filopodia) (Scita G et al, 2000). The concept of affinity maturation is important in terms of integrin activation as intercellular signals can modulate ligand associated properties, thereby, affecting the binding activity of integrins to the ECM (Hughes PE et al, 1998). It is now thought that the expression of integrins on the cell surface is in a low-affinity conformation, which requires interaction with cytoplasmic components to become activated (Fernandez C et al, 1998). Affinity maturation is thought to be modulated by pathways linked to the G-protein coupled receptors of the Ras family (Aplin AE et al, 1998) or through cytoskeleton manipulation by the Rho family of GTPases (Hall A, 1998; Schwartz MA et al, 2000).

Section 1.8.4-II

Cell-Cell Adhesion: Sensing thy neighbours

The principle molecule responsible for the formation of direct contacts between epithelial cells is E-Cadherin (Steinberg MS et al, 1999). Adhesions via cadherins between cells are mediated via adherens junctions formed in the presence of calcium ions which allows cell-cell adhesion (Troyanovsky SM, 1999). Like integrins, cadherins bind to the actin cytoskeleton, through a complex of cytosolic adaptor proteins (Radisky D et al, 2002). Integrin signaling can also modulate cadherin function through the adaptor integrin-linked kinase (ILK). Activation of ILK, down regulates E-cadherins and results in the epithelial to mesenchymal transition of cells (Dedhar S, 2000). Cadherins also interact directly with signal transduction pathways that impact on cell proliferation and differentiation (Hazan RB et al, 1998). Cadherins may also indirectly affect the wnt signaling pathway by sequestering β-catenin which activates genes linked to wnt (Ben-Ze'ev A et al, 2000). Wnt

signaling is important in regulating epithelial cell proliferation and is often dysregulated in CRC (Peifer M et al, 2000).

Therefore, the expression of cell-cell and cell-matrix adhesion molecules is complex and integrated both in normal functioning cells and tissues and in malignancy. Changes within ECM composition may both effect cell-matrix interactions and thereby cell growth, but also cell-cell adhesion.

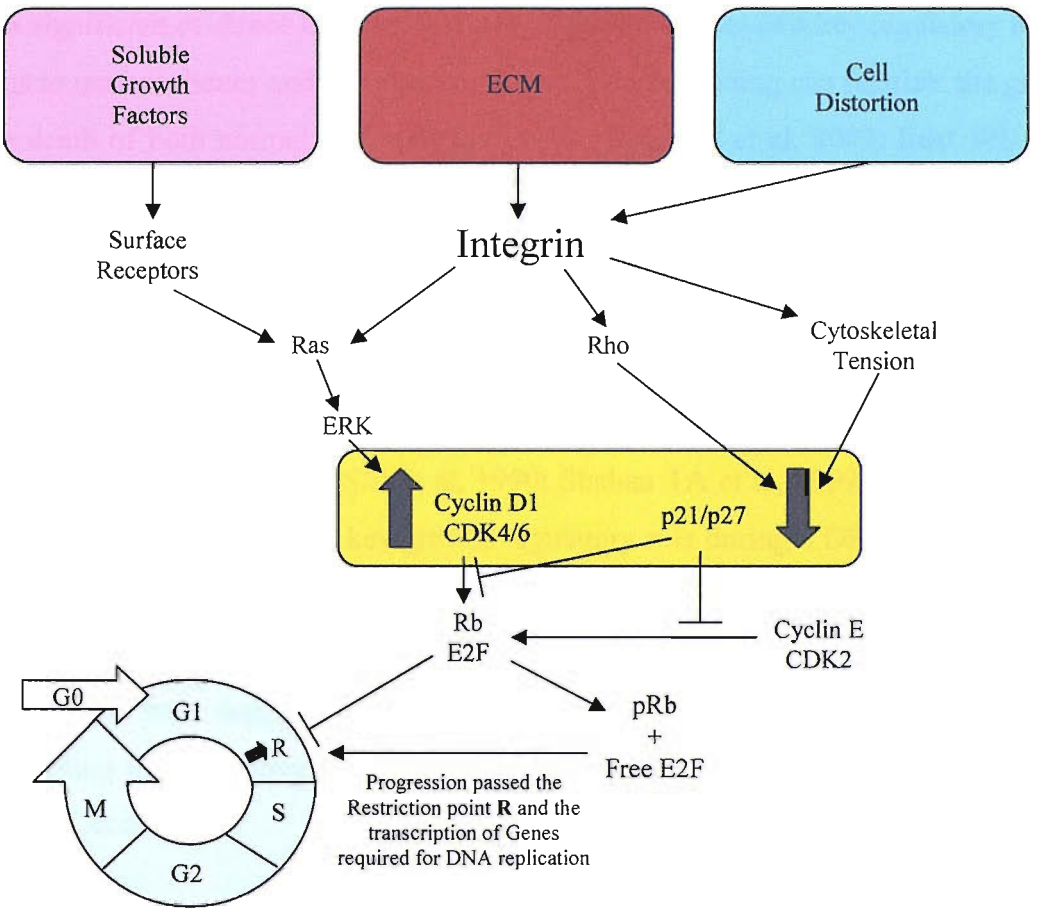
Section 1.8.4-III

Interaction between Cell Shape, Growth factor expression and Integrin ligation

This is illustrated in Figure 7, and shows the complex interaction between all these factors. Simplistically, the control of cell cycle regulation cannot be defined solely in terms of the presence or absence of particular mitogens, integrin binding and mechanical forces. Instead all these factors are interconnected and contribute to the progression of cells from resting G_0 state to G_1 and then to undergo mitosis (Cell cycle stages see - Appendix 1b). However, is this new understanding consistent with what is seen in vivo?

Well, in the case of bone this governing principle is known as "Wolff's law" which states that a bone develops the structure most suited to resist the forces acting upon it (updated by Huang S et al, 1999). Also, in recent genetic studies on the effect of growth factors in lung morphogenesis in both the drosphilia respiratory system and mammalian lung it was concluded that the mechanisms controlling the growth of new branches in these organs is due to the localized production of several soluble growth factors (Metzger RJ et al, 1999), which drive growth and govern the site of these new branches at the tissue level, whereas localised changes in ECM turnover and composition and thereby integrin ligation and cytoskeletal mechanics determine precisely which cells will respond to these factors (Huang S et al, 1999).

Figure 7: The interactions between Integrins, Growth factors and Cell Shape.



This figure demonstrates how cell distortion, soluble growth factors and the extracellular matrix (ECM) regulate progression of cells from G1. The control of late G_1 arrest is under the influence of the level of phosphorylation of the retinoblastoma protein (pRb), which upon phosphorylation allows cell entry past the late G1 Restriction point (R) and entry into S phase and thereby the process of cell division. Cyclins (D and E) and cyclin dependent kinases (CDK's) control cells passage past the restriction point, due to their regulation of the level of phosphorylation of the retinoblastoma protein. Cyclin and CDKs actions are influenced by cyclin binding, their level of phosphorylation and the presence of cyclin inhibitor proteins p21 and p27. Cyclin D and E control progression of cells by assembling CDK partners which when activated, phosphorylate and thereby inactivate Rb proteins, releasing E2F. The late G1 restriction point marks the end for the need for external growth factors in the cell cycle. This figure reproduced and adapted from Huang S et al, 1999.

Section 1.9

The role of Integrins in Cancer

There is significant evidence from both in vivo and vitro studies of a key regulatory role of integrins in normal tissues and many cancers, that integrin binding can regulate the growth and the death of both normal and malignant cells (Hood JD et al, 2002; Rust WL et al, 2002). Up-regulation of these adhesion molecules has been associated with adverse outcome in many cancers. For example αvβ3 and αvβ5 integrins, both in vitro and in vivo studies have long been associated as a key mediators in melanoma and other cancers, because ligation of these integrins are associated with the stimulation of growth promoting pathways, angiogenesis and reduced rates of cellular apoptosis (Petticlerc E et al, 1999; Brooks PC et al, 1994; Albelda SM et al, 1990; Shahan TA et al, 1999; Kumar CC et al, 2001). αvβ3 integrin may play a key growth regulatory role during a DR as its ligands are often only revealed after matrix turnover (Davis GE, 1992; Xu J et al, 2001). That is why this integrin is viewed as a key mediator in the repair of tissue after injury. In vitro studies using CRC lines have suggested that up-regulating αvβ5 integrins, may play an important role in shifting the malignant phenotype of CRC to a more aggressive and invasive type (Schramm K et al, 2000).

Down regulation of some integrins may also be poor prognostic factor in cancers. In prostate cancer down-regulation of certain β1 integrin isotypes (Perlino E et al, 2000), α6 and β4 (Bonaccorsi L et al, 2000) integrins have been shown to be poor prognostic indicators, and in melanoma high β1 expression has been shown to be a good prognostic indicator (Vihinen P et al, 2000). However, the effects of β1 integrin do not always follow the same pattern; instead they appear to be tissue specific and reliant on many other factors. In vitro studies in breast and ovarian cancer cell lines showed that up-regulation of β1 integrins increased the cells invasiveness (Arboleda MJ et al, 2003) and in bowel and breast cancer specimens up-regulation of β1 integrin were a poor prognostic indicator associated with metastatic spread (Fujita S et al, 1995; Morini M et al, 2000).

Section 1.10

The possible Role of Matrix Metalloproteinases in CRC liver metastases

Matrix metalloproteinases (MMPs) have been shown to play a crucial role in the development of CRC liver metastases (Garbett EA et al, 1999; McDonell S et al, 1999; Theret N et al, 1997). They form a group of zinc proteinases whose main function is the degradation of the extracellular matrix (ECM) which occurs in tissue formation and remodelling (Matrisian LM, 1990). Invasion of the basement membrane and extracellular matrix is a critical step in the growth of a tumour and the process of metastatic spread (Egelbad M et al, 2002). MMPs are present in normal healthy individuals and play an important role in the turnover of the ECM (Vu TH et al, 2000). Their normal regulation is altered in pathological conditions such as tumour invasion and metastases resulting in the loss of ECM function and compromised matrix boundaries (Chang C et al, 2001; Egelbad M et al, 2002). MMPs are strictly regulated at several levels; including their synthesis at a gene level; their secretion as pro-enzymes and subsequent activation; and their inhibition by natural inhibitors the tissue inhibitors of matrix metalloproteinases (TIMPs) (Sternlicht MD et al, 2001; Gomez DE et al, 1997).

In cancer, proteinases are thought to enhance cancer invasion by catalysing the degradation of ECM components. Such degradation occurs at several levels of the metastatic cascade, including angiogenesis, local invasion and intravasation (Chang C et al, 2001). For this to take place a number of different proteinases are needed. That is why over 25 different MMPs have now been recognized (Egelbad M et al, 2002).

Section 1.11

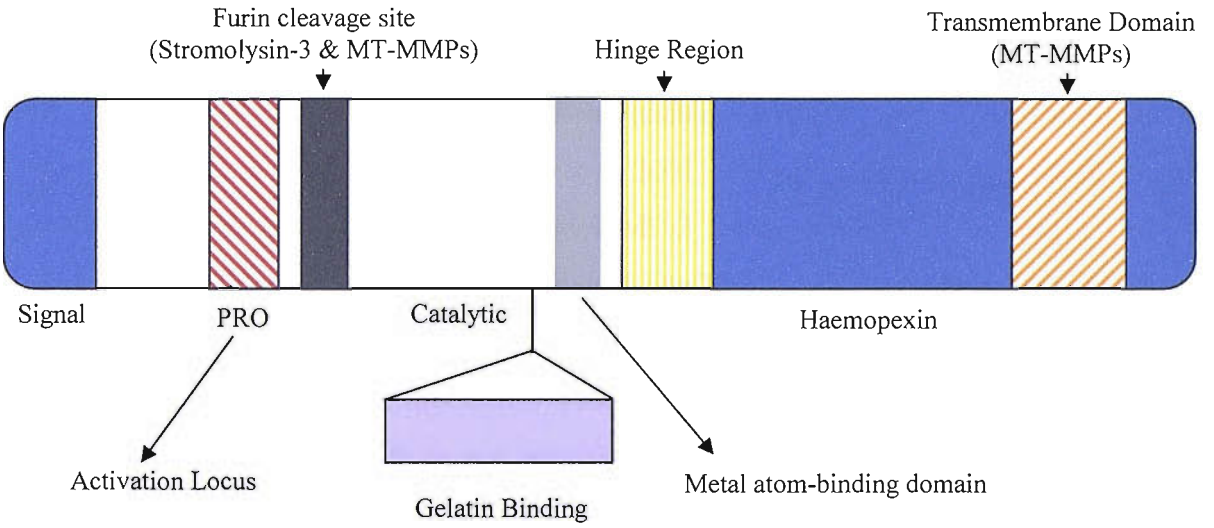
Classification and Structure of Matrix Metalloproteinases

MMPs were originally sub-divided into groups based upon their substrate specificity. However, with the increased numbers of MMPs identified, they are now grouped according to their structure. By this classification there are now eight distinct structural classes of MMPs (Egelbad M et al, 2002). All MMPs have broadly the same basic structure, consisting of 4 domains (Yu AE et al, 1997). These are a propeptide domain (N-terminal) which is involved in the maintenance of enzyme latency and is removed during enzyme activation (Van Wart HE et al, 1990). A signal peptide domain which directs the

translational product of the endoplasmic reticulum and the catalytic domain which contains a highly conserved site in which 3 histidine residues co-ordinate Zn ion binding in the activated protease (Yu AE et al, 1997). The fourth domain is called the C-terminal and shows homology for vitronectin and haemopexin in all MMPs except the smallest MMP, Matrilysin (MMP-7) where it is very truncated (Libson AM et al, 1995). In others the C-terminal is considerable larger and plays an important role along with the catalytic domain in determining specifity for each MMP (Shapiro SD, 1998; Nagase H et al, 1999). The C-terminal is also important in that its loss results in inactivation of the MMP. However, there are two exceptions; these are MMP-2 and 9, the gelatinases. They possess an additional domain in comparison to the other MMPs and even when the C-terminal is bound by their natural inhibitors, they retain matrix degrading activity (Murphy G et al, 1992). In addition the C-terminal domain in these two MMPs can also can be used for cell binding, pro-enzyme activation and binding TIMP-2 (Yu AE et al, 1997; Emmert-Buck et al, 1995; Kleiner DE et al, 1992).

Most MMPs are secreted as pro-enzymes with the exception of Stromelysin 3 (MMP-11) and the MT-MMPs, which are activated intracellularly by furins (furins are intracellular proteases) via a furin enzyme recognition site (Sternlicht MD et al, 2001). MMP synthesis is regulated by growth factors and cytokines and by the matrix via integrin-linked pathways (Vu TH et al 2000; Riikonen T et al, 1995). Via this latter route the matrix can directly regulate its own homeostasis. Once secreted MMPs can exist in latent and active forms and can be held in complexes with or without their natural inhibitors (TIMPs) (Egelbad M et al, 2002). Only free unbound active MMPs are biologically active. MMP latency is maintained by a "cysteine switch" formed by the interaction between the sulphadryl group of a conserved cysteine residue within the propeptide domain and the catalytic zinc within the catalytic domain. This interaction blocks zinc-dependent activation of a water molecule required to mediate the nucleophilic attack on peptide bonds (Van Wart HE et al, 1990). The activation of a number of MMPs is mediated via the plasminogen activation system and the action of other MMPs (Irigoyen JP et al, 1999; Murphy G et al, 1999). With regards MMPs that are able to degrade collagen, MMPs 1, 2, 8, MT1-MMP (MMP-14) and MMP-13 in rodents are the only ones able to degrade native or non-denatured collagen (Benyon RC et al, 2001; Aimes RT et al, 1995; Holmbeck K et al, 1999; Ohuchi E et al, 1997).

Figure 8: The structure of MMPs



This figure illustrates the domain structure of Matrix Metalloproteinases. Each member has at least 4 domains. (1) Signal peptide sequence, which localizes the translation product to the endoplasmic reticulum. (2) Pro-peptide domain (PRO), which is lost during enzyme activation. (3) Catalytic domain, which contains the conserved sequences (VAAHEXGHXXGXXH), where the 3 histidine (H) residues co-ordinate the active Zn site. (4) C-terminal domain, which has sequence homology with haemopexin and vitronectin, which is severely truncated in matrilysin (MMP-7) and contains a transmembrane domain in MT-MMPs. There is a fifth domain in MMP-2 and 9, which shares homology with type II fibronectin repeats; this domain binds gelatin. Reproduced from Yu AE et al, 1997.

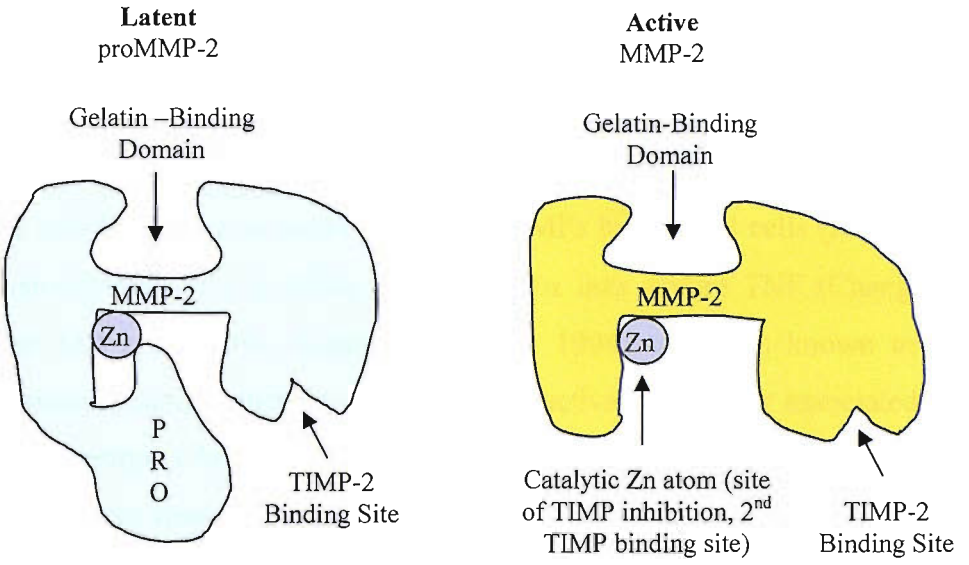
Section 1.12

The Tissue Inhibitors of Metalloproteinases (TIMPs)

There are four human TIMPs, which have been identified and all have closely related structures and size (Gomez DE et al, 1997). All TIMPs act by binding to most active MMPs to deactivate them, forming a reversible complex with TIMP:MMP in a stochiometric ratio of 1:1 (Sternlicht MD et al, 2001). However, TIMP 1 specifically binds proMMP-9 (Goldberg GI et al, 1992) and TIMP-2 binds proMMP-2 (Howard EW et al, 1991) at an additional site at the C-terminal. When TIMPs bind to latent forms of these 2 MMPs, these complexes can still be activated and retain some protease activity (Murphy G et al, 1992). The reason for this is that MMPs 2 & 9, have 2 TIMP binding sites. This additional TIMP binding site is not specific for any particular TIMP and can only be revealed after cleaving the pro-enzyme fragment (Yu AE et al, 1997). Therefore in summary the balance between active MMPs and free TIMPs determines the total amount of ECM degradation.

TIMPs have other roles than purely inhibiting MMPs. It is known that high levels of TIMP 1 and 2 inhibit angiogenesis; probable by interfering with the production of angiogenic factors induced by the release of MMPs as angiogenesis was believed to follow the same pathway as seen during metastatic spread (Johnson MD et al, 1994; Schnapper HW et al, 1993). Consisting of attachment, proteolysis and migration of new vessels, with the last 2 highly MMP dependent, therefore high TIMP levels are likely to interfere with this process. Evidence for the importance of MMPs in angiogenesis was suggested by the fact that high levels of MMP-2 encourage angiogenesis, although if MMP-2 levels are excessively elevated they appear to have an inhibitory action on angiogenesis (Schnapper HW et al, 1993), perhaps mediated via the generation of anti-angiogenic factors (i.e. tumstatin and PEX) as discussed in section (1.8.3-IId). Another, newly discovered function of TIMPs is that they may have growth promoting properties and inhibit cellular apoptosis, both by MMP dependent and independent means (Murphy FR et al, 2002; Guedez L et al, 1998; Guedez L et al, 2001). In CRC it has been shown that high TIMP 1 and 2 in liver metastases are very poor prognostic indicators (Howell RD et al, 2002), which might be attributable to these recently discovered functions.

Figure 9: The domain structure and interactions with TIMP of gelatinases (MMP-2,9)



This figure schematically illustrates the domain structure of the gelatinases and their interactions with TIMP-2. ProMMP-2 has 3 essential domains. (1) The prodomain (PRO) with a conserved cysteine residue, which binds with a zinc (Zn) ion in the active site to maintain enzyme latency. (2) The gelatin binding domain (homolgous to the gelatin binding domain of fibronectin). (3) The TIMP-2 binding site in the c-terminus of the enzyme. Through this site pro-MMP-2 forms a tight complex with TIMP-2. This complex as well as TIMP-2 free proMMP-2 can be activated by removal of the pro-fragment to generate active enzyme or active enzyme-TIMP-2 complex. A 2nd TIMP binding site (not specific for TIMP-2) becomes available on cleavage of the pro-fragment during enzyme activation and is responsible for the MMPs deactivation. Reproduced from Yu AE et al, 1997.

Section 1.13

The Transmembrane Matrix Metalloproteinases (MT-MMPs)

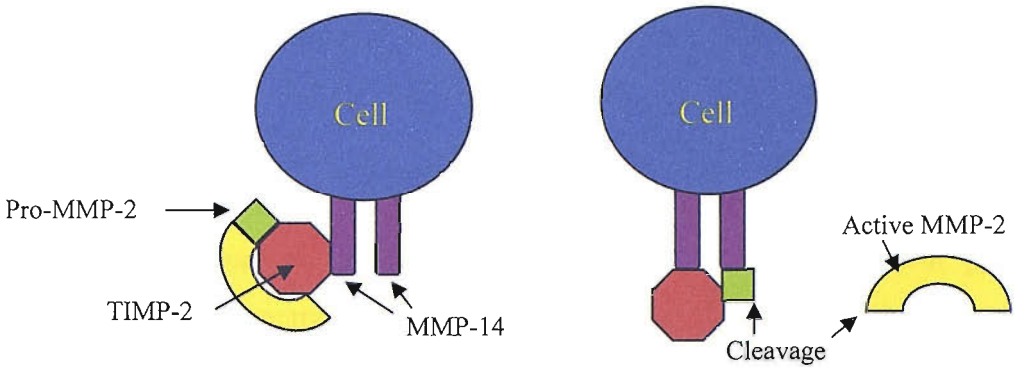
These are the membrane bound MMPs (MT-MMPs), which have been shown to play an important role, in the activation of MMPs (Sato H et al, 1994; Werb Z, 1997; Knauper V et al, 1996). At present seven MT-MMPs have been identified, with the most widely studied being MT1-MMP (MMP-14), which activates MMP-2 and 13 (Sato H et al, 1994, Knauper V et al, 1996)). All have the same basic structure as MMPs and are membrane bound. There are 3 classes of MT-MMPs; a) those that possess a transmembrane domain within the C-terminal (MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP); b) Glycosyl phosphatidyl anchored MT-MMPs (MT4-MMP and MT6-MMP) and c) an N-terminal signal anchored MT-MMP (MMP-23) (Eglebad M et al, 2002). MT-MMPs are secreted in complexes with TIMPs (Imai K et al, 1996; Yu AE et al, 1997) and activated via furins in

a similar manner to Stromelysin 3 (MMP-11) (Sternlicht MD et al, 2001). Binding with TIMPs deactivates most MT-MMPs, with the exception of TIMP-1 which is unable to inhibit MT1-, MT2- and MT5-MMP (Will H et al, 1996).

Both tumour and stromal cells produce MT1-MMP, with TNFα known to potently up-regulate MT1-MMP production as well as other MMPs by stromal cells (Migita K et al, 1996). Activated MMPs and ADAMs process TNFα into mature TNF (Chang C et al, 2001; d'Ortho MP et al, 1997; Gearing AJ et al, 1994), which is known to elicit a subsequent stromal reaction; thereby recruiting and activating tumour associated stromal cells. There is therefore a feedback mechanism formed which is believed important in the pathogenesis of cancer spread (DeClerck YA, 2000).

Over expression of MT1-MMP has been associated in cervical cancer with the development of nodal metastases (Gilles C et al, 1996) and in the case of breast cancer the presence of lymphatic and distant metastases (Ishigaki S et al, 1999). It has now been demonstrated that MMP-2 is activated through a multi-step pathway involving TIMP-2 and MT1-MMP. TIMP-2 acts as a bridging complex binding to MT1-MMP via its amino acid terminus and pro-MMP-2 via its carboxyl terminus (figure 10a). This then allows a non-inhibited MT1-MMP molecule to cleave the bound 72 kDa pro-MMP-2 and results in higher levels of intermediate 66 kDa pro-MMP-2 (Sato H et al, 1994; Strongin AY et al, 1995). The MT1-MMP does not completely activate the MMP-2; instead the 66 kDa pro-MMP-2 interacts with αvβ3 integrin and an already fully activated MMP-2, which results in the creation of the fully activated 62 kDa enzyme (Deryugina EI et al, 2001). An important feature of this regulation of MMP-2 activity is that it concentrates MMP-2 activity at sites of active peri-cellular matrix degradation and may protect the MMP-2 from the inhibitory effects of extracellular TIMPs. This is a possible explanation of the findings in breast cancer of high levels of TIMP-2 being associated with tumour recurrence (Remacle A et al, 2000) as it acts as part of this complex. However, the level of TIMP-2 is important in this growth complex, with MT1-MMP and TIMP-2 levels needing to be approximately equimolar to achieve effective pro-MMP-2 capturing and thereby increased active MMP-2 levels. With higher levels of TIMP-2 the effect is to inhibit proteolysis because it blocks MT1-MMP activity and thereby reduces the level of active MMP-2 (Ward RV et al, 1991). The process of MMP-2 activation is shown below.

Figure 10a: Activation of MMP-2, involves MMP-14 and TIMP-2



This figure illustrates how MMP-2 is activated. A TIMP-2 and Pro-MMP-2 molecule are linked together in complex with MT1-MMP (MMP-14). The pro-MMP-2 molecule is then cleaved by another MMP-14 molecule and following further processing releases the activated MMP-2 enzyme.

Section 1.14

Matrix Metalloproteinase Inhibitors (MMPIs)

There is considerable evidence to show a role for MMP/TIMP imbalance in the development of colorectal cancer liver metastases (Theret N et al, 1997; Swallow CJ et al, 1996; Musso O et al, 1997); it was therefore postulated that inhibiting MMP's would have potent anti-tumour growth properties. Batimistat and Marimistat (MMPIs) in animal models of CRC and liver metastases produced exciting results, in that administration of these drugs slowed the progress of tumour spread and led to a reduction in size of metastatic deposits in most of the animals, which was attributed to these drugs reducing the net amount of proteolysis and inhibiting angiogenesis (Aparicio T et al, 1999; Primrose JN et al, 1999). Marimistat and other MMPI's therefore entered clinical trials and were hoped to be a *"Magic Bullet"* in the treatment of metastatic disease in patients with CRC and other advanced gastrointestinal tumours. Unfortunately in clinical trials this class of drugs was shown to be of little benefit and the trials have now been stopped (Zucker S et al, 2000; Coussens LM et al, 2002).

Section 1.15

The complexities of MMP and TIMP interactions

We do not know precisely why MMPIs failed, however we do need to increase our knowledge with regard the interactions of MMPs and TIMPs in cancer progression. It is now understood that MMP and TIMP interactions are far more complex than previously thought. Depending on the circumstances MMPs and TIMPs can have both growth promoting and inhibitory properties. For example MMPs can be growth promoting because of their protease activity leading to the release of growth factors such as TGF-α (Peschon JJ et al, 1998) from membrane bound pre cursors or insulin like growth factors from their binding protein (Manes S et al, 1997); matrix degradation may also reveal neo-epitopes in the ECM which may promote growth and encourage angiogenesis (Petitclerc E et al, 1999; Albelda SM et al, 1990, Shahan TA et al, 1999; Kumar CC et al, 2001); the action of MMPs may also degrade receptors (FAS ligand) for the death receptor apoptosis activation pathway on cancer cells (Mitsiades N et al, 2001). MMPs can have growth inhibitory effects by altering ECM composition, thereby degrading growth promoting and survival promoting epitopes expressed by the matrix, which results in altered integrin signaling (Agrez M et al, 1994; Egelbad M et al, 2002). Also MMPs may liberate TGF-β from its binding protein, which also inhibits cell growth (Dernyck R et al, 2001). TIMPs by contrast have growth inhibitory effects by simply inhibiting MMPs, thereby reducing the rate of proteolysis and therefore affecting the growth promoting effects of MMPs already described (Egelbad M et al, 2002). TIMPs can also have growth promoting effects by inhibiting apoptosis pathways by MMP dependent and independent mechanisms (Murphy FR et al, 2002; Guedez L et al, 1998; Guedez L et al, 2001). Ongoing work (Dr A Jamel) at the Liver group in Southampton demonstrates the complexity of MMP/TIMP effects on cell growth as the growth and rate of apoptosis of HSC may be profoundly influenced by the level of MMP-2, with low levels being pro-proliferative and higher levels are pro-apoptotic. The complexities of MMP and TIMP interactions are illustrated in the figure below.

Figure 10b: The complexities of function of Matrix metalloproteinases and their inhibitors

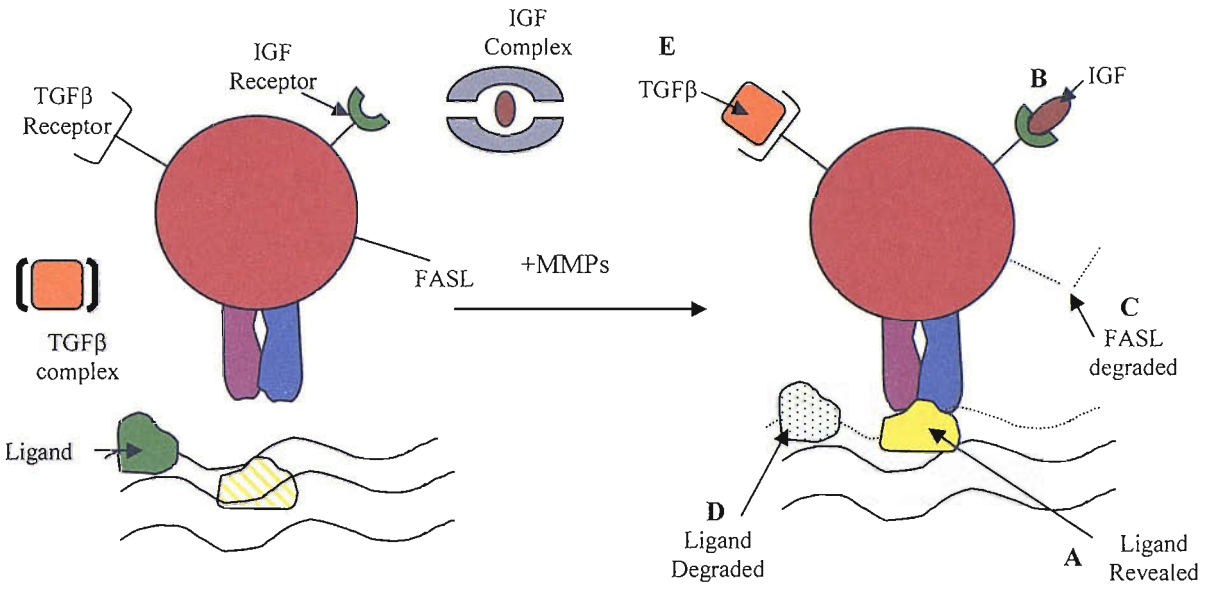


Figure 10b illustrates the many functions of Matrix Metalloproteinases (MMPs). MMPs may have growth promoting effects by; A) matrix turnover, which reveals binding epitopes for integrins expressed by the cancer cells; B) by releasing Insulin Like growth (ILG) factors from complexes with their binding proteins, the released IGF then ligates its receptor; C) they reduce the rate of apoptosis by degrading FAS ligands (FASL), which are important ligands in the death receptor apoptosis pathways. MMPs may have growth suppressing and pro-apoptotic functions by; D) altering ECM composition, and thereby affecting integrin mediated growth; E) by releasing Transforming Growth Factor β (TGFβ) from its complex. The released TGFβ binds to its receptor which inhibits cell growth. The effects on tumour growth of TIMPs are in many circumstances the opposite of MMPs, therefore the balance between the two may favour tumour growth or apoptosis for the reasons already shown. However, in addition TIMPs may act as cell survival factors as by yet not fully elucidated mechanisms and TIMPs may be growth promoting because of the growth complex formed, as shown in figure 10a. Re-produced and modified from Eglebad M, 2002.

Section 1.16

The Source of Metalloproteinases

The interactions of MMPs, TIMPs and MT-MMPs are likely to play a critical role in the development and growth of colorectal cancer liver metastases. By regulating the growth environment around both the primary tumour and metastatic deposits (Theret N et al, 1997; Swallow CJ et al, 1996; Musso O et al, 1997).

The major source of many of the MMPs and TIMPs contrary to initial theories was not the tumour itself; instead they are produced by stromal cells (Egelbad M et al, 2002; Yu AE et al, 1997). This was illustrated by the fact that in the liver MMP production was induced by the primary tumour before being seeded by a CRC metastases, even when the presence of micrometastases have been excluded (Theret N et al, 1997). Numerous studies have shown the association of MMPs -1,-2,-7,-9 and more recently MT1-MMP in the development of CRC liver metastases (Sunami E et al, 2000; Mook OR et al, 2003; Zeng ZS et al, 2002; Bendaraf R et al, 2003; Zucker S et al, 2004). TIMP-2 (Musso O et al, 1997) and MT1-MMP (Theret N et al, 1998) were predominantly produced by stromal cells. The source of MMP-1, 2 and 9 is still a matter of some debate; however it is apparent that these MMPs may be produced by both tumour and stromal cells (McDonell S et al, 1999; Sato H et al, 1994; Sunami E et al, 2000; Mook OR et al, 2003). As the DR is rich in interstitial collagens, the key MMPs in CRC in terms of matrix turnover are therefore likely to be MMPs-1, 2 and 14 due to their ability to degrade intact, non-denatured collagens (Section 1.10).

Section 1.17.1

The Hepatic Stellate Cell

Stromal cells in the liver are derived from Hepatic Stellate cells (HSCs). The stimulation of HSCs by a variety of different factors leads to them adopting a myofibroblast phenotype, so-called "activation". Activated HSCs are known to express a wide variety of MMPs and TIMPs and deposit fibrillar collagens and therefore HSCs play a key role in matrix turnover both in health and pathological conditions such as liver fibrosis and perhaps even tumours (Musso O et al, 1997; McCrudden R et al, 2000). Therefore it is likely that recruitment and activation of HSCs by CRC metastases plays a critical role in

facilitating the spread of the tumour, mediated via HSC derived MMPs, TIMPs and by the deposition of fibrillar collagens as part of a desmoplastic reaction. The ability of the tumour to recruit and activate HSC offers it several advantages; Firstly, in the liver there are numerous HSCs in an unactivated state, therefore recruitment of these HSCs by the tumour would allow it to magnify several fold the quantity of MMPs and TIMPs created than could be produced by the tumour itself, particularly in the early stages of metastatic deposit development. Secondly, stromal cells are positioned in the peri-sinusoid space adjacent to liver tumours (Musso O et al, 1997). This was likely to help the tumour deposits disseminate through the sinusoidal network once the HSCs are activated and they can be primed even before being seeded by the metastases.

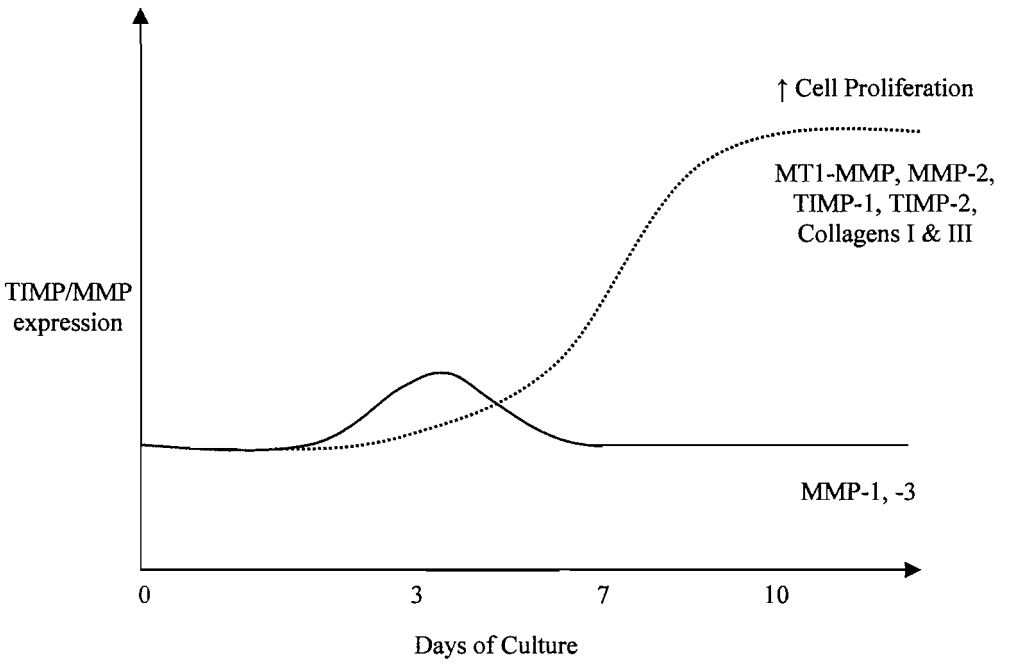
Section 1.17.2

Hepatic Stellate Cell Activation

HSC activation leading to them adopting a myofibroblast phenotype has been studied most extensively in the field of liver fibrosis (Benyon RC et al, 2001). During progressive activation in culture, HSCs produce significant amounts of MMP-1 (13 in rats, as rats do not express MMP-1) and MMP-3, but no TIMPs during the first 3 days (Vyas SK et al, 1995; Iredale JP et al, 1996). As time passes, MMP-1 and 3 levels diminish and TIMP 1 and 2 levels significantly increase as do MMP-2 and MT1-MMP levels (Arthur MJ et al, 1989; Benyon RC et al, 1999; Theret N et al, 1999). The higher levels of MMP-2 are presumable mediated via the ternary complex of pro-MMP-2/MT1-MMP/TIMP-2. The elevated levels of MMP-2 contribute to the proliferation of HSCs due to the catalytic activity of this activated enzyme (Benyon RC et al, 1999). TIMP-1, TIMP-2 and MMP-2 and MT1-MMP are progressively expressed by activated HSC over many cell passages. Studies with fibrotic liver tissue suggest HSC activated in vivo also express these proteins (Iredale JP et al, 1995; Benyon RC et al, 1996; Knittel T et al, 2000; Nieto N et al, 2001). This classic pro-fibrogenic phenotype of HSCs results in the degradation of collagen/ECM but overall the net accumulation of fibrillar collagen (types I and III) produced by the HSC to replace the degraded basement membrane (Benyon RC et al, 2001). At this stage if the cause of the injury persists, further activation and proliferation of HSCs occurs. However, if the stimulus is removed HSCs undergo apoptosis and collagenlysis predominates and

fibrosis resolves (Hammel P et al, 2001). These changes are represented graphically in the figures below.

Figure 11a: How MMP/TIMP expression changes during HSC activation

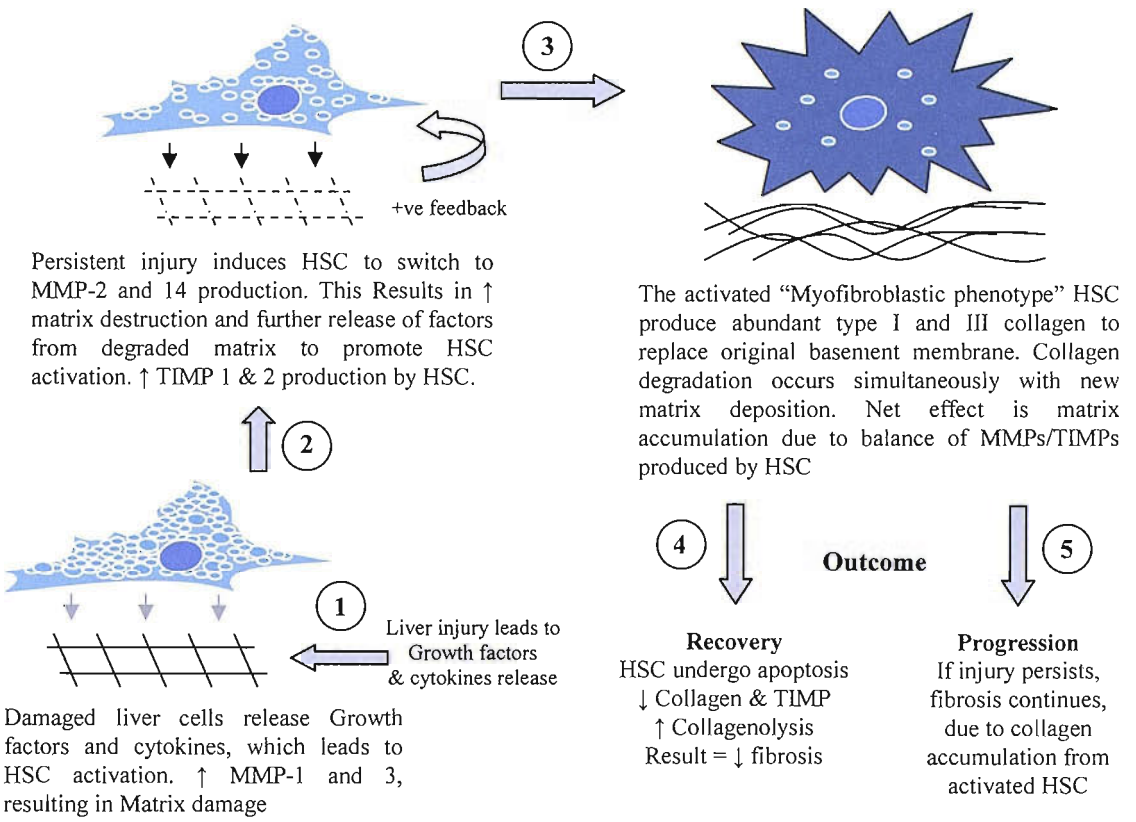


This figure illustrates the classical HSC response in terms of MMP/TIMP expression after activation. As can be seen MMP-2, MMP-14 and TIMP-2 levels increase as the duration of culture increases, accompanied by matrix accumulation and HSC proliferation.

Activated HSC express αSMA and other intermediate filaments, possess increased mobility and deposit interstitial matrix (Friedman SL et al, 1992 and 2000). Many factors have been identified as activation factors for HSC. Cytokines including TNF-α, interleukin 1 are known to activate HSC, with perhaps PDGF and TGFβ the most extensively studied (Le Pabic H et al, 1999; Borkham-Kamphorst E et al, 2004, Uemura M et al, 2005). What all these cytokines have in common is that they are released during tissue injury and inflammation. TGFβ1 plays an important role in liver fibrogenesis, as it inhibits MMP-1 synthesis (Uria JA et al, 1998) but induces expression of MMP-2, -13 and TIMP-1, which results in collagen synthesis, matrix remodeling and the net accumulation of new interstitial matrix (Benyon RC, 2001). HSC activation and proliferation is also

under the influence of the matrix itself with collagen IV being inhibitory to growth and collagen I increasing activation and proliferation of HSC (Sohara N et al, 2002; Gaca MD et al, 2003), with αvβ3 integrin playing a crucial role in regulating these matrix mediated effects on HSC (Zhou X, 2004).

Figure 11b: The HSC response to activation in terms of MMP/TIMPs



This figure aims to show the effects of HSC activation, following liver damage. As can be seen the possible outcomes are recovery or the progression of fibrosis. Although not shown, even when the stimulus is removed, once the fibrosis has become well established, full recovery may not be possible. Re-produced and adapted from Benyon RC, 2001.

Section 1.18

If we believe that HSC and CRC cell interactions are important, which signaling pathways may be important?

What factors could be involved in CRC and HSC interaction

If there is a link between tumour cells, HSCs specific membrane bound or soluble factors may be important. A possible implicated factor could be EMMPRIN (Extracellular Matrix MetalloPRoteinase Inducer (CD147)). This is a recently described glycosylated protein found on tumour cells (Breast cancer and Gliomas) and is known to potently induce MMP's from adjacent stromal cells (Li R et al, 2001; Sun J et al, 2001). How this is achieved however, is not known and is a matter for continued research.

What is known about EMMPRIN is that it potently upregulates MMP production (Especially MMP-1, 2 and 9), but it also tends to bind these MMPs to the tumour surface via an MMP/TIMP/MT-MMP complex, which further increases the tumours invasiveness (Sun J et al, 2001; Guo H et al, 2000).

Cytokine stimulatory pathways have also been implicated in stromal - cancer cell interactions, mediated by amongst others; Tumour Necrosis Factor Alpha (TNF-α), Interleukin-1 (IL-1), PDGF and Hepatocyte Growth Factor (HGF) (Zucker S et al, 2000; Shimizu S et al, 2000), although no definitive studies in this area have yet been published in the area of CRC and HSC interactions. What we do know is that these cytokines can potentiate both stromal cell and cancer cell activation and proliferation (Zucker S et al, 2000; Shimizu S et al, 2000; Balbin M et al, 1999; Uria JA et al, 1998) and therefore may be produced by both CRC and HSC, in a paracrine and autocrine fashion. In addition as described earlier activated HSC produce TIMP-1 which we now know may offer cancer cells a growth and survival advantage and may also be involved in these interactions.

HYPOTHESES

The program of research in this thesis investigates the following hypotheses:

a) That deregulated matrix turnover, characterized by alterations in the distribution of collagen IV within the ECM and its replacement with fibrillar collagen as part of a desmoplatic reaction is important in the development of colorectal cancer liver metastases.

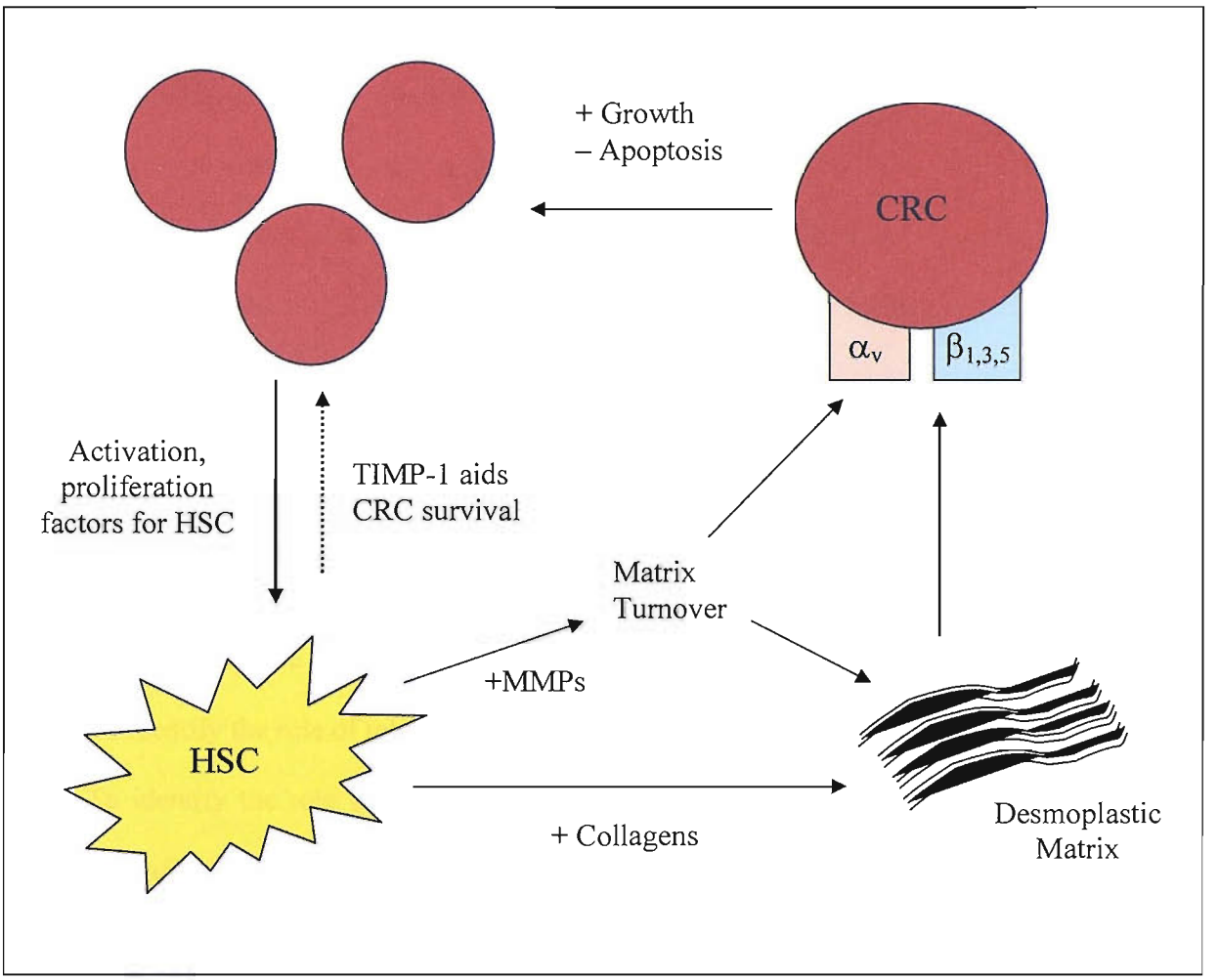
b) That the turnover of established and new matrix is a dynamic process and occurs simultaneously with new matrix deposition and that the cancer gains a growth and survival advantage from this deregulated matrix turnover, by integrin mediated pathways. If our hypothesis is correct, plan to dissect the mechanisms underlying these observations.

c) The newly formed matrix and many of the MMPs & TIMPs, which are important in matrix turnover are produced by activated Hepatic Stellate cells (HSCs).

d) The mechanism underlying HSC activation and production of MMPs I hypothesize is linked to the production of soluble factors by the Cancer cells.

e) That HSC produce factors which may directly affect the growth of CRC metastases.

Figure 12: Proposed model for Cancer and matrix interactions



The desmoplastic reaction (DR) results in alterations in the composition of the matrix. The deposition of fibrillar collagen and matrix degradation is a dynamic process occurring simultaneously. Integrins (β1 & αvβ3 and αvβ5) expressed by the Cancer cells then interact with epitopes revealed in the DR matrix, providing the cancer with a growth and survival advantage. Hepatic stellate cells (HSC) under the influence of the cancer cells are key mediators in altering the matrix composition by producing fibrillar collagen and Metalloproteinases which degrade the new and existing matrix. TIMP-1 produced by activated HSC may also act as a survival signal for the cancer cells.

AIMS

Therefore this study aims to answer the following questions.

1) To determine whether alterations in the composition of the extracellular matrix plays a role in the spread of CRC liver metastases.
2) To identify how alterations in matrix composition as part of a desmoplastic reaction affects the rates of growth and apoptosis of Colorectal cancer cell lines.
    a) Namely the accumulation of collagens I & III
    b) The alterations in the distribution of collagen IV
    c) The potential loss of the known growth regulatory isotypes of collagen IV
3) To identify the mechanisms underlying these differences, concentrating on integrin expression by the cancer cells and ligands by the matrix.
4) To identify the role of intercellular signaling pathways underlying these findings.
5) To identify the role of matrix turnover in revealing additional integrin ligands, which may affect colorectal cancer cell growth and apoptosis.
6) To identify the role of HSCs in matrix turnover and alterations in matrix composition. In particular HSC expression of MMPs/TIMPs and fibrillar collagen.
7) To identify if CRC cell lines induce or suppress MMP, TIMP and fibrillar collagen production by HSC.
8) To identify the mechanisms by which this is achieved, in terms of signaling pathways.
9) To identify if HSC influence growth and apoptosis of CRC cell lines by the expression of TIMP or growth factors.

The overriding aim is to determine how deregulated matrix turnover as part of a desmoplastic reaction encourages CRC growth and how these results may inform the design of new novel treatments for patients with CRC.

Chapter 2

Methods

Section 2.1

Cell Culture

All Tissue Culture Plasticware (TCP) were provided by Greiner Bio-one (Greiner Bio-One LTD, Stonehouse, Gloucestershire, UK) and all Cell culture media, antibiotics and foetal calf serum (FCS) were provided by Invitrogen (Invitrogen Ltd, Paisley, UK) unless otherwise stated. All tissue culture solutions were warmed for 30 minutes to 37°C before use. All other laboratory reagents were provided by Sigma UK (Sigma, Poole, Dorset, UK), unless otherwise specified.

Cells were cultured under standard conditions using a Nuaire incubator; set to 37°C (Humidified) and 5% CO_2, with cell culture media changed on alternate days. The cells were grown in the appropriate media supplemented with 10% Foetal calf serum (Serum Enriched media), Penicillin G ($1x10^5$ units/l), Streptomycin ($1x10^5$ mg/l) and L-Glutamine (Final concentration 2mmol/l).

The HT-29 cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) and the KM cell line series, a kind gift from Proferssor IJ Fidler (MD Anderson Centre, USA), were cultured in Minimum Essential Media (MEM), supplemented with MEM Vitamins (0.2%), MEM Non-essential Amino Acids (0.1%) and Sodium Pyruvate (1 mmoles/l).

Cells were passaged by removing the media and washing the cells x 2 with warmed Hanks Balanced Salt Solution minus calcium (HBSS-Ca^{2+}). The cells were gently trypsinized using 1x Trypsin in HBSS-Ca^{2+} (Trypsin 0.25% and EDTA 1mmol/l) at 37°C for approximately 5 minutes.

The trypsin in the resultant cell mix was neutralised by the addition of serum enriched media, and the mixture was then gently spun at 1,000rpm for 5 minutes. The supernatant was then removed and the pellet re-suspended using serum enriched media and then transferred into flasks (Using an approximate 1:10 split) and the cells cultured as described above.

Section 2.2

Assessment of cell number and viability

The viability of cells immediately after passaging was routinely determined by the exclusion of Trypan blue dye, at a concentration of 0.05% weight per volume (w/v). Viable cells stay small, round and refractile, whilst non-viable cells become enlarged and swollen and also stain dark blue. Both the percentage of viable cells and the total number of cells/ml were determined, the latter using a haemocytometer with Neubauer ruling. Cell viability was on all occasions >95%.

Section 2.3

Cell Lines

HT-29 (Poorly metastatic cell line) was obtained from the European Cell and Culture Collective (Porton Down, Wiltshire, UK) at passage number 140.

KM12 Cell line series (3 cell lines); The KM12c cell line was established from a Dukes B carcinoma (Prof IJ Fidler). The progressively more aggressive liver metastising variants were established by intra-splenically injecting the parent line (KM12c) into nude mice and extracting cells from the resultant liver metastases. This was repeated several times to establish each metastatic cell line.

KM12c – Poorly-metastatic cell line, passage 12.

KM12L4a – Moderately metastatic cell line, passage 14.

KM12SM – Highly metastatic cell line, passage 21.

All cell lines were allowed to recover from the residual effects of cryopreservation for at least 1 passage after defrosting, before being used in experiments and all cells were discarded before completing 15 passages after being defrosted. Cells were frozen, using a standard freezing protocol, reducing the temperature by 1°C each hour. The freezing media used consisted of 90% FCS and 10% Dimethyl Sulphoxide (DMSO).

Section 2.4

Serum Free Media

Serum free media was produced, by reducing the FCS component to 0.5% unless otherwise specified. To compensate for the loss of buffering capacity that reducing the FCS concentration causes, HEPES was added (25mmol/l). During validation experiments 0.5% FCS was chosen as being optimal. The KM12 cell line series appeared to be more serum dependent than the HT-29 cell line and therefore, in some circumstances (prolonged culture for 48-72 hours under serum free conditions) 1% serum was used, which was found during the validation experiments not to adversely affect outcome.

Section 2.5.1

Collagen

All collagens used were acid soluble.

Collagen I from Rat tail (CI), stored at 4°C

Collagen III from calf skin (CIII), stored at 4°C

Collagen IV from Human Placenta (CIV), stored at -20°C

Type I r/r collagen and Wild Type control (w/t) were extracted from mouse tails as per protocol (Appendix 3), stored at -20°C following freeze drying, then 4°C once in solution.

Section 2.5.2

Collagen Preparation

a) Plating

All collagens were dissolved in sterile 0.1mmoles/l acetic acid (Fisher Scientific, Loughborough, Leicestershire, UK) to a final concentration of 1mg/ml and plated out at a concentration of 15μg/cm^2. To ensure uniform coverage of the flask or plate, the collagens were spread out using a cell scraper or pipette tip. The acetic acid was then allowed to evaporate for 30 minutes at room temperature under aseptic conditions, before being

refrigerated (4-8^{0}C) for up to 48 hours before use. In each experiment a control was provided by tissue culture plastic (TCP) or the appropriate IgG isotype antibody control.

b) Washing the Collagen

For all experiments, to ensure the collagen films were free from residual acetic acid, the collagen films were washed twice with the appropriate media (DMEM or MEM). Following which, to prevent non-specific binding, a solution containing 0.1% Bovine Serum Albumin (BSA) in the appropriate media was added to each flask or well and left in situ for 1 hour. This was then replaced with HBSS-Ca^{2+} until the cells were ready to be plated out.

Section 2.6

Mycoplasma Screening

All cell lines were ensured to be mycoplasma free by routinely screening every 8 weeks for mycoplasma contamination, using the ATCC Mycoplasma (American Type Culture Collection, Manassas, VA, USA) detection kit, version 2. The cell lines were grown for 2 weeks without antibiotics, and passaged by scraping before being screened. The cells were then lysed using the lysis buffer provided. The kit involves the use of a nested PCR reaction with both stage primers provided. Two positive controls of known Mycoplasma Strains (*A Laidlawii* and *M Pirum*) were provided in the kit, and as a negative control the cell lysis buffer was used. The taq polymerase (Platinum taq, Invitrogen Ltd, Paisley, UK), was used as the taq polymerase buffer provided in the kit, was already optimised for use with this hot-start taq. After completing the 2 stage PCR, using a Thermohybaid PX2 cycler, the samples were loaded onto a 2% agarose (Promega) gel (+ Ethidium Bromide) and run at 100v for 1 hour. The gels were then imaged under UV fluorescence.

Section 2.7.1

Tinctorial staining and Immunohistochemical staining sample preparation

1) Cutting and deparaffinizing the slides

Samples were obtained after appropriate consent from 10 consecutive patients who underwent liver resection for metastatic colorectal cancer (LREC No: 215/99). After identifying the appropriate paraffin blocks, 5μm thick tissue sections were cut and applied onto APES coated glass slides. The sections were deparaffinized using 2 changes of xylene (5 minutes each). The sections were then rehydrated by the use of graded alcohols; 100% ethanol twice (5 minutes each) and 70% ethanol (5 minutes). All steps were undertaken at Room Temperature, unless otherwise specified.

Section 2.7.2

2) Tinctorial Staining with sirius red

The slides were washed twice in dH_20 (5 minutes each) then treated with 0.2% phosphomolybdic acid for 5 minutes (to aid dye binding). The slides were then covered with a 0.1% Sirius Red (Sirius Red dye 0.1 g in Picric Acid 100mls) solution for 2 hours. The slides were then rinsed briefly with 0.01% HCl and then washed for 1 minute in dH_20. The slides were then stained with Mayers Haemalum blue for 2 minutes and then washed in tap water for 5 minutes. The sections were then dehydrated using graded alcohols; Ethanol 70% (1 minute), then Ethanol 100% twice (2 minutes each). Finally the sections were washed 3 times with xylene (3 minutes each) as a clearing agent, the excess xylene after the final wash was wiped off and the slides were mounted using DPX and a cover slip. The slides were then visualized using light microscopy: Nuclei appear blue, collagenous fibers appear red and the other tissue elements appear bright yellow. Sirius red predominantly stains fibrillar collagens, in particular types I, II and III collagen.

Section 2.7.3

3) For Immunohistochemical staining

The following antigen unmasking techniques were employed, either individually or in combination (The specific treatments for each stain used are shown below). In all techniques the slides were not allowed to dry out at any stage.

a) Microwave; Two different buffers were employed during microwave antigen retrieval.

(i) Citrate Buffer – a solution of 0.01M citrate buffer made up with dH_20 and adjusted to pH=6 with 1M NaOH.

(ii) EDTA Buffer – a solution of 1mM EDTA buffer was made up and adjusted to pH=8 with 0.1M NaOH.

The appropriate buffer solution and slides were placed into a microwaveable container and microwaved on medium power (≈400W) for 25 minutes. The container was removed from the microwave and run under cold water for 5 minutes. Following which the slides were washed twice with Tris Buffered Saline (TBS) (5 minutes each wash).

b) Enzyme digestion (Pronase); the slides were placed into a trough in a water bath (37^0C) containing ultra-pure water. A 0.05% pronase solution (Dako, Ely, Cambridgeshire, UK) was placed in a separate trough, in the same water bath and the warmed slides were then immersed in the pronase solution. The trough containing the slides was removed from the water bath and left for 10 minutes at room temperature. The slides were then removed from the pronase solution and washed twice with TBS (5 minutes each wash).

Section 2.7.4

4) Staining using an Avidin Biotin Complex

The slides after being rehydrated were blocked with a 0.5% hydrogen peroxidase in methanol solution for 10 minutes to inhibit endogenous peroxidase activity. Following which the slides were washed twice in TBS (2 minutes each wash). The slides were treated with the appropriate antigen unmasking technique. The slides were then rinsed twice with TBS (5

minutes each rinse). The slides were then drained and an avidin blocking solution (Vector Laboratories Ltd, Peterborough, UK) applied for 20 minutes, following which the slides were rinsed 3 times (2 minutes each rinse) with TBS. The slides were then drained and the biotin blocking solution (Vector Laboratories Ltd, Peterborough, UK) was applied for 20 minutes, following which the slides were rinsed 3 times (2 minutes each rinse) with TBS. The slides were drained and the blocking medium containing 1% BSA and 20% FCS applied for 20 minutes. The slides were drained and the primary antibody solution made up in TBS was applied at appropriate dilutions and incubated overnight at 4^0C. The following day, the slides were rinsed 3 times with TBS (5 minutes each rinse) and then after draining the slides the biotinylated secondary antibody solution made up with TBS was applied for 30 minutes. The slides were then rinsed 3 times with TBS (5 minutes each rinse) and then the Steptavidin Biotin-Peroxidase complex (Dako, Ely, Cambridgeshire, UK) in TBS at appropriate dilutions was applied for 30 minutes. The slide was then rinsed 3 times with TBS (5 minutes each rinse). The slides were drained and the coloured product of the enzyme was developed using an appropriate chromogen, 3,3'-diaminobenzidine tetrahydrochloride (DAB, Biogenex, San Ramon, CA, USA) which was applied for 5 minutes and then the slides were rinsed with TBS and then tap water for 5 minutes. The slides were counterstained using Mayers haematoxylin blue, then rinsed under tap water and then dehydrated using graded alcohols; Ethanol 70% (1 minute), then Ethanol 100% twice (2 minutes each). Finally the sections were washed 3 times with xylene (3 minutes each) as a clearing agent, the excess xylene after the final wash was wiped off and the slides were mounted using DPX and a cover slip.

Sirius red and Immunohistochemical staining was analysed as to the intensity and distribution of staining identified by these techniques, by two blinded pathologists (Dr A Bateman and Dr T Kendall).

In appendix 2a, the protocols for APES coating of slides and buffer formulas used are listed.

Section 2.7.5

Immunohistochemical Staining: For each antibody studied the pre-treatment techniques and antibody concentrations were optimised. A negative control was included of non-immune IgG. Please find listed below details of the primary antibodies used (clone, manufacturer and concentration) and which antigen unmasking technique was required. For mouse derived primary antibodies the biotinylated rabbit anti-mouse secondary antibody (Dako, Ely, Cambridgeshire, UK), was used at a concentration of 1:200. For rabbit derived primary antibodies the biotinylated swine anti-rabbit secondary antibody (Dako, Ely, Cambridgeshire, UK), was used at a concentration of 1:400.

1) **Alpha-Smooth Muscle Actin (αSMA) staining**. The mouse monoclonal primary antibody (Clone 1A4, Sigma, Poole, Dorset, UK) was used at a concentration of 1:40,000 (Microwave, citrate buffer).

2) **Collagen I (CI)**. The mouse monoclonal primary antibody (Clone Col-1, Abcam, Cambridge, UK) was used at a concentration of 1:100 (Microwave, citrate buffer).

3) **Collagen III (CIII)**. The rabbit polyclonal primary antibody (Clone CL50311AP, Cedarlane, Ontario, Canada) was used at a concentration of 1:1,000 (Pronase).

4) **Collagen IV (CIV)**. The mouse monoclonal primary antibody (Clone CIV 22, Dako, Ely, Cambridgeshire, UK) was used at a concentration of 1:150 (Pronase).

5) **Beta-1 Integrin (β1)**. The mouse monoclonal primary antibody (Clone 4B7R, Santa Cruz, CA, USA) was used at a concentration of 1:400 (Pronase).

6) **αvβ3 Integrin (αvβ3)**. The mouse monoclonal primary antibody (ab7167, Abcam, Cambridge, UK), was used at a concentration of 1:50 (Microwave, EDTA buffer).

7) **αvβ5 Integrin (αvβ5)**. The mouse monoclonal primary antibody (Clone 2Q1009, United States Biological, Swampscott, MA, USA), was used at a concentration of 1:50 (Microwave, EDTA buffer).

Section 2.8

Cell Survival assays

The next stage of this study aimed to determine the effect of different matrix components on long-term cell survival in response to chemotherapy.

Clonogenic Assay

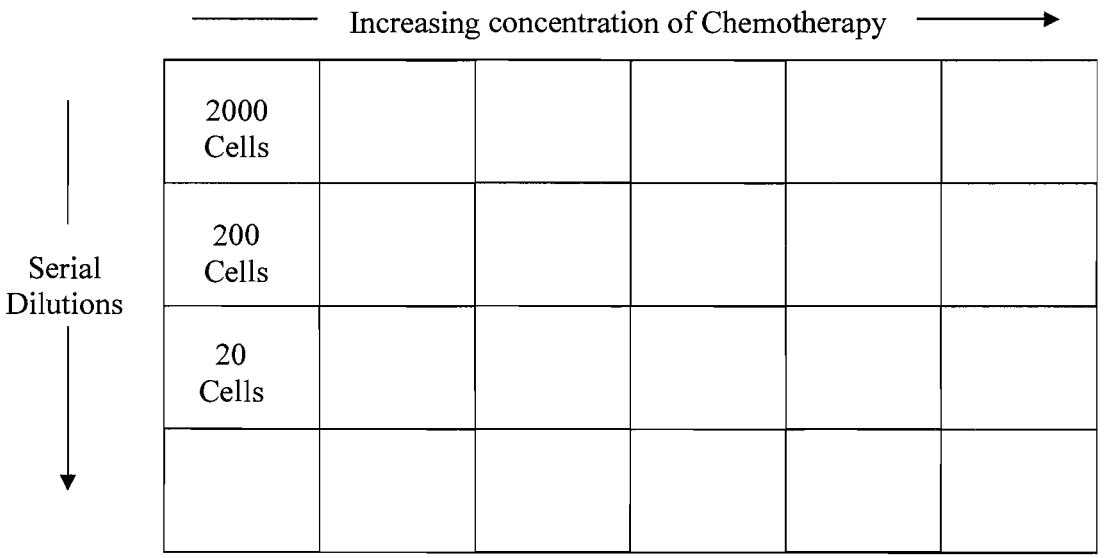
To undertake this part of the study a technique called the clonogenic assay was employed. A Clonogenic assay provides an index of clonal cell survival and was originally established by Rosenblum ML et al, 1975 to monitor the effectiveness of chemotherapy treatments in malignant brain tumours.

The collagen was plated out into 25 cm^2 flasks and prepared as previously described (TCP vs CI vs CIII vs CIV). The CRC cell lines were passaged and $1x10^6$ cells were added to each flask in 10ml of serum enriched media. The next morning, the supernatant was removed, and replaced with warmed Hanks Balanced Salt solution plus calcium ($HBSS+Ca^{2+}$) for 5 minutes; this was then replaced with serum free media. 8 hours later this supernatant was removed and replaced with either 10ml of serum free media (Controls) and various concentrations of 5-Fluorouracil (5-Fu, Faulding DBL Cambridge, UK), 0.25 – 10 μg/ml on each matrix. The cells were then cultured under standard conditions for 48-56 hours. The cells were then trypzinised as previously described and counted using a haemocytometer.

The next step involved plating out the cells at each particular concentration on each matrix using serial dilutions from 2000, 200 and 20 cells per well into a 24 well plate in 1,000 μl of serum enriched media, as shown in figure 13.

A separate plate was employed for each matrix (including controls), repeated in triplicate, with a serial dilution for each different concentration of chemotherapy used.

Figure 13: Plating out the Clonogenic Assay.



The plates were the cultured under standard conditions (with the media changed as or when required) for approximately 10 – 14 days, until colonies were formed, with >16 cells.

The plate was then processed, once colonies were established by removing the supernatant and then washing each well twice with 0.5ml of warmed Phosphate Buffered Saline (PBS). This was then removed and replaced with 250µl of Methanol for 15 minutes to fix the cells, the methanol was then removed and the wells allowed to dry overnight. To stain the colonies the next morning 250 µl of Giemsa stain was added for 10 minutes. The Giemsa stain was removed and the plate washed in a large container of dH_20, with 3 water changes and then the plates were allowed to dry.

The next stage was to count the number of colonies, which was undertaken using light microscopy. The 200 cell dilution were counted on all plates and a colony contained >16 cells. The number of colonies on each matrix at each concentration was averaged from the triplicate. The clonogenic index could then be worked out for each matrix and each concentration of chemotherapy used including controls. Comparisons were then made between different matrices.

$$\text{Clonogenic index (CI)} = \frac{\text{Number of Colonies Counted}}{\text{Number of cells plated /well (200 cells)}}$$

Cell survival is a complex phenomenon, the factors which determine whether a cell survives and thrives or undergoes apoptosis is a multifaceted process. Two important factors contributing to cell survival are the rate at which a cell grows and undergoes apoptosis. We have studied the rate of cellular proliferation for CRC grown on different matrix components and the rate of cellular apoptosis on these matrices, after the cells were treated with chemotherapy.

Section 2.9.1

Cellular Proliferation

There are many different ways of studying cellular proliferation, perhaps the most widespread used technique is to quantify cellular proliferation in terms of DNA synthesis by measuring the rate of ^{3}H Thymidine incorporation. This technique is often combined with a simultaneous quantification of DNA content in cells, using the Pico green assay.

Section 2.9.2

Proliferation Assay

a) ^{3}H Thymidine

Preparation: Using a 24 well plate and the appropriate matrix coated as described above (TCP vs CI, CIII and CIV), the cell lines HT-29, KM12c, KM12L4a and KM12SM were studied. A triplicate of wells was used for each condition and this technique was combined with the pico green assay as illustrated in figure 14.

After preparing the matrix as described above, the cell lines were passaged as per protocol and counted. A cell mix containing 1.5 x 10^4 cells/ml was made up using 10% serum enriched media for each cell line. 500µl of this cell mix containing 0.75 x10^4 cells was added to each well. The plates were then placed in an incubator, set to standard conditions and the cells allowed to adhere overnight. After approximately 16 hours, the supernatant was removed, the

wells washed once with HBSS+Ca^{2+}. This was then removed and replaced with serum free media and the plates returned to the incubator for eight hours at which stage ^{3}H Thymidine (1microCi/well) was added to each well. The plates were again placed in the incubator and the samples processed after 12 – 18 hours.

Processing: The media from the wells was discarded and each well washed with 0.75ml HBSS+Ca^{2+} 3 times. For each wash the wells containing cold HBSS+Ca^{2+} were placed on ice on a rocking platform (Sorvall) for 15 minutes, set at speed 4.

After discarding the supernatant following the third wash with HBSS+Ca^{2+}, 0.5ml of ice cold methanol was added to each well and the plates were left for at least 1 hour in a freezer, to fix the cells. The washing stage as described above was repeated after discarding the methanol.

After discarding the supernatant following the third wash, 0.25ml of cell dissolution solution was added to each well, and the plates were placed on a rocking platform at Room Temperature (speed 4) for 20 – 30 minutes. Then 15 µl of 5M HCL was added to each well, to neutralize the NaOH in the cell dissolution solution.

0.75ml of OptiPhase HiSafe 3 Scintillation fluid (Wallac, Turku, Finland), was placed in each well of a Wallace 24 well scintillation counter plate and the dissolved cell mixture added (Approximately 265µl). The wells were covered using a non-sterile plastic adhesive film, to prevent well to well contamination and spillage.

The plates were counted using a Wallac MicroBeta 1450 Scintillation Counter (Perkin Elmer LAS, Beacon Field, Buckinghamshire, UK). Each well was counted for 2 minutes and the rate of ^{3}H thymidine incorporation expressed in terms of Counts/ Minute (CPM). These results were then combined with those obtained using the pico green assay and the proliferation rate was expressed in terms of CPM/ng DNA (The counting protocols are shown in Appendix 2b).

Section 2.9.3

Pico Green Assay to measure cellular DNA

This assay utilizes a nucleic acid dye staining technique to quantify double stranded DNA. The cyanine dye was produced by Molecular Probes (Invitrogen Ltd, Paisley, UK). Until the processing stage the technique is exactly the same as described for ^{3}H Thymidine, except no radioisotope was added. Once, the plates were ready for processing, the supernatant was removed and 0.5ml of sterile filtered PBS was added and the wells scraped using a cell scraper. The supernatant was then removed and placed into a 1.5ml microfuge tube. This scraping step was repeated once more, and then the tube was spun at 13,000 rpm for 10 minutes, resulting in the cells precipitating out at the bottom of the tube. The supernatant was then removed and cells were then resuspended in 100µl of Sterile TE Buffer (pH = 7.5). The tubes were then sonicated in a water-bath sonicator for 10 minutes, to breakdown the cell membranes. At this stage the samples were frozen.

To work out the DNA concentration for each well, the concentration of the specimens were compared against a standard curve produced using known DNA concentrations of herring sperm DNA, from 50,000ng/ml – 97.9ng/ml of DNA, in TE buffer. The quantity of DNA of each sample was then be quantified by comparing the emission results of the specimens with those obtained from the standard curve. 100 µl of each control and TE buffer were plated out in triplicate as were the samples on a nunc maxisorp 96 well plate. A solution of Pico green was then made up using a 1:200 dilution of the dye with TE buffer. Once made up this mix was protected from the light and used as soon as possible. 100µl of Pico Green mix was added to each well and the plate placed in a fluorescence spectrophotometer (Cytofluor, Perkin Elmer LAS, Beacon Field, Buckinghamshire, UK). With an excitation wavelength of 480nm, and an emitted wavelength of 530nm (Full details of parameters - Appendix 2b). The proliferation assay results were expressed by combining the ^{3}H thymidine and Pico Green results, and expressed in terms of CPM/ng DNA, to take into account any difference in cell numbers within the cell monolayer for each experiment.

In addition it was established that there was no effect on ^{3}H Thymidine or Pico green incorporation, from the matrices plated out.

Figure 14; Plate set-up for proliferation assay

← ^{3}H Thymidine →			← Pico Green →		
TCP					
CI					
CIII					
CIV					

Section 2.10.1

Determination of protein expression using western blotting

The rate of cellular apoptosis was studied by looking at the rate of Poly-ADP Ribose Polymerase cleavage, detected by western blotting.

However, before going into detail on this specific technique, I will describe the general technique used to study and prepare protein samples for this and later experiments.

Section 2.10.2

Protein Preparation for western blotting

1) Cell scraping and lysis buffer:

A standard method of protein extraction was used in all experiments. After preparing flasks ($75cm^2$) in the standard way with respect to coating with different matrices and matrix preparation as described earlier. Cell lines used were passaged and counted on a hameocytometer, $1x10^6$ cells were added to each flask in serum enriched media. The following morning, the supernatant was removed and the cells washed with 10ml of HBSS+Ca^{2+} for 5 minutes. This was then removed and replaced with serum free media. In those experiments in which chemotherapy was to be added, the cells remained in serum free media for 8 hours, before replacing this with serum free media (Controls) or serum free media plus chemotherapy. In all cases after the cells had been allowed to grow for the set time allowed (see later), the flasks were scraped and the supernatants removed, and placed in 50ml conical tubes. The flask was then washed with 10ml of PBS and scraped again and the supernatant transferred to the tube. The tubes were placed on ice until all were ready for the next step. The tubes were spun at 1,000 rpm for 5 minutes, the supernatant removed and the cell pellet resuspended in the remainder of the residual supernatant. This cell mix was then transferred to a microfuge tube, the 50ml conical tubes were washed with 0.5ml of PBS, this supernatant was then removed and added to the microfuge tube and the samples placed on ice. The microfuge tubes were then spun at 13,000 rpm for 10 minutes, following which the supernatant was removed, until all that remained was the cell pellet. The protein was then extracted by the addition of a lysis buffer, in all cases this was the Radio-Immunoassay

Precipitation Buffer (RIPA), containing 1 part/100 of mammalian protease inhibitors. A volume of the RIPA lysis buffer of between 20-50µl was added to the cell pellet dependent on the pellet size, and the pellet thoroughly mixed with the lysis buffer, using the pipette and then vortex spun. The samples were then placed on ice for 30 minutes. The samples were then spun at 13,000rpm for 10 minutes and all the supernatant was removed (protein lysate) and placed into labeled microfuge tubes and frozen at -20^{0}C for use the next day or -80^{0}C for later use.

2) Determination of protein concentration by Bicinchoninic Acid Protein Assay kit

Protein concentrations of all cell extracts were determined by the use of the Bicinchoninic acid protein assay kit (BCA-1, Sigma, Poole, Dorset, UK). Proteins reduce alkaline Cu (II) to Cu (I) in a concentration dependent manner (Lowry OH et al, 1951). Bicinchoninic acid (BCA) is a highly specific chromogenic reagent for Cu (I) forming a purple complex with maximum absorbance at 562 nm. 10 µl of protein extracts in a range of dilutions with lysis buffer were added into wells of a 96 well plate in triplicate. 200 µl of the BCA mix (BCA mix = Bicinchoninic Acid Solution: 4% Copper (II) Sulfate, in a ratio of 50:1) was added to each well. The protein concentration was ascertained relative to a standard curve using standards containing 0 to 1.6 mg/ml of bovine serum albumin (BSA) dissolved in ultra-pure water. In each individual assay a triplicate of each control was used, and samples of ultra-pure water and lysis buffer were also measured. The chromogenic output was adjusted for the optical densities of the ultrapure water (BSA controls) and RIPA (samples) and the standard curve in all cases produced a correlation coefficient (R^2) >99%, from which to work out the samples protein content.

3) Sample Preparation

All agents were provided by Invitrogen Ltd, Paisley, UK. Between 10-75µg of protein was loaded for each sample, dependent on which western blot was being undertaken, in a volume of between 10 – 25µl. NuPage LDS sample buffer was used and made up ¼ of the final volume. For reduced samples NuPage reducing agent was added and made up $^1/_{10}$ of the final volume. The appropriate volume of sample was added to achieve the desired protein concentration. To make up the volume to the total needed, ultra-pure water was added (this is

summarized in table 2). Samples were then heated in a heating block (70^0C for 10 minutes), following which samples were centrifuged for 5 minutes at 13,000rpm.

Table 2: Sample Preparation

e.g. for each 10µl	***Reduced***	***Unreduced***
Sample	*x*	*x*
Reducing Agent	1µl	0
LDS sample Buffer	2.5µl	2.5µl
Ultra-pure water	Up to 10µl	Up to 10µl

Section 2.10.3

SDS-Polyacrylamide (SDS-PAGE) gel electrophoresis and Western Blotting

a) Electrophoresis: All agents were provided by Invitrogen Ltd, Paisley, UK unless otherwise stated. All alcohols were provided by Fisher (Fisher Scientific, Loughborough, Leicestershire, UK). SDS-PAGE gels of between 4-20% were made using standard protocols and loaded into Nupage gel cassettes. Alternatively, bis-tris (Novex Nupage) 4-12% pre-cast gels were purchased and run using a MOPS/SDS running buffer + anti-oxidant for reduced samples. Samples were run at 200V for 45 minutes. A Multimark rainbow marker (5µl) and Magicmark (2.5µl) were run on each gel. The Rainbow marker was used to assess quality of transfer and to cut the membrane as necessary. Magicmark was used for detection of band size after western blotting. Details of all western blotting reagents are shown in Appendix 2c.

b) Transfer: The samples were then transferred using a XCELL II blot module onto PVDF membranes (0.45µm Invitrolon pre-activated with 100% methanol), using transfer buffer containing 10% Methanol (1 gel) or 20% methanol (2 gels) and anti-oxidant for reduced samples. Transfer for 1 gel was undertaken at 30V for 1 hour and for 2 gels 30V for 2 hours or overnight at 4^0C at 15V.

c) Western Blotting: After completing the transfer the PVDF membranes were placed into an appropriate container on a rotating platform set to 1 revolution/second, which was used for all subsequent steps and at all times it was ensured that the membranes were completely covered with the appropriate reagent. All steps were undertaken at room temperature unless otherwise specified. The membranes were washed twice (5 minutes each) with autoclaved ultra-pure water to remove any SDS and residual gel. This was then removed and the membranes were then blocked using 5% BSA for 1 hour, followed by two washes with ultra-pure water as before. The primary antibody was then applied in a 3% BSA solution for 1 hour at Room temperature or overnight at 4^0C. The primary antibody was then removed and 3 washes with wash buffer (5 minutes each) were undertaken. After the last wash, the secondary antibody in 3% BSA was placed onto the membrane for 30 minutes. The membrane was then washed again 3 times with wash buffer as before and then twice with ultra-pure water (2 minutes each). The membranes were then removed from the container and placed onto a clear sheet of plastic. 2.5ml of the chemiluminiscent substrate was then applied to each membrane ensuring complete membrane coverage and left for 5 minutes. The excess substrate was then blotted using whatman paper and another clear sheet of plastic placed on top of the membrane to make a sandwich, all air bubbles were removed.

The blots were then imaged using a Biorad Fluor-S max multi-imager (Biorad life sciences, Hemel Hempstead, Hertfordshire, UK) with exposure times from 30 seconds to 15 minutes. The images were digitally recorded.

Section 2.10.4

Measurement of Apoptosis

Poly-ADP Ribose Polymerase (PARP) Cleavage assay

PARP is a protein involved in DNA repair. However, it can be used as a measure of apoptosis, because when caspases are activated during the induction of apoptosis, Caspase 3 cleaves PARP. In intact viable cells, uncleaved PARP (116 kDa) is identified by western blotting whereas in apoptotic cells, cleaved PARP (85 kDa) is identified. The balance between cleaved and uncleaved PARP can be used to compare rates of cellular apoptosis.

75cm^2 flasks with the appropriate matrices (Pl vs CI vs CIII vs CIV) were prepared as previously described. Then 1 x 10^6 CRC cells were plated out per flask in serum enriched media. The next morning the cells were washed with 10ml of HBSS+Ca^{2+} for 5 minutes, this was then replaced with serum free media (0.5% FCS; HT-29 and 1%; KM12 cell lines) for 8 hours. Then 5-Fu in the appropriate serum free media at concentrations of between 10-75μg/ml were added to the appropriate flask, for each of the matrices studied and fresh serum free media was also added to the control flasks. The cells were exposed to chemotherapy for 48-56 hours and then processed as described above.

The assay used the standard western blotting technique as previously described. Samples were prepared under reducing conditions and 35μg of protein was loaded per well. The protocol as described above was used, with the PARP primary antibody (Clone C2-10, R&D systems, Abingdon, Oxon, UK) used at a concentration of 1:1,500. The rabbit anti-mouse secondary antibody (Invitrogen Ltd, Paisley, UK) was used at a concentration of 1:10,000. A positive control consisted of loading samples of lysates from the Breast Cancer cell line (MCF-7) treated with etoposide, and a negative control consisted of omitting the primary antibody and proceeding with the blotting protocol. Although not strictly necessary for use in analysing PARP cleavage, β-actin expression was also detected by western blotting to show the equal loading of samples being compared.

After imaging, the ratio of cleaved to total PARP expressed by CRC cells exposed to different doses of chemotherapy was determined by volume analysis of the blots using the Quantity One program (Biorad life sciences, Hemel Hempstead, Hertfordshire, UK).

Section 2.10.5-I

Cell cycle regulators expression by CRC cells

75cm^2 flasks with the appropriate matrices as previously described were prepared. Then 6 x 10^5 cells were plated out per flask in serum enriched media. The next morning the cells were washed with 10ml of HBSS+Ca^{2+} for 5 minutes, this was then replaced with serum free media (0.5% FCS). The cells were allowed to grow for a further 36 hours, before processing.

Lysates were prepared under reducing conditions and 25µg of protein loaded per well. The standard western blotting technique as previously described was used. All primary antibodies were supplied at 0.2µg/µl concentration and the rabbit anti-mouse secondary antibody (Invitrogen Ltd, Paisley, UK) used at a concentration of 1:10,000. A positive control consisted of whole cell lysates from the Jurkat cell line (sc-2204, Santa Cruz, CA, USA).

i) PCNA - Mouse Monoclonal 1° antibody concentration 1:3000 (Clone PC-10, Santa Cruz, CA, USA).

ii) Cyclin D1 - Mouse Monoclonal 1° antibody concentration 1:500 (Clone DCS-6, Santa Cruz, CA, USA).

iii) p21 - Mouse Monoclonal 1° antibody concentration 1:350 (Clone 187, Santa Cruz, CA, USA).

iv) p27 - Mouse Monoclonal 1° antibody concentration 1:350 (Clone DCS-6, Santa Cruz, CA, USA).

Section 2.10.5-II

Apoptosis regulators expression by CRC cells

The same protocol as previously described (section 2.10.4) for measuring PARP cleavage was used. The blots from the PARP cleavage assays were re-probed after first being stripped or new blots prepared as described above, loading between 25µg-35µg of protein per well. The standard western blotting technique as previously described was used. All santa cruz primary antibodies were supplied at 0.2µg/µl concentration and the BD biosciences antibodies were supplied at a concentration of 0.1µg/µl. The rabbit anti-mouse secondary antibody (Invitrogen Ltd, Paisley, UK) used at a concentration of 1:10,000.

i) bcl-2 - Mouse Monoclonal 1° antibody concentration 1:350 (Clone C-2, Santa Cruz, CA, USA). A positive control consisted of whole cell lysates from the Jurkat cell line (sc-2204, Santa Cruz, CA, USA).

ii) bcl-xl - Mouse Monoclonal 1° antibody concentration 1:350 (Clone H-5, Santa Cruz, CA, USA). A positive control consisted of whole cell lysates from the BJAB cell line (sc-2207, Santa Cruz, CA, USA).

iii) mcl-1 - Mouse Monoclonal 1° antibody concentration 1:400 (Clone 559027, BD Biosciences, USA). A positive control consisted of whole cell lysates from the K-562 cell line (ATCC CCL-443).

iv) bad - Mouse Monoclonal 1° antibody concentration 1:350 (Clone C-7, Santa Cruz, CA, USA). A positive control consisted of whole cell lysates from the Hela cell line (sc-2200, Santa Cruz, CA, USA).

v) bak - Mouse Monoclonal 1° antibody concentration 1:400 (Clone 559027, BD Biosciences, USA). A positive control consisted of whole cell lysates from the Hela cell line (sc-2200, Santa Cruz, CA, USA).

vi) bax - Mouse Monoclonal 1° antibody concentration 1:350 (Clone 2D2, Santa Cruz, CA, USA). A positive control consisted of whole cell lysates from the Jurkat cell line (sc-2204, Santa Cruz, CA, USA).

Section 2.10.6

β-Actin detection

Western blotting for β-actin was undertaken to compare equal loading of samples. After probing the membrane with the antibody of interest the membrane was washed twice in wash buffer (5 minutes each) and once in ultra-pure water (5 minutes) and then the western protocol as described above performed from the blocking stage. The β-actin antibody (Clone AC15, Sigma, Poole, Dorset, UK) was used at a concentration of 1:10,000 and left on for 30 minutes. The rabbit anti-mouse secondary antibody (Invitrogen Ltd, Paisley, UK) was used at a concentration of 1:10,000. The remainder of the protocol proceeded as described above. In some circumstances the membranes were cut to allow simultaneous estimation of β-actin expression and the protein of interest.

Section 2.11.1

Integrin Mediated effects

To identify if the differences observed were Integrin mediated the following experiments were undertaken;

Section 2.11.2

Western Blotting for integrins expressed by CRC cells

The standard technique was used as described above. Unless otherwise specified the samples were prepared unreduced and 25μg of protein loaded per well. All antibodies were supplied at 1μg/μl concentration. The rabbit anti-mouse 2° antibody (Invitrogen Ltd, Paisley, UK) was used at a concentration of 1:10,000.

i) β1 integrin – Mouse Monoclonal 1° antibody concentration 1:1000 (Clone JB1A, Chemicon, Chandlers Ford, Hampshire, UK). Positive control consisted of lysates from the SW480 cell line.

ii) αv integrin – Mouse Monoclonal 1° antibody concentration 1:1000 (Clone AV1, Chemicon, Chandlers Ford, Hampshire, UK). Positive control consisted of Human platelet lysates.

iii) β3 integrin – Mouse Monoclonal 1° antibody concentration 1:1000 (Clone BB10, Chemicon, Chandlers Ford, Hampshire, UK). 75μg of protein loaded per well. Positive control consisted of Human platelet lysates.

iv) β5 integrin – Mouse Monoclonal 1° antibody concentration 1:500 (Clone 343.11D1, Calbiochem, Beeston, Nottingham, UK). Positive control consisted of lysates from the HT-29 cell line.

Section 2.11.3

Adhesion Assay

A standard adhesion assay was used, which was first optimised as to the number of cells to be added and the length of both pre-incubation and incubation time. A standard protocol was then used for each different antibody.

The matrices were plated out into the wells of a 24 well plate and prepared as described before. After routine passaging and counting, $5x10^4$ cells were to be added per well in serum enriched media, after first pre-incubating the cells with the blocking antibody, IgG isotype control or no antibody for 15 minutes at 37°C. Serial dilutions of the blocking antibody were used and all samples were plated out in triplicate. During the pre-incubation stage the cells were vortexed twice to ensure complete mixing with the antibodies. After plating out the cells, the 24 well plates were placed into an incubator for 2 hours 15 minutes. At this stage the supernatant was removed and the wells were washed with 0.5ml of warmed PBS, to remove non-adherent cells from the cell monolayer. The cells were then fixed for 1 hour using 4% Formaldehyde at room temperature. The wells were then washed once with dH_2O, following which 250µl of 1% methylene blue was added to each well and the plates were then placed on a rotating platform for 20 minutes. This was then removed and the wells were washed 4 times with 1ml of dH_2O to remove any excess methylene blue which had not stained the cells. The wells then had 0.25ml of 0.5M HCL added to release the methylene blue within the cells. These supernatants were then added to the wells of a Nunc Maxisorp 96 well plate and viewed under a colormetric plate reader at 620nm.

Section 2.11.4-I

β_1 Neutralising Antibody

A sodium azide free version of this antibody was obtained from Beckman Coulter (Clone 4B4-azide free, Beckmann Coulter, High Wycombe, Buckinghamshire, UK). The experiments were set out as described above in section 2.11.3. The antibody was provided at a concentration of 2.4µg/µl. The antibody was used at a concentration of 1, 5, 10 and 15µg/ml

for these experiments, and 500µl of the cell mix containing $5x10^4$ cells were added per well for the adhesion assay. The IgG isotype control was provided by Ancell (Bayport, MN, USA).

Section 2.11.4-II

Assay of β_1 Integrin Neutralising Antibody effects on CRC proliferation

The standard ^3H Thymidine proliferation/Pico green assay were used (section 2.9.2), with the exception that $1.5x10^4$ cells were added per well and the ^3H thymidine was then added 6-8 hours after switching to serum free media as previously described. The blocking antibody and control were used at a concentration of 5µg/ml and the cells were pre-incubated with the neutralising antibody as described in the adhesion assay in section 2.11.3.

Section 2.11.5-I

$\alpha v\beta_3$ & $\alpha v\beta_5$ Integrin Neutralising Antibodies

Sodium azide free versions were obtained of the αvβ3 integrin (Clone LM609, Chemicon, Chandlers Ford, Hampshire, UK) and αvβ5 integrin (Clone P1F6, Chemicon, Chandlers Ford, Hampshire, UK) neutralising antibodies. The experiments were set out as described above in section 2.11.4. These antibodies were provided at a concentration of 1µg/µl and were used at concentrations of between 5 and 20µg/ml for the adhesion assay as previously described.

Section 2.11.5-II

Assay of $\alpha v\beta_3$ & $\alpha v\beta_5$ Neutralising Antibody effects on CRC proliferation

The standard ^3H Thymidine proliferation/Pico green assay was used (sections 2.9.2 and 2.11.4) with the following modifications. $1.5x10^4$ cells were added per well and the cells were allowed to adhere for 8-12 hours, then washed with HBSS+Ca^{2+} and the medium was switched to serum free medium which was left on overnight. The following morning the supernatant was removed and serum free medium containing the neutralising antibody or the appropriate IgG control was added, then 8 hours later the ^3H Thymidine was added. The blocking antibody and control were used at a concentration of 5 and 10µg/ml.

Section 2.11.6

Matrix metalloproteinase resistant Type I r/r Collagen effects on proliferation

The standard ^{3}H Thymidine proliferation/Pico green assay was used (section 2.9.2), after plating out and processing the appropriate matrices; collagen I (rat tail), type I r/r collagen and w/t type collagen control. These experiments were also repeated with the addition of $\alpha v\beta_3$ & $\alpha v\beta_5$ integrin neutralising antibodies as previously described (section 2.11.5).

Section 2.12

Gelatin Zymography to detect MMP-2 & MMP-9 expression in CRC conditioned media

The role MMP-2 and 9 in our studies were determined using gelatin zymography. The matrices (collagen I and IV) were plated out into 25 cm^2 flasks and prepared as previously described. $1x10^6$ cells were added to each flask in 5 ml of serum enriched media. The next morning the cells were washed with warmed HBSS+Ca^{2+} for 5 minutes, and then the supernatant was replaced with 2 ml of serum free media. After a further 36 hours in culture, the supernatants were removed, centrifuged to remove cell debris and then frozen. The flasks were scraped after adding 2ml of PBS (repeated x1), the supernatants removed were centrifuged, and the resulting cell pellets were lysed using RIPA buffer. The lysates protein concentration was determined using the BCA assay, which allowed normalisation for different cell numbers within the cell monolayer in each flask, when preparing zymogram samples. Supernatants were defrosted, and samples were prepared using 2x sample buffer and 20µl loaded per well. A positive control of 0.5% FCS (which contains MMP-2 and 9) was loaded on each gel along with Multimark rainbow marker. Pre-cast 10% Tris-glycine-gelatin zymogram gels (Invitrogen, Paisley, UK) were run using a standard technique. The gels were run at 125V for 90 minutes, following which the gels were placed into an appropriate container, on a rotating platform set to 1 revolution/second at room temperature (unless otherwise specified), which was used for all subsequent steps. It was ensured the gels were always totally immersed in the buffers used. Following electrophoresis, SDS was removed from the gel and the gels were renatured using renaturing buffer for 30minutes, this was then removed and replaced by developing buffer for 30 minutes. At this stage the developing buffer was replaced with fresh developing buffer and placed into a rotary shaker, set to 37°C

and was left overnight to allow MMPs to degrade the gelatin within the gels. The following morning, the gels were stained for 6 hours using Colloidal blue stain (Invitrogen, Paisley, UK). The gels were then washed several times with distilled water, before being mounted between cellulose sheets and air dried.

Details of all reagents used, not listed elsewhere are shown in Appendix 2d.

Section 2.13

Statistical Analysis

All data were analysed using a paired student t-test, assuming a two tailed distribution. This method of statistical analysis was decided upon after discussion with the statistician, Dr J Goddard, University of Southampton. The results were expressed as means ± 95% Confidence intervals, after calculation using Microsoft Excel. A $p<0.05$ was taken as being of significance.

Chapter 3

The Desmoplastic Reaction and pattern of Integrin expression within Colorectal cancer liver metastases

Section 3.1.1

The Desmoplastic reaction and pattern of integrin expression within Colorectal cancer liver metastases

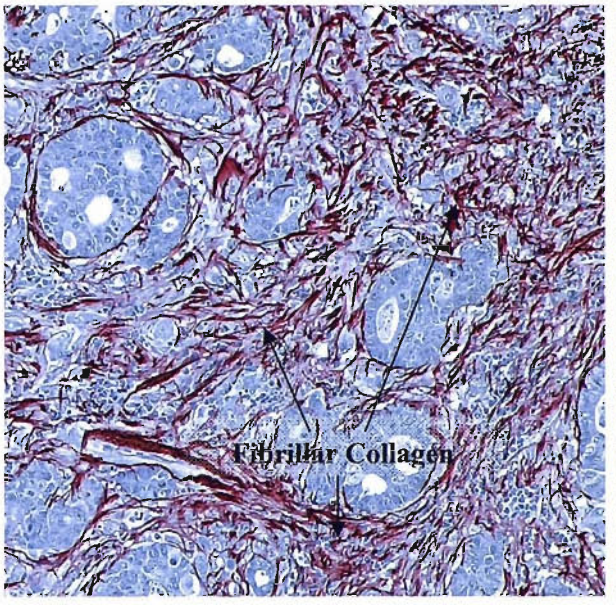
A Desmoplastic Reaction is characterised by deregulated matrix turnover, which results in the deposition of fibrillar collagens produced by activated stromal cells and alterations in the pattern of collagen IV distribution. The alterations in matrix composition associated with a DR in several cancers, have been suggested to offer the cancer a growth advantage and resistance to chemotherapy. Studies in resected primary CRC have suggested a pronounced DR was a poor prognostic indicator in terms of the development of cancer recurrence and metastatic spread. These studies therefore aimed to determine if a pronounced desmoplastic reaction was observed, within resected specimens of patients with CRC liver metastases. Studies in several different cancers have suggested that the level of expression of different integrin sub-types may be associated with different phenotypic properties of the cancer, as integrins play an important role in regulation of cell growth and apoptosis. Furthermore, we went on to characterise the pattern of integrin sub-type expression within the metastases and normal liver and if the malignant phenotype of the CRC cells had any influence on their pattern of integrin expression.

Results

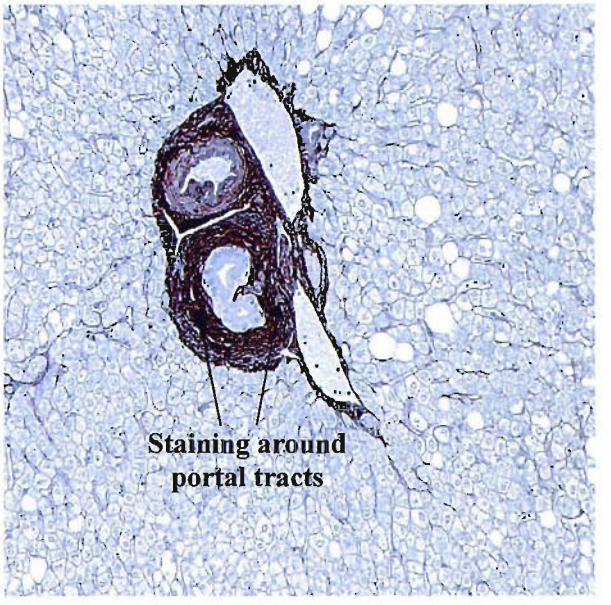
Sirius red and α-SMA staining

The following experiments were performed as described in section 2.7. In all the sections studied, there was a significant increase in the deposition of fibrillar collagens within Colorectal Cancer liver metastases, compared to sections of normal liver from the same patient. Within the liver metastases, there was abundant staining for deposited fibrillar collagens (Figure 15a). In contrast in the normal sections there was very little staining for fibrillar collagens, with the exception of staining located around the portal tracts, in the walls of the vessels (Figure 15b). Interestingly the deposition of fibrillar collagens was at its most dense in poorly differentiated areas of the tumour, with the fibrillar collagens often enveloping individual tumour cells (Figure 15c). Representative examples of these staining patterns are shown below.

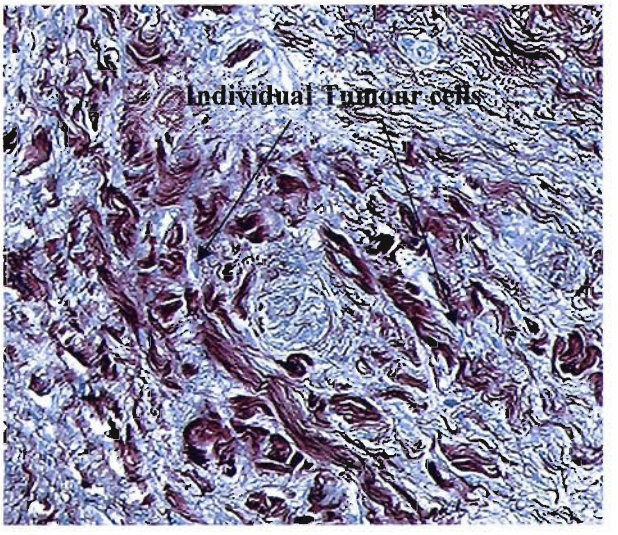
Figure 15: Sirius red staining of colorectal cancer liver metastases



a: Tumour



b: Normal Liver



c: Poorly Differentiated Tumour

This figure demonstrates the increased deposition of fibrillar collagens within CRC liver metastases (15a) compared to normal liver (15b). Within normal liver, fibrillar collagen staining was limited to around the portal tracts. In poorly differentiated areas of the CRC metastases it was apparent that the deposited collagens were more densely packed often enveloping individual cancer cells (15c). All images were 100 x objective magnification, with the exception of figure 15c (200x objective magnification). These images are representative staining patterns identified in 10 different patients.

Staining for αSMA showed a similar pattern to that of Sirius red staining. There were very few if any activated HSC within areas of normal liver tissue (Figure 16a), in contrast within the tumour stroma, there were abundant activated HSC associated with the deposited fibrillar collagens (Figure 16b). Representative examples of these staining patterns are shown below (αSMA +ve Cells are stained brown).

Figure 16: α–Smooth Muscle Actin (αSMA) staining in colorectal cancer liver metastases

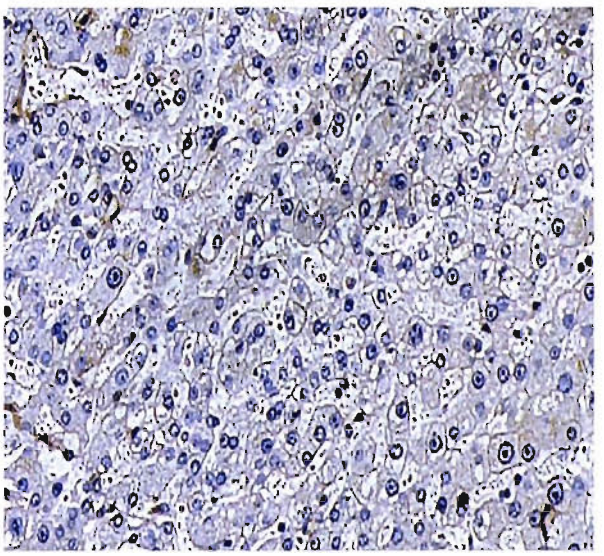


Fig16a: Normal Liver

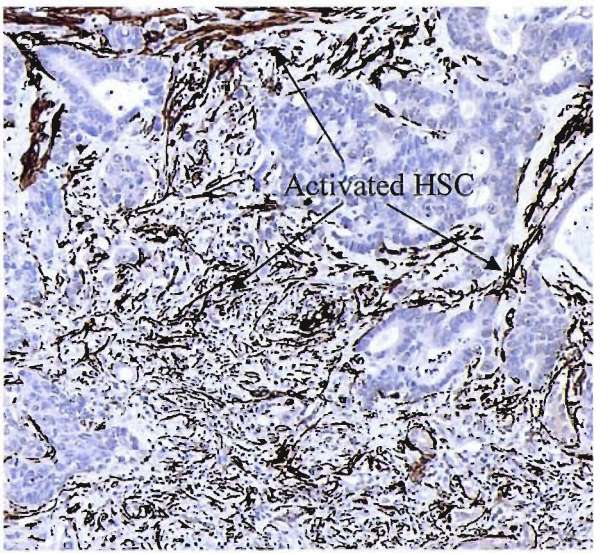


Fig 16b:Tumour

This figure demonstrates the close association of increased numbers of αSMA positive hepatic myofibroblasts, with the deposited fibrillar collagens found within CRC liver metastases, compared to normal liver. All images are 100x objective magnification. Sections treated with non-immune IgG instead of specific antibody, showed no staining. These images are representative staining patterns identified in 10 different patients.

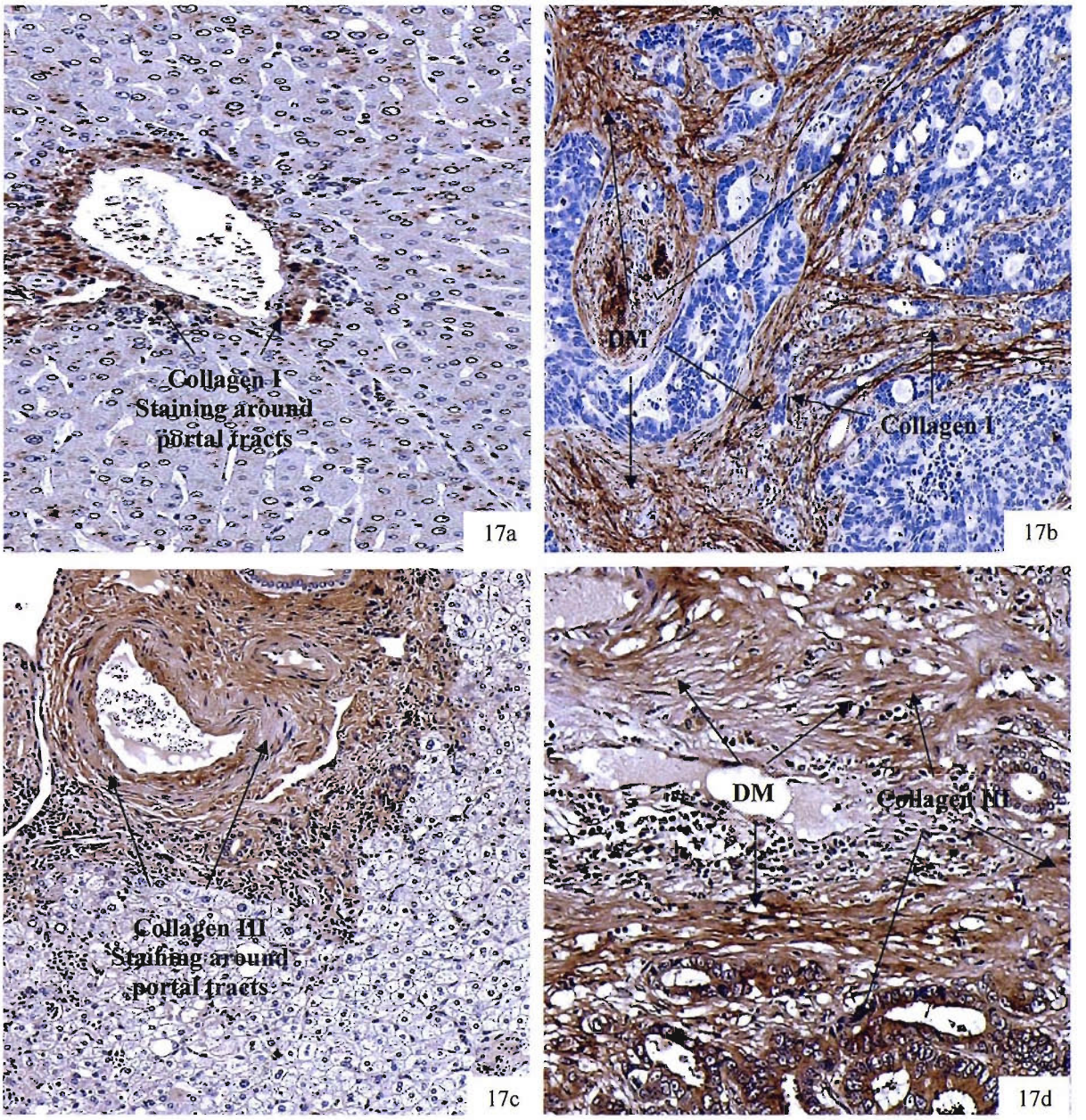
Section 3.1.2

Staining patterns for collagens I, III and IV within CRC liver metastases

Staining for collagens I & III followed the same pattern as identified with Sirius red staining, with extensive staining within the deposited desmoplastic matrix (DM). In most areas, the amount of staining for collagen I was greater than for collagen III. Staining for both collagens

was most dense in poorly differentiated areas of the metastases. In the figure below are representative staining patterns for collagens I and III.

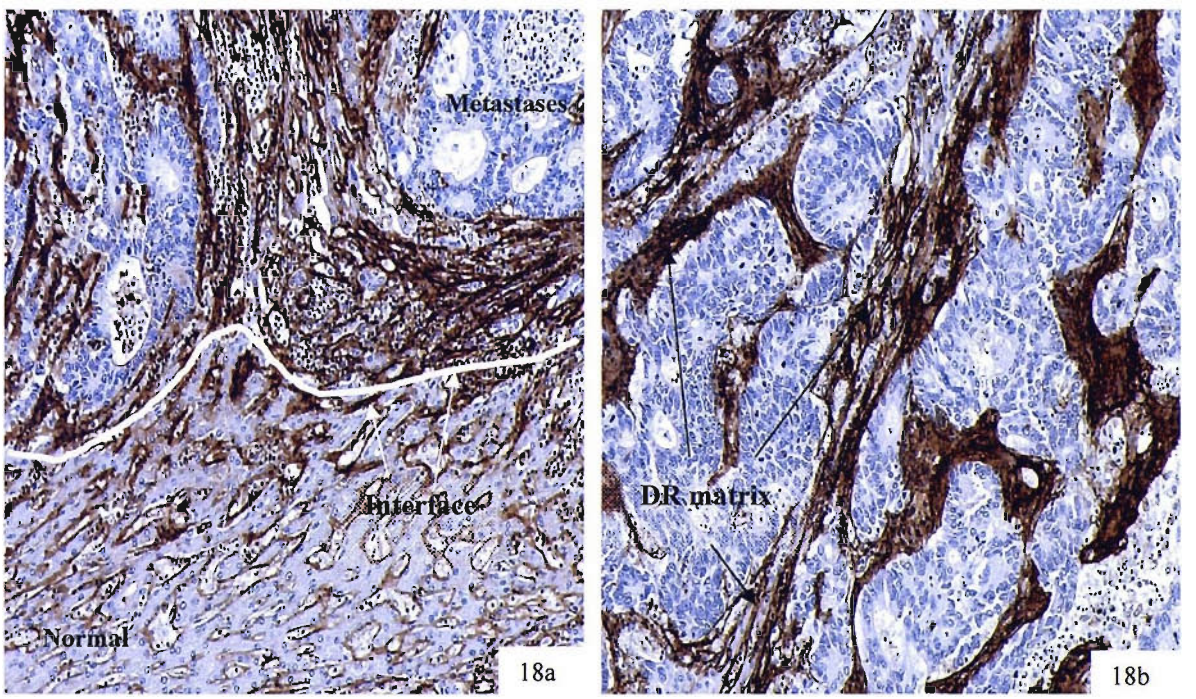
Figure 17: Staining for Collagens I and III within Colorectal liver metastases & normal liver



This figure demonstrates the increased deposition of collagens I and III within CRC liver metastases, as was previously demonstrated by Sirius red staining. The staining pattern for collagen I is shown in figure 17a (normal) & 17b (tumour), and for collagen III is shown in figure 17c (normal) & 17d (tumour). There was much higher background staining produced using the polyclonal collagen III antibody. Sections treated with non-immune IgG instead of specific antibody showed no staining. These images are representative staining patterns identified in 10 different patients. All images are 100x objective magnification.

Staining patterns for collagen IV are shown in the figures below. Interestingly as can be seen in the normal liver the pattern of staining was regularly distributed, whereas in contrast within the metastases, the collagen IV was arranged in a more haphazard manner, illustrated by the loss of the normal regular basement membrane distribution within the metastases. Instead, the collagen IV often appears to be incorporated into the desmoplastic matrix. The antibody used recognises an epitope exposed on the triple helix formed from the α-1 and α-2 chains of collagen IV. Staining for the specific growth regulatory isotypes (α3 chain) of collagen IV has proved to be very difficult and as of yet has not been optimised.

Figure 18: Staining for collagen IV, within Colorectal liver metastases and normal liver



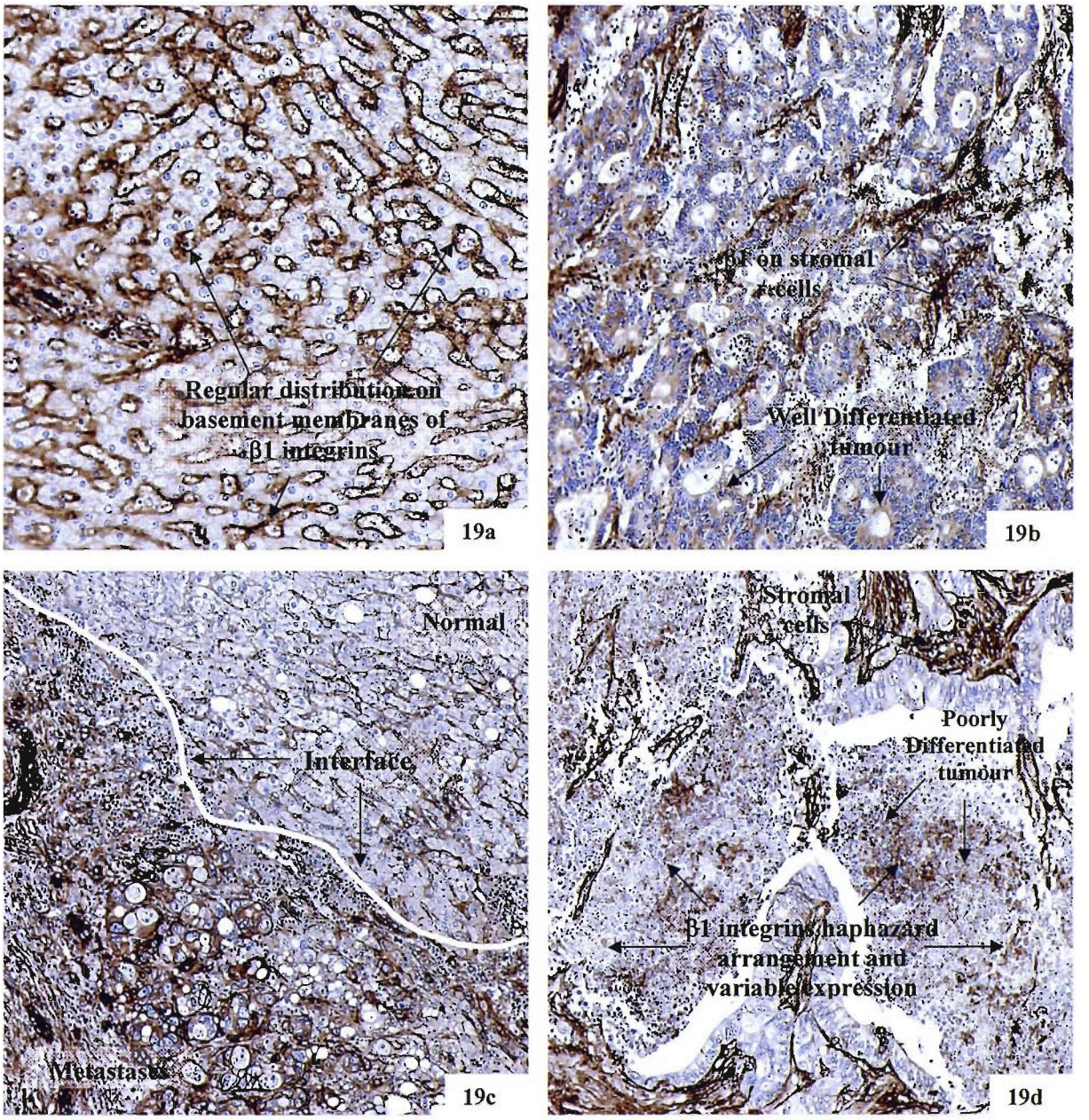
In figure 18, representative staining patterns for collagen IV are shown. The pattern of distribution of collagen IV at the interface between the tumour and normal liver is demonstrated in figure 18a and within the metastases in figure 18b. The most striking feature is the loss of the organised basement membrane structure and therefore collagen IV distribution within the metastases compared to normal liver, presumably due to matrix degradation of collagen IV as part of the DR. Interestingly, within the metastases there appears to be newly deposited collagen IV, associated with the deposited fibrillar collagen rich desmoplastic matrix, presumably produced by activated stromal cells. All images 100x objective magnification. Sections treated with non-immune IgG instead of specific antibody showed no staining, these images are representative staining patterns identified in 10 different patients.

Section 3.1.3

The pattern of β1 integrin expression within CRC metastases

In the figures below the staining patterns for β1 integrins are illustrated. The most striking feature is the alteration in β1 integrin expression and distribution between the CRC metastases and normal liver. Compared to the organised and localised distribution in normal liver of β1 integrins, within the metastases this integrin was expressed in a more haphazard manner. Overall, β1 integrins were more highly expressed by CRC cells compared to hepatocytes. However, this was not a uniform finding with β1 integrins being expressed at very high levels in some areas of the metastases, whilst in others this integrin was expressed at very low levels. This pattern of staining was particularly noticeable in poorly differentiated parts of the tumour, where β1 integrins often appeared to be down-regulated. A possible reason for this will be illustrated by the staining for αv integrins in the next section. In addition to CRC expressing β1 integrins within the metastases, there was also extensive staining of activated stromal cells as well. These features are illustrated in figure 19 below.

Figure 19: Staining for β1 integrins within Colorectal cancer liver metastases & normal liver.



The pattern of β1 integrin is clearly demonstrated in this figure. β1 integrins were highly expressed along the basement membrane of normal liver (19a). Within the CRC metastases (19b) and at the interface between normal liver and the metastases (19c), β1 integrins were highly expressed by both CRC cells and hepatocytes, with the highest level of expression found within the metastases on both CRC and stromal cells. However, there was often a haphazard pattern of distribution of β1 integrins within the metastases; in particular expression within poorly differentiated areas of the metastases was often at much lower levels than the rest of the metastases (19d). All images are 100x objective magnification. Sections treated with non-immune IgG instead of specific antibody showed no staining. These images are representative staining patterns identified in 10 different patients.

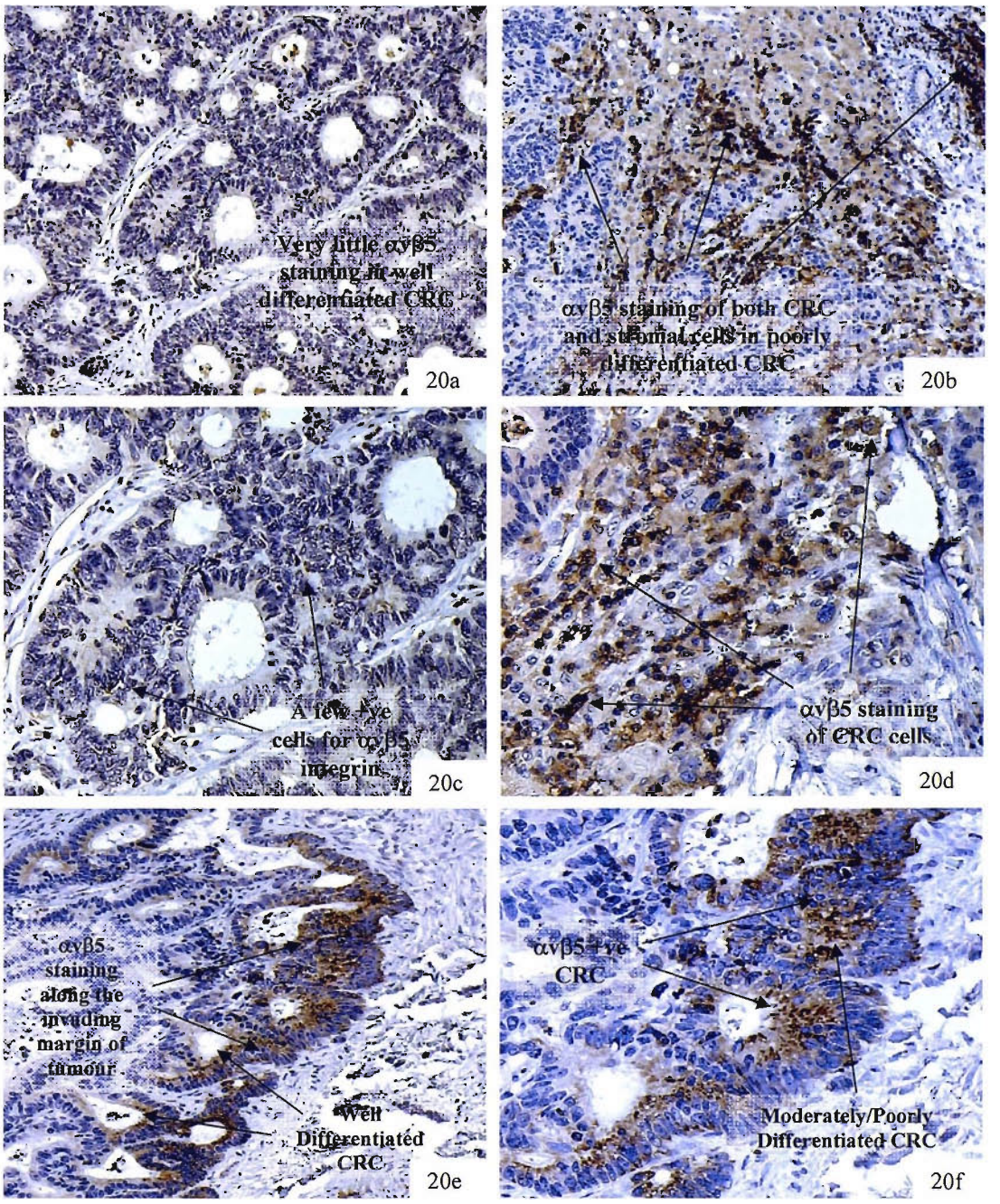
Section 3.1.4

The pattern of αv integrin expression within CRC metastases

Staining for alpha v integrins proved to be very difficult, when identification of the whole complex, rather than the different integrin sub-units was attempted. However, using different pre-treatment techniques as described in section 2.7.3, it was possible to successfully detect both αvβ3 and αvβ5 integrin expression in our sections and controls.

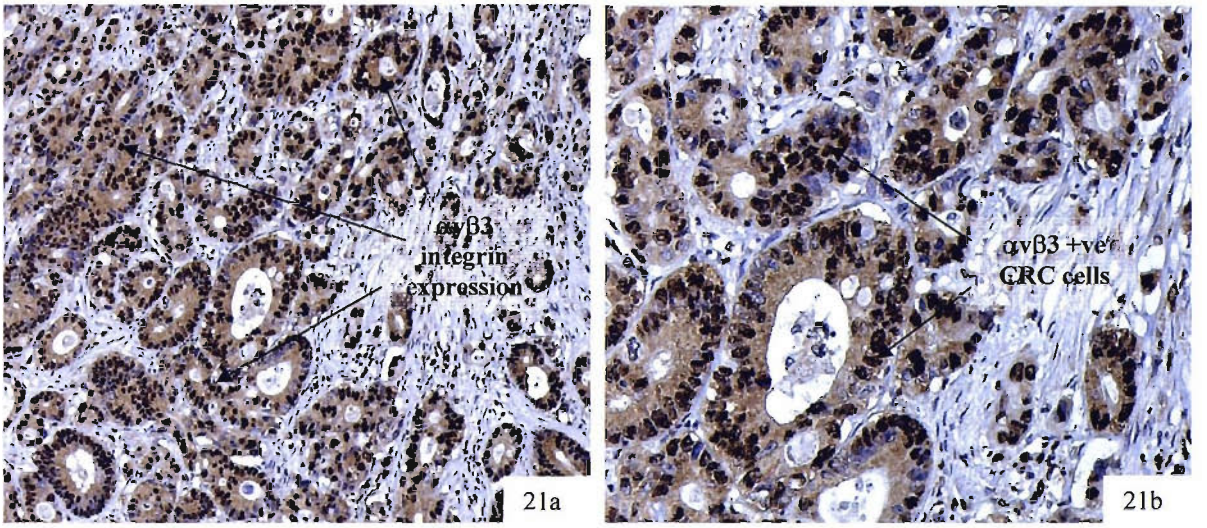
Both CRC and stromal cells express αvβ3 and αvβ5 integrins, however the most interesting finding was the differential pattern of αvβ5 integrin expression in well and poorly differentiated areas within CRC liver metastases. In contrast to β1 integrin expression, αvβ5 expression was highest on CRC in poorly differentiated areas of the metastases, with low levels of expression of this integrin on CRC in well differentiated tumour areas. The highest level of expression of αvβ5 integrin was demonstrated at the invading margin of poorly differentiated areas of the metastases. In well differentiated areas of CRC there was little αvβ5 integrin staining identified, with the exception of the invading margin of the metastases, where this integrin was upregulated. However in most sections this was not to the levels seen in poorly differentiated areas of the metastases. The staining pattern for αvβ3 integrins were less clear cut than those identified for αvβ5 integrin. αvβ3 integrin was expressed in both well and poorly differentiated areas of the metastases, by both cancer and stromal cells. Although there may be higher expression of αvβ3 integrins in poorly differentiated areas of the metastases, there was not as profound a difference as demonstrated by staining for both β1 and αvβ5 integrins. The staining patterns for both αvβ5 and αvβ3 integrin expression are illustrated in figure 20 and 21 respectively.

Figure 20: Staining of CRC liver metastases for αvβ5 integrin



This figure illustrates the pattern of αvβ5 integrin expression within CRC metastases. Figure 20a & c shows the low level of αvβ5 integrin expression in well differentiated areas of the metastases. In contrast in figure 20 b & d, αvβ5 integrin was highly expressed in poorly differentiated areas of the metastases, with αvβ5 integrin expressed by both CRC and stromal cells. Figures 20 e & f illustrates that αvβ5 integrin was highly expressed along the invading margin of the metastases. Figures 20 a, b & e are at 100 x objective magnification and figures 20 c, d & f are at 200x objective magnification. Sections treated with non-immune IgG instead of specific antibody showed no staining. These images are representative staining patterns identified in 10 different patients.

Figure 21: Staining of CRC liver metastases for αvβ3 integrin



This figure clearly demonstrates that αvβ3 integrins are expressed by both CRC and stromal cells. As can be seen, this integrin was highly expressed throughout the metastases, perhaps more highly expressed in poorly differentiated areas, but the differences are not as clear cut as those identified with αvβ5 integrin. Sections treated with non-immune IgG instead of specific antibody showed no staining. These images are representative staining patterns identified in 10 different patients.

Section 3.1.5

Discussion – The desmoplastic reaction and pattern of integrin expression within CRC liver metastases

These studies clearly demonstrate that CRC liver metastases evoke a desmoplastic reaction characterised by dysregulated matrix turnover. This results in the degradation of the normal basement membrane rich in type IV collagen and the accumulation of fibrillar collagens, in particular type I collagen. The alteration in the pattern of type IV collagen distribution are important findings as the unique composition of the basement membrane is thought to confer tissue specificity, epithelial polarity and functionality (Kalluri, R, 2003). In pancreatic cancer a key and prognostic event is the loss of basement membrane integrity and invasion of malignant cells into the interstitial matrix. This exposes malignant cells to interstitial type I collagen of the DR (Mollenhauer J et al, 1987 and Lee CS et al, 1984). In the development of CRC liver metastases degradation of the normal basement membrane plays a key role in the development of a metastatic deposit. This in turn allows the CRC to come into contact with fibrillar collagens produced by activated stromal cells and as will be shown later this offers the cancer distinct survival and growth advantages. However, this underestimates the importance of type IV collagen, which possesses its own intrinsic growth regulatory properties. In primary CRC, it has been demonstrated that alterations in the pattern of $\alpha 1$ & $\alpha 2$ chains distribution was dependent on the degree of differentiation of the CRC, with very little collagen IV expression in poorly differentiated areas of the CRC (Oka Y et al, 2002).

It has previously been established that CRC cells induce an inflammatory response in the liver (Yoong KF et al, 1999) which in turn leads to stromal cell activation. In vitro studies have suggested a contributory role for CRC and stromal cell interactions in the development of primary colorectal cancers (Hauptmann S et al, 2003). Using an in vivo animal model it was suggested that the redistribution and expansion of myofibroblasts plays an important role in the formation of established CRC liver metastases (Higashi N et al, 2002). The source of myofibroblasts has long been assumed to be the HSC. Many studies have suggested the HSC are an important source of MMPs and TIMPs for the establishment of CRC liver metastases (Musso O et al, 1997; Sunami E et al, 2000; Mook OR et al, 2003) and it was postulated that CRC and HSC interactions were mediated by cytokines such as PDGF (Shimizu S et al,

2000). With the extensive work undertaken on the important role of HSC in fibrogenesis resulting in cirrhosis (Benyon RC et al, 2001) it would seem likely that HSC are the source of the deposited desmoplastic matrix, as both CRC and activated HSC are found in close proximity with the deposited matrix.

A DR is a classical wound healing response in response to inflammation, characterised by the activation of stromal cells to a myofibroblastic phenotype, it was initially postulated as the body's response to wall off the tumour. However, studies from the Southampton Liver group and others in primary breast and pancreatic cancers (Armstrong TA, et al, 2004; Buchholz M et al, 2003; Meng L et al, 2001) and this work in metastatic CRC, it appears that the successful tumour clones which expand in the liver, have adapted to this response and actually use the desmoplastic matrix to their own advantage. In recent studies it has now been established that a dense DR in the primary CRC was an independent poor prognostic indicator in terms of the development of metastatic disease and increased mortality (Sis B et al, 2005 and Nishimura R et al, 1998). Therefore, it appears apparent that the DR may just be a classical wound healing reaction in response to cancer cells, which leads to the production of factors that activate stromal cells. The successful cancer is able to adapt to the DR and use it to its advantage. As the clonal expansion of the cancer cells produces cells with more or less malignant phenotypes, the more aggressive malignant cells survive and outgrow the less malignant cells. Therefore an alternative and perhaps more interesting explanation for the CRC response to the DR, is that as the cancer adopts a progressively more aggressive malignant phenotype, it may induce a desmoplastic reaction by producing specific factors that activate stromal cells, and benefit from a growth and survival advantage from being in contact with a desmoplastic matrix.

Therefore the observation that the deposited DR matrix was often most dense within poorly differentiated areas of the tumour, often enveloping individual cancer cells may be an expected finding. It could therefore be assumed the denser the DR the faster the CRC proliferate and the greater the advantage gained by the cancer. However, as will be demonstrated later the advantage gained in terms of survival and growth by the highly metastatic CRC clones was less than for the poorly metastatic clones. Indeed, in a lung cancer

mouse model, it was demonstrated that the desmoplastic reaction was most dense in the poorly metastatic cancer cell lines (Nakanishi H et al, 1994) and in studies in primary colorectal cancers, although there was evidence of increased collagen I deposition, the fibrils deposited were small and rarely cross-linked (Bode M et al, 2000). Cross-linking stabilizes collagen I fibres and makes them resistant to the action of MMPs and is believed to play an important role in preventing resolution of cirrhosis (Issa R et al, 2004). Therefore, the density of the deposited desmoplastic matrix may be only one factor in a complex tumour microenvironment where other factors, such as the size of the deposited fibrillar collagen fibres, whether the collagen fibres are cross-linked, and the presence of cytokines and growth factors may also be important. In addition as will be expanded upon in later sections, the pattern of integrin expression between CRC clones with different metastatic phenotypes, may also play a crucial role and that the deposited DR should be viewed as a highly dynamic process with new Desmoplastic matrix deposited and continually turned over, this provides a replenishable supply of new ligands for integrin liagtion.

My data indicates the importance of β1 integrins in the development of CRC liver metastases. Overall β1 integrins were most highly expressed by CRC and stromal cells within the metastases. However it was observed that in poorly differentiated areas of the metastases, β1 integrin expression showed a haphazard distribution pattern and frequently was expressed at much lower levels compared to the remainder of the metastases. There are several possible reasons for this observation; firstly these findings may be artefactual due to technical problems with the IHC, this was unlikely to be the case as this appeared to be a consistent finding. Instead, as a second possibility, our studies have demonstrated that there was also a differential pattern of αv integrin expression. Typically in areas where β1 integrins were poorly expressed αv integrins appeared more highly expressed. As will be demonstrated in later sections, as CRC adopt a more aggressive malignant phenotype, αv integrins appear to play an increasingly important growth regulatory role and this may explain the different patterns of integrin staining within the metastases.

Which particular β1 integrins are likely to be important in CRC development is a matter for speculation, however, from previous studies in both CRC (Lotz MM et al, 1990; Koretz K et

al, 1994; Fujita S et al, 1995; Roela RA et al, 2003) and other tumours (Morini M et al, 2000; Sawai H et al, 2003; Li X et al, 2004) it is likely that α2β1, α3β1, α5β1, α6β1 and αvβ1 are likely to be of interest. Of particular interest are those integrins that are known to play an important role in regulating cell adhesion to collagens, these include the α1β1 and α2β1 integrins (Jokinen J et al, 2004). From these results it is tempting to conclude that low β1 integrin expression may be poor prognostic indicator in CRC, as it is in prostate cancer and melanoma. But this may not be a uniform finding applicable to all cancers such as primary bowel and breast cancers, where high β1 integrin expression was a poor prognostic indicator. This apparent paradox between primary and metastatic CRC, could be explained in terms of high β1 integrin expression being important in the process of metastatic spread. Once the metastatic deposit has become established other integrin types may become increasingly important. Perhaps the reason why β1 integrin expression does not always follow the same pattern is that their expression appears to be tissue and cancer specific and also reliant on the particular integrin heterodimer expressed by the cancer. Further evidence for this can be concluded by studies in human colon cancer cell lines, which demonstrated that k-ras mutations and thereby protein expression affected specific β1 integrins expression. Namely, high α1β1 and α5β1 integrin expression was associated with a non-transformed phenotype; in contrast α3β1 expression was associated with a transformed metastatic phenotype (Schramm K et al, 2000). In addition increased α2β1 and α3β1 integrin expression appears to correlate with a poorly differentiated phenotype in both CRC (Buda A et al, 2003) and breast cancer (Morini M et al, 2000) respectively.

The pattern of αvβ5 integrin expression within CRC liver metastases appears to reinforce the hypothesis that the more invasive and aggressive cancers rely on different integrin sub-types to regulate their growth. Staining for αvβ5 integrin was highest in poorly differentiated areas of CRC metastases, especially along the invading margin of the tumour. In contrast, within well differentiated areas of the CRC metastases, αvβ5 integrin expression was absent with the exception of the invading margins of the tumour. These data suggest αvβ5 integrin may be a key integrin in the development of CRC liver metastases, especially as CRC adopt a more aggressive and invasive malignant phenotype. These data would in keeping with in vitro

studies using CRC lines, which have suggested that up-regulating αvβ5 integrins shifts the malignant phenotype of CRC to a more aggressive and invasive type (Schramm K et al, 2000). The differences identified in αvβ3 expression were less clear, but showed a similar pattern as seen with αvβ5 integrin expression and would be in keeping with the results obtained in melanoma, where αvβ3 integrin plays a key role in regulating cellular proliferation, apoptosis and angiogenesis (Shahan T et al, 1999; Petitclerc E et al, 1999; Mitjans F et al, 2000 & Kumar C et al, 2001).

Chapter 4

The effects of the Extracellular Matrix on Colorectal cancer proliferation and apoptosis

Section 4.1.1

Effect of the Extracellular Matrix on CRC Proliferation and Apoptosis

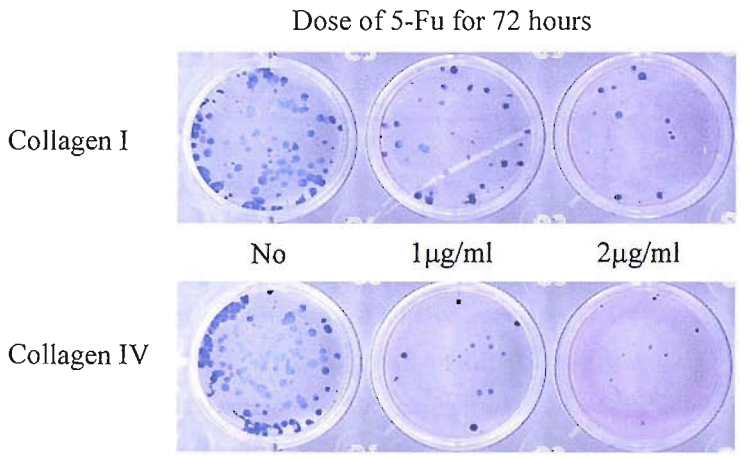
The in vivo studies demonstrated evidence of a pronounced DR within CRC liver metastases. In this section the effect of different matrix components on CRC survival, proliferation and apoptosis were studied. It has been previously established that normal and malignant cell growth may be influenced by changes in the composition of the ECM. The changes accompanying a DR have been suggested to offer a growth and survival advantage to several different cancers as discussed in section 1.7. These studies therefore illustrate how the change in matrix composition from a collagen IV rich ECM found in normal liver to a fibrillar collagen rich Desmoplastic matrix influenced CRC growth.

Results

Clonogenic Assay

Clonogenic assays were performed as described in section 2.8. These studies clearly demonstrate that the Colorectal Cancer cell Lines, HT-29, KM12c and KM12SM gain a significant survival advantage when grown on collagen I relative to collagen IV. The clonogenic index for each cell line grown on TCP was for; HT-29 (0.433); KM12c (0.50) and KM12SM (0.52). To reduce the clonogenic index on TCP by approximately 50%, for the HT-29 cells required 1μg/ml of 5-Fu for 56 hours and for the KM12c and KM12SM cell lines respectively required 0.125μg/ml and 0.25μg/ml of 5-Fu for 48 hours. Figure 22 demonstrates a representative clonogenic assay for HT-29 cells grown on collagens I & IV.

Figure 22: An example of a clonogenic assay, using HT-29 cells.



To allow comparisons between each matrix and groups of experiments, the number of colonies that grew on each matrix were expressed relative to the control plastic (TCP) for each experiment. These data are summarised in table 3a, along with the dose of 5-Fu used to reduce the clonogenic index for each cell line by approximately 50% of that seen on TCP when CRC were grown without the addition of chemotherapy. The results are expressed as the mean of the percentage number of colonies relative to TCP (100%) at each concentration of 5-Fu ±95% Confidence Intervals (95% CI). A paired t-test was applied to this data and the *p*-values are shown in table 3b.

Table 3a: Clonogenic assay

Cell Line	Matrix	Dose of 5-Fu and duration	Mean± 95% CI
HT-29	Collagen I	None	99.5±6.4
		1 μg/ml for 56 hours	158.6±13.5
	Collagen IV	None	101.8±14
		1 μg/ml for 56 hours	73.3±13.4
KM12c	Collagen I	None	108±5.8
		0.125 μg/ml for 48 hours	150.5±15.5
	Collagen IV	None	97.3±8.1
		0.125 μg/ml for 56 hours	91.4±10.3
KM12SM	Collagen I	None	109±7.2
		0.25 μg/ml for 56 hours	134.2±6.85
	Collagen IV	None	96.6±2.4
		0.25 μg/ml for 56 hours	83.8±12.6

The mean percentage number of colonies formed relative to control TCP (100%) at each different concentration of 5-Fu, for HT-29, KM12c & KM12SM cells grown on different matrices. The dose of 5-Fu given reduced the clonogenic index on TCP for each cell line by 50%, compared to no treatment on TCP. Data representative of at least 3 independent experiments for each cell line.

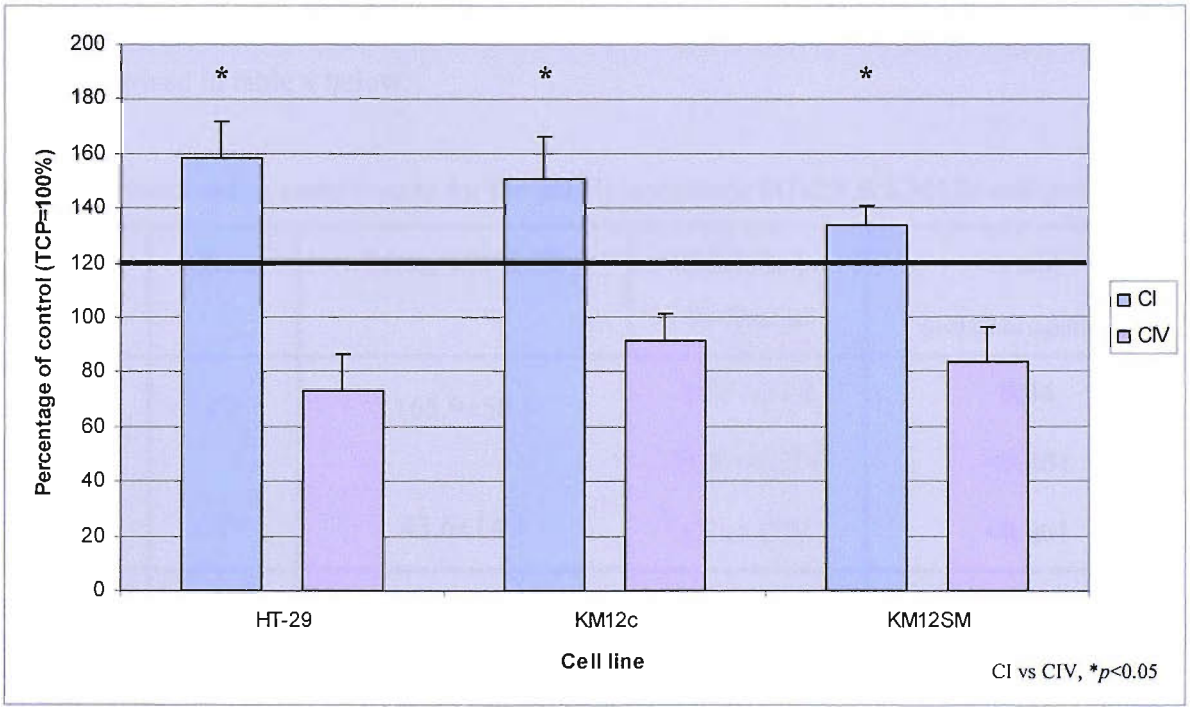
Table 3b: Statistical analysis performed on results from Table 3a.

Cell Line	5-Fu concentration and duration	Matrices	t-test (p<0.05=significant)
HT-29	None	TCP vs CI	0.88
		TCP vs CIV	0.81
		CI vs CIV	0.775
	1 μg/ml for 56 hours	TCP vs CI	**0.013**
		TCP vs CIV	**0.023**
		CI vs CIV	**0.023**
KM12c	None	TCP vs CI	0.109
		TCP vs CIV	0.58
		CI vs CIV	0.21
	0.125 μg/ml for 48 hours	TCP vs CI	**0.023**
		TCP vs CIV	0.24
		CI vs CIV	**0.021**
KM12SM	None	TCP vs CI	0.13
		TCP vs CIV	0.11
		CI vs CIV	0.082
	0.25 μg/ml for 48 hours	TCP vs CI	**0.010**
		TCP vs CIV	0.124
		CI vs CIV	**0.004**

There were no differences in cell survival identified on any matrix for any cell line without the addition of chemotherapy. However, as can be clearly seen, collagen I relative to collagen IV offers HT-29, KM12c and KM12SM CRC a significant survival advantage, when the cells were exposed to chemotherapy. This was illustrated for HT-29 cells grown on collagen I (results expressed as mean % number of colonies relative to TCP ±95% CI) and exposed to 1μg/ml of 5-Fu for 56 hours; 158.67 ±13.55 compared to collagen IV; 73.33 ±13.4, $p<0.05$, n=3.

Interestingly for the highly metastatic CRC line (KM12SM), the survival benefits of growing on collagen I compared to collagen IV although still significant, was reduced in magnitude in comparison to poorly-metastatic CRC. In figure 23, the matrices effects on cell survival are illustrated for all the CRC lines used when grown on collagens I and IV.

Figure 23: Clonogenic assays results for the CRC cell lines HT-29, KM12c and KM12SM



CRC gain a significant survival advantage when grown on collagen I compared to collagen IV and exposed to 5-Fu. Results expressed in relation to TCP (100%) as means and 95% Confidence Intervals. Data representative of at least 3 independent experiments for each cell line.

Section 4.1.2

Proliferation assays

Proliferation assays were performed as described in section 2.9. For all the CRC cell lines studied, collagen I relative to collagen IV was always significantly growth promoting. Collagen IV was significantly inhibitory to growth relative to TCP, with the exception of the highly metastatic CRC cell lines, where this difference was not observed. Shown below are the proliferation assay results for the HT-29 and KM12c, poorly-metastatic cell lines. The percentage of the control (TCP) was calculated for each cell line on each matrix from the individual experiments. The background CPM readings attributable to collagens with no cells growing on them was very small (<0.1%) of the lowest recorded values. So no deduction was made for background tritium levels in the calculations.

As can be seen for each experiment with the HT-29 and KM12c cell lines the most striking pattern identified was that, collagen I was strongly growth promoting relative to collagen IV and that collagen IV was significantly inhibitory to growth compared to TCP. These results are summarised in table 4 below.

Table 4: Proliferation assay results for the poorly metastatic HT-29 & KM12c cell lines.

Cell Line	Matrix	Mean ±95% CI	Matrix to be compared	t-test (p<0.05 as significant)
HT-29 (n=7)	CI	165.9±50.3	TCP vs CI	**0.04**
			TCP vs CIV	**<0.001**
	CIV	43.6±14.5	CI vs CIV	**<0.001**
KM12c (n=5)	CI	176.2±66.6	TCP vs CI	**0.047**
			TCP vs CIV	**<0.002**
	CIV	64.6±12.9	CI vs CIV	**<0.019**

Poorly metastatic CRC proliferate at a higher rate when grown on collagen I compared to collagen IV. Results expressed with respect to control (TCP=100%) for each cell line on each matrix.

The rate of proliferation for the two metastatic cell lines KM12L4a and KM12SM on collagens, were similar to those previously identified with the poorly metastatic cell lines. Namely collagen I was always significantly growth promoting relative to TCP and collagen IV. However, the inhibitory effects on cell growth for collagen IV for these cell lines were reduced compared to the differences seen with the poorly-metastatic variants. Most notably the rate of proliferation for the highly metastatic cell line KM12SM on collagen IV, although being significantly less than that seen on collagen I, when compared to TCP the inhibitory effect on cellular proliferation for collagen IV were ablated. The proliferation assay results for the moderately metastatic KM12L4a and highly metastatic KM12SM cell are shown in table 5.

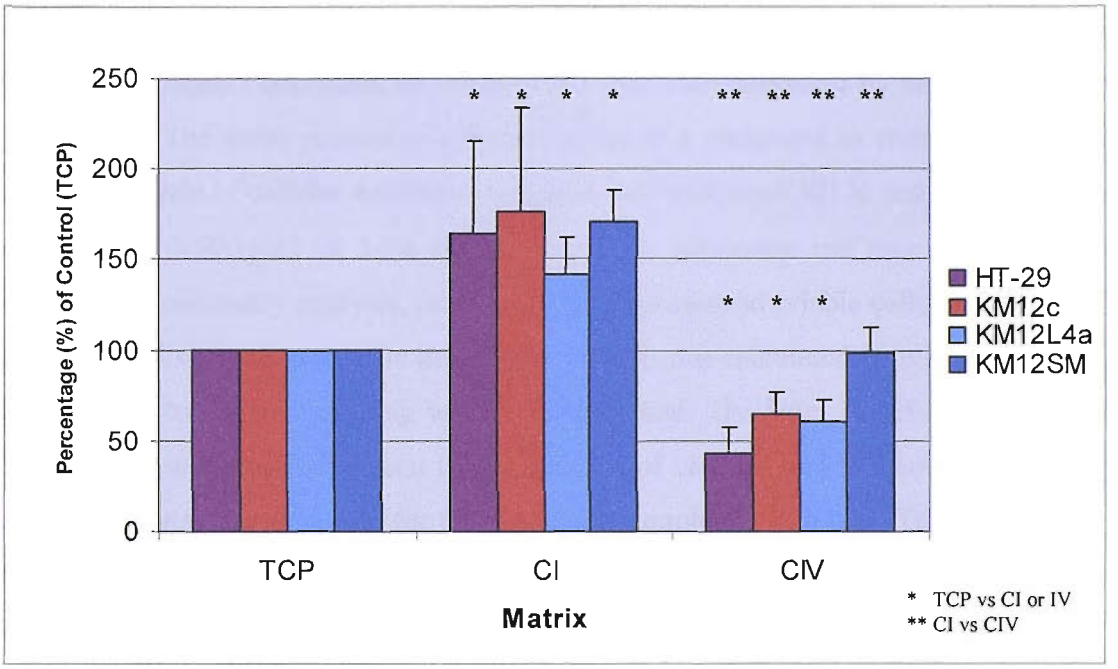
Table 5: Proliferation assay results for KM12L4a and KM12SM cell lines.

Cell line		Mean ±SD±95% CI	Matrix to be compared	t-test (p<**0.05** as significant)
KM12L4a (n=5)	CI	142.2±12.8	TCP vs CI	**0.001**
			TCP vs CIV	**<0.001**
	CIV	60.8±11.3	CI vs CIV	**0.002**
KM12SM (n=6)	CI	171.3±17.3	TCP vs CI	**<0.001**
			TCP vs CIV	0.83
	CIV	98.3±14.3	CI vs CIV	**0.002**

Collagen I was always significantly growth promoting for the metastatic CRC lines relative to TCP and collagen IV. These data clearly demonstrate that for the KM12SM cell line, no significant difference was now identified when comparing TCP and CIV. Illustrating that the inhibitory influence on cellular proliferation of collagen IV were reduced. Results expressed with respect to control (TCP=100%) for each cell line on each matrix.

The results for the effects of matrices on cellular proliferation are summarised in the figure below (Figure 24) using the poorly-metastatic HT-29 and KM12c cell lines, the moderately metastatic KM12L4a and highly metastatic KM12SM cell lines.

Figure 24: The effect of the different matrix components on cellular proliferation for metastatic vs poorly-metastatic cell lines



The striking features are that collagen I was always significantly growth promoting for CRC growth and as CRC adopt a more aggressive metastatic phenotype the inhibitory effects on cellular proliferation of collagen IV are reduced. Results expressed as means ± 95% Confidence intervals (* = $p<0.05$). Data representative of at least 5 independent experiments for each cell line.

Section 4.1.3

The effects of the matrix on the rate of cellular apoptosis

Western blotting for the cleaved and uncleaved forms of Poly-ADP Polymerase (PARP) were undertaken to assess the role of the matrix, in protecting colorectal cancer cells from apoptosis induced by chemotherapy as described in section 2.10.4. Using the HT-29 cell line and doses of chemotherapy from 25 – 40μg/ml of 5-Fu for 56 hours, there was clear evidence of a chemoprotective effect in terms of reduced rates of cellular apoptosis when cells were grown on collagen I compared to collagen IV. This was confirmed by densitometric analysis of the blots. The same protective effect of collagen I compared to collagen IV in terms of reducing the rate of cellular apoptosis, was also seen with the KM12c and KM12SM cell lines exposed to 10-50μg/ml of 5-Fu for 48 hours. To determine the rate of PARP cleavage, following densitometry analysis, the percentage of uncleaved (viable cells) and cleaved PARP (apoptotic cells) compared to the total PARP density was calculated for each experiment. A β-actin control to compare loading was also undertaken. The latter is not strictly necessary as comparisons were made between the percentage of cleaved and uncleaved PARP fractions and the total PARP expression for that particular sample on each blot. The ratio of uncleaved or cleaved PARP fractions for each cell line comparing collagens I and IV was then determined for each blot.

The percentage of uncleaved and cleaved PARP for each experiment are summarised in table 6 and the ratio of uncleaved or cleaved PARP fragments for CRC grown on collagen I compared to collagen IV are demonstrated in table 7, including statistical analysis. In figure 25 the ratio of uncleaved (a) and cleaved (b) PARP, respectively are illustrated for CRC cell lines grown on collagen I compared to collagen IV.

Table 6: The percentage of uncleaved and cleaved PARP for each experiment for each cell line are shown when grown on collagen I compared to collagen IV.

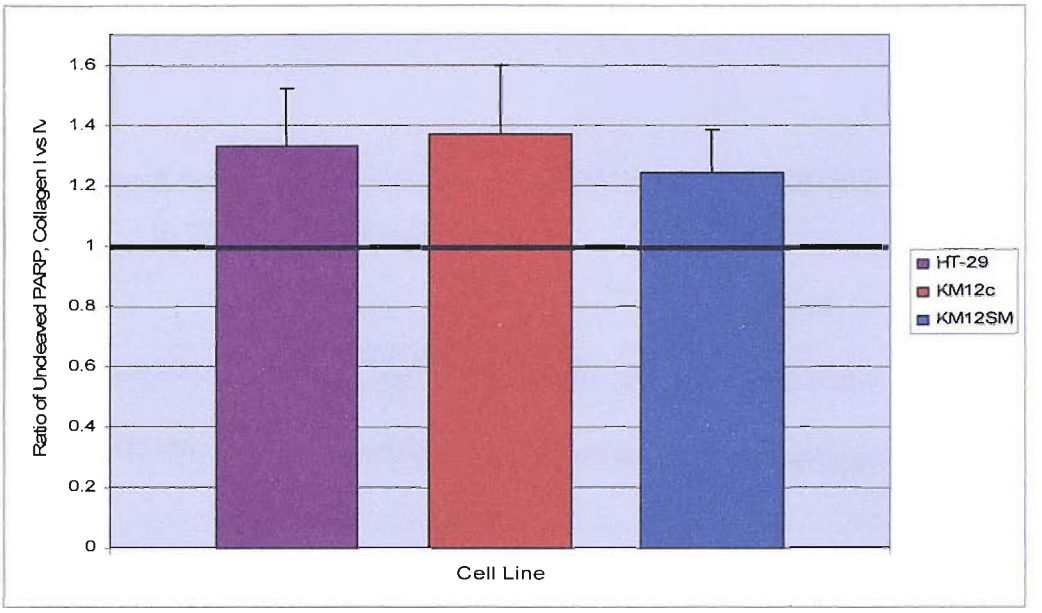
Cell Line	Uncleaved PARP		Cleaved PARP	
	CI	CIV	CI	CIV
HT-29 (n=5)	52	40.5	48	59.5
	28	22.5	72	77.5
	37	33.5	63	66.5
	66	39	34	61
	56	42	44	58
KM12c (n=5)	52	38.5	48	61.5
	50.8	42.2	49.2	57.8
	37	33	63	67
	46.3	33	53.7	67
	34	29	66	81
KM12SM (n=3)	45.5	36.6	54.5	63.4
	19	14	81	86
	44.8	40.3	55.2	59.7

Table 7: The ratio of uncleaved or cleaved PARP for HT-29, KM12c and KM12SM cells grown on collagen I compared to collagen IV.

PARP Ratio	Cell Line		
	HT-29	KM12c	KM12SM
Uncleaved CI vs CIV	1.33±0.195	1.37±0.23	1.24±0.14
Cleaved CI vs CIV	0.8±0.14	0.84±0.06	0.91±0.05
p-value	**0.041**	**0.006**	**0.048**

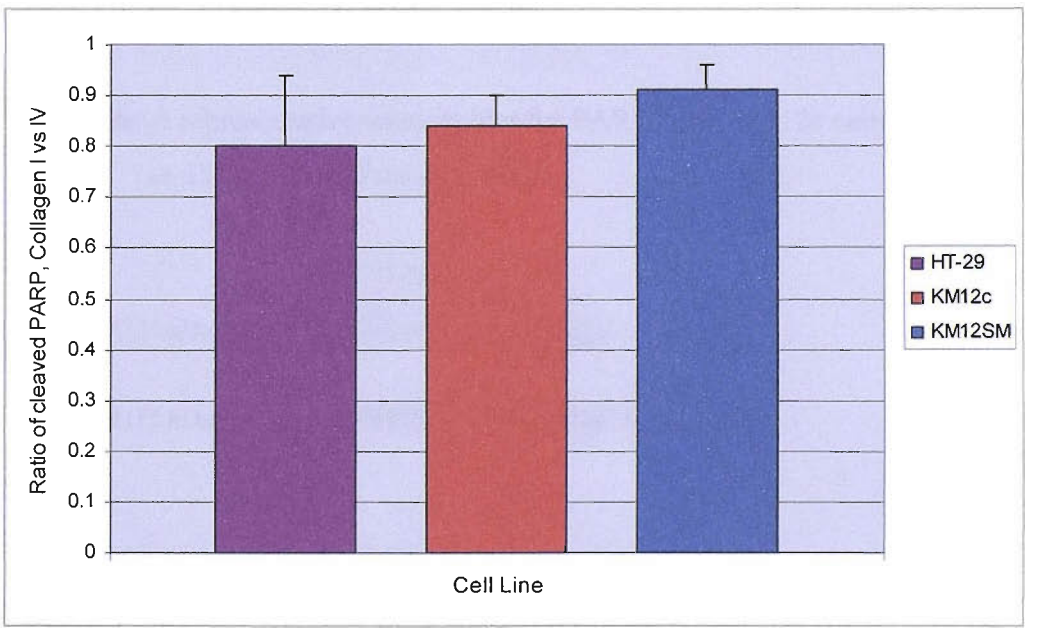
CRC grown on collagen I compared to collagen IV had increased chemoresistance. Results expressed as means ± 95% CI. Data representative of at least 3 independent experiments for each cell line.

Figure 25a: The ratio of uncleaved PARP for CRC cells grown on collagen I compared to collagen IV.



CRC grown on collagen I compared to collagen IV had increased chemoresistance. Means ± 95% Confidence intervals. A ratio of 1 equals no difference, representative of at least 3 independent experiments for each cell line.

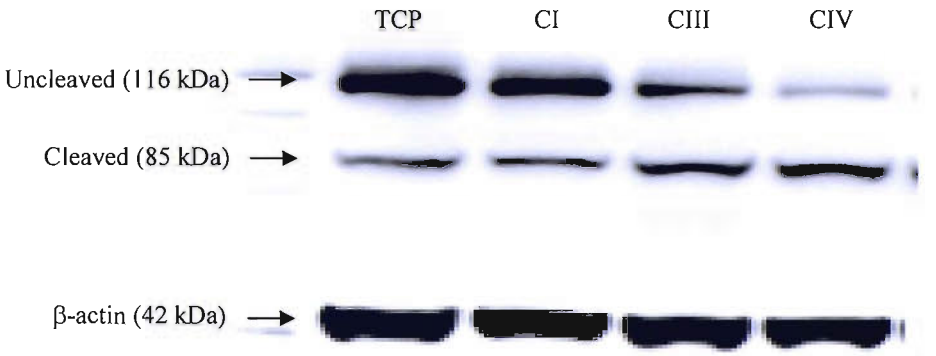
Figure 25b: The ratio of cleaved PARP for CRC cells grown on collagen I compared to collagen IV.



The reduction in cellular apoptosis obtained when grown on collagen I compared to collagen IV was greatest for poorly metastatic CRC. Means ± 95% Confidence intervals. A ratio of 1 equals no difference, data representative of at least 3 independent experiments for each cell line.

A representative western blot for PARP cleavage for each of the CRC cell lines used exposed to 5-Fu on different matrices are shown in Figure 26a (HT-29); b (KM12c) and c (KM12SM).

Figure 26a: A representative western blot for PARP, with HT-29 cells, exposed to 25μg/ml of 5-Fu for 56 hours.



These blots clearly show that for HT-29 and KM12c (poorly metastatic CRC) cells grown on collagen I compared to collagen IV there is a significantly reduced rate of cellular apoptosis (approximately 30%) in response to chemotherapy.

Figure 26b: A representative western blot for PARP, with KM12c cells, exposed to 15μg/ml of 5-Fu for 48 hours.

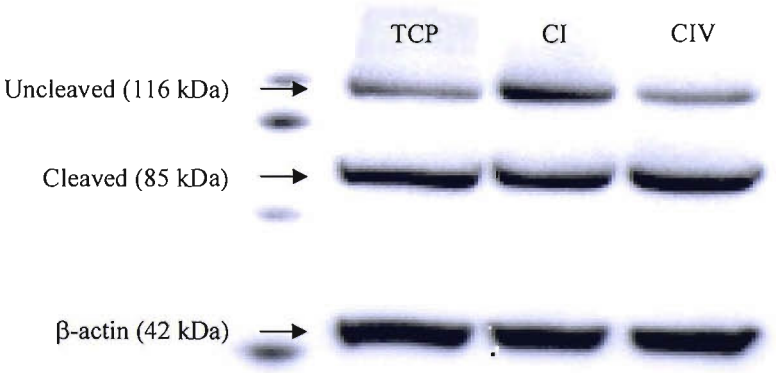
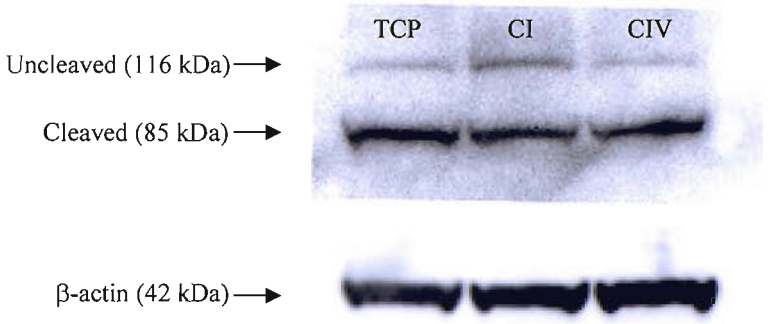


Figure 26c: A representative western blot for PARP, with KM12SM cells, exposed to 30μg/ml of 5-Fu for 48 hours.



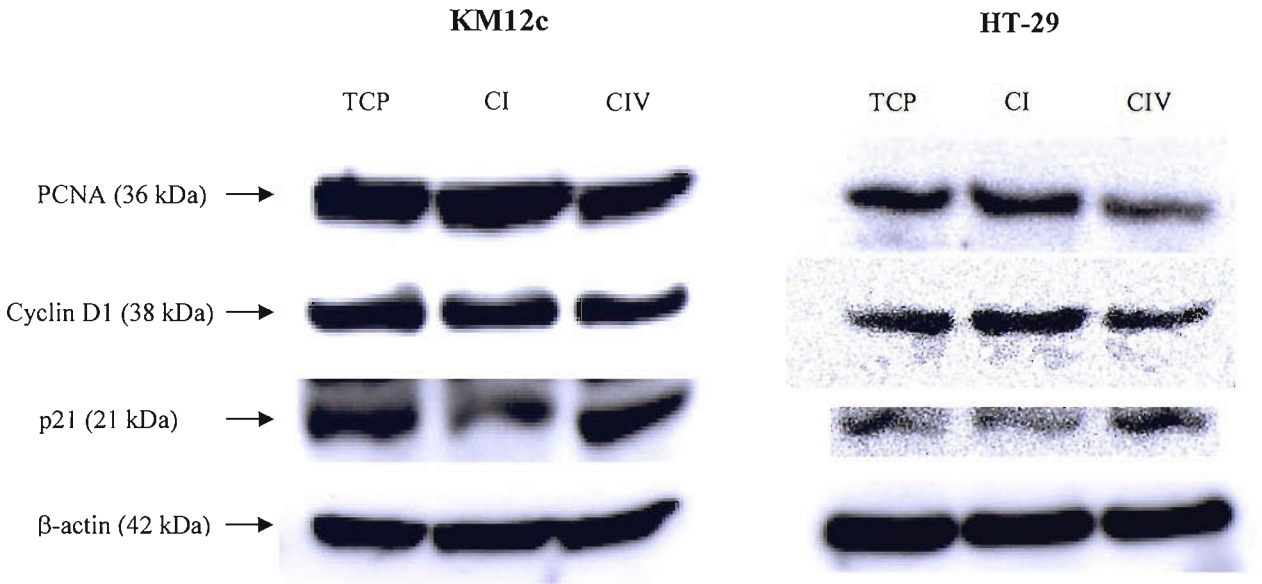
As this figure demonstrates, when the highly metastatic KM12SM cell line was grown on collagen I compared to collagen IV, there was a significantly reduced rate of cellular apoptosis in response to chemotherapy, although not to the same extent as that seen with the poorly metastatic CRC.

Section 4.1.4

Cell cycle regulators

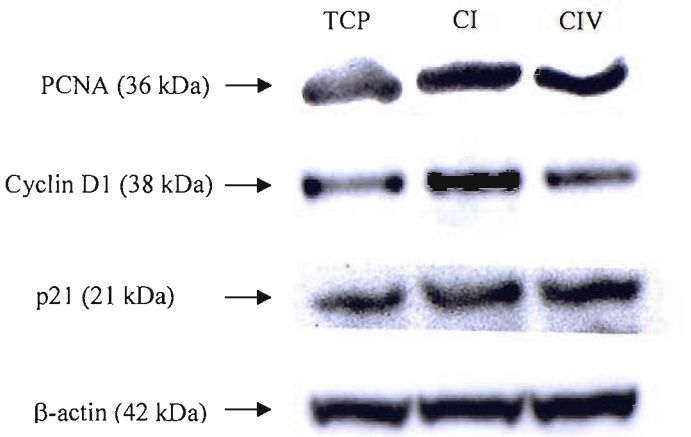
To determine the intracellular changes that accompanied the growth promoting effects of collagen I compared to collagen IV and TCP, western blotting for the cell cycle regulators PCNA, Cyclin D1, p21 and p27 were undertaken as described in section 2.10.5. For all the cell lines used, both PCNA and cyclin D1 were more highly expressed by CRC grown on collagen I compared to collagen IV. A reciprocal down-regulation in p21 was also identified for the poorly metastatic HT-29 and KM12c cell lines. Representative examples of blots for PCNA, Cyclin D1 and p21 are shown below.

Figure 27a: Cell cycle regulator expression for the KM12c and HT-29 cell lines.



This figure clearly demonstrates that when KM12c and HT-29 CRC were grown on collagen I compared to collagen IV, the expression of the pro-proliferative cell cycle regulatory proteins PCNA and Cyclin D1 were up-regulated. In contrast, expression of the growth inhibitory p21 protein was reciprocally down-regulated. These data appear to reinforce the proliferation assay experiments results, that KM12c and HT-29 CRC grown on collagen I proliferate at a faster rate and suggests the intracellular changes that may accompany this. This blot is representative of at least 2 independent experiments for each cell line.

Figure 27b: Cell cycle regulator expression for the KM12SM cell line



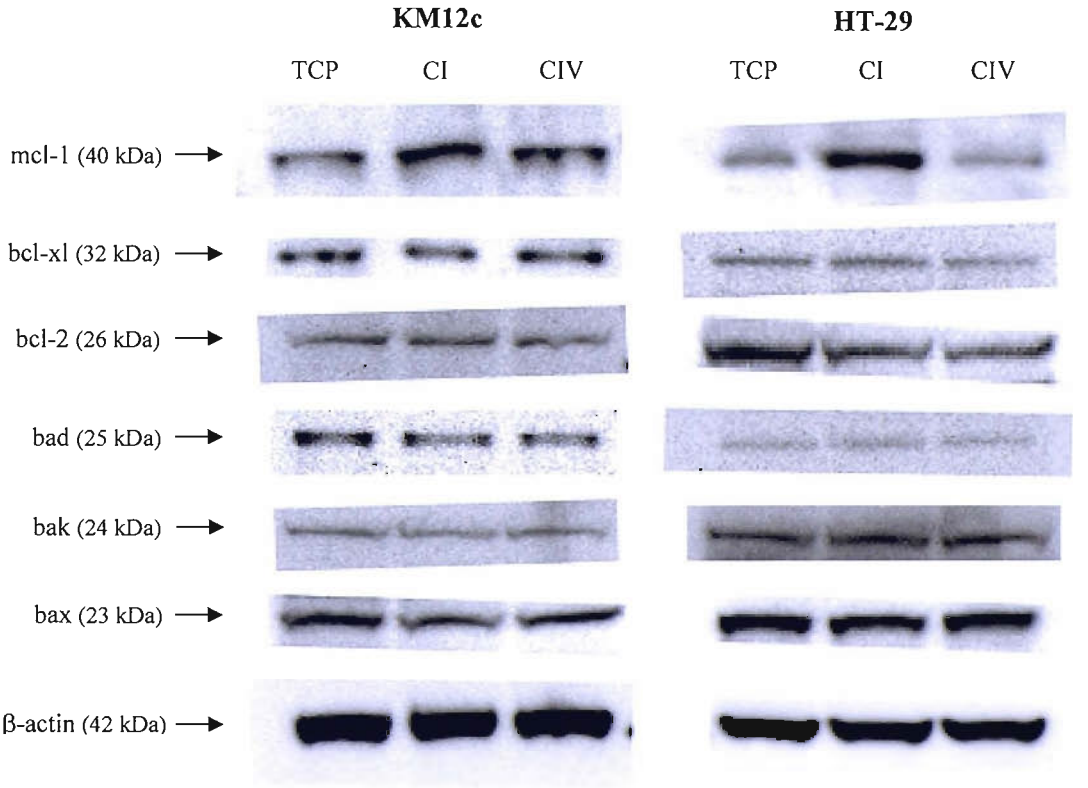
This figure clearly demonstrates that when the highly metastatic KM12SM CRC were grown on collagen I compared to collagen IV, the expression of the pro-proliferative cell cycle regulatory proteins PCNA and Cyclin D1 were up-regulated. In contrast, the matrix had no influence on the expression of the growth inhibitory p21 protein. These data appear to reinforce the proliferation assay experiments results, that KM12SM CRC grown on collagen I proliferate at a faster rate and suggests the intracellular changes that may accompany this. This blot is representative of at least 2 independent experiments.

Section 4.1.5

The effect of the ECM on the regulators of Apoptosis

The influence of the matrix in regulating cellular apoptosis was determined by PARP cleavage as previously shown. However, by western blotting we aimed to determine the possible intracellular mechanisms underlying these findings as described in section 2.10.5. Interestingly, no differences were identified in the expression of bax, bad, bak, bcl-2 and bcl-xl for any of the cell lines used. However, in the poorly metastatic KM12c and HT-29 cell lines, we were able to show that the mcl-1 anti-apoptotic protein was more highly expressed in these cell lines when grown on collagen I compared to collagen IV.

Figure 28: Anti-apoptotic protein expression in the KM12c and HT-29 cell lines



These data are representative of at least 2 independent studies for each cell line. The most striking feature was that the anti-apoptotic protein mcl-1 was upregulated in these CRC when they were exposed to chemotherapy. This blot is representative of at least 2 independent experiments for each cell line.

Section 4.1.6

Discussion – The influence of the extracellular matrix on CRC growth and apoptosis

These studies have demonstrated that a major component of the desmoplastic matrix, namely collagen I relative to collagen IV, offer the CRC cell lines a significant survival and growth advantage and confer a degree of resistance to chemotherapy. The survival advantage the CRC gained was greatest for the poorly metastatic CRC. Interestingly, for all the cell lines studied using a standard proliferation assay, collagen I relative to collagen IV was always significantly growth promoting. The inhibitory effects of collagen IV on cellular growth varied according to the metastatic phenotype of the CRC cells. As CRC adopted a more aggressive metastatic phenotype, the differences identified between collagens I and IV, although still significant, were reduced in magnitude. CRC grown on collagen I compared to collagen IV had reduced rates of cellular apoptosis, as measured by PARP cleavage when CRC cells were exposed to chemotherapy. Again these protective effects of the desmoplastic matrix component collagen I was greatest for the poorly metastatic CRC lines. These results would be in keeping with studies in lung (Sethi T et al, 1999), breast (Meng L et al, 2001) and pancreatic cancer (Armstrong T et al, 2004; Buchholz M et al, 2003), showing growth benefits and reduced rates of cellular apoptosis for cancer cells grown on fibrillar collagens. Perhaps the most intriguing finding was the influence the malignant phenotype of the CRC, had on the magnitude of the benefits gained, with poorly metastatic CRC gaining the greatest benefits.

A possible explanation for this was the fact that the collagen IV used contained predominantly the α-1 & α-2 chains and possibly the α-3 chain as well. The α-2 and 3 chains are known to be growth regulatory as has been previously described (section 1.5.3). In highly metastatic cell lines the inhibitory effects of collagen IV on cellular growth are reduced, possibly due to differences in integrin expression by the metastatic CRC lines. This occurs as a consequence of additional genetic mutations, as CRC adopt a more aggressive phenotype altering integrin expression and adhesiveness and possible integrin expression may also be altered in response to the composition of the matrix (Schramm K et al, 2000). Therefore the highly metastatic CRC express a wide variety of integrins, which allows them to grow at a higher rate on type

IV collagen. This will be further expanded upon in the sections studying the effects of integrin neutralising antibodies and the role of matrix turnover on CRC growth.

Previous studies involving CRC and different matrix components produced highly variable results in terms of the effect of matrix components on CRC growth and apoptosis (Zvibel I et al, 1998; Kouniavsky G et al, 2002). The most recent of these studies by Kourniavsky et al, suggested that stromal derived ECM components may offer CRC benefits in reducing CRC apoptosis in response to chemotherapy. Why such differences exist is a matter of speculation; however the experimental design was significantly different from our studies. In particular, the collagens were prepared differently and used at different concentrations and it was not documented if the same volumes of different matrices were used, which would effect the thickness of collagen films formed. The process of dry heating the matrices to 37°C may potentially damage and cause contraction of the collagen films. There was also no evidence that the matrices were washed to remove residual acetic acid or blocked with BSA to inhibit non-specific binding and some experiments were undertaken using 10% FCS enriched media rather than serum free media. Our studies demonstrated consistent and reproducible results for the effects of collagens on CRC survival, growth and apoptosis. Further evidence supporting our results originate from a study by Brabletz T et al, 2004, in which they demonstrated that collagen I, through β1 signalling and down-regulation of Cdx2, is associated with a loss of CRC differentiation and indicates a role for the tumour microenvironment in malignant tumour progression.

The intracellular changes accompanying the changes in cell growth and apoptosis previously identified were not unexpected. As the CRC were proliferating at a higher rate it might be expected that there would be increases in cyclin D1 and PCNA. Perhaps the most interesting finding was that p21 expression was down-regulated in poorly metastatic CRC lines. Whereas in the highly metatstatic CRC KM12SM, no apparent difference was identified. Although these studies offer a limited view of the intracellular workings of the cell, the p21 findings may illustrate an important feature of metastatic behaviour. For p21 to be expressed and thereby have a growth inhibitory effect requires functioning wild type p53 (El-Deiry W et al,

1994). However, as described earlier (Section 1.2) as CRC adopt a more aggressive metastatic phenotype, genetic mutations in p53 are increasingly common and genetic mutations accumulate. Therefore p21 may play a less important growth regulatory role in the highly metastatic CRC, as this growth inhibitory pathway no longer functions. However, it should not be overlooked that a limitation of all these western blotting studies are that they determine protein expression at a set time point. Unlike PARP cleavage where the cleaved PARP accumulates intracellulary, differences in the expression of the cell cycle regulators may be missed as this is a dynamic process. With the phosphorylation level of many of the proteins involved in cell cycle regulation playing a crucial role, with the absolute protein levels perhaps remaining relatively constant or differences not being detectable by the techniques used in this study. Although there was no identifiable difference in total protein expression, this does not mean that the matrix has no influence on these pathways.

These arguments are equally applicable to the regulators of apoptosis. However, the mcl-1 upregulation was a consistent finding especially for the poorly metastatic CRC. This would be in keeping with studies that have suggested that mcl-1 up-regulation plays an important role in reducing the rate of cellular apoptosis in leukemias (Michels J et al, 2004) and pancreatic cancer (Armstrong T et al, 2004). As our knowledge of these regulators of the cell cycle and apoptosis improve and the methods to detect them in a more dynamic fashion are created, this will be an interesting area for further study.

Section 4.2.1

The effects of collagen III on CRC growth and survival

Preliminary studies using the HT-29 cell line suggested that collagen III effects on cell survival were somewhere between that of TCP and CIV following the addition of chemotherapy. However, the only significant differences identified in terms of cell survival were identified between collagens I and III after the addition of 1μg/ml of 5-Fu for 56 hours (p=0.003). These results are summarised in table 8.

Table 8: Clonogenic Assay

HT-29	Dose of 5-Fu for 56 hours (μg/ml)	
	0	1
Collagen I (CI)	99.5 ± 6.4	158.66 ± 13.6
Collagen III (CIII)	98 ± 14	96.66 ± 6.6
Collagen IV (CIV)	101.8 ± 14.1	73.3 ± 13.4

The mean percentage number of colonies formed relative to control TCP, for HT-29 cells grown on different matrices and exposed to 5-Fu. Results expressed as means ± 95% CI, data representative of at least 3 independent experiments.

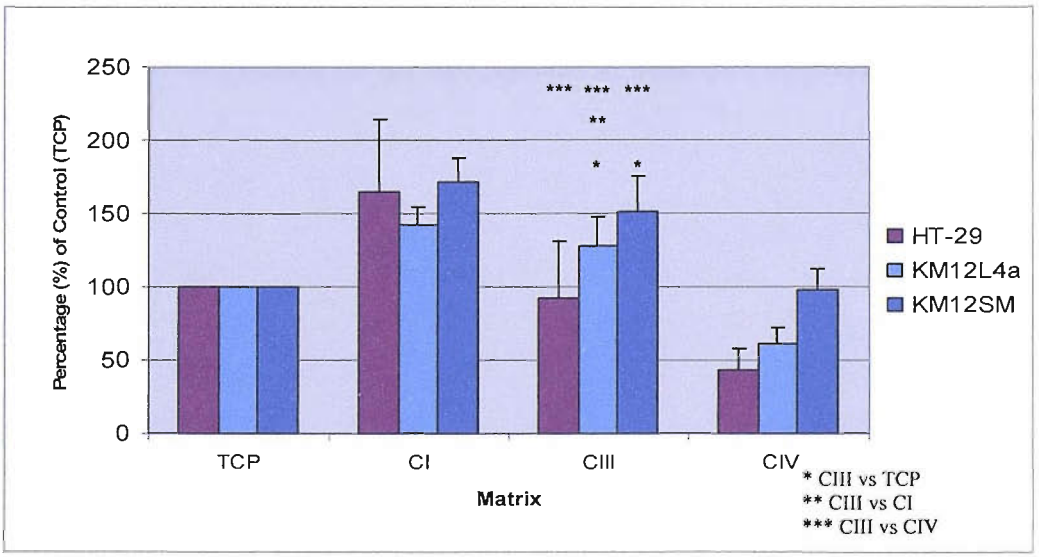
When the effects of collagen III on CRC proliferation were studied a consistent proliferative advantage was demonstrated as CRC adopted a more aggressive metastatic phenotype compared to TCP. For the poorly metastatic HT-29 cell line collagen III's effect on cellular growth was somewhere between collagen IV and TCP, whereas for the moderately metastatic KM12L4a and highly metastatic KM12SM cell lines collagen III's effects on CRC growth approached that seen on collagen I. These data are shown in table 9 and illustrated in figure 29.

Table 9: Proliferation assay results for HT-29, KM12L4a and KM12SM on different matrices

Cell Line	Matrix	Mean ±SD±95% CI	Comparison	t-test
HT-29 (n=7)	Collagen I (CI)	165.9±50.3	TCP vs CIII	0.70
	Collagen III (CIII)	92.1±39.1	CI vs CIII	0.07
	Collagen IV (CIV)	43.6±14.5	CIII vs CIV	**0.03**
KM12L4a (n=5)	Collagen I (CI)	142.2±12.8	TCP vs CIII	**0.049**
	Collagen III (CIII)	127.6±19.8	CI vs CIII	**0.049**
	Collagen IV (CIV)	60.8±11.3	CIII vs CIV	**0.002**
KM12SM (n=6)	Collagen I (CI)	171.3±17.3	TCP vs CIII	**0.008**
	Collagen III (CIII)	151.2±24.9	CI vs CIII	0.33
	Collagen IV (CIV)	98.3±14.3	CIII vs CIV	**0.008**

Results expressed with respect to control (TCP=100%) as means ± 95% CI to illustrate the effect of collagen III on CRC proliferation, a $p<0.05$ was taken as being of significance.

Figure 29: The effect of the different matrix components on cellular proliferation for metastatic vs non-metastatic cell lines.



Collagen was significantly growth promoting for metastatic CRC. Results expressed as means ±95%Confidence Intervals, $p<0.05$*. Data representative of at least 5 independent experiments.

Section 4.2.2

Discussion

The effects on cell growth of collagen III in these limited studies have been shown to be somewhere between that seen for collagens I and IV, with the growth promoting effects of collagen III being most noticeable for the highly metastatic CRC cells lines, it could be postulated for the same reasons as proposed for collagen IV. Namely, that the highly metastatic CRC express a wide variety of integrins and therefore can grow successfully on many different matrix types. Due to the magnitude of the differences identified on cell growth between collagens I and IV, we initially chose to concentrate on the role of these two matrix components, to try and illustrate the effect on cell growth and survival of a desmoplastic reaction in the development of CRC liver metastases.

There have been far fewer studies on the role of type III collagen on cancer cell growth. Pancreatic cancer cells grown on collagen III had increased rates of cellular proliferation and adopted a migratory phenotype (Menke A et al, 2001). Studies on CRC and collagen III are very limited, but it has been shown that high collagen III levels are associated with advanced disease in primary CRC (Basso D et al, 2001[†]) and in vitro, metastatic CRC cells appear to increase fibroblast collagen III production (Basso D et al, 2001[‡]). Therefore the preliminary IHC and in vitro studies performed as part of this project may be an interesting area for further research and be a marker for the development of metastatic disease in CRC.

Chapter 5

Regulation of CRC adhesion and proliferation by Integrins

Section 5.1.1

Regulation of CRC adhesion and proliferation by integrins

As previously discussed in section 1.9, integrins play a key role in the development of many cancers. Integrins are cell surface receptors which upon ligating ligands expressed by the matrix can effect the survival and growth of normal and malignant cells. Up-regulation of different integrin types are associated with different phenotypic changes in cancers and these studies therefore aimed to determine how integrin expression changed between poorly and highly metastatic CRC and how different integrin sub-types effect CRC adhesion and growth.

Finally we aimed to identify if any correlation exists between our in vivo and in vitro studies with regards the role of integrins in CRC liver metastases development.

Results

Western Blotting for Different integrin isoforms

By western blotting, using the technique as described in section 2.11 it has been demonstrated that the expression of different integrin isoforms varies between poorly and highly metastatic CRC cell lines.

This technique demonstrated that there was an up-regulation of alpha-v (α_v) integrin expression by the highly metastatic (KM12SM) cell line compared to the poorly-metastatic (KM12c) cell line (figure 30a). The two bands seen represent two isoforms of alpha-v integrin (150 & 165 kDa) representing differentially glycosylated forms. Interestingly, the highest expression for alpha-v integrins was seen for KM12SM cells grown on collagens I and IV.

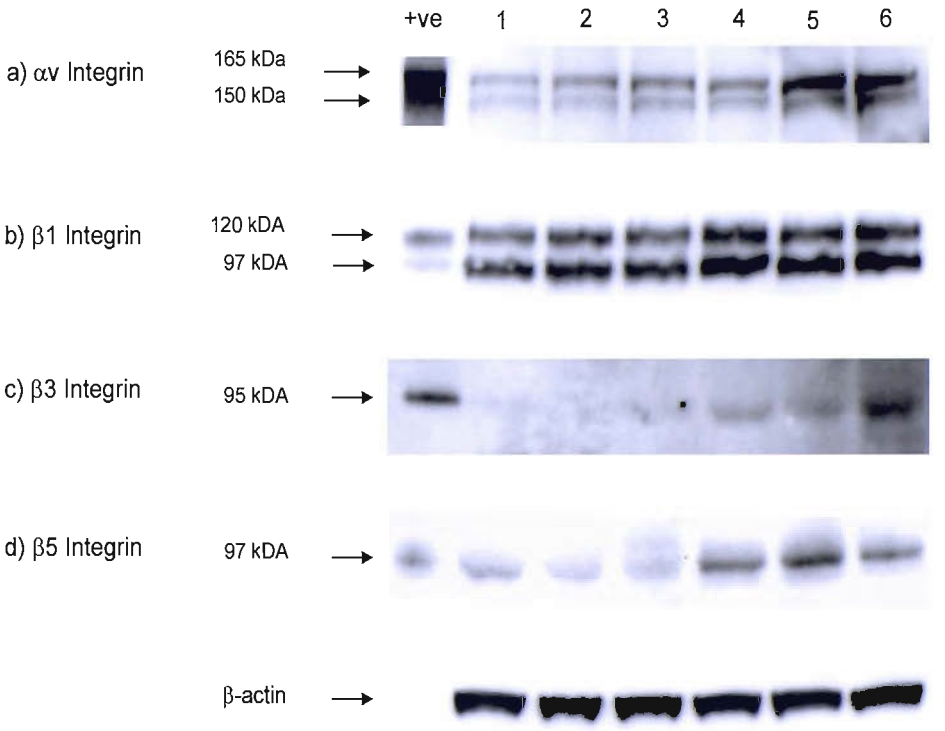
Two isoforms of beta-1 (β_1) integrin (120 and 97 kDa) were identified by western blotting, again representing differentially glycosylated forms, which were highly expressed by both poorly and highly metastatic cell lines (figure 30b). However, it appears that 97 kDa isoform of β_1 integrin, was perhaps more highly expressed by the highly metastatic (KM12SM) cell line.

There was very low expression of beta 3 (β_3) integrins (95 kDa) detected using this technique, particularly by the poorly-metastatic (KM12c) cell line (figure 30c). Nonetheless, it was demonstrated that β_3 integrins were more highly expressed by the highly metastatic CRC (KM12SM) compared to the KM12c cell line. However, 75μg of protein had to be loaded to obtain reproducible measurable levels of β_3 integrin expression even for the highly metastatic cell line. For further analysis of β_3 integrin expression flow cytometric analysis would need to be undertaken. Nevertheless, there seems to be a similar pattern of expression for β_3 integrins as for the α_v integrins, where the highest level of expression occurs on the highly metastatic cell line, particularly when the CRC were grown on collagen IV. Although, unlike αv integrin

expression there was no clear difference identified in terms of β_3 integrin expression when comparing TCP and collagen I.

Finally by western blotting it was shown that beta-5 (β_5) integrin (97 kDa) expression was up-regulated on the highly metastatic KM12SM cell line (figure 30d). Interestingly as with the αv integrins, the highest level of β_5 integrin expression was seen for cells grown on collagen I. All blots shown are representative of 3 independent experiments and a loading control was provided by β-actin (42kDa).

Figure 30: Representative western blots illustrating, how the level of integrin expression varies between the poorly metastatic (KM12c) & highly metastatic (KM12SM) CRC.



Results for the KM12c cell line grown on TCP (1), collagen I (2) and collagen IV (3) and for the KM12SM cell line grown on TCP (4), collagen I (5) and collagen IV (6) are shown. In all blots equal loading was determined by the level of β-actin (42 kDa) expression. For αv (a) and β3 (c) integrins (+ve control, human platelet lysates) and for β1 and β5 integrins (+ve control, HT-29 cell lysates). αv, β3 and β5 integrins are more highly expressed by metastatic CRC, especially when grown on collagens. These blots are representative of at least 3 independent experiments.

Section 5.1.2

Effect of β1 blocking antibodies on cell adhesion

Using the adhesion assay as described in section 2.11, it was shown that Beta-1 integrin neutralising antibodies (1-15μg/ml) significantly reduced the adhesion of all the cell lines used to collagen I and IV in a dose dependent manner with no effect on the adhesion of CRC cells to TCP.

Shown below in Table 10 are the results of the adhesion assays using HT-29, KM12c and KM12SM cell lines and 5 & 10μg/ml of β1 integrin neutralising antibody. The results are expressed relative to the IgG_1 isotype control (100%) and are expressed as mean ± 95% Confidence Intervals (95% C.I) along with t-test values ($p<0.05$ was taken as being of significance). Although not shown, there was no effect of the IgG_1 control antibody on cancer cell adhesion relative to omitted antibody.

For all cell lines, the β1 integrin neutralising antibody reduced the adhesion of the cancer cells to both collagen I and IV in a dose dependent manner, without any effect on the adhesion of the cells to TCP.

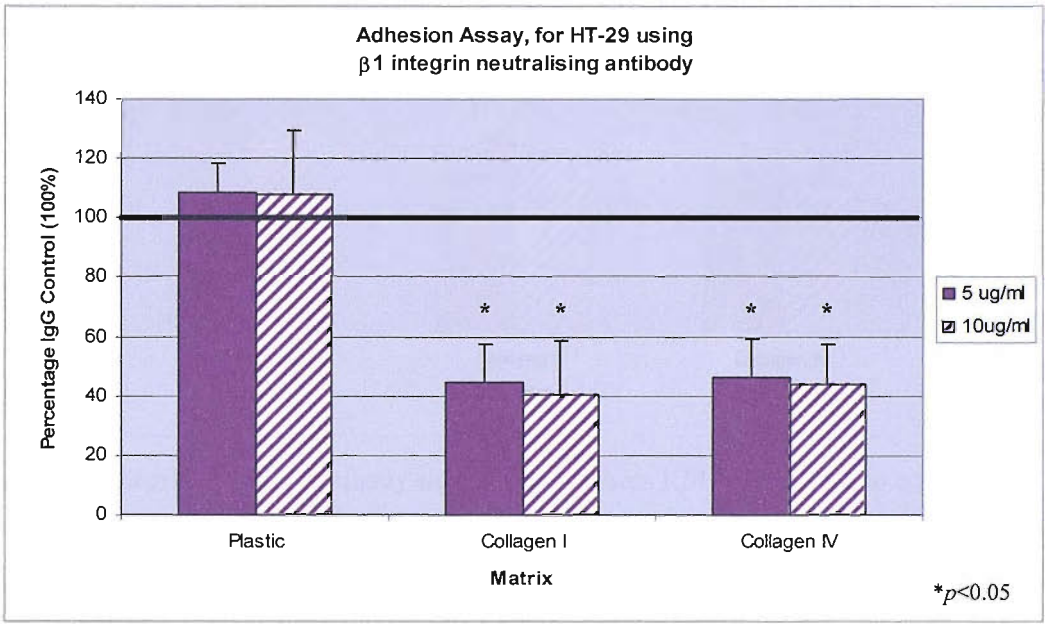
Table 10: Results comparing the effect of individual sub-cellular matrices on CRC adhesion using 5-10μg/ml of β1-integrin blocking antibody in comparison to IgG control.

Cell Line	Matrix	Concentration μg/ml	Mean % of isotype control ± 95% C.I	*p*-value
HT-29	TCP	5	108.5±9.6	0.175
	CI		44.25±13.2	**0.003**
	CIV		46.1±12.9	**0.004**
HT-29	TCP	10	107.5±22.2	0.547
	CI		40.5±17.97	**0.007**
	CIV		44±13.6	**0.004**
KM12c	TCP	5	101±11.5	0.873
	CI		60.75±12.7	**0.008**
	CIV		48.25±11.9	**0.003**
KM12c	TCP	10	97.25±12.3	0.685
	CI		53.88±18.3	**0.015**
	CIV		43.75±13.6	**0.004**
KM12SM	TCP	5	96.5±11	0.57
	CI		52.75±14	**0.007**
	CIV		48.75±27.9	**0.035**
KM12SM	TCP	10	100.75±14	0.92
	CI		47.25±2.2	**<0.001**
	CIV		42.5±20	**0.011**

As can be seen there is a dose dependent reduction in cellular adhesion to collagens for all the cell lines used. Data representative of 4 independent experiments, $p<0.05$ was taken of being of significance.

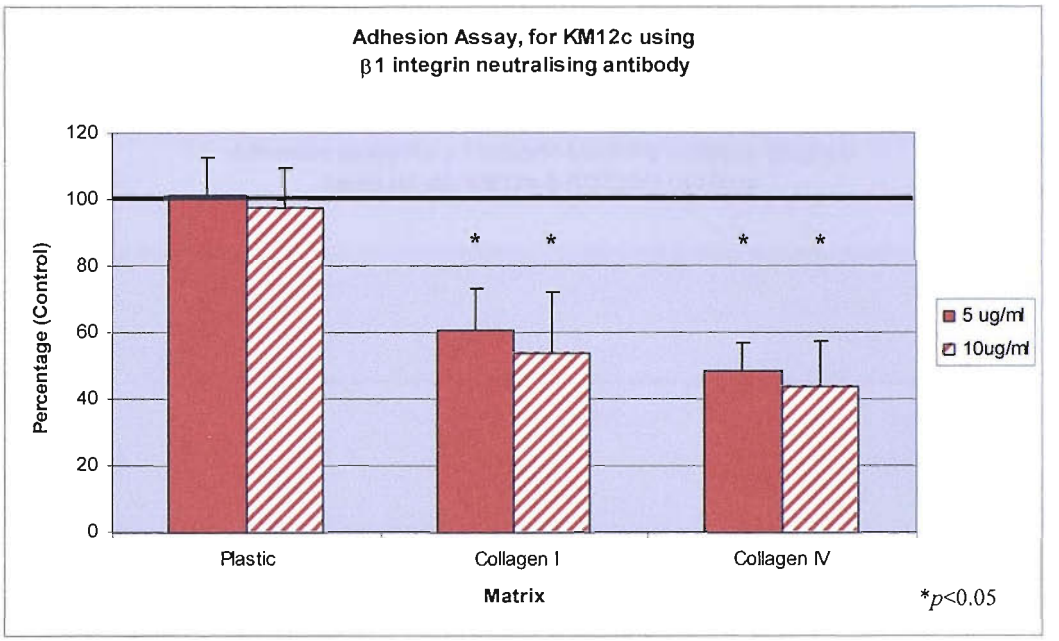
These results are illustrated in the figures below; Figure 31a - HT-29; b - KM12c; c-KM12SM and combined in Figure 31d. Data expressed as mean ± 95% Confidence Intervals (95% C.I), $p<0.05$=*.

Figure 31a: The effect of 5-10μg/ml of β1 integrin blocking antibody on cellular adhesion of HT-29 cells on specific sub-cellular matrices, relative to IgG isotype control (100%).



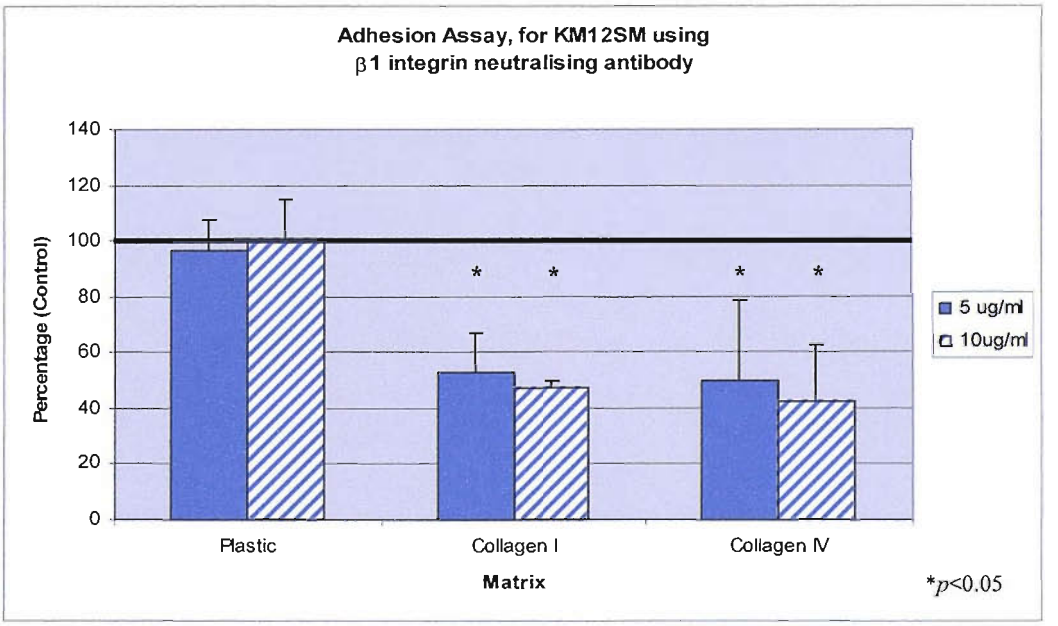
β1 integrin blocking antibody significantly reduces HT-29 adhesion to collagens

Figure 31b: The effect of 5-10μg/ml of β1 integrin blocking antibody on cellular adhesion of KM12c cells on specific sub-cellular matrices, relative to IgG isotype control (100%).



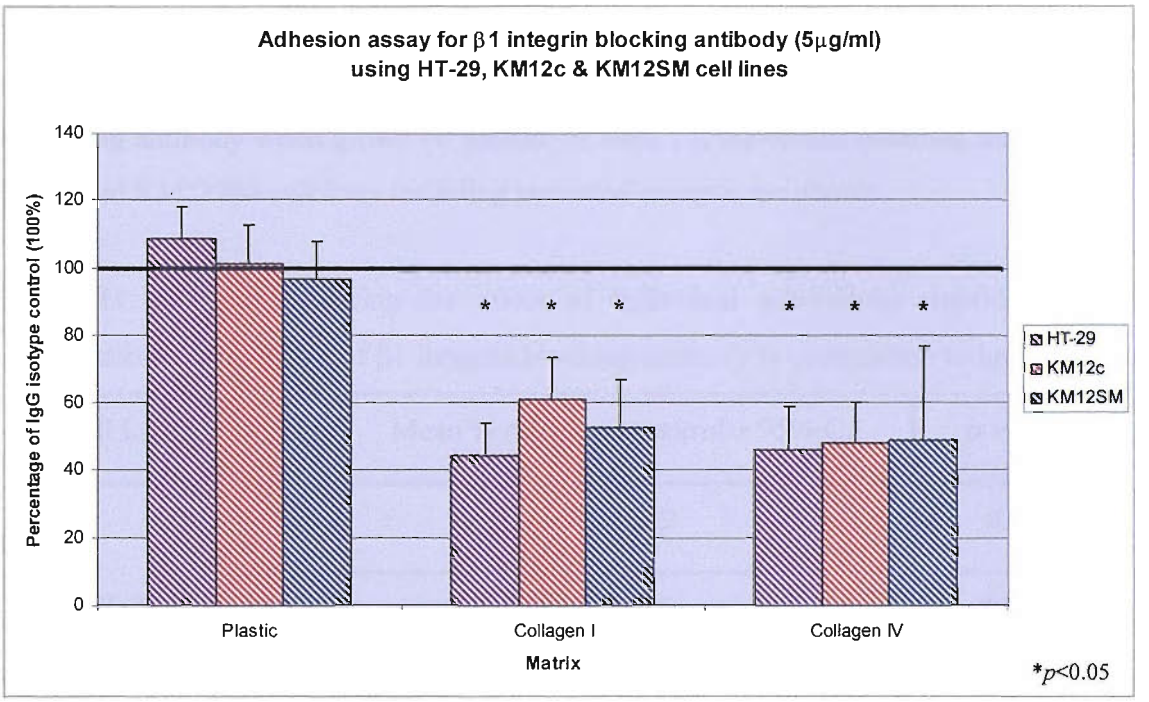
β1 integrin blocking antibody significantly reduces KM12c adhesion to collagens

Figure 31c: The effect of 5-10μg/ml of β1 integrin blocking antibody on cellular adhesion of KM12SM cells on specific sub-cellular matrices, relative to IgG isotype control (100%).



β1 integrin blocking antibody significantly reduces KM12SM adhesion to collagens

Figure 31d: A comparison of the effect on CRC cellular adhesion on different sub-cellular matrices using 5μg/ml of β1 integrin blocking antibody, relative to IgG isotype control (100%).



For all the CRC lines β1 integrin blocking antibodies significantly reduced cellular adhesion to a similar extent to collagens, with no affect on adhesion to TCP. Data representative of at least 4 independent experiments.

Section 5.1.3

The effect of β1 blocking antibodies on cellular proliferation

This was assessed as described in section 2.11. A concentration of 5μg/ml of the β1 integrin neutralising antibody, reduced the rate of cellular proliferation as determined by the ^{3}H thymidine proliferation assay on both collagen I and IV for all the CRC cell lines used. There was no significant effect on CRC proliferation for any cell line using the β1 integrin neutralising antibody when grown on plastic. In table 11, the results obtained for the HT-29, KM12c and KM12SM cell lines including statistical analysis are shown.

Table 11: Results comparing the effect of individual sub-cellular matrices on CRC proliferation using 5μg/ml of β1-integrin blocking antibody in comparison to IgG control.

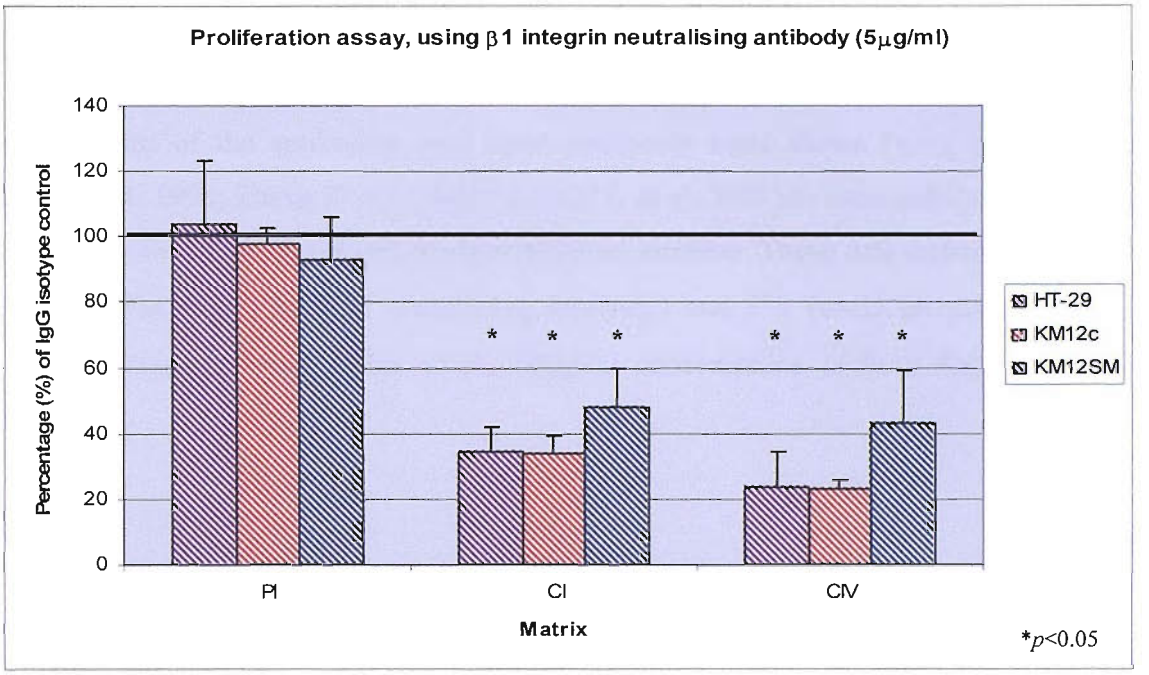
Cell Line	Matrix	Mean % of isotype control ± 95% C.I	*p*-value
HT-29 (n=3)	TCP	104±19	0.72
	CI	35±7.5	**0.003**
	CIV	24±11	**0.005**
KM12c (n=4)	TCP	98±4.9	0.47
	CI	34±5	**<0.001**
	CIV	23±2.3	**<0.001**
KM12SM (n=3)	TCP	93.3±13	0.41
	CI	48±11.7	**0.01**
	CIV	43±16	**0.02**

There was a significant reduction in cellular proliferation on collagens for all the CRC used, by blocking β1 integrins, with no effect on proliferation on TCP. Data representative of at least 3 independent experiments, $p<0.05$ was taken as being of significance.

These data clearly demonstrate that in the HT-29, KM12c and KM12SM cell lines, proliferation was significantly reduced in comparison to IgG control. The effect of β1 integrin blocking antibodies on cellular adhesion will have contributed significantly to the differences seen.

However, there are also likely to be effects on cellular growth in addition to the adhesion effects already noted mediated by β1 integrins, as ^{3}H thymidine incorporation was adjusted to take into account the number of cells remaining adherent in the monolayer, i.e. incorporation was calculated as cpm/ng of cellular DNA content. This was given further weight by additional experiments (data not shown), in which the cells were allowed to adhere prior to adding the blocking antibody and proliferation was still reduced compared to control. This suggests that β1 integrin ligation also has a growth regulatory role for the CRC cells studied; however, it is likely that regulation of CRC adhesion by β1 integrins plays a crucial role in mediating these growth regulatory effects. In figure 32 below, the effect on cellular proliferation of β1 integrin neutralising antibody is demonstrated.

Figure 32: The effect of 5μg/ml of β1 integrin neutralizing antibody on CRC proliferation on specific sub-cellular matrices, relative to IgG isotype control = 100%.



There was a significant reduction in cellular proliferation on collagens, with no effect on TCP for all the CRC used by blocking β1 integrins. Results expressed as mean ± 95% Confidence Intervals (95% C.I), data representative of at least 3 independent experiments, $p<0.05$*.

Of interest in comparing results between the poorly and highly metastatic cell lines, the reduction in the rate of cellular proliferation as determined by the proliferation assays with the addition of β1 blocking antibody, was of greater magnitude for the poorly metastatic cell lines (HT-29 & KM12c) than that seen for the highly metastatic cell line (KM12SM). Although it has to be emphasized that for all cell lines the β1 integrin neutralizing antibody significantly reduced the rate of cellular proliferation on all the matrices except plastic. It must also be noted that care must be taken when comparing between different cell lines, as the experiments were undertaken at different times, although using an identical protocol.

Section 5.1.4

The effects of αvβ3 and αvβ5 neutralising antibodies on cellular adhesion

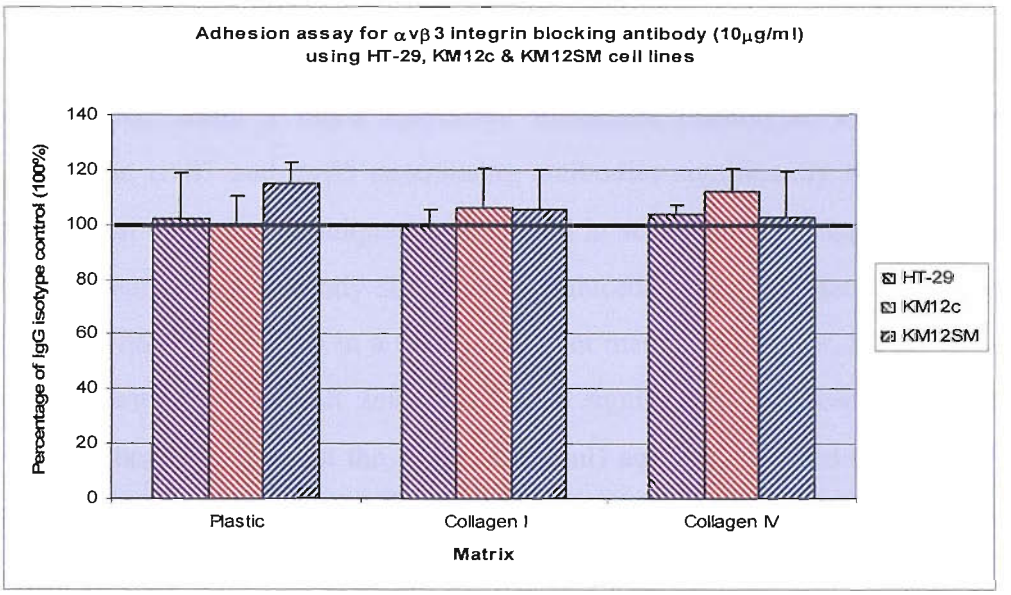
Using αvβ3 & αvβ5 neutralising antibodies at concentrations of 5 – 20μg/ml did not significantly reduce the rate of cellular adhesion for any of the CRC cell lines used. The concentrations of the antibodies used have previously been shown (www.chemicon.com; Sung V et al, 1998; Zheng D et al, 2000; Kumar C et al, 2001) to successfully block cellular adhesion for several different cell types to different matrices. These data summarized in table 12, figure 33a (αvβ3 integrin neutralising antibody) and 33b (αvβ5 integrin neutralising antibody) demonstrate the effect of the 10μg/ml concentration of both these neutralising antibodies.

Table 12: Results comparing the effect of individual sub-cellular matrices on CRC adhesion using 10μg/ml of αvβ3 & αvβ5-integrin blocking antibody in comparison to IgG control.

Cell Line	Matrix	Mean±Standard Deviation ±95%Confidence Intervals	*p*-value
HT-29 αvβ3	TCP	102.3±16.73	0.81
	CI	100±5.5	0.91
	CIV	103.5±3.5	0.18
HT-29 αvβ5	TCP	93.83±19.3	0.59
	CI	100.66±6.57	0.86
	CIV	110±20.24	0.43
KM12c αvβ3	TCP	99.83±10.8	0.98
	CI	106.2±14.4	0.49
	CIV	112±8.9	0.12
KM12c αvβ5	TCP	101.3±22.2	0.9
	CI	103.3±17.97	0.7
	CIV	96.2±13.6	0.73
KM12SM αvβ3	TCP	115±7.5	0.06
	CI	105±14.2	0.52
	CIV	102.5±16.6	0.79
KM12SM αvβ5	TCP	95.5±8.75	0.41
	CI	103±11.3	0.74
	CIV	100.5±2.6	0.29

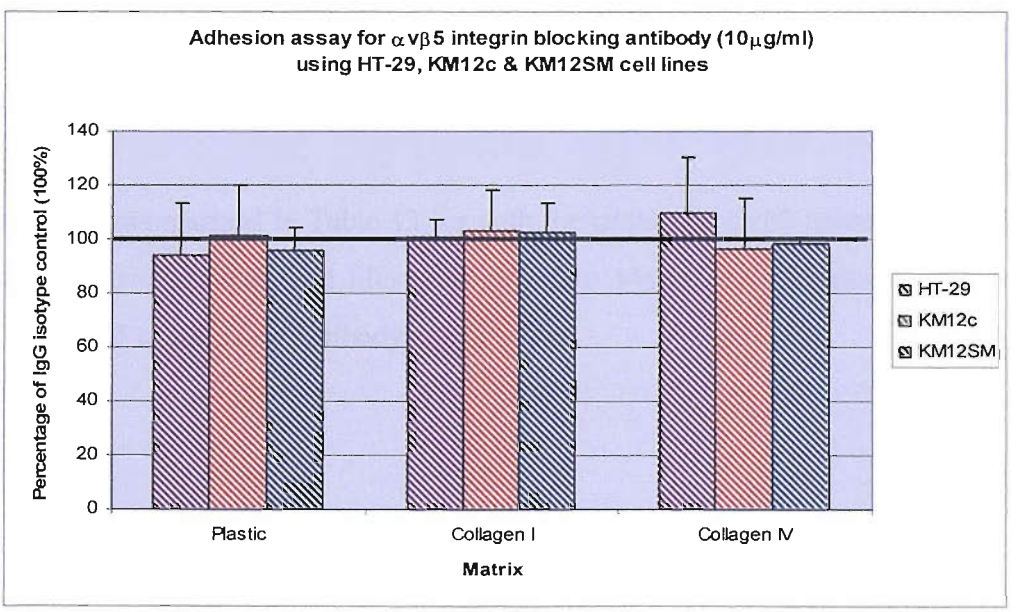
No significant reduction in cellular adhesion using either antibody was apparent for any of the cell lines used. Data representative of at least 3 independent experiments.

Figure 33a: This graph illustrates that 10μg/ml of αvβ3 integrin neutralizing antibody had no effect on cellular adhesion of CRC to different sub-cellular matrices.



αvβ3 blockade had no effect on CRC adhesion. Results shown as means ± 95% CI in comparison to IgG isotype control (100%), data representative of 3 independent experiments.

Figure 33b: This graph illustrates that 10μg/ml of αvβ5 integrin neutralizing antibody had no effect on cellular adhesion of CRC to different sub-cellular matrices.



αvβ5 blockade had no effect on CRC adhesion. Results shown as means ± 95% CI in comparison to IgG isotype control (100%), data representative of 3 independent experiments.

Section 5.1.5

The effects of αvβ3 and αvβ5 neutralising antibodies on cellular proliferation

As shown previously the αv, β3 and β5 integrin sub-types appeared to be more highly expressed as CRC adopt a more aggressive metastatic phenotype. Proliferation assays demonstrated that αvβ3 and αvβ5 neutralising antibodies significantly reduced the rate of CRC proliferation using the techniques as described in section 2.11. Using 5-10μg/ml of the αvβ3 integrin neutralizing antibody significantly reduced cellular proliferation of the highly metastatic CRC line (KM12SM) in a dose dependent manner. However, the proliferation of the poorly metastatic CRC cell lines were not significantly reduced using the αvβ3 neutralising antibody. In contrast the αvβ3 (10μg/ml) antibody reduced the rate of cellular proliferation of KM12SM CRC by 35% and 32%, when grown on collagens I and IV respectively, with no significant effect on proliferation on plastic.

αvβ5 neutralising antibody (5-10μg/ml) significantly reduced cellular proliferation in both poorly and highly metastatic CRC on collagens, by between 20-50%. The magnitude of the reduction in cellular proliferation on collagens was greatest using the αvβ5 neutralising antibody (10μg/ml) and the highly metastatic KM12SM cell line, where proliferation was reduced by 50.2% and 49.5% on collagens I and IV respectively, with no significant effect on proliferation on plastic.

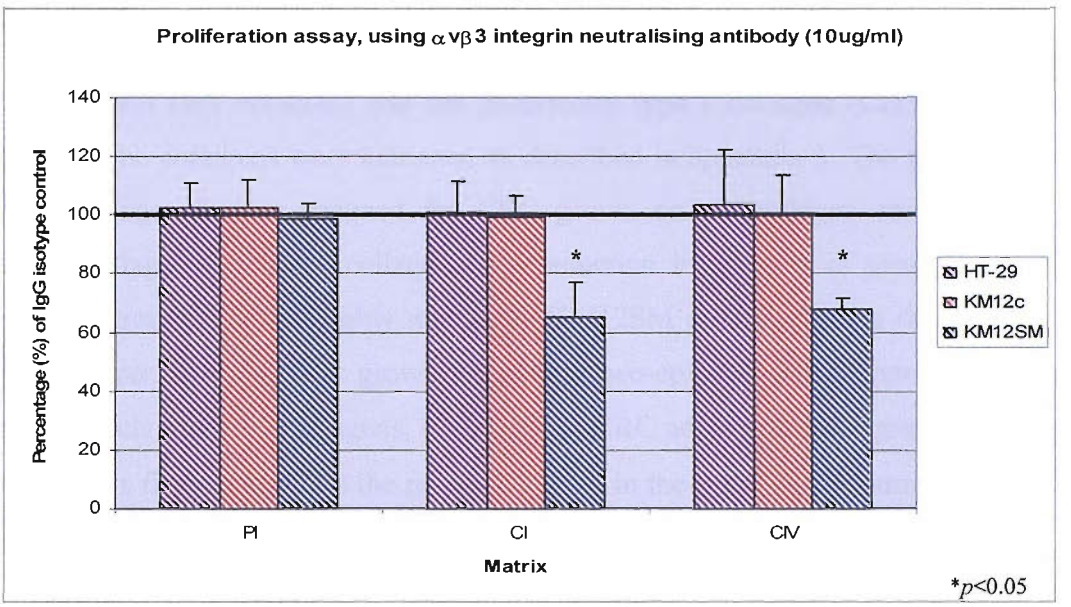
These data are summarized in Table 13 for both the αvβ3 and αvβ5 neutralising antibodies, including statistical analysis and illustrated in figure 34a (αvβ3 neutralising antibody) and figure 34b (αvβ5 neutralising antibody).

Table 13: Results comparing the effect of individual sub-cellular matrices on CRC proliferation using 5-10μg/ml of αvβ3 & αvβ5-integrin blocking antibody in comparison to IgG control.

Cell Line	Matrix	Concentration μg/ml	Mean± 95% Confidence Intervals	*p*-value
HT-29 αvβ3 integrin	TCP	10	102±8.9	0.66
	CI		100.5±10.9	0.93
	CIV		103.5±18.7	0.73
KM12c αvβ3 integrin	TCP	10	102.4±9.4	0.65
	CI		99±7.8	0.81
	CIV		100±13.5	0.97
KM12SM αvβ3 integrin	TCP	5	101.2±2.7	0.48
	CI		82.32±5.8	**0.026**
	CIV		82±4.2	**0.013**
KM12SM αvβ3 integrin	TCP	10	98.6±5.2	0.66
	CI		65±12.2	**0.029**
	CIV		68±3.7	**0.003**
HT-29 αvβ5 integrin	TCP	10	98±3.5	0.32
	CI		80±6.7	**0.03**
	CIV		76±6.4	**0.02**
KM12c αvβ5 integrin	TCP	10	97.5±6.8	0.52
	CI		70.4±5.95	**0.002**
	CIV		75.6±6.74	**0.005**
KM12SM αvβ5 integrin	TCP	5	99.5±4.4	0.84
	CI		80.3±8.3	**0.02**
	CIV		71.6±13.8	**0.03**
KM12SM αvβ5 integrin	TCP	10	96.1±6.8	0.34
	CI		49.2±21	**0.017**
	CIV		50.5±20	**0.017**

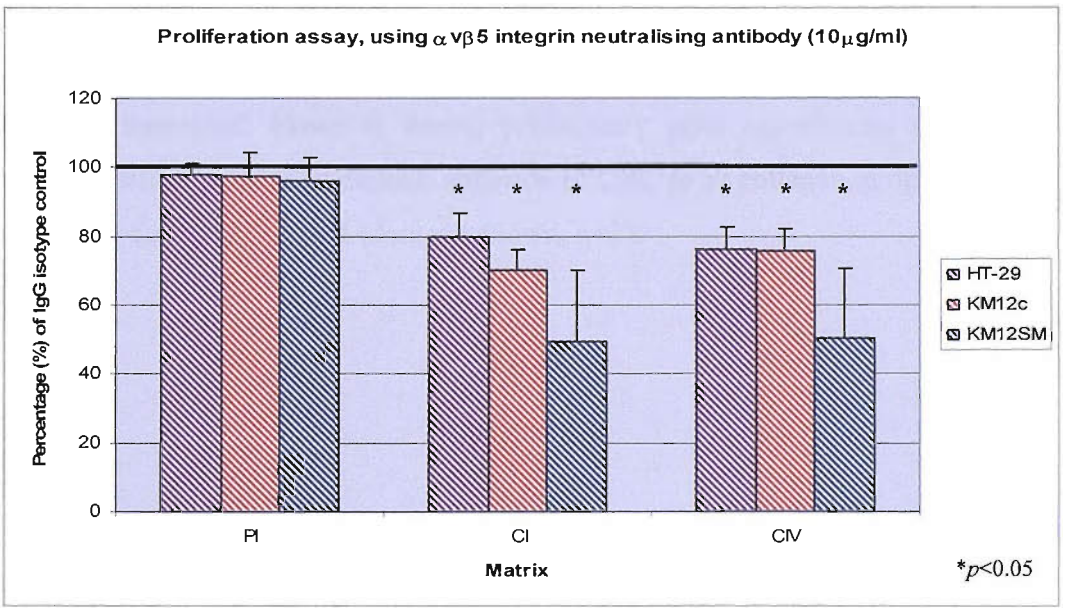
αvβ3 and αvβ5 neutralising antibodies produce a significant dose dependent reduction in cellular proliferation in the highly metastatic KM12SM cell line. Data representative of 4 independent experiments, $p<0.05$ was taken as being of significance. There was also a significant reduction in cellular proliferation although of less magnitude to that seen in the KM12SM line using the poorly-metastatic cell lines and 10 μg/ml of the αvβ5 neutralising antibody.

Figure 34a: The effect of 10μg/ml of αvβ3 integrin neutralizing antibody on CRC proliferation on specific sub-cellular matrices.



Only with the highly metastatic KM12SM cell line can we demonstrate a significant reduction in the rate of cellular proliferation. Means ± 95% Confidence intervals compared to IgG control (100%), data representative of 4 independent experiments.

Figure 34b: The effect of 10μg/ml of αvβ5 integrin neutralizing antibody on CRC proliferation on specific sub-cellular matrices.



A significant reduction in cellular proliferation was demonstrated in all the cell lines used. This effect was most marked in the highly metastatic KM12SM line where we were able to reduce the rate of cellular proliferation by 50% on collagens. Means ± 95% Confidence intervals compared to IgG control (100%), data representative of 4 independent experiments

Section 5.1.6

The effect of type I r/r collagen on CRC cellular proliferation

The effect of type I r/r (r/r collagen) collagen on CRC proliferation was compared to mouse wild type control (w/t collagen) and the proprietary type I collagen (CI) as described in section 2.11. The collagens were extracted as described in appendix 3. The pro-proliferative effects of collagen I were reduced for CRC grown on r/r collagen compared to both proprietary collagen I and w/t collagen. The reduction in the rate of proliferation on r/r collagen was greatest for the highly metastatic KM12SM cell line. These data suggest that collagen turnover revealing new growth regulatory neo-epitopes is important in the growth promoting effects of type I collagens, especially as CRC adopt a more aggressive metastatic phenotype. This finding supports the results obtained in the αv integrin neutralising antibody proliferation experiments shown in figures 34a and 34b.

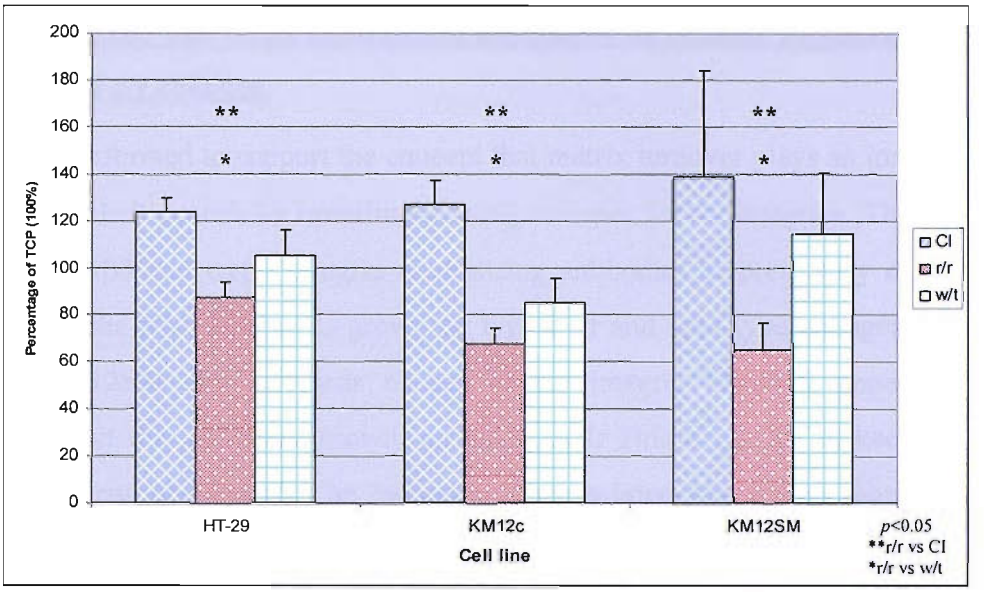
These data are summarized in table 14 and figure 35a. In addition to aid comparisons between CRC grown on r/r collagen versus wild-type collagen I these data are shown in figure 35b. The reason for including this as a separate figure was that w/t collagen because it is extracted from a mouse from the same breed and strain and parental origin as r/r collagen; it was the best control for these experiments. Differences in cellular adhesion which could affect the rate of CRC proliferation were compensated for by expressing the results in terms of cpm/ng DNA as previously described. However during preliminary pilot experiments there was little difference identified in terms of cellular adhesion of CRC to r/r collagen, proprietary collagen I and wild-type collagen I control (data not shown, n=2).

Table 14; Summary of the proliferation assay results using different types of collagen I (including statistical analysis).

Cell Line	Matrix	Mean ±SD±95% CI	Comparison	%	t-test
HT-29	Collagen I (CI)	123.6±5.7	r/r vs CI	70.8±10.4	**0.01**
	r/r Collagen	87.3±7.8	r/r vs w/t	83.1±4.1	**0.004**
	w/t Collagen	105±11.2			
KM12c	Collagen I (CI)	122.1±9.9	r/r vs CI	56.2±8.5	**0.008**
	r/r Collagen	67.9±6.2	r/r vs w/t	79.9±2.2	**0.005**
	w/t Collagen	85.3±10.1			
KM12SM	Collagen I (CI)	138.9±44.7	r/r vs CI	49.3±9.3	**0.04**
	r/r Collagen	65.2±11.3	r/r vs w/t	58.2±14	**0.018**
	w/t Collagen	114.3±26.3			

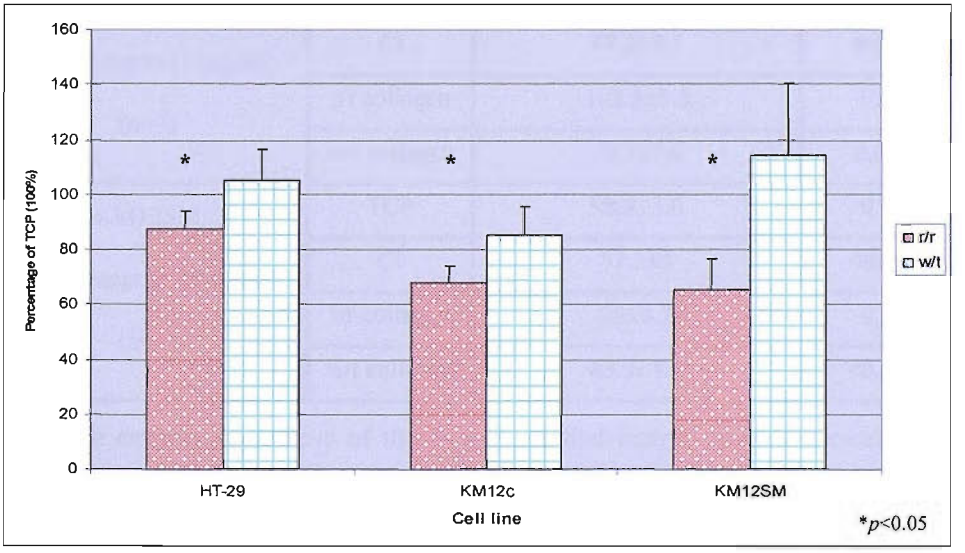
These data indicate that matrix degradation is important in promoting the growth of CRC. All results are expressed in relation to TCP (100%) for each cell line as Means ±95% Confidence intervals. Data representative of 4 independent experiments for each cell line, $p<0.05$ was taken as being of statistical significance.

Figure 35a: This graph illustrates the effect on cellular proliferation of the different types of collagen I



A significant reduction in cellular proliferation was demonstrated in all the CRC cell lines when they are grown on type I r/r collagen compared to proprietary collagen I and wild-type collagen I control. This effect was most marked in the highly metastatic KM12SM line. These data suggest that matrix turnover promotes CRC growth on collagen I. Results shown as means ± 95% Confidence intervals in comparison for IgG isotype control (100%), data representative of at least 4 independent experiments.

Figure 35b: This graph illustrates the effect on cellular proliferation for CRC grown on r/r and wild-type collagen I.



For each cell line, there were differences between r/r collagen I and wild-type collagen I control. All CRC proliferate at a lower rate when in contact with r/r (non-degradable) collagen I.

Section 5.1.7

The effects of αvβ3 and αvβ5 neutralising antibodies on cellular proliferation for CRC grown on type I r/r collagen

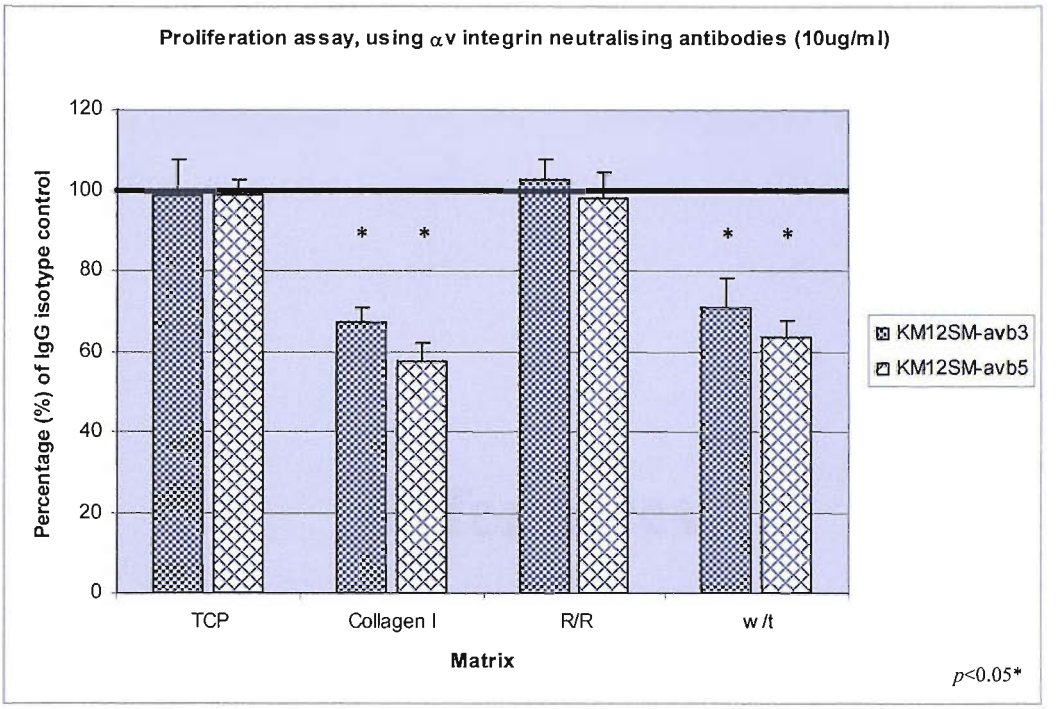
Studies were performed to support the concept that matrix turnover plays an important role in regulating CRC cell growth by revealing binding epitopes for αv integrins. The proliferation assays using αvβ3 and αvβ5 integrin neutralising antibodies as previously described were repeated using the KM12SM cells grown on type I r/r and wild type collagens. The highly metastatic KM12SM cell line was chosen as αv integrin blockade appeared to more profoundly affect this cell lines growth on collagen I. This would be in keeping with our hypothesis that αv integrins play an increasingly more important growth regulatory role, as CRC adopt a more aggressive metastatic phenotype. These data are summarized in the table below and illustrated in figure 36.

Table 15; Summary of the proliferation assay results using different types of collagen I and αvβ3 & αvβ5 neutralising antibodies.

Cell Line	Matrix	Mean± 95% Confidence Intervals	*p*-value
KM12SM αvβ3 integrin (10μg/ml) (n=3)	TCP	98.7±8.83	0.8
	CI	67.3±3.5	**0.003**
	r/r collagen	102.3±5.5	0.48
	w/t collagen	70.7±7.4	**0.016**
KM12SM αvβ5 integrin (10μg/ml) (n=4)	TCP	98.8±3.6	0.54
	CI	57.3±5	**<0.001**
	r/r collagen	98±6.2	0.57
	w/t collagen	63.3±4.4	**<0.001**

These data are entirely supportive of the hypothesis that matrix turnover reveals αv integrin binding ligands and thereby promotes the growth of CRC. All results are expressed in relation to IgG isotype control (100%) for each cell line on each matrix as Means ±95% Confidence intervals. $p<0.05$ was taken as being of statistical significance. Data representative of at least 3 independent experiments.

Figure 36: This graph illustrates the effect of αvβ3 and αvβ5 neutralising antibodies on cellular proliferation on r/r and wild type collagen I.



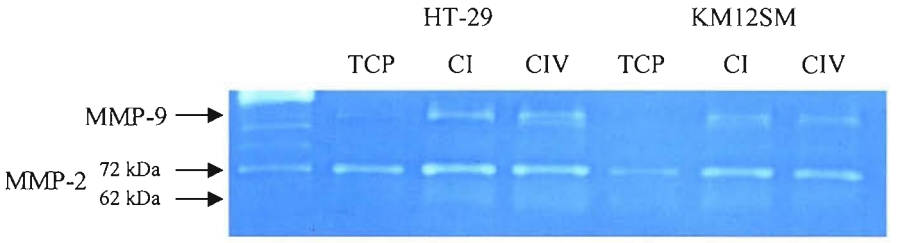
As can be clearly seen, αv neutralizing antibodies have no effect on CRC proliferation on r/r collagen, but significantly reduce CRC proliferation on proprietary and wild type collagen I. Results shown as means ± 95% Confidence intervals in relation to IgG isotype control (100%) on each matrix. Data representative of at least 3 independent experiments.

Section 5.1.8

Zymography

The data from the r/r collagen studies suggested that one or more proteases were released from CRC to degrade the collagen. To help determine, which MMPs may play an important role in matrix turnover, gelatin zymography was performed as described in section 2.12 on the supernatants extracted from CRC grown on different matrices. From a search of the literature, it was likely that MMP-2 would be a key MMP in CRC progression and by inference MMP-14 because of its role in activating MMP-2. The zymography studies showed that total & active MMP-2 levels were higher in conditioned media of CRC grown on collagens.

Figure 37: Gelatin zymography, using CRC grown on different matrices,



This figure demonstrates that MMP-2 was up-regulated when CRC were grown on collagens compared to TCP. Data representative of at least 3 independent experiments.

Section 5.1.9

Discussion

These studies of integrin expression by CRC may help explain the functional consequences of the pattern of staining obtained by IHC of CRC liver metastases. In these studies it was demonstrated that β1 integrins appear to be up-regulated throughout the CRC liver metastases. However, their pattern of expression was not uniform, in poorly differentiated areas of the metastases β1 integrins showed a haphazard pattern of distribution, with areas in which this integrin was barely expressed. Instead, αv integrins appeared to be more highly expressed within these poorly differentiated areas of the metastases. The tissue culture studies in this chapter have demonstrated that in CRC β1 integrins play a key role in regulating both cellular adhesion and proliferation for all the CRC lines studied. Which β1 integrin sub-types are important would be a matter of speculation, however those integrins that are known to play an important role in regulating cell adhesion to collagens, these include the α1β1 and α2β1 integrins (Jokinen J et al, 2004) are expected to be the most likely candidates. In addition, β1 integrins also regulated cellular proliferation even within the adherent cell population when the differences in cell adhesion were compensated for by using the pico

green assay to relate ^{3}H thymidine incorporation to monlayer cell DNA. There are several possible reasons to explain this reduction in CRC cellular proliferation. The most likely was that the very β1 integrins that regulate adhesion, effect proliferation as for example the α1β1 and α5β1 receptors both control cell cycle progression depending on substrate adhesion (Wary K et al, 1996 and Varner J et al, 1995). An alternative explanation could be that by blocking all β1 integrins, some of the reduction in cellular proliferation may be attributable to blocking different β1 integrin heterodimers, which may be more important in regulating cell growth, such as α3β1 integrin. Higher expression of this integrin in melanoma was associated with a metastatic and invasive phenotype (Natali P et al, 1993). To address this point, further studies might characterise α subunit partners of β1 integrins in our CRC cell lines, using flow cytometry and appropriate antibodies. In addition by utilising neutralising and labelled antibodies to these integrin heterodimers in vitro and within our IHC sections it will be possible to further characterise the specific β1 integrins of interest.

An interesting finding from both the β1 and αv integrin western blotting was the presence of two isoforms of each integrin. The lower of the two bands probably represents an unglycosylated immature form of the αv and β1 integrins (Von Lampe B et al, 1993). The immature unglycosylated forms of these integrins in normal cells do not form heterodimeric pairs and remain intracellularly (Akiyama SK et al, 1987). However, it has been shown in CRC cells that these immature unglycosylated forms of the integrin do form heterodimeric pairs and be expressed by the cell membrane (Von Lampe B et al, 1993; Akiyama SK et al, 1989). It has therefore been postulated that changes in the level of glycosylation of the integrins expressed by the cancer cell, may have important functional significance in encouraging a more aggressive phenotype (Fujita S et al, 1995). Varying the level of glycosylation of these proteins, may have functionally important effects on cell growth and metastatic spread, which may explain why there was higher expression of the different iso-forms of αv and β1 integrins by the CRC cell lines. Interestingly, although these studies suggest β1 integrins are perhaps more functionally important for poorly metastatic CRC in regulating cell growth, our western blotting studies demonstrated that β1 integrins were more highly expressed by highly metastatic CRC. Therefore studies using specific $\alpha_{sub\text{-}type}\beta1$

antibodies would help us further dissect how the profile of different β1 integrin types varies between poorly and highly metastatic CRC and what functional significance this may have, because as discussed in previous sections different β1 integrin sub-types may be associated with a more aggressive invasive phenotype.

From the αv integrin western blotting data, it became apparent that there was differential expression of this integrin between poorly and highly metastatic CRC cell lines. To identify which αv integrin sub-type may be important as the CRC adopt a more aggressive metastatic phenotype, a thorough literature search was undertaken. Several possible candidate αv integrins of interest were identified, these included αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 integrins. However, it became apparent from previous studies in CRC that αvβ5 integrin appeared to be important in the development of CRC, especially as CRC adopted a more aggressive invasive phenotype (Schramm K et al, 2000) and that αvβ3 and αvβ5 integrins are believed to play role an important role in the development of many cancers including melanoma (Shahan T et al, 1999; Petitclerc E et al, 1999; Mitjans F et al, 2000 & Kumar C et al, 2001) and breast cancer (Sung V et al, 1998 and Bartsch J et al, 2003). By western blotting it was clearly demonstrated that as CRC adopted a more aggressive metastatic phenotype both β3 and β5 integrins were more highly expressed especially when grown on collagens. The most striking differences were demonstrated in the level of β5 integrin expression especially for highly metastatic CRC grown on collagen I. Therefore the αvβ3 and αvβ5 integrins were chosen as candidates for further study.

The standard adhesion assay were performed using the HT-29, KM12c and KM12SM cell lines utilising concentrations of the αvβ3 and αvβ5 integrin neutralising antibodies that have been shown to inhibit cancer cell adhesion and proliferation on a wide variety of matrices (www.chemicon.com; Sung V et al, 1998; Zheng D et al, 2000; Kumar C et al, 2001). Despite the altered pattern of integrin expression by the CRC there was no effect on cellular adhesion for any of the CRC used with either of the antibodies used. There could be several possible explanations for this: Firstly, the αvβ3 and αvβ5 integrins are not required for CRC adhesion; Secondly, possible limitations of the western blotting results are that they look at the level of

expression of all αv integrins rather than the particular heterodimers of interest. Therefore, comparing the adhesion assay and western blotting data might be inappropriate. From previous studies, the αvβ3 and αvβ5 integrins are known to regulate cell growth, apoptosis and adhesion to fibronectin and vitronectin (Kemperman H et al, 1997; Sung V et al, 1998; Schramm K et al, 2000; Zheng D et al, 2000; Kumar C et al, 2001). However, an important requirement for αv integrin regulated cell growth on collagens has been demonstrated to be that of matrix turnover of collagens I and IV (Davis GE, 1992; Xu J et al, 2001), as the ligands for these αv integrins are not exposed in non-denatured collagens. Therefore it was decided to perform the proliferation assays using the αvβ3 and αvβ5 neutralising antibodies.

In contrast to the lack of effect on CRC adhesion, a dose dependent reduction in cellular proliferation was produced for all the CRC using the αvβ5 neutralising antibody. However, the magnitude of the reduction in proliferation was in the region of 20-30% on collagens in the poorly metastatic CRC lines (HT-29 and KM12c) compared to a 50% reduction in proliferation for the highly metastatic KM12SM cell line. The αvβ3 neutralising antibody, did not significantly reduce cellular proliferation on collagens using either of the poorly metastatic CRC. In contrast a significant dose dependent reduction in cellular proliferation was demonstrated using the αvβ3 neutralising antibody and the highly metastatic KM12SM CRC. As to how αv integrins may regulate cancer cell proliferation, Cruet-Hennequart S et al, 2003 demonstrated that αv integrins regulated ovarian cancer cell growth via Integrin Linked Kinases (as previously discussed in section 1.8.3).

These proliferation assay data suggest as CRC adopt a more aggressive metastatic phenotype αv integrins (specifically αvβ3 and αvβ5) have an important role in regulating CRC growth on collagens. A possible explanation for this could be that matrix turnover reveals key growth regulatory epitopes that can be ligated by αv integrins expressed by the CRC. Therefore, one possiblility is that these integrins had no effect in regulating CRC adhesion in our model as these ligands were not present during the short duration of the adhesion assay, but become exposed during the rather more protracted proliferation assay, due to MMP mediated collagen degradation. An alternative explanation could be that during the duration of the proliferation

assay, immature matrix components (fibronectin and vitronectin) are incorporated into the collagens films. These immature matrix components are known to possess binding epitopes (RGD sequences) that can be ligated by these αv integrins. It has become well established that αvβ3 and αvβ5 integrins may ligate the RGD sequences expressed by fibronectin, vitronectin (Sanders R et al, 1998; Pijuan-Thompson V et al, 1997; Kemperman H et al, 1997) and that αvβ3 integrins may also ligate RGD sequences expressed by denatured collagens I and IV (Pijuan-Thompson V et al, 1997). However, only one paper has demonstrated the ability of αvβ5 integrins to ligate collagen I directly (Pedchenko V et al, 2004). This was somewhat surprising as studies by Cheresh D et al, 1987 suggested that the specificity for Arg-Gly-Asp recognition lies with the α integrin chain. As there were no differences seen in the rate of CRC proliferation on TCP, in which immature matrix components would be incorporated. That would suggest that it would be wrong to assume the only reason for the proliferation differences identified with the αvβ3 and αvβ5 integrin neutralising antibodies was because the well established ligands expressed by fibronectin and vitronectin were not ligated. However, it could equally be argued that immature matrix components bind better to collagens than TCP. Irrespective of this though, these in vitro studies have tried to reproduce what occurs in vivo, in which immature matrix components are incorporated into the deposited fibrillar collagen matrix. Therefore, to further investigate the hypothesis that matrix turnover specifically of collagens was also important in regulating CRC growth the r/r collagen experiments were undertaken.

The r/r collagen data supports the neutralizing antibody experiments and reinforces the importance of matrix turnover as CRC adopt a more aggressive metastatic phenotype. For all the CRC grown on type I r/r collagen, the rate of cellular proliferation was significantly less than for CRC grown on proprietary and wild type collagen I. Interestingly, the rate of CRC proliferation was not as great on wild-type collagen I as the proprietary collagen I. Possible explanations for this include, differences in purity between the two collagens, although using SDS PAGE electrophoresis the purity between the samples appeared similar (Appendix 3). An alternative explanation could be that the wild-type and r/r collagen were mouse derived and the proprietary collagen was rat derived and perhaps contained different growth

regulatory ligands, or the stability of the triple helix was different. However, the same pattern of reduced CRC proliferation on r/r collagen was still identified when comparisons were made with either of the controls used. Again, the greatest reduction in proliferation was consistently identified for the highly metastatic CRC KM12SM grown on r/r collagen compared to either of the collagen controls. As r/r collagen is non-degradable by MMPs (Wu H et al, 1990) this confirms the role of matrix turnover in regulating CRC growth especially as CRC adopt a more aggressive metastatic phenotype. Matrix turnover has now become established as a key concept in regulating cancer cell growth (Hornebeck W et al, 2003) and this study adds further weight to this hypothesis.

To examine the role of αvβ3 and αvβ5 integrins in regulating proliferation on the different sub-cellular matrix forms of collagen I, the proliferation assays using KM12SM were repeated with cognate integrin neutralising antibodies. The results supported the role of matrix turnover in regulating CRC growth due to the revealing of growth regulatory sequences, which are ligated by αvβ3 and αvβ5 integrins. Although an alternative explanation could be put forward that r/r collagen alters cell proliferation by an effect on matrix binding of fibronectin or an integrin ligand. However, the key ligands are likely to be revealed RGD sequences which are known to play a key growth regulatory role in melanoma development and are expressed by both denatured collagen I and IV (Davis GE, 1992; Pijuan-Thompson V et al, 1997; Xu J et al, 2001). Few studies have concentrated on the role of αvβ5 integrins in regulating cancer cell growth in comparison to αvβ3 integrins. However, these integrins may act in a complementary manner (Wayner E et al, 1991; Sung V et al, 1998; Pidgeon G et al, 2003) regulating cellular shape, migration, proliferation and apoptosis. Therefore it should be no surprise that they play an important role in CRC development and could be potentially targeted therapeutically in patients with CRC.

The zymography data suggest that both total and active MMP-2 expression were up-regulated by both poorly and highly metastatic CRC grown on collagens I and IV. A possible explanation for this is the role of the Discoidin Domain receptor 2 (DDR-2) which is a receptor tyrosine kinase which has been suggested to play a role in regulating MMP-2 release

from cells. These studies used stably transfected HSC expressing wild-type, constitutively active or kinase dead DDR-2, with the HSC grown on collagen I and demonstrated that MMP-2 release and thereby activity was up-regulated, linked to increased DDR-2 activity (Olaso E et al, 2001). For DDR-2 -/- fibroblasts a similar mechanism was postulated for the loss of collagen degrading activity and thereby reduced invasion through matrigel (Olaso E et al, 2002). Collagen IV forms a major component of matrigel, which has been suggested to replicate the basement membrane more closely than collagen IV itself. It does however have many disadvantages, including high cost and it possess many intrinsic growth factors which may cause problems in interpreting results and was why this was not used in our studies. However, these previous DDR-2 findings on collagen I and matrigel may explain why MMP-2 levels were increased on both collagens I and also collagen IV.

By inference, if MMP-2 levels are higher it would seem likely that MMP-14 expression may also be up-regulated, due to its important role in activating MMP-2 (Section 1.11). The possible importance of MMP-14 in cancer development was illustrated by the work of Hotary K et al, 2003 who were able to demonstrate the key role this MMP plays in regulating cellular geometry and thereby proliferation in a 3D collagen I matrix. However, there are also several other possible candidate MMPs, which would require studying as previously discussed in section 1.16, although initially concentrating on MMPs-1, 2, 8 and -14 which are able to degrade native or non-denatured collagen. This data does suggest at least two possible candidate MMPs (MMP-2 & -14), which would degrade the matrix to reveal the growth regulatory ligands which may regulate CRC growth identified in the previous sections.

Chapter 6

General Discussion and Future Work

It has long been known that CRC contain a predominant stromal component, however there are few previous studies looking specifically at the role of the stroma and the Desmoplastic reaction in the development of colorectal cancer liver metastases. These studies provide clear support for the importance of a desmoplastic reaction consisting of types I and III collagen and activated myofibroblasts in the development, growth and chemoresistance of colorectal cancer liver metastases. Our studies indicate the CRC derive a growth and survival advantage and increased CRC chemoresistance as a result of contact with collagen I, a major component of this DR.

Immunostaining of resected CRC liver metastases provides clear evidence of a DR, consisting of predominantly type I collagens which form discrete fibrils throughout the tumour stroma. Interestingly, the DR was often at its most dense in the poorly differentiated areas within the CRC metastases. A possible explanation for this is that CRC cells provoke an inflammatory reaction within the liver, characterised by lymphocyte recruitment (Yoong KF et al, 1999). In consequence the hepatic repair response becomes activated with the transformation of Hepatic stellate cells to fibrogenic myofibroblasts which produce an interstitial matrix. In vitro studies have also suggested a contributory role for CRC and stromal cell interactions in the development of primary colorectal cancers (Hauptmann S et al, 2003). Using an in vivo animal model it was suggested that the redistribution and expansion of myofibroblasts plays an important role in the formation of established CRC liver metastases (Higashi N, 2002), and a recent study using micoarrays in a nude mouse CRC model has demonstrated the presence of activated hepatic stellate cells along the invading margin of the tumour (Bandapalli O et al, 2006). A DR in these circumstances can therefore be viewed as a fibrogenic response to inflammation. Successful CRC cell clones in this context may have been selected by the ability to respond to this new milieu with both enhanced proliferation and survival. Indeed studies in several different primary cancers (Meng L et al, 2001; Buchholz M et al, 2003), including studies in pancreatic cancer (Armstrong TA et al, 2004) suggest that this response to the DR may be a feature of aggressive cancer.

The immunohistochemistry studies of section 3.1 indicate that the DR in the liver was populated with αSMA positive myofibroblasts which are likely to be the key matrix producing cells. It is important to realise that although we have concentrated on the role of types I and IV collagen in our studies, other matrix components including fibronectin and vitronectin may be expressed and may regulate the CRC phenotype. The expression of these components were not determined during this study, but both are deposited in liver fibrosis induced by chemical hepatoxins in rats (Richter HB et al, 1998; Zhou X et al, 2000) and human liver fibrosis of various aetiologies (Koukoulis GK et al, 1995; Koukoulis GK et al, 2001; Kershenobich Stalnikowitz D et al, 2003). Previous studies investigating CRC responses to different matrix components in terms of proliferation and apoptosis have produced highly variable results (Zvibel I et al, 1998; Kouniavsky G et al, 2002). These studies demonstrate a consistent and reproducible effect of interstitial collagens on CRC growth. Within the DR in vivo, the alterations in the distribution of collagen IV may also be important to the CRC phenotype. The immunohistochemical staining was specific for the α1 & 2 chains of collagen IV and consistently showed dysregulated secretion of collagen IV. Type IV collagen possesses many inhibitory sequences to cell growth, these sequences have been identified on the α2, 3 & 6 chains of collagen IV and have been shown to inhibit the proliferation, migration and MMP production in other cancer cells (Petticlerc E et al, 2000; Maeshima Y et al, 2000). A further interesting observation was that for all CRC clones studied, although collagen I was always significantly growth promoting, the inhibitory effects of collagen IV on CRC proliferation depended on the metastatic potential of the CRC clone. We demonstrated that the poorly metastatic but not highly metastatic CRC clones proliferate at a much slower rate when cultured on collagen IV, therefore the change in matrix from ordered type IV collagen to type I collagen may promote phenotypic qualities that enhance the malignant behaviour of carcinomas. Alternatively, suppressive signals from type IV collagen in the DR might become overwhelmed by proliferative/ survival signals from type I collagen.

It can be postulated that the differential effects on CRC growth of collagen IV could be partly explained by altered patterns of integrin expression between CRC clones of different

metastatic potential. In the in vitro studies β1 integrin positively regulated both cellular adhesion and growth for all CRC clones. However, for poorly metastatic CRC, β1 integrins are more effective in regulating CRC growth. In contrast highly metastatic CRC express αv integrins at higher levels, and αvβ3 and αvβ5 appear to have a key growth regulatory role. Through this different pattern of integrin expression, the highly metastatic CRC clones, can respond to a wide variety of matrix components and are able to thrive on both collagen I and IV. The studies utilising CRC cultured on non-degradable r/r collagen suggest that degradation of both collagen I and IV reveals the key growth regulatory RGD sequences, which can be ligated by these αv integrin sub-types. Generation of these ligands has also been shown to be important in the development of malignant melanoma (Shahan T et al, 1999; Petitclerc E et al, 1999; Mitjans F et al, 2000 & Kumar C et al, 2001). CRC up-regulation of αvβ5 integrins, has been suggested to shift the malignant phenotype of CRC to a more aggressive and invasive type (Schramm K et al, 2000). RGD sequences are expressed by degraded collagens, fibronectin and vitronectin (Davis GE et al, 1992; Xu J et al, 2001). In vivo IHC studies shown in section 3.1 would reinforce these tissue culture findings with different areas of the tumour expressing different integrin sub-types dependent on their degree of differentiation.

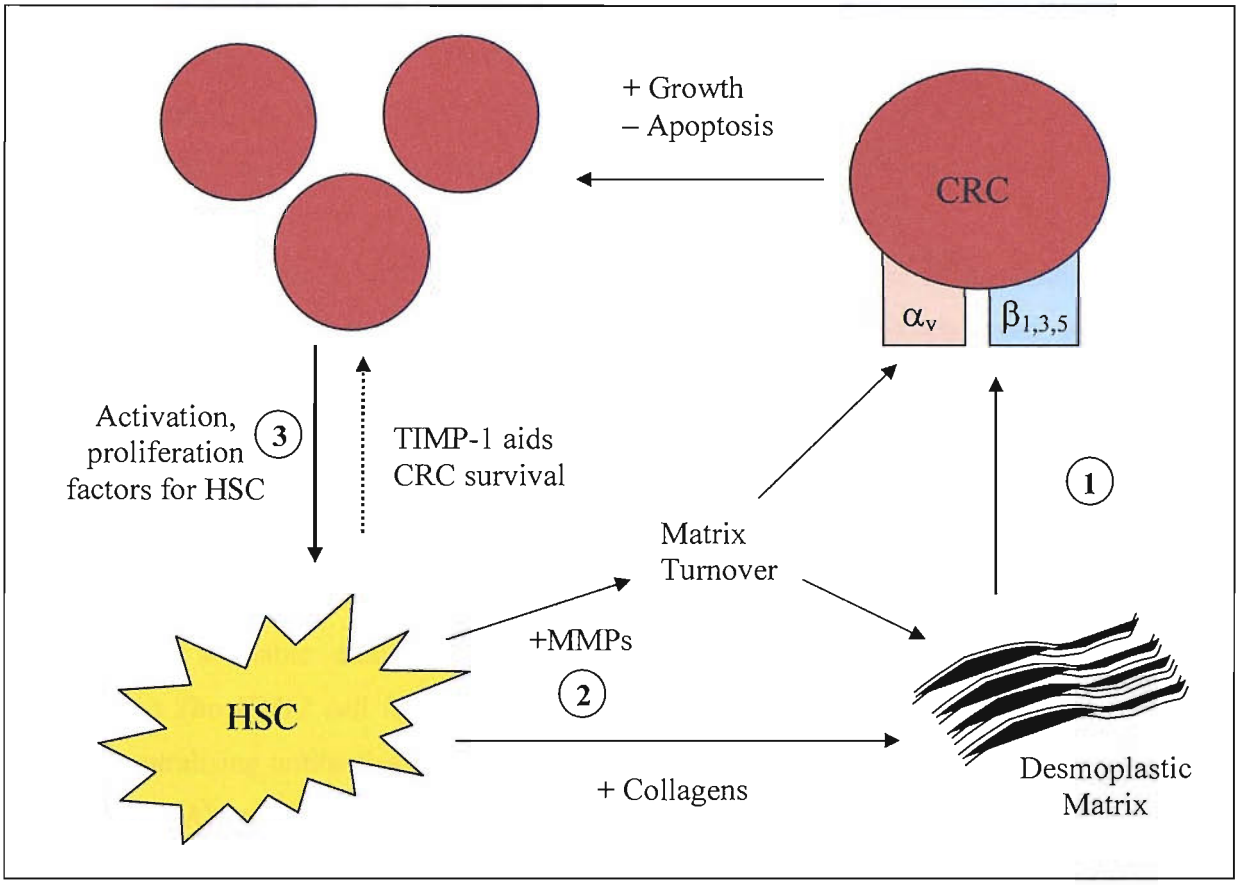
It has been recently shown for CRC that presence of abundant collagen I in the DR is associated with a more aggressive poorly differentiated phenotype (Brabletz T et al, 2004). There may therefore be a complex interplay between different matrix components and CRC with integrins acting as key intermediates. Alterations in integrin expression, conformation and adhesiveness are under the control of *ras* signalling pathways (inside-out signalling) (Miyamoto S et al, 1998; Boudreau NJ et al, 1999), but can also be influenced by the ECM (outside-in signalling) (Boudreau NJ et al, 1999; Radisky D et al, 2002). *ras* mutations are common in CRC and are also found in our CRC cell lines, but crucially the studies of section 5.1 suggest the components of the desmoplastic matrix could, in turn modify the pattern and adhesive properties of integrins expressed by CRC.

Studies to identify candidate MMPs involved in the development of CRC liver metastases have been undertaken previously and MMPs-2, 7, 9 & 14 produced by both stromal and CRC cells are believed to be important (Sunami E et al, 2000; Mook OR et al, 2003; Zeng ZS et al, 2002; Bendaraf R et al, 2003; Zucker S et al, 2004). The gelatin zymography studies of section 5.1, have demonstrated that the CRC produce both active MMP-2 & 9, whilst the activated myofibroblast (the HSC) are known to express MMP-2, 9 and 14 (Benyon RC et al, 1999). The importance of matrix remodelling by MMPs in generating matrix derived proliferative signals to CRC is illustrated by studies showing that MMP-resistant r/r collagen provides a less effective proliferative stimulus compared to normal type I collagen. The type I r/r collagen mouse possesses a mutation in the Col1a-1 gene, which encodes the α1 chain of collagen I (Vuorio E et al, 1990). This mutation at amino acids 775 and 776 of the Col1a-1 gene renders the synthesised collagen highly resistant to collagenases and therefore prevents the initial cleavage (Wu H et al, 1990) and unwinding that reveals the αv binding epitopes. By demonstrating a reduction in proliferation in response to r/r collagen we have further reinforced our αv integrin neutralising antibody experiments, complementing them with a model using a dominant negative ligand. This experimental approach is particularly robust as it obviates the need to inhibit specific MMPs involved in the process of matrix remodelling.

These data suggest that the DR is a dynamic phenomenon, with degradation of both the normal basement membrane rich in type IV collagen, occurring simultaneously with that of deposited fibrillar collagens. The DR arises as a consequence of the process of metastatic infiltration, invoking a classical wound healing response with activation of hepatic myofibroblasts and results in the accumulation of matrix, which provides a rapidly replenishable resource of neo-epiptopes which may be ligated by growth regulatory integrins expressed by CRC, including the β1 sub-types. CRC expressing the collagen I binding integrins will be positively selected. Then, as CRC and activated hepatic myofibroblasts degrade the basement membrane rich in collagen IV and the newly laid down desmoplastic matrix rich in collagen I, this reveals key growth regulatory neo-epitopes which are ligated by αv integrins. The differential integrin expression appears to characterise a more aggressive malignant phenotype in which the growth inhibition effects of collagen IV is lost. The

proposed model for the development of colorectal cancer liver metastases development is illustrated in the figure below.

Figure 38: Hypothesised model for Colorectal cancer liver metastases development



To further characterise this model, it is planned to further study the matrix changes accompanying a DR (1) by immunohistochemical staining. Particularly concentrating on how the distribution of the known growth regulatory isotypes of type IV are altered as part of a DR. The expression of immature matrix components as part of a DR will also be characterised and if possible stains for denatured collagens I, III and IV employed, to confirm the hypothesis, that the DR is a highly dynamic process. Further studies will aim to characterise which Metalloproteinases (2) are important in the development of CRC liver metastases, as thus far these studies have only concentrated on the potential role of MMP-2. The next key area to be studied would be to characterise the role of the HSC, the key matrix producing cells

in the liver. Preliminary data, using a boyden chamber co-culture model has suggested that CRC gain a significant proliferative advantage when cultured with HSC, with little effect on HSC proliferation. In contrast HSC activation and MMP-2 expression were up-regulated using this co-culture model. By the use of neutralising antibodies to soluble cytokines (PDGF, HGF, and TGFβ) it will be possible to further characterize stromal-CRC interactions. Direct co-culture techniques could also be employed to determine the importance of cell to cell contact in regulating cancer and stromal cell growth, and the rate of HSC production of fibrillar collagens would be determined by the use of Taqman PCR for procollagen I.

Perhaps the most exciting area for further research would be the use of an animal model, using intra-splenic injection of CRC cells. Two possible models could be used: a) *The r/r collagen mouse model* – however the main difficulty is that a nude r/r collagen mouse would have to be bred to use the human cell lines studied (approximately 18 months - 2 years to breed). An alternative would be to use a C57 Black derived mouse CRC cell line, after characterising the cell lines responses to the matrix. The main disadvantage of using a mouse line are that thus far all the work has been undertaken with human CRC lines and there are no commercially available mouse lines, which possess variants with different metastatic potential; b) *The KM12 cell line series* – injected into a nude mouse, with the addition of integrin neutralising antibodies, to determine if the rate of CRC metastatic development can be obtunded. Alternatively, vector based RNAi to specific integrins to be studied could be employed. This has the advantage of being able to down-regulate integrin expression for a prolonged period of time, as the RNAi is replenished in subsequent CRC clones. The main disadvantages are that using the integrin neutralising antibodies would require several doses to be given and using the RNAi technique it would only be possible to knock all the β1 or αv integrins not the specific integrin heterodimers of interest. There are also concerns with regard RNAi as to the non-specific effects it may have within the cell, although with appropriate controls many of these problems can be overcome.

To answer the obvious criticisms that accompany the use of cell lines, it may be possible to establish primary cultures from extracted CRC liver metastases or primary CRC cancers, to re-inforce our cell line studies. It would also be possible to determine if the expression of

different integrins correlates with survival and the development of metastatic disease from resected primary CRC and also determine how integrin expression changes between resected primary CRC and those that have undergone liver resection for metastatic disease. If possible, using matched samples from the original primary CRC resection and hepatic resection.

Therefore there are exciting opportunities for continuing this project and these data also suggest that desmoplastic matrix synthesis or turnover could offer new novel therapeutic options in patients with CRC. In particular the two main areas of therapeutic interest are; a) *Blocking CRC binding to the matrix combined with existing therapies (i.e chemotherapy)* – thereby interfering with the pro-proliferative and anti-apoptotic benefits CRC gained by being in contact with the DR matrix and potentially increasing the efficacy of chemotherapy. The disadvantages are that several integrins may need to be targeted and these integrins are important for normal cell functioning and wound healing. A targeted RNAi system would offset and may avoid many of these problems. However, the technology to achieve this is still being developed; b) *Preventing the production of DR matrix* – This could be achieved by targeting the HSC. However, further characterisation of the role of HSC and hepatic myofibroblasts will need to be undertaken. This technique is also likely to have the disadvantage of targeting all activated myofibroblasts, with obvious problems with impaired wound healing a possible side-effect.

Chapter 7

References

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Chapter 8

Appendices

Appendix 1a

Dukes Classification for Colorectal Cancer

The Dukes staging system is based upon the pathological characteristics of the resected cancer specimen and is shown in Table 16. Originally there were only three Dukes stages; Dukes A, B and C. However, it is now customary to include an additional stage; Dukes D. The importance of the Dukes classification is that it is used to decide if additional adjuvant treatments are needed. Interestingly even in so called early disease (Dukes A), a considerable number of patients still die from Colorectal cancer.

Table 16: Dukes Classification system

Dukes Stage	Pathological Features
A	The tumour is limited to the bowel wall with no lymph node involvement
B	The tumour extends through the wall of bowel, with no lymph node involvement
C	Lymph node involvement; C1 nodes adjacent to the bowel involved C2 apical nodes within the mesentery involved.
D	Presence of Distant metastases

The original Dukes classification, was described by Dukes CE in the British Journal of Surgery in 1930 (it has been modified several times).

Appendix 1b

The Cell Cycle

The cell cycle can be divided into four phases. These are illustrated in the figure below.

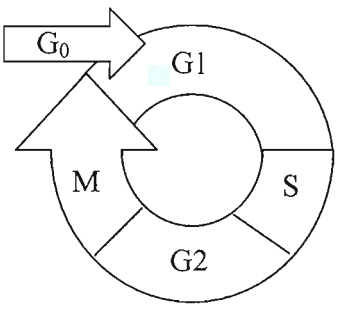


Figure 39: The stages of the Cell cycle.

1) Synthesis (**S**) - Two key events during this stage, these are the;

a) Replication of chromosomal DNA

b) The duplication of the centrosomes

2) **G_2**.

This stage is the interval between the end of DNA synthesis and the onset of Mitosis. The duplicated centrosomes still function as a single microtubule-organising centre. However, as the G_2 phase ends and the cell enters M-phase the centrosomes separate.

3) **M** = Mitosis;

The process of cellular division. Mitosis can be divided into 5 stages;

a) **Prophase**; The Chromatin produced during S-phase condenses into well defined chromosomes and the centrosomes migrate apart.

b) **Prometaphase**; Microtubules sprout from the centrosomes and the nuclear membrane breaks down.

c) **Metaphase**; The chromomsomes congress on an equatorial plane, called the metaphase plate.

d) **Anaphase**; The sister chromatids are then pulled to opposite poles of the cell.

e) **Telophase**; Once the chromatids reach the poles, the nuclear envelopes reform.

4) **G_1/G_0**. This stage occurs after completing M-phase and before S-phase. The cell has the correct number of chromosomes and then has the potential to enter the cell cycle for another round of cell division or can enter a so called resting stage (G_0).

Appendix 2a

Reagents used for immunohistochemical staining

APES (3-AminoPropyltriEthoxySilane) coating of slides

Place slides in a large rack. Immerse slides in 2% 3-aminopropyltriethoxysilane dissolved in dry acetone, for 5 minutes. Rinse in dry Acetone twice. Wash in ultra-pure water, twice and air dry overnight. Store covered at room temperature until needed.

Mayers Haemalum

In 1L dH_2O
Haematoxylin 1g
Potassium alum 50g
Citric acid 1g
Chloral hydrate 50g
Sodium iodate 0.2g

Microwave pre-treatment

a) 0.01M Citrate buffer (pH=6)

2.1g Citric acid crystals in 1L dH_2O, adjust to pH =6 with 1M NaOH.

b) 1mM EDTA Buffer (pH=8)

0.292g of EDTA in 1L dH_2O, adjust to pH=8 with 0.1M NaOH.

Pronase pre-treatment

Make a 1% pronase stock, by dissolving 100mg in 10mls of TBS. Then freeze at -20°C. Dilute with TBS to working concentration, by adding 100µl of stock to 1.9mls of TBS.

1M Tris HCL, pH =7.6

36.3g Tris base, in 200ml dH_2O. pH adjust with 5M HCL and make up to 300ml with dH_2O.

Tris Buffered Saline (TBS), PH=7.6

8g NaCl (137mM)
20ml 1M Tris HCL, pH=7.6 (20mM)
Make up to 1L with dH_2O, and pH adjust.

Blocking Medium

DMEM + 20% Foetal Calf Serum (FCS) and 1% Bovine Serum Albumin (BSA)

Endogenous Peroxidase Inhibitor

0.5% Hydrogen Peroxide solution made up with;
0.2ml Hydrogen Peroxide (30%)
11.8ml Methanol (100%)

Diaminobenzidine (DAB)

DAB bought in kit form from Menanni Diagnostics. For 5mls, take 0.5ml of 10x substrate buffer and add to 4.5ml of ultrapure H_2O. Then add 4 drops of DAB and 2 drops of Hydrogen peroxide (30%). The substrate is then ready to use.

Appendix 2b

Counting Protocol for ^{3}H Thymidine incorporation

Plate	Wallac (24 wells)
Time per well	120 seconds
Precision	0.2%
Display	Printer output

Counting Protocol for Pico Green

Plate	Nunc Maxisorp (96 wells)
Mix time	5 seconds
Reads/well	3
Centre count	yes
Excitation	485/20nm
Emission	530/25nm
Gain	70-80

Appendix 2c

Reagents used for SDS-PAGE electrophoresis/Western Blotting

LDS sample buffer (4x)

4g Glycerol
0.682g Tris base
0.666g Tris HCl
0.8g LDS
0.006g EDTA
0.75ml of a 1% solution of Serva Blue G250
0.25ml of a 1& solution of Phenol red
Made up to 10ml with ultrapure water.

Reducing Agent (10x)

0.5M DTT

For pre-cast Nupage Novex 4-12% Gradient Gels

3-(N-morpholino) propane sulfonic acid/Sodium Dodecysulpahte (MOPS/SDS) Running Buffer

Make up 20x stock
104.6g MOPS
60.6 g Tris Base
3g EDTA
10g SDS or 100ml of 10% SDS.
Make up to 500ml with ultrapure water

Make up to 1x MOPS/SDS running buffer with dH_2O. Final concentration of MOPS (1.05%)/ SDS (0.1%), pH =7.7. Add anti-oxidant for reduced samples (500µl/l), purchased from Invitrogen.

Transfer Buffer

Make up 20x Stock
40.8g Bicine
52.4g Bis-Tris (free base)
3g EDTA
Make up to 500ml with dH_2O.

Use 1x transfer buffer, final concentration of Bicine (25mM), Bis-tris (25mM) and EDTA (0.05mM), pH = 7.2. To make 1x transfer buffer, add Methanol to a final concentration of 10% (1 gel) or 20% (2 gels) and anti-oxidant (1000µl/l) for reduced samples. Make up to final volume with dH_2O.

For Casting Gels

The following reagents were used;

A gel stock solution was made using 30% Acylamide (w/v)/0.8% bis acrylamide (w/v) purchased from Biorad life sciences (Hemel Hempstead, Hertfordshire, UK). 1.5M Tris-HCL (PH=8.8) and 20% SDS were also used.

In addition a 10% (w/v) Ammonium Persulphate (APES) solution 0.1g/1ml and N,N,N',N'-tetramethlyenedianine (TEMED) from Sigma were also needed.

To make a Separating gel mix

Take 10ml of appropriate % gel mix (see table 17 below), add 33µl of 10% APES and 3.8µl TEMED.

To make a Stacking gel

Take 5ml of stacking gel stock, add 16.7µl of 10% APES and 3.8µl TEMED.
In the table below are the proportions of each used to make the stock gel solutions.

Reagent	Percentage gel			
	7.5%	**10%**	**15%**	**Stacking**
30% Acrylamide/ 0.8% bisacrylamide	22.5ml	30ml	45ml	12.5ml
1.5M Tris-HCl, pH=8.8	22.5ml	22.5ml	22.5mls	25mls
dH_2O	44.6ml	37.1ml	22.1ml	62ml
20% (w/v) SDS	0.45ml	0.45ml	0.45ml	0.5ml

Table 17: Reagents needed for varying % separating gels and the stacking gel, stock solutions.

1.5M Tris HCl (pH=8.8)

54.45g Tris base, in 200ml dH_2O. pH adjust with 5M HCl and make up to 300ml with dH_2O.

Running Buffer (x1)

For each 5 litres, made up with dH_2O
15.15g Tris base (0.025M)
72g Glycine (0.192M)
25ml 20% SDS (0.1%)
pH to 8.3 with HCl.

Transfer Buffer (x1)

For each 5 litres, made up with dH_2O.
15.15g Tris base (0.025M)
72g Glycine (0.192M)
1000ml Methanol (20%)
Do not pH adjust (should be 8-8.3)

For Western Blotting

Blocking Buffer

5% Bovine Serum albumin (BSA), 5g/100ml BSA in TTBS.

Primary & Secondary antibody solutions

3% BSA, 3g/100ml BSA in TTBS.

Wash Buffer (Tween-TBS (TTBS) = Tris Buffered Saline (TBS) and 0.05% Tween)

8.8g NaCl
0.2g KCl,
3g Tris base
500µl of Tween-20.
Add to 800ml dH_2O, pH adjust to 7.4, then make up to 1litre.

Appendix 2d

General Reagents

Phosphate Buffered Saline (PBS)

PBS tablets were purchased from Sigma, and one tablet added to each 200ml dH_2O.
This produces a 0.01M phosphate buffer.

1.5M Tris HCl (PH=8.8)

54.5g Tris base in 200 ml dH_2O, pH adjust with HCL and make up to 300ml with dH_2O.

Radioimmunoprecipitation Buffer (RIPA)

1X RIPA	5X RIPA	5X RIPA (100ml)
0.15M NaCl	0.75M NaCl	15ml of 5M NaCl
1% NP40	5% NP40	5ml 100% NP40
0.5% Sodium Deoxycolate	2.5% DOC (Na^+)	25ml 10% DOC (Na^+)
0.1% SDS	0.5% SDS	2.5ml 20% SDS
0.05% Tris HCl; pH 8	0.25M Tris	25ml 1M Tris HCl; pH 8
Ultra-pure H_2O		27.5ml

RIPA x1, supplemented with 1:100 (w/v) mammalian protease inhibitors (Sigma)

5M HCL

Use 32% Concentrated HCL (56.9ml) and 43.1ml of dH_2O. (NB. Add acid to water, in the fume cupboard)

20% SDS

200g of SDS per litre of dH_2O

5M NaOH

20g NaOH to 100ml dH_2O

EDTA (1M)

29.2g/100ml dH_2O

Cell Dissolution Solution

100ml total volume
5ml 5M NaOH
2ml 10% SDS
93 ml dH_2O

Tris-EDTA (TE) Buffer (pH=7.6)

10ml 1M Tris HCl (pH=8)
400µl 0.25M EDTA
Make up to 1 litre with dH_2O, after pH adjusting.

Gelatin Zymography

Novex Zymogram gels (10%) containing 0.1% gelatin were purchased from Invitrogen, or alternatively, standard bis-acrylamide Tris HCl gels were cast (see appendix 2c) with the modification that in the running gel component, 0.1% gelatin (1 mg/ml) was added from a stock solution of 10mg/ml gelatin dissolved in dH_2O.

In either case, 1x running buffer was used (appendix 2c) to run the gels at 120v for 90 minutes.

Sample buffer (2x)

2.5ml 0.5M Tris HCl (pH=6.8)
2ml Glycerol
4ml 10% (w/v) SDS
0.5ml of 0.1% bromophenol blue
dH_2O up to 10ml

Zymogram renaturing buffer (10x)

Triton X-100, 25% (v/v) in dH_2O

Zymogram developing buffer (10x)

12.1g Tris base
63g Tris HCl
117g NaCl
7.4g $CaCl_2$
0.2% Brij 35
dH2O to 1L

Appendix 3

Extracting type 1 r/r collagen or wild type control collagen

This technique is based on that first described by Cawston TE and Barrett AJ, in 1979. The protocol is as described below.

Day 1

1) Take about 40-50g of rat tails, cut into small pieces with knife and then process, with a food processor. Then add a small amount of 0.9% NaCl + 0.03% Toluene in dH_2O.
2) Wash mixture with 300ml of 0.9% NaCl + 0.03% Toluene in dH_2O. (4x) for 30 minutes each time in a cold room (4°C), with regular stirring. Filter through a 125μm nybolt mesh each time.
3) Extraction of collagen, with 250ml of 0.5M Acetic Acid + 0.03% Toluene, stirring overnight in a cold room. Then Filter.

Day 2

4) Repeat step (3)

Day 3

5) Spin extracts at 7,500g for 2 hours at 4°C.

6) Remove trace of lipid from surface.

7) Dialyse the supernatant against 2 changes over 2 days with 5% NaCl in 0.1M Acetic acid (5L) overnight in cold room (All dialysis steps require continuous stirring)

Day 5

8) Spin at 7,500g for 30 minutes at 4°C.

9) Resuspend pellet with 500ml 0.5M Acetic acid, then dialyse overnight in cold room against 0.5M acetic acid (5L).

Day 6

10) Then dialyse against 4 changes of 0.02M disodium hydrogen phosphate (5L), over 3 days.

Day 9

11) Spin at 7,500g for 30 minutes at 4°C.

12) Resuspend pellet with 500mls 0.5M Acetic acid.

Day 10

13) Add 25g of NaCl to make up to 5% (w/v) in above solution

14) Spin at 7,500g for 30 minutes at 4°C.

15) Wash pellet once with 20% NaCl

16) Spin at 7,500g for 30 minutes at 4°C.

Day 11

17) Resuspend pellet with 500ml 0.5M Acetic acid, stirring overnight in cold room.

Day 12

18) Spin at 30,500g for 1hr at 4°C

19) Dialyse overnight against 0.1M acetic acid (5L) overnight.

Day 13

20) Freeze Dry

21) Store in Freezer

Quantify using UV spectrophotometer 750nm, using Biorad D_c protein assay reagent (Biorad life sciences, Hemel Hempstead, Hertfordshire, UK) or the Bicinchoninic acid (BCA) protein assay as described in section 2.10.2. Assess extracted collagens purity by SDS PAGE electrophoresis and then staining the gel using colloidal blue stain for the α 1 & 2 chains of collagen I.

For use resuspend in 0.1mmol/l acetic acid as described earlier in section 2.5.2, then store at 4°C.

Solutions

1) 0.9% NaCl + 0.03% Toluene in dH_2O

NaCl 9g/litre + 0.3g/litre of Toluene

Toluene = 99.5%, 99.5g/100ml. For 0.03%, require 0.3g/litre.

Therefore add 300µl/litre

2) 0.5M Acetic Acid + 0.03% Toluene

Acetic acid = 99.8%, 99.8g/100mls. MW = 60.05.

1 mole = 60.05 grams

Therefore for 1mol/l = 60mls/litre

For 0.5M = 30ml acetic acid per litre + 300µl Toluene/litre

3) 5% NaCl in 0.1M Acetic acid

NaCl 50g + 6ml acetic acid/litre

4) 0.02M disodium hydrogen phosphate

0.02 moles/litre

MW = 142, therefore for 1mole = 142g. For 0.02moles = 2.84g.

6) 20% NaCl

NaCl 200g/litre

Dialysis Tubing Preparation

Dialysis tubing (BiodesignDialysis tubing™) was obtained from BDH laboratory supplies (VWR international, Poole, Dorset, UK).

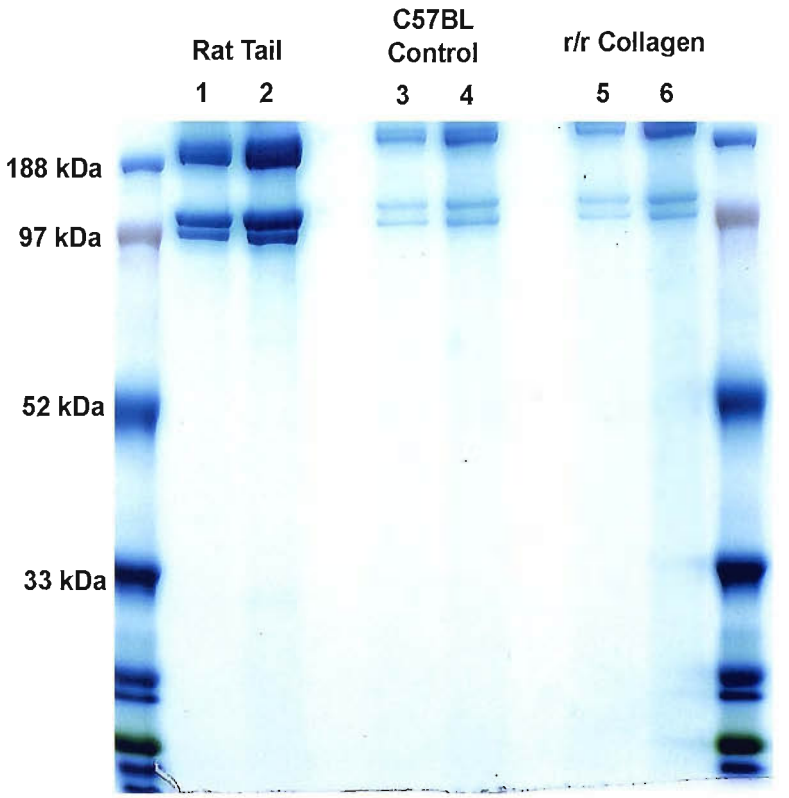
The tubing was pre-treated in 1mmol/l EDTA for 30 minutes, to remove heavy metal ions.

EDTA disodium, MW = 372.2

1 mole/litre = 372.2g/litre. For 1mmol, require 0.372g/litre.

The tubing was then washed several times in distilled water.

Figure 40: Purity of extracted collagens



SDS-PAGE electrophoresis and colloidal blue staining to determine the purity of extracted type I collagens. Lanes 1 and 2, contain proprietary rat tail collagen I at concentrations of 0.5mg and 1mg/ml respectively. It is unlikely, using this technique, to be able to resolve the seperate α-1 (138 kDa) and α-2 (129 kDa) chains that form collagen I. Instead, the two bands identified on the gel are most likely to represent different levels of glycosylation of the α-chains that form collagen type I. Collagen I extracted from the wild-type control (lanes 3 and 4) and r/r collagen (lanes 5 and 6) are demonstrated at two different concentrations for each collagen type.